Properties and Regulation of Valine-sensitive Acetolactate Synthase from Mitochondria of *Euglena gracilis*

By YUJI ODA, YOSHIHISA NAKANO AND
SHOZABURO KITAOKA*

Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 591,
Japan

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Acetolactate synthase from mitochondria of *Euglena gracilis* was markedly activated by ATP and feedback-inhibited by valine and, to a lesser extent, by other branched-chain amino acids. 2-Oxobutyrate, a substrate converted to acetohydroxybutyrate, had a much higher affinity for this enzyme than pyruvate and markedly inhibited the formation of acetolactate from pyruvate. The inhibition of growth of *E. gracilis* by threonine and the relief of this inhibition by valine are explained by these characteristic properties of *E. gracilis* acetolactate synthase, the key enzyme in the biosynthesis of branched-chain amino acids.

INTRODUCTION

Acetolactate synthase (EC 4.1.3.18), or acetohydroxy acid synthase, is one of the enzymes involved in the common biosynthetic pathway of isoleucine, valine and leucine (Umbarger, 1978). Since this enzyme, which catalyses the formation of precursors of these branched-chain amino acids, is under the control of the end-products, intensive studies have been made on the biochemical and genetical regulation of the enzyme from bacteria (Iaccarino *et al.*, 1978; De Felice *et al.*, 1979). Acetolactate synthase has been studied less intensively in eukaryotic organisms, probably because of the lability of the eukaryotic enzymes (Magee & de Robichon-Szulmajster, 1968; Mifflin, 1971; Glatzer *et al.*, 1972; Takenaka & Kuwana, 1972).

In previous papers we have reported that exogenous threonine inhibits the growth of *Euglena gracilis* (Oda *et al.*, 1982*b*) and that the inhibition is due to a disturbance in the conversion of 2-oxo acids to 2-aceto-2-hydroxy acids catalysed by acetolactate synthase. This was related to isoleucine–valine biosynthesis in mitochondria (Oda *et al.*, 1982*a*). The present paper reports the unique properties and regulation of valine-sensitive acetolactate synthase from mitochondria of *E. gracilis*.

METHODS

Organism and culture. *Euglena gracilis* SM-ZK (a streptomycin-bleached mutant derived from strain z) was cultured in a cyanocobalamin-limited medium (50 ng l⁻¹), as reported previously (Oda *et al.*, 1982*a*).

Preparation of the mitochondrial fraction as enzyme. A cell homogenate prepared by the method of Tokunaga *et al.* (1976) was centrifuged at 10000*g* for 10 min. The pellet of physiologically intact mitochondria was washed and suspended in 10 mM-3-(*N*-morpholino)propanesulphonic acid (MOPS)/KOH buffer (pH 7.5) containing 0.25 M-sucrose. This suspension (5.0 mg protein ml⁻¹) was used as the enzyme.

Enzyme assay. The activity of acetolactate synthase was assayed by the method of Takenaka & Kuwana (1972). The reaction mixture, containing 50 mM-sodium pyruvate, 1 mM-thiamin pyrophosphate (TPP), 0.5 mM-ATP, 100 mM-potassium phosphate buffer (pH 7.5) and enzyme (0.5 mg protein) in a total volume of 1.0 ml, was incubated at 25 °C for 30 min. The reaction was stopped by the addition of 0.1 ml of 3 M-H₂SO₄ and the acetolactate synthesized was converted into acetoin (acetyl methylcarbinol) by maintaining the mixture at 40 °C

for 1 h. After centrifuging, acetoin in the supernatant was determined by the method of Westerfeld (1945). The specific activity of enzyme was expressed as μmol acetolactate formed h^{-1} (mg protein) $^{-1}$. Acetoin was not formed directly from pyruvate during the enzyme reaction since no acetoin was detected in the supernatant when the reaction was stopped by adding 0.1 ml of 2.5 M-NaOH (Glatzer *et al.*, 1972). Acetohydroxybutyrate was determined by the microbiological method of Leavitt & Umbarger (1960). Protein was determined by the method of Bradford (1976).

Identification of reaction products. The acidic supernatant (obtained after the addition of H_2SO_4 to the enzyme reaction as described above) containing acetoin (acetylmethylcarbinol) formed from acetolactate, or acetoin and acetylmethylcarbinol formed from acetohydroxybutyrate, was neutralized with 0.3 mmol $\text{Ba}(\text{OH})_2$ and the mixture was agitated vigorously. After centrifuging, the supernatant was passed through columns of Dowex 1-X8 (HCOO^- form) and then Dowex 50-X8 (H^+ form); the columns were washed with distilled water. The carbinols in the effluent were converted into the corresponding pteridine derivatives and separated on a thin-layer of cellulose powder by the method of Wagner *et al.* (1959). Authentic acetolactate and acetohydroxybutyrate were prepared by the methods of Krampitz (1957) and Kiritani & Wagner (1971), respectively.

RESULTS

Stability of E. gracilis acetolactate synthase

During attempts to solubilize *E. gracilis* acetolactate synthase, the enzyme activity was completely lost by treatment of the mitochondrial fraction at 4 °C with Triton X-100, Brij 35, sodium deoxycholate or sodium dodecyl sulphate (all at 0.2%, w/v), or KI or KClO_4 (0.4 M) for 5 min, or by sonication in 1 M-phosphate buffer (pH 7.5) for 20 s. Emulgen 910 and Tween 20 also failed to solubilize the enzyme; after treatments at 4 °C for 5 min only 65 and 18% of the original activity remained.

The untreated mitochondrial fraction was therefore employed as the enzyme preparation throughout the following experiments. Acetolactate production was directly proportional to the enzyme concentration in the reaction mixture up to 1.5 mg protein ml^{-1} . Storing the enzyme preparation at 4 °C for 24 h caused a decrease in enzyme activity to 46% of the original activity.

Co-factor requirements

Table 1 summarizes the co-factor requirements of *E. gracilis* acetolactate synthase. The activity depended on ATP and, to a lesser extent, on ADP, but not on AMP and other nucleotides. When potassium phosphate buffer was replaced by MOPS/KOH buffer, the activity was reduced to 65%, indicating that P_i stimulates the activity.

Acetolactate synthase from most organisms requires TPP or FAD (Szentirmai *et al.*, 1970; Grimmering & Umbarger, 1979), but omission and addition of these co-factors did not affect the activity of the *E. gracilis* enzyme. The activity was slightly inhibited by 2 mM- Mg^{2+} , but not by 1 mM-EDTA. Dithiothreitol (5 mM) and, to a lesser extent, 2-mercaptoethanol stimulated the activity about 1.34-fold, but these SH-reagents strongly interfered with the Westerfeld reaction (Glatzer *et al.*, 1972).

Even when the mitochondrial fraction was dialysed against MOPS/KOH buffer for 24 h, the co-factor requirements of the enzyme (as in Table 1) did not change, although 83% of the original enzyme activity was lost.

Optimum pH and temperature

The optimum pH of *E. gracilis* acetolactate synthase was 7.5 in 100 mM-potassium phosphate buffer; in the presence of valine the activity was reduced and the optimum pH was shifted to higher values (Fig. 1).

The activity increased with temperature up to 50 °C, but the valine sensitivity of the enzyme was highest at about 25 °C. High temperature might cause a change in enzyme structure with consequent loss of the valine sensitivity.

Table 1. *Co-factor requirements of acetolactate synthase*

Adenine nucleotide (0.5 mM), MgSO_4 (1.0 mM) or FAD (2.0 μM) was added to the reaction mixture as indicated.

Alteration(s) to reaction mixture		Specific activity [$\mu\text{mol h}^{-1} (\text{mg protein})^{-1}$]
Omission	Addition	
—	—	1.82
ATP	—	0.225
ATP	ADP	1.25
ATP	AMP	0.243
TPP	—	1.81
—	Mg^{2+}	1.65
—	FAD	1.77
P^*	—	1.18

* Phosphate buffer was replaced by MOPS/KOH buffer.

Fig. 1

Fig. 2

Fig. 1. Effect of pH on acetolactate synthase and its inhibition by valine. Enzyme activity was determined without L-valine (O) and with 0.4 mM (●) and 5.0 mM (□) L-valine.

Fig. 2. ATP saturation curves for acetolactate synthase. Enzyme activity was determined without L-valine (O) and with 0.2 mM-L-valine (●).

Table 2. *Effect of L-valine and other related amino acids on acetolactate synthase*

Each amino acid was added to the reaction mixture at 5.0 mM.

Addition to reaction mixture	Inhibition (%)
None	0
L-Valine	89
D-Valine	1.1
L-Leucine	23
L-Isoleucine	32
L-2-Aminobutyrate	21
L-Threonine	6.6

Inhibition by valine

L-Valine inhibited the activity of *E. gracilis* acetolactate synthase (Table 2), and the percentage inhibition as a function of valine concentration showed a hyperbolic curve, unlike the enzymes in *Neurospora crassa* (Takenaka & Kuwana, 1972) and *Pseudomonas*

Fig. 3. Formation of acetohydroxy acids from pyruvate in the presence and absence of 2-oxobutyrate by acetolactate synthase: acetolactate (O) formed in the absence of 2-oxobutyrate; acetolactate (□), acetohydroxybutyrate (■) and total acetohydroxy acids (●) formed in the presence of 2.0 mM-2-oxobutyrate.

aeruginosa (Arfin & Koziell, 1973). This inhibition was specific for the L-isomer. Isoleucine, leucine and 2-aminobutyrate were not effective inhibitors. At a concentration of 0.2 mM, valine, isoleucine and leucine inhibited the enzyme by 29, 9 and 21%, respectively, while inhibition by combinations of these amino acids (0.2 mM for each L-amino acid), valine + isoleucine, valine + leucine, isoleucine + leucine and valine + isoleucine + leucine, was 27, 48, 27 and 51% respectively.

The enzyme followed normal Michaelis–Menten kinetics toward pyruvate, giving a K_m of 2.1 mM. Valine inhibited the enzyme non-competitively; the K_i was 0.28 mM.

Increasing the concentration of ATP activated the enzyme in the presence or absence of valine (Fig. 2). The ATP concentration which gave the half-maximal activation was 20 μ M. These kinetics indicate that this enzyme is regulated allosterically by ATP, and that valine has no effect on the allostericity.

Effect of 2-oxobutyrate

The carbinols produced from acetohydroxy acids by *E. gracilis* acetolactate synthase were identified as described in Methods. Only acetoin (acetylmethylcarbinol) was detected in the reaction mixture containing 50 mM-pyruvate alone; acetylethylcarbinol, in addition to acetoin, was detected when 10 mM-2-oxobutyrate was added to the reaction mixture containing 50 mM-pyruvate.

Figure 3 shows formation by acetolactate synthase of acetolactate and acetohydroxybutyrate from different concentrations of pyruvate and 2.0 mM-2-oxobutyrate. 2-Oxobutyrate not only reduced the total amount of acetohydroxy acids produced but also caused a preferential production of acetohydroxybutyrate when less than 3.2 mM-pyruvate was present. From the Lineweaver–Burk plots in the presence and absence of 0.4 mM-2-oxobutyrate the inhibition by 2-oxobutyrate was shown to be competitive toward pyruvate, giving a K_i of 0.13 mM.

DISCUSSION

Purification of acetolactate synthase has been difficult because of its lability (Umbarger, 1978) except with the enzyme from *Pseudomonas aeruginosa* (Arfin & Koziell, 1973). Recently, Grimminger & Umbarger (1979) have succeeded in purifying the enzyme from

Escherichia coli K12 using glycerol and a buffer of high ionic strength. In eukaryotic cells purification of the enzyme is complicated by the requirement to extract it from mitochondria where it is located (Umbarger, 1978). Acetolactate synthase in permeabilized cells of *Saccharomyces cerevisiae* is effectively inhibited by valine, but some physical and chemical treatments of the enzyme reduced its valine sensitivity (Magee & de Robichon-Szulmajster, 1968). A valine-sensitive acetolactate synthase was also extracted from *N. crassa* mitochondria by sonication in 1 M-potassium phosphate buffer (Kuwana & Date, 1975); this enzyme appears to be stabilized by P_i . However, the *E. gracilis* enzyme was not solubilized under the similar conditions. It seems that the activity and valine sensitivity of the *E. gracilis* enzyme are immediately lost when the enzyme is extracted from the mitochondrial membrane.

Acetolactate synthase from *E. gracilis* mitochondria required ATP as a co-factor. Takenaka & Kuwana (1972) reported that inhibition of the *N. crassa* enzyme by valine was relieved by ATP or ADP and that the enzyme was activated by ATP, ADP or P_i , but the *E. gracilis* enzyme was activated allosterically by ATP, keeping the valine sensitivity unchanged.

Other co-factors reported to be necessary for acetolactate synthase from other organisms (Szentirmai *et al.*, 1970; Glatzer *et al.*, 1972; Grimminger & Umbarger, 1979) were either slightly inhibitory or were ineffective with the *E. gracilis* enzyme; the co-factor requirements of the *E. gracilis* enzyme are similar to those reported for the *S. cerevisiae* enzyme (Magee & de Robichon-Szulmajster, 1968). TPP is essential in some bacteria (Szentirmai *et al.*, 1970) as the carrier of a hydroxyethyl group to form acetohydroxy acids, and ineffectiveness of exogenous TPP in *E. gracilis* may indicate that TPP is tightly bound to the *E. gracilis* enzyme protein.

Escherichia coli (Grimminger & Umbarger, 1979), *Salmonella typhimurium* (O'Neill & Freundlich, 1972) and *Aerobacter aerogenes* (Asada *et al.*, 1976) are known to contain multiple acetolactate synthases, whose activities are under the control of inhibition and repression by the end-products, valine, isoleucine and leucine. In eukaryotic organisms such as *N. crassa* (Takenaka & Kuwana, 1972) and ripening peas (Davies, 1964), two types of acetolactate-forming enzyme have been found, one insensitive to valine with an optimum pH 6.0 and the other sensitive to valine with an optimum pH 7.4 to 8.5. The *E. gracilis* acetolactate synthase showed the highest activity at pH 7.5 and had no apparent separate activity. The activity of the *E. gracilis* enzyme was lowered by valine with a shift of the optimum pH, indicating that it is analogous to the valine-sensitive enzyme in *Thiobacillus acidophilus* (Proteau & Silver, 1978). Harding *et al.* (1970) showed that the valine-insensitive activity at pH 6.0 in *N. crassa* mitochondria was included in the pyruvate dehydrogenase complex.

Formation of acetolactate from pyruvate by acetolactate synthase was inhibited in the presence of 2-oxobutyrate, whereas production of acetohydroxybutyrate was stimulated. Acetolactate synthase in some micro-organisms (Szentirmai & Horváth, 1976) has two binding sites for the two substrates – in the synthesis of acetolactate both sites bind pyruvate, while in the synthesis of acetohydroxybutyrate one of the two sites binds pyruvate to form a hydroxyethyl group and the other binds 2-oxobutyrate (Szentirmai & Horváth, 1976). Since pyruvate and 2-oxobutyrate compete for the binding sites and the *E. gracilis* enzyme has a much higher affinity for 2-oxobutyrate than pyruvate, acetohydroxybutyrate is the major product at low concentrations of pyruvate and the total production of acetohydroxy acids was reduced in the presence of 2-oxobutyrate (Fig. 3). Szentirmai *et al.* (1970) suggested that the difference in affinities of acetolactate synthase for pyruvate and 2-oxobutyrate is an important regulatory adaptation for the balanced synthesis of isoleucine and valine, since cells would be expected to have higher concentration of pyruvate than 2-oxobutyrate under normal conditions. Previously we reported that growth inhibition of *E. gracilis* by threonine was due to lowered production of valine, since threonine is converted to 2-oxobutyrate in the cytosol and this 2-oxo acid both stimulates isoleucine synthesis and inhibits valine synthesis in

mitochondria (Oda *et al.*, 1982*a*). This conclusion is supported by the present results; acetolactate synthase from *E. gracilis* mitochondria has a much higher affinity for 2-oxobutyrate than for pyruvate, and in the presence of the two 2-oxo acids production of acetohydroxybutyrate, an intermediate of the isoleucine synthesis, is preferred to that of acetolactate. Acetolactate synthase thus plays a central role in the growth inhibition by threonine in *E. gracilis*.

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