Biochimica et Biophysica Acta, 321 (1973) 348–355

BBA 67000

ACETOLACTATE SYNTHASE OF *PSEUDOMONAS AERUGINOSA*

I. PURIFICATION AND ALLOSTERIC PROPERTIES

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(Received March 13th, 1973)

SUMMARY

Acetolactate synthase (acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18, formerly also known as acetohydroxy acid synthetase) from *Pseudomonas aeruginosa* was purified approx. 380-fold to homogeneity. Feedback inhibition by all three branched-chain amino acids was observed and was potentiated at low temperatures. Plots of percent inhibition *vs* inhibitor concentration were sigmoid for all three ligands. At subsaturating levels, inhibition by combinations of branched-chain amino acids appears to be cumulative.

INTRODUCTION

Acetolactate synthase (acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18, formerly also known as acetohydroxy acid synthetase) catalyzes the first step unique to valine (and ultimately, leucine) biosynthesis and the second step of isoleucine biosynthesis[1]. Feedback inhibition by valine of the enzyme from *Escherichia coli*[2] was reported shortly after the first description of this phenomenon. Subsequently, valine has been shown to be a feedback inhibitor of the acetolactate synthases of *Salmonella typhimurium*[3], *Aerobacter aerogenes*[4] and *Saccharomyces cerevisiae*[5]. All of these studies have been carried out with relatively crude enzyme preparations and little is known of the structure, mechanism of action or mechanism of allosteric inhibition of acetolactate synthase.

The present paper describes the purification of acetolactate synthase from *Pseudomonas aeruginosa* and some properties of the enzyme. While this work was in progress two preliminary reports appeared[6,7] describing two isozymes of acetolactate synthase in *E. coli* and *S. typhimurium*, one of which is sensitive to feedback inhibition by all three branched-chain amino acids, as described below for the enzyme from *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals

Amino acids, sodium pyruvate, thiamine pyrophosphate, FAD, FMN and riboflavin were all purchased from Calbiochem. Calcium phosphate gel and Agarose 0.5 m, were from Bio-Rad Laboratories. Polyethylene glycol 6000 was from J. T. Baker Co. All other reagents were the highest quality available commercially.

Buffer

The main buffer used, Buffer A, contained 50 mM potassium phosphate (pH 7.0), 10 mM mercaptoethanol, 1 mM $MgCl_2$ and 10 μM thiamine pyrophosphate.

Organism and growth conditions

P. aeruginosa (ATCC 7700) was grown in the minimal salts medium of Davis and Mingioli[8] containing 8 g/l of isoleucine assay medium (Difco) and 0.5% glucose in place of citrate. Cultures were grown at 37 °C on a gyrotory shaker and harvested in early stationary phase by centrifugation at 2 °C. The sedimented cells were washed with 50 mM phosphate buffer (pH 7.0) and either used immediately or stored frozen at −20 °C.

Acetolactate synthase assay

The standard assay mixture contained: 100 mM potassium phosphate (pH 8.0), 40 mM sodium pyruvate, 10 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 0.025 mM FAD and a limiting amount of enzyme. The usual volume was 1.0 ml. Assay mixtures were incubated at 37 °C for 10 min. The reaction was terminated by the addition of 1.0 ml of 50% H_2SO_4 and the precipitated proteins were removed by centrifugation. The amount of α-acetolactate formed was determined as described by Magee and de Robichon-Szulmajster[5].

A unit of enzyme is defined as that amount which will produce 1.0 μmole of α-acetolactate in 1 min. Specific activity is expressed as enzyme units per mg of protein as determined by the procedure of Lowry *et al.*[9].

Resolution of FAD

1 ml of enzyme solution containing 4 mg of protein was mixed with 0.5 ml of 4.6 M KBr and stirred for 30 min at 0 °C. Aliquots of this mixture were then diluted 20-fold with Buffer A and assayed for activity with and without added flavins.

Ultracentrifugation

Sedimentation velocity experiments were performed with a Spinco Model E ultracentrifuge equipped with schlieren optics and a rotor temperature indicator control unit.

RESULTS AND DISCUSSION

Purification of enzyme

Step 1. Crude Extract. Cells are suspended in 3 vol. of Buffer A and disrupted by sonic oscillation for 3 min at 2–10 °C, with a Branson Sonifier at 20 kcycles and

maximum power. Cell debris is removed by centrifugation at 15 000 rev./min for 40 min.

Step 2. Treatment with ce tyl trimethyl ammonium bromide. One-fifth volume of 2% (w/v) cetyl trimethyl ammonium bromide is added to the crude extract. The suspension is stirred for 15 min and the inactive precipitate is removed by centrifugation and discarded.

Step 3. First fractionation with $(NH_4)_2SO_4$. $(NH_4)_2SO_4$, saturated at 4 °C and neutralized to pH 7.5 with NH_4OH, is added to the supernatant solution from Step 2 to give a final saturation of 58%. The precipitate is collected by centrifugation and dissolved in a volume of Buffer A equal to one-fourth the original crude extract volume.

Step 4. Fractionation with polyethylene glycol 6000. An equal volume of a solution of 25% (w/v) polyethylene glycol 6000 in distilled water is added to the redissolved $(NH_4)_2SO_4$ pellet. The precipitate is collected by centrifugation and redissolved in Buffer A to a final concentration of 12–16 mg protein/ml.

Step 5. Calcium phosphate gel adsorption and elution. Enough calcium phosphate gel suspension (80 mg dry wt/ml) is added to the enzyme solution obtained in Step 4 to give a gel to protein dry weight ratio of 1. The suspension is stirred for 15 min and the gel and adsorbed protein collected by centrifugation. The gel is washed with 10 ml of Buffer A and then eluted two times with 10 ml of 200 mM potassium phosphate (pH 7.0)–10 mM mercaptoethanol–1 mM $MgCl_2$–10 μM thiamine pyrophosphate. The two eluates are combined.

Step 6. Second fractionation with $(NH_4)_2SO_4$. Neutralized saturated $(NH_4)_2SO_4$ is added to the combined eluates to 55% saturation. After centrifugation, the precipitate is resuspended in 5 ml of 1.2 M $(NH_4)_2SO_4$ in Buffer A and stirred for 10 min. The precipitate is collected by centrifugation and dissolved in 1–2 ml of Buffer A.

Step 7. Gel filtration. The solution from Step 6 is applied to a column (2.5 cm × 85 cm) of Bio-Gel A-0.5 m, 200–400 mesh, equilibrated with Buffer A, and eluted with the same buffer. The activity is eluted from the column between 150 and 180 ml of eluant. The active fractions are pooled and concentrated by vacuum dialysis.

The purified enzyme loses only about 20% of its activity when stored in Buffer A at 0–4 °C for one month.

A typical purification is summarized in Table I.

TABLE I

SUMMARY OF THE PURIFICATION OF ACETOLACTATE SYNTHASE

Step	*Volume (ml)*	*Total units*	*Total protein (mg)*	*Specific activity*	*Yield*
1. Crude extract	1330	1500	22 800	0.066	100
2. Cetyl trimethyl ammonium bromide treatment	1410	1530	11 600	0.132	100
3. First $(NH_4)_2SO_4$ fractionation	335	1040	7 270	0.143	69.3
4. Polyethylene glycol 6000 fractionation	50	925	840	1.10	61.7
5. Calcium phosphate gel treatment	36	683	203	3.36	45.5
6. Second $(NH_4)_2SO_4$ fractionation	1.2	728	28.8	25.3	48.5
7. Bio-Gel A – 0.5 m column	18	410	16	25.6	27.3

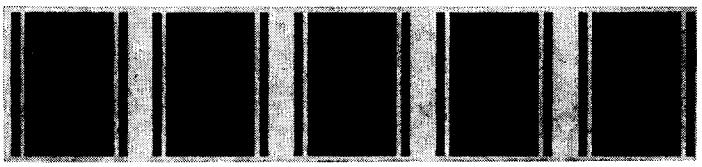


Fig. 1. Ultracentrifugal patterns of acetolactate synthase at 8 mg protein/ml in Buffer A. Centrifugation was at 40 000 rev./min in a Spinco Model E ultracentrifuge at 25 °C. The pictures were taken at 8-min intervals. The direction of sedimentation is from left to right.

Homogeneity of the purified enzyme

The enzyme emerges from the Bio-Gel A-0.5 m column as a single symmetrical peak of constant specific activity. Fig. 1 shows the ultracentrifuge sedimentation pattern of highly purified enzyme. A single symmetrical peak with a sedimentation constant, $s_{20,w}$, of 16.9 S was obtained. A more extensive analysis of the physical properties of the enzyme is presented in the accompanying paper[17].

Cofactor requirements

Thiamine pyrophosphate and Mg^{2+} have been demonstrated to be essential cofactors for α-acetolactate and α-aceto-α-hydroxybutyrate formation by the acetolactate synthases of the *enterobacteriacae*. This requirement is readily understandable in light of the necessity for the intermediate formation of hydroxyethylthiamine pyrophosphate. In addition, Stormer and Umbarger[10] have also demonstrated a requirement for FAD in acetolactate formation by partially purified enzymes from *E. coli* and *S. typhimurium*. In contrast, no cofactor requirements could be demonstrated for acetolactate formation by dialyzed extracts of *S. cerevisiae*[5]. Acetolactate synthesis by extracts of a wild type strain of *P. aeruginosa* are not stimulated by the addition of FAD, but Loutit and Davis[11] have isolated isoleucine–valine requiring mutants of *P. aeruginosa* strain 1 which have acetolactate synthases which show a requirement for FAD. The purified enzyme from *P. aeruginosa* (ATCC 7700) is stimulated by both Mg^{2+} and thiamine pyrophosphate, but added FAD does not lead to further stimulation (Table II). However, if the purified enzyme is treated with KBr as described in Materials and Methods, the enzyme by itself has only about 40% of the original activity. Upon the addition of FAD, such a preparation shows 80% of the original activity. No increase in activity is observed when FMN or riboflavin is added in place of FAD (Table III).

TABLE II

COFACTOR REQUIREMENTS FOR ACETOLACTATE SYNTHASE FROM *P. AERUGINOSA*

The data refer to 1.0 ml of a standard reaction mixture (see Materials and Methods) and 10 μg of purified enzyme protein.

Omission	*α-Acetolactate formed (μmoles/10 min)*
None	2.25
Thiamine pyrophosphate	0
FAD	2.10
$MgCl_2$	0.35

TABLE III

ACTIVITY OF PARTIALLY RESOLVED ACETOLACTATE SYNTHASE WITH FAD, FMN OR RIBOFLAVIN

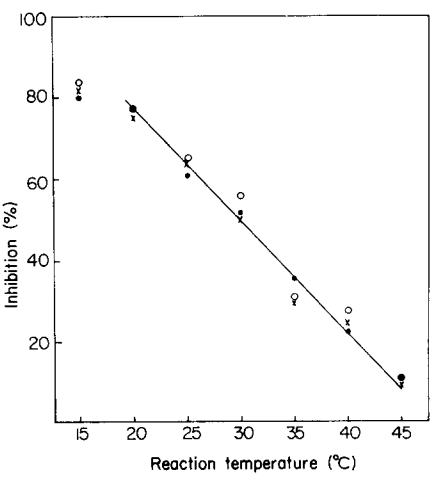
The KBr-treated enzyme was assayed under standard conditions. Flavins were added at a final concentration of 20 μg/ml. Activity is expressed as percent of the activity of untreated enzyme.

Flavin	*Activity (%)*
None	40
Riboflavin	40
FMN	40
FAD	82

The apoenzyme is apparently unstable, since attempts to isolate it from the KBr mixture by precipitation with $(NH_4)_2SO_4$ or polyethylene glycol 6000 gave preparations which could not be reactivated by added FAD.

Allosteric inhibition of enzyme activity

Initial experiments in which the inhibition of acetolactate synthase by branched-chain amino acids was examined, showed that the enzyme was inhibited by all three branched-chain amino acids and that the inhibition was markedly temperature dependent. Fig. 2 shows that at a constant inhibitor concentration, percent inhibition varies 4.5-fold over a temperature range of 15–45 °C. The shape of the curve is similar for all three amino acids, although the concentration of each necessary to give a similar degree of inhibition varies considerably. Under the assay conditions the enzyme is stable throughout this temperature range. The percent inhibition is charac-



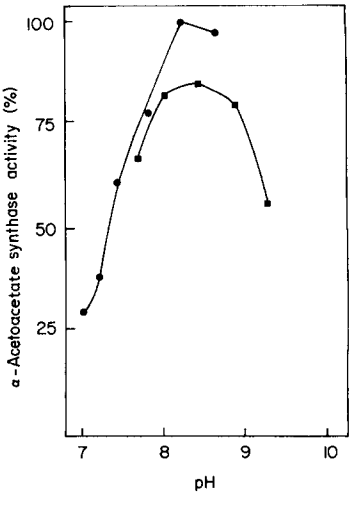


Fig. 2. Effect of reaction temperature on inhibition of acetolactate synthase by branched-chain amino acids. Reaction time was 10 min at pH 7.4. ●, $5 \cdot 10^{-6}$ M valine; ○, $2 \cdot 10^{-4}$ M isoleucine; ×, 10^{-3} M leucine.

Fig. 3. pH dependence of the initial rate of α-acetolactate formation. The standard reaction mixture was used. ●, 0.1 M potassium phosphate buffer; ■, 0.1 M bicine buffer.

teristic of the temperature at which the assay is performed and is not affected by preincubation of enzyme *plus* inhibitor at higher or lower temperatures. Other workers have observed that temperature strongly influences the regulatory properties of some allosteric enzymes[12,13]. In these cases it has also been observed that decreasing temperature favors binding of the allosteric effector.

The effect of pH on reaction velocity and on branched-chain amino acid inhibition is shown in Figs 3 and 4. The pH optimum of the catalytic reaction covers the range from 8.0 to 8.8. Enzyme activity falls rapidly on either side of this pH range (Fig. 3). The inhibitory effect of the three branched-chain amino acids strongly depends on pH and increases with decreasing pH. The pattern for all three amino acids is similar (Fig. 4).

Fig. 5 shows that the inhibition of acetolactate synthase activity by each of the branched-chain amino acids is sigmoidal, suggesting cooperativity in the binding of these inhibitors. The inhibition is noncompetitive with respect to pyruvate in each case. Saturation kinetics with respect to pyruvate are hyperbolic in the presence or absence of branched-chain amino acids. Replots of the data of Fig. 5 according to a modified Hill equation give straight lines with a slope (n) of about 1.4 for each branched-chain amino acid. The K_i values, determined according to Dixon[14], are $4.4 \cdot 10^{-6}$ M, $2.7 \cdot 10^{-4}$ M and $1.8 \cdot 10^{-3}$ M for valine, isoleucine and leucine, respectively.

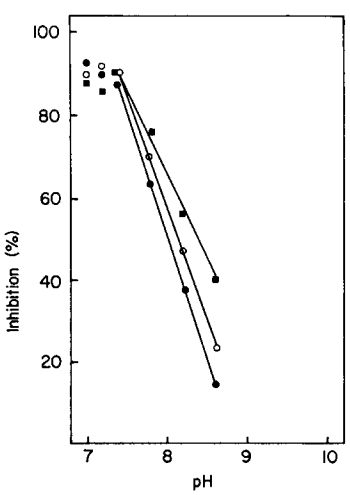


Fig. 4. pH dependence of allosteric inhibition by branched-chain amino acids. The standard reaction mixture was used. The buffer was 0.1 M potassium phosphate. ○, 10^{-2} M leucine; ■, $3 \cdot 10^{-3}$ M isoleucine; ●, $3 \cdot 10^{-5}$ M valine.

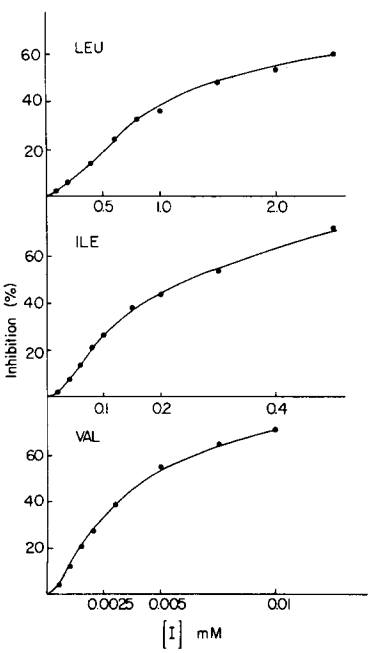


Fig. 5. Effect of branched-chain amino acid concentration on inhibition of acetolactate synthase activity. Incubation conditions were as described in Materials and Methods. Percent inhibition equals $(V_0 - V_i)/V_0$.

TABLE IV

CUMULATIVE INHIBITION OF ACETOLACTATE SYNTHASE BY BRANCHED-CHAIN AMINO ACIDS

The data refer to a standard reaction mixture containing the indicated final concentrations of branched-chain amino acids.

Inhibitor			*Inhibition (%)*		
$2 \cdot 10^{-6}$ *M Val*	$8 \cdot 10^{-5}$ *M Ile*	$6 \cdot 10^{-4}$ *M Leu*	*Observed*	*Calculated for cumulative*	*Calculated for Additive*
+	−	−	28		
−	+	−	29		
−	−	+	34		
+	+	−	47	49	57
−	+	+	49	53	63
+	−	+	49	52	62
+	+	+	63	66	91

When combinations of the branched-chain amino acids are tested for inhibition at less than saturating levels, the inhibition appears to be cumulative (Table IV). The similarity of the pH and temperature dependence for inhibition by all three branched-chain amino acids suggests that they are acting at similar if not identical sites on the enzyme. Initial attempts to desensitize the enzyme to inhibition by one or more branched-chain amino acid have been unsuccessful. Whatever the molecular basis, the concentrations of branched-chain amino acids likely to be present *in vivo* should, in combination, give rise to cumulative inhibition.

Varga and Horvath[15] showed that when crude extracts of *P. aeruginosa* are chromatographed on Sephadex G-200, four of the enzymes involved in branched-chain amino acid biosynthesis are eluted in the void volume. These workers suggested that the enzymes of branched-chain amino acid biosynthesis may exist as an aggregate in this organism. More recently, Marinus and Loutit[16] have shown that in *P. aeruginosa* α-acetolactate synthase and α-acetolactate isomeroreductase are subject to coordinate repression and derepression by the end products of the pathway but that the synthesis of the other enzymes is constitutive. This lack of coordinate synthesis amongst all the enzymes in the pathway would clearly place restraints on the constancy of structure of a multienzyme aggregate. These workers also showed that both α-acetolactate synthase and α-acetolactate isomeroreductase activities both appear in the void volume when crude extracts of *P. aeruginosa* are chromatographed on Sephadex G-200. However, the relative specific activities of the two enzymes differ considerably in the various fractions. We have found that α-acetolactate synthase and α-acetolactate isomeroreductase activities in crude extracts of *P. aeruginosa* ATCC 7700 copurify through the first fractionation with $(NH_4)_2SO_4$ in our purification scheme but that the α-acetolactate isomeroreductase is not found in the fraction precipitating between 0 and 12.5% polyethylene glycol 6000 but is quantitatively recovered in the fraction precipitating between 12.5 and 25% polyethylene glycol 6000.

These findings do not eliminate the possibility that the two enzymes are in fact complexed to one another *in vivo*. It is however, equally possible that both activities appear in the void volume after gel filtration of crude extracts on Sephadex

G-200 because both proteins are excluded by this gel. In the accompanying paper[17] we present evidence that the α-acetolactate synthase has a molecular weight of about 600 000. Although the size of the α-acetolactate isomeroreductase of *P. aeruginosa* is unknown, the enzyme from *S. typhimurium* has a molecular weight of 220 000 (ref. 18). If the *P. aeruginosa* enzyme is of a similar size then both enzymes would be expected to be excluded on a Sephadex G-200 column.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service research grant 1R01 AM14102 from the National Institutes of Arthritis and Metabolic Diseases.

REFERENCES

1 Umbarger, H. E. (1969) in *Current Topics in Cellular Regulation* (Horecker, B. L. and Stadtman, E. R., eds), Vol. 1, pp. 57–76, Academic Press, New York
2 Umbarger, H. E. and Brown, B. (1958) *J. Biol. Chem.* 233, 1156–1160
3 Bauerle R. H., Freundlich, M., Stormer, F. C. and Umbarger, H. E. (1964) *Biochim. Biophys. Acta* 42, 142–149
4 Halpern, Y. S. and Umbarger, H. E. (1959) *J. Biol. Chem.* 234, 3067–3071
5 Magee, P. T. and de Robichon-Szulmajster, H. (1968) *Eur. J. Biochem.* 3, 507–511
6 Blatt, J. M., Pledger, W. J. and Umbarger, H. E. (1972) *Biochem. Biophys. Res. Commun.* 48, 444–450
7 O'Neill, P. and Freundlich, M. (1972) *Biochem. Biophys. Res. Commun.* 48, 437–443
8 Davis, B. D. and Mingioli, E. S. (1950) *J. Bacteriol.* 60, 17–28
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
10 Stormer, F. C. and Umbarger, H. E. (1964) *Biochem. Biophys. Res. Commun.* 17, 587–592
11 Loutit, J. S. and Davis, P. F. (1970) *Biochim. Biophys. Acta* 222, 222–225
12 Jensen, R. A. and Nester, E. W. (1966) *J. Biol. Chem.* 241, 3373–3380
13 Paulus, H. and Gray, E. (1967) *J. Biol. Chem.* 242, 4980–4986
14 Dixon, M. (1953) *Biochem. J.* 55, 170
15 Varga, J. M. and Horvath, I. (1965) *J. Mol. Biol.* 13, 596–599
16 Marinus, M. G. and Loutit, J. S. (1969) *Genetics* 63, 557–567
17 Arfin, S. M. and Koziell, D. A. (1973) *Biochim. Biophys. Acta* 321, 356–360
18 Arfin, S. M. and Umbarger, H. E. (1969) *J. Biol. Chem.* 244, 1118–1127