Two Forms of Herbicide-Sensitive Acetolactate Synthase under Different Control by 2-Oxobutyrate in *Rhodospirillum rubrum*

Jobst-Heinrich Klemme and Irmgard Schneider

Institut für Mikrobiologie und Biotechnologie der Universität Bonn, Meckenheimer Allee 168, 5300 Bonn, Bundesrepublik Deutschland

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Regulation of acetolactate synthase (ALS, EC 4.1.3.18) in the phototrophic prokaryote *Rhodospirillum rubrum* was studied. In cell free extracts of 5 strains investigated, enzyme activity was very labile (about 80% loss of activity within 12 h during storage at 4 °C) but was stabilized to some extent by 10 μM FAD and 20 vol.% glycerol. By filtration of extracts through Superose 6 HR gels (FPLC technique), ALS activity of all strains was separated in two fractions of 200 and 600 kDa, respectively. The enzyme fractions had about the same affinity to pyruvate (K_m = 1.6–1.8 mM), the same sensitivity to L-valine (50 and 65% inhibition by 0.1 mM valine in the standard test mixture) and the herbicide sulfometuron methyl (90 and 92% inhibition by 1 μM herbicide), but differed greatly in their sensitivity to inhibition by 0.4 M NaCl. In culture media with 2-oxobutyrate (2-OB), growth began only after a lag-phase of several days (5 days with 1 mM of the inhibitor). Cells grown in the presence of 2-OB had a reduced total ALS activity and did not contain the 200 kDa fraction. The inhibition of ALS by valine was noncompetitive in respect to pyruvate (K_i = 0.1 mM). From other branched-chain amino acids tested (L-leucine, L-isoleucine, norvaline, norleucine) only isoleucine was inhibitory (K_i = 3.1 mM).

Introduction

Acetolactate synthase (ALS, EC 4.1.3.18) is the enzyme which initiates the biosynthetic routes leading to the branched chain amino acids leucine, isoleucine and valine. In enteric bacteria, up to three different isozymes of ALS (forms I, II and III) have been found and studied in structural, regulatory and genetic respect [1–3]. The isozymes are coded by separate operons (*ilv*NB, *ilv*MG and *ilv*HI), have similar cofactor requirements and subunit compositions [4], but differ in their sensitivities to inhibition by L-valine and herbicides (sulfonylureas, imidazolinones and sulphonanilides) [5].

In a previous paper [6], we have shown that growth of the phototrophic bacterium *Rhodospirillum rubrum* is strongly inhibited by the herbicides sulfometuron methyl and chlorsulfuron, even in the absence of L-valine which is required for growth inhibition of enteric bacteria by sulfonylurea compounds [7]. This finding suggested the occurrence of only one type of ALS in *R. rubrum*. We now show that this phototrophic prokaryote contains at least two forms (isozymes?) of ALS which differ in respect to (i) their molecular weight (200 and 600 kDa forms), (ii) inhibition by high salt concentrations, and (iii) regulation of biosynthesis (only the 200 kDa form being repressed during growth in 2-oxobutyrate containing media).

Non-standard abbreviations: AHB, α-acetohydroxybutyrate; AL, α-acetolactate; ALS, acetolactate synthase; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; 2-OB, 2-oxobutyrate.

Reprint requests to Prof. J.-H. Klemme.

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Materials and Methods

The *Rhodospirillum rubrum* strains used in this study (S1 = ATCC 11170; Ha = DSM 107; DSM 50914 = ATCC 27048; FR1; 1761-1a) are kept in the culture collection of the institute. The bacteria were grown photosynthetically at 30 °C in a defined malate-ammoniumsulfate medium [8]. Where indicated, the medium was supplemented with filter-sterilized L-amino acids (1 mM) or 2-oxobutyrate (1–5 mM). Cell extracts were prepared by ultrasonic treatment at 4 °C of washed cell suspensions in 20 mM K-phosphate, pH 7.2, followed by a 40,000 × *g* centrifugation for 30 min at 4 °C.

ALS activity of cell extracts or partially purified enzyme preparations was assayed at 30 °C in centrifuge tubes. Activities are expressed in nkat. 1 nkat = 0.06 units (μmol/min). The reaction mixtures (1 ml) contained the following components: 20 mM K-phosphate, pH 7.8; 0.45 mM thiaminepyrophosphate-HCl; 20 mM Napyruvate; and, for the assay of acetohydroxybutyrate (AHB)-forming activity, 10 mM 2-oxobutyrate (Na-salt). The reaction was started by the addition of extract or partially purified enzyme (0.67 nkat at maximum) and stopped by addition of 0.1 ml 50% (w/v) trichloroacetic acid for the assay of acetolactate (AL)-forming activity or 0.1 ml 1 N NaOH plus 0.1 ml 10% (w/v) $ZnSO_4$ for the assay of AHB-forming activity. The precipitates were centrifuged off and the supernatants were used for AL or AHB determinations. AL was determined as acetoin according to [9]. AHB was assayed microbiologically. This assay is based on the fact that the inhibition of *E. coli* K 12 by L-valine can be compensated by L-isoleucine or one of its C_6-precursors. Using defined conditions, the cell yields of *E. coli* K 12 in valine-containing media are proportional to the amounts of isoleucine or AHB added. Under our culture conditions (glucose-ammonium-medium supplemented with 4 mg/ml L-valine; incubation for 7 h at 37 °C after inoculation with cells from the early stationary phase plus addition of 100–250 μl of a filter-sterilized sample containing isoleucine or AHB) the cell yields of *E. coli* K 12 in valine-media were proportional to the concentration of isoleucine (or AHB) up to 50 μM (OD_{546}-turbidity values of 0.5 obtained with 20 μM isoleucine).

Protein concentrations of cell extracts and partially purified ALS preparations were determined according to [10]. Gel filtrations were performed on Superose 6 HR columns (1 × 30 cm) of Pharmacia-LKB, Freiburg (FPLC technique) equilibrated with 100 mM Tris-HCl, pH 7.8, supplemented routinely with 10 μM FAD and 20 vol.% glycerol. In some filtration experiments, the FAD concentration was varied (5 or 20 μM) or 0.2 M NaCl was added to the standard elution buffer. The molecular weights of ALS fractions were determined by calibrating the Superose column with a set of marker proteins (ferritin, catalase, aldolase, bovine serum albumin).

Marker proteins for molecular weight determinations were obtained from Boehringer, Mannheim; FAD, glycerol and thiaminepyrophosphate-HCl from Serva, Heidelberg; all other chemicals from E. Merck, Darmstadt. Sulfometuron methyl was a gift of Dr. P. Babczinski, Bayer AG, Leverkusen.

Results and Discussion

Acetolactate synthase activities in cell extracts of five different *R. rubrum* strains (S 1, Ha, FR 1, 50914, 1761-1 a) grown photosynthetically in a malate-ammoniumsulfate medium ranged from 0.33 to 0.66 nkat/mg protein (20 to 40 nmol/min × mg protein) when assayed in the standard test system (AL-forming reaction). Cultivation of strain S 1 in a medium supplemented with the branched-chain amino acids L-leucine, L-isoleucine, and L-valine (each at a concentration of 400 mg/l) reduced the specific activity of total ALS in extracts to about 35% of the control value. In extracts of all strains tested, ALS activity was strongly inhibited by sulfometuron methyl (about 90% inhibition at a herbicide concentration of 1 μM).

Half life of ALS activity in cell extracts prepared in 20 mM K-phosphate (pH 7.2) was only about 12–14 h during storage at 4 °C (about 2 days at −18 °C) for all *R. rubrum* strains investigated. However, the enzyme activity was stabilized by addition of FAD (10 μM) and 20 vol.% glycerol. In the presence of these substances, half life of ALS activity in extracts (stored at 4 °C) was about 2 days.

When cell extracts were filtered through a Superose 6 HR column (equilibrated with the stabilization buffer noted before), two ALS fractions were obtained with all *R. rubrum* strains studied (data for strain S 1 shown in Fig. 1). Calibration of the column with standard marker proteins indicated mol. wts. of the two enzyme fractions of about 600×10^3 and 200×10^3, respectively. It may be noted that variation of the FAD (5 to 20 μM) and the salt concentration (up to 0.2 M NaCl) of the elution buffer did not significantly change the elution behaviour of the two ALS forms. When the ALS fractions were filtered through Superose again, each fraction yielded only one activity band with an elution position identical to that observed during the first separation. Further purification of

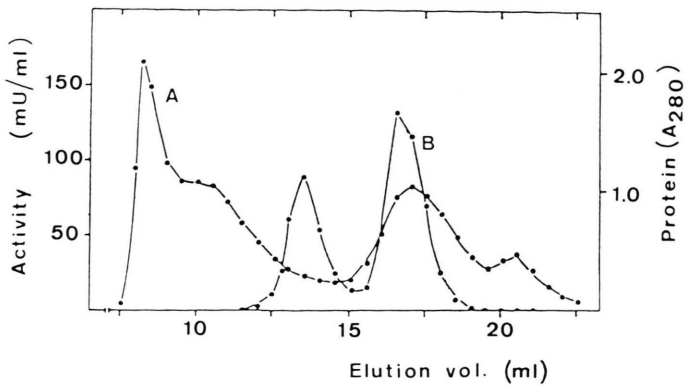

Fig. 1. Separation of two acetolactate synthase fractions of *R. rubrum* S1 by gelfiltration through Superose 6. 500 µl extract (containing 9.2 mg protein) was applied to a Superose 6HR column (1 × 30 cm) equilibrated with 100 mM Tris-HCl (pH 7.8) + 10 µM FAD + 20 vol.% glycerol. The column was eluted at a pressure of about 25 MPa with a flow rate of 15 ml/h (fraction volume = 0.47 ml). Specific ALS activities (acetolactate-forming reaction) in peak fractions were 5.50 (fraction at 13.5 ml) and 2.33 (fraction at 16.4 ml) nkat/mg protein (= 330 and 140 mU/mg protein, respectively). A: protein (A_{280}); B: ALS activity (mU/ml). The figure shows a typical separation experiment. Variation of data from 4 different filtrations is within a 15% range.

the two ALS forms by following a protocol for the isolation of FAD-stabilized ALS II from *Salmonella typhimurium* [3] was made impossible by the instability of the enzymes.

As shown in Table I, the two ALS fractions from strain S1 had about the same affinity for pyruvate, about the same sensitivity to L-valine and the sulfonylurea herbicide sulfometuron methyl, but differed considerably in their responses to inhibition by 0.4 M NaCl. Another difference (data not shown in the table) between the 600 and 200 kDa fraction concerned the activity with pyruvate plus 2-oxobutyrate (AHB forming reaction). With our experimental setup (direct colorimetric assay of AL and indirect, microbiological determination of AHB), the 600 kDa fraction had a higher AHB forming activity than the 200 kDa fraction. The activity ratios (rate of AHB-forming reaction/rate of AL-forming reaction) were 1.6–1.8 (600 kDa) and 0.6–0.8 (200 kDa), respectively. Likewise, the ALS isozymes of enteric bacteria differ in their catalytic activities with pyruvate and pyruvate plus 2-OB, respectively [11, 12].

The mechanism of inhibition of ALS by L-valine was analyzed more closely with the 200 kDa fraction. L-Valine acted as a noncompetitive inhibitor in respect to pyruvate (AL-forming reaction) with a K_i-value of 0.1 mM. From other branched-chain amino acids tested (L-isoleucine, L-leucine, norleucine, norvaline) only L-isoleucine was found to be inhibitory (K_i = 3.1 mM). 2-Oxobutyrate, substrate of ALS in the AHB forming reaction, was reported to be toxic to enteric bacteria [13] although the molecular mechanism of the toxicity is unclear. Besides from being an inhibitor of the acetolactate-forming activity of ALS [12], the substance in-

Table I. Properties of two acetolactate synthase fractions isolated from *Rhodospirillum rubrum* S1.

Properties	600 kDa-Fraction	200 kDa-Fraction
K_m (pyruvate)	1.8 ± 0.2 mM	1.6 ± 0.2 mM
Inhibition by 0.1 mM L-valine*	50 ± 5%	65 ± 5%
Inhibition by 1 µM sulfometuron methyl*	92 ± 5%	90 ± 5%
Inhibition by 0.4 M NaCl*	85 ± 8%	5 ± 1%

* Refers to the standard test system (AL-forming activity) with 20 mM Na-pyruvate. Data shown are average values from 3 different experiments.

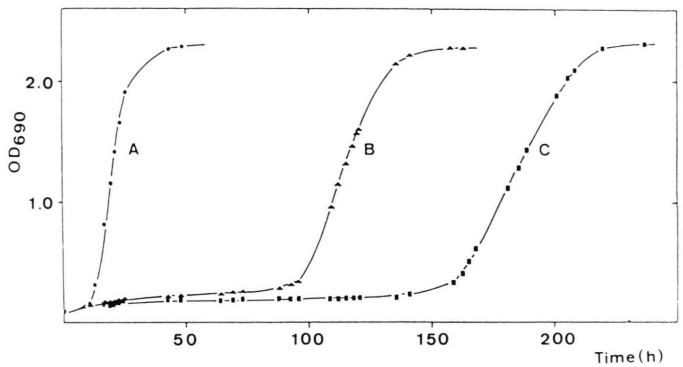

Fig. 2. Inhibition of photosynthetic growth of *R. rubrum* S1 by 2-oxobutyrate. Growth in 13 ml-screw cap tubes (malate-ammonium-sulfate-medium) was monitored by directly measuring the turbidity at 690 nm (OD_{690}). A: Control without inhibitor; B: + 1 mM 2-OB; C: + 5 mM 2-OB. The figure shows a typical experiment. Variation of data from 3 different growth experiments is within a 20% range.

terferes with the formation of aminoacyl-tRNAs [14], pantothenate biosynthesis [11], formation of aspartate [15], and other metabolic processes.

2-Oxobutyrate (2-OB) inhibited acetolactate-formation catalyzed by the 200 kDa fraction of *R. rubrum* AHA synthase (60% inhibition by 20 mM of the inhibitor in the standard assay with 20 mM pyruvate; the 600 kDa fraction not tested) and had a strong inhibitory effect on growth of *R. rubrum* resulting in a very pronounced lag-phase of several days (its duration being dependent on the concentration of 2-OB) (Fig. 2). Growth rates in the "recovery phase" of inhibited cultures were only slightly lower than in control cultures. Cells in the "recovery phase" of 2-OB-inhibited cultures did not carry a stable genetic mutation as indicated by the fact that a lag-phase of growth was also observed after transfer of "recovered" cells to fresh media containing the inhibitor. The molecular nature of the "recovery phenomenon" is unknown yet.

Extracts from *R. rubrum* S1 cells grown in the presence of 2-OB (1 mM) had a reduced ALS activity (0.167–0.250 nkat/mg protein). These extracts contained only the 600 kDa enzyme fraction. The different responses of the two ALS fractions to repression by 2-OB could be an indication of the existence of ALS isozymes in *R. rubrum*. However, the possibility that the two enzyme fractions represent different oligomeric forms of only one enzyme (compare ref. [16]) can not entirely be excluded.

Repressive control of an ALS fraction by 2-oxobutyrate, as found in *R. rubrum*, is in contrast to the regulatory pattern observed for the *ilv* gene clusters of enteric bacteria in which ALS isozyme I is derepressed by 2-oxobutyrate [17]. The insensitivity of the L-threonine dehydratase (EC 4.2.1.16) to feedback inhibition by isoleucine in *R. rubrum* [18] may be part of the regulatory network controlling the biosynthesis of isoleucine, leucine and valine in this phototrophic prokaryote.

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