Specificity of plant acetohydroxyacid synthase: formation of products and inhibition by herbicides

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Abstract

Plant acetohydroxyacid synthase (EC 4.1.3.18) or AHAS catalyses either the condensation of two molecules of pyruvate or one molecule of pyruvate with one molecule of 2-ketobutyrate. 2-Aceto-2-hydroxybutyrate and 2-acetolactate are formed when the pyruvate concentration is ten times greater than the 2-ketobutyrate concentration. This result indicates that plant AHAS has a preference for 2-ketobutyrate in the second substrate site. A similar inhibition by sulfometuron methyl was observed for the two reactions catalysed by the enzyme. These studies were developed by using a new chromatographic method based on the formation of 2,4-dinitrophenylhydrazone of acetoin and 2,4-dinitrophenyl-hydrazone of 3-hydroxy-2-pentanone (compounds resulting of decarboxylation of the enzyme products).

INTRODUCTION

Acetohydroxy acid synthase (AHAS, EC 4.1.3.18, or ALS) catalyses the first common step in branched-chain amino acid biosynthesis which is specific to plants and microorganisms (Umbarger, 1978). Special attention has been given to this pathway since AHAS has been identified as the target of two classes of powerful herbicides, the sulfonylureas (Larossa and Schloss, 1984) and the imidazolinones (Shaner et al., 1984). The enzyme catalyses either the condensation of two molecules of pyruvate to form acetolactate (a precursor of leucine and valine) or a molecule of pyruvate with a molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (an intermediate in the isoleucine biosynthesis) (*fig.* 1). These reactions proceed via a common mechanism well accepted for all thiamine pyrophosphate-catalyzed decarboxylation: addition of the first substrate (pyruvate) on the cofactor, decarboxylation of the formed intermediate and fixation of the second substrate (pyruvate or 2-ketobutyrate). A quantitative estimate of the

relative contribution to isoleucine versus valine-leucine synthesis at given substrates concentrations was investigated by Barak et al. (1987) in the case of AHAS isozymes of enteric bacteria. Concerning the inhibition of AHAS, a comparative study for sulfonylureas in the way of inhibiting acetolactate and 2-aceto-2-hydroxybutyrate formations may be of interest in the understanding of inhibitors binding-site.

In this paper, the two reactions catalysed by AHAS are considered for the plant enzyme from both catalysis and inhibition points of view. A new chromatographic method was developed to carry out these studies.

RESULTS AND DISCUSSION

HPLC method

So far, methods to determine the two possible reactions of AHAS simultaneously were limited by either low sensitivity or tedious procedures
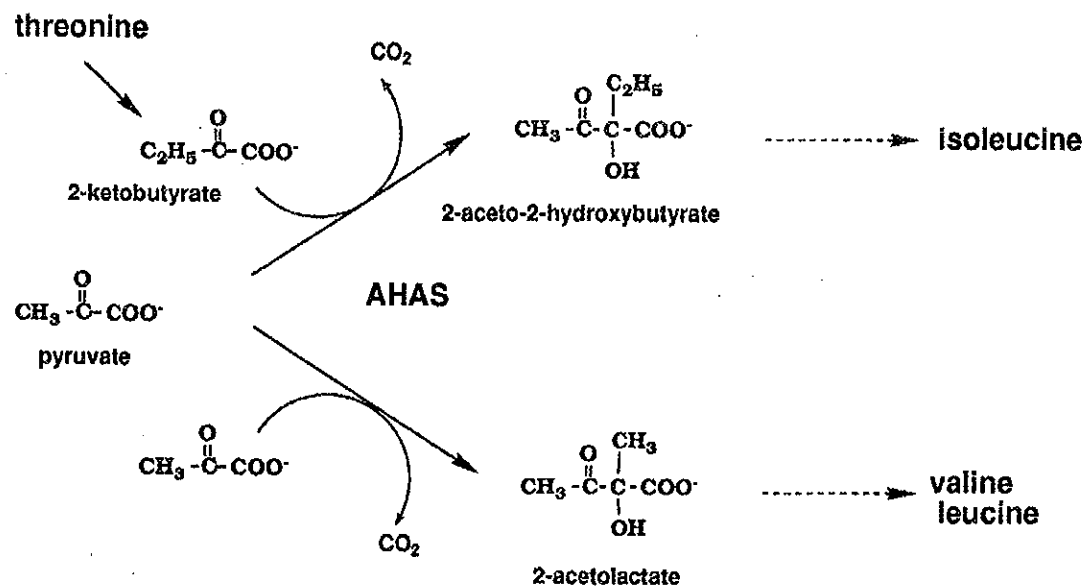
threonine

Figure 1. *The biosynthetic pathway to the branched-chain amino acids.*

(Shaw and Berg, 1980; Gollop *et al.*, 1987; Adam *et al.*, 1989). Our method is based on the formation of acetoin and 3-hydroxy-2-pentanone-2,4-dinitrophenylhydrazone derivatives, the two compounds obtained after decarboxylation of the enzyme products. 3-Hydroxy-2-pentanone was synthesized by oxidation of the enol ether of 2-pentanone (Rubotton *et al.*, 1974). A problem was the high concentrations of substrates (pyruvate and 2-ketobutyrate) in the enzymic reaction mixture. These keto-acids can react with 2,4-dinitrophenylhydrazine to yield four 2,4-dinitrophenylhydrazone derivatives (Katsuki *et al.*, 1972) which may interfere with the resolution of hydroxy-ketones DNP derivatives in the HPLC analysis. The ketoacid DNP derivatives are slightly soluble in water. Partial precipitation of the ketoacid DNP derivatives and removal of the precipitate were therefore realized by using a mixture acetonitrile/water (35/65, v/v). In these conditions, a satisfactory HPLC analysis was obtained.

Mixtures of known amounts of authentic acetoin and 3-hydroxy-2-pentanone were derivatized as described above in order to obtain the calibration curves of figure 2. The concentrations of the compounds were determined from calibration curves of the authentic DNP derivatives (data not shown). A direct measurement of the efficiency of conversion of the hydroxyketones to the corresponding DNP derivatives
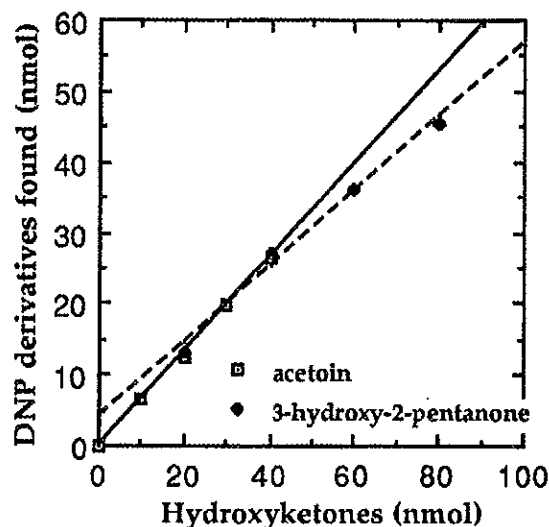

Figure 2. *Relation between quantities of DNP derivatives measured and hydroxyketone in the assay.*

is therefore possible. The yield of these reactions is 65% for acetoin and 63% for 3-hydroxy-2-pentanone. Reliable measurements can be made at very low conversions of substrate, the sensitivity of the method being 1 nmol of acetoin and (or) hydroxypentanone formed in 1 ml of reaction media.

Specificity on the products formation

Different ratios of pyruvate and ketobutyrate were tested. Acetoin and 3-hydroxy-2-pentanone formed were converted after work up to DNP derivatives and analyzed by HPLC.

Table 1. *Dependence of acetolactate and 2-aceto-2-hydroxybutyrate on the pyruvate and 2-ketobutyrate concentration.* Barley AHAS activity was measured after 1 h at 37°C by HPLC measurement (determined from the corresponding DNP derivatives). s_1 and s_2 are respectively 2-ketobutyrate and pyruvate concentrations, $s_1 + s_2 = 20$ mM. R is AHAS specificity ratio.

	s_1/s_2				
	0	0.005	0.026	0.053	0.111
Acetoin formed (*nmol*)	285.5	261.8	203.5	149	116
3-hydroxy-2-pentanone formed (*nmol*)	0	5.1	48.6	81.3	130.9
R		9.9	10.2	10.5	10.2

The results of table 1 show that plant AHAS has a preference for 2-ketobutyrate in the second substrate site. The specific ratio R was calculated by the method of Barak *et al.* (1987):

$$R = \frac{v_1/v_2}{c_1/c_2}$$

with v_1 and v_2 being respectively acetohydroxybutyrate and acetolactate rates of formation and c_1 and c_2 being 2-ketobutyrate and pyruvate concentrations. Except for the small concentrations of 2-ketobutyrate, the ratio was constant and close to 10. Equivalent rates of the two products formation occur when c_2/c_1 is equal to R. This value is different from those obtained with bacterial isozymes of AHAS: R = 1 for AHAS I, R = 180 for AHAS II and R = 60 for AHAS III (Barak *et al.*, 1987). It appears that AHAS II and plant AHAS have not the same behaviour considering the formation of products (Mazur *et al.*, 1987). In plants, the low specificity of AHAS for one reaction over the other is consistent with the fact that only one isozyme is instrumental in valine, leucine and isoleucine biosynthesis.

Inhibition by herbicides

Sulfonylureas are strong inhibitors of AHAS, the enzymic activity being determined by dosage of acetoin (Shaner *et al.*, 1984). The inhibition is time-dependent, initial and final steady-state levels of

Table 2. *Inhibition of AHAS activity by sulfometuron methyl (10 nM for barley AHAS and 20 nM for spinach AHAS).* Barley and spinach AHAS reactions were measured at 37°C respectively with 19 mM pyruvate, 1 mM 2-ketobutyrate and 19.5 pyruvate, 0.5 mM 2-ketobutyrate. A, inhibition of acetolactate formation; B, inhibition of 2-aceto-2-hydroxybutyrate formation. The enzymatic activity was 0.28 mU for 70 min reaction and 1 mU for 20 min reaction (barley) and 0.2 mU (spinach).

Source	Time of reaction (*min*)	A (*% inhibition*)	B (*% inhibition*)
Barley	20	18 ± 4	21 ± 2
Barley	70	51 ± 4	59 ± 4
Spinach	75	76 ± 5	74 ± 6

inhibition can be determined. The measurement of inhibition of AHAS activity at 20 and 70 min showed an increase of inhibition with time for acetolactate and 2-aceto-2-hydroxybutyrate synthesis (*tab.* 2). Nevertheless, the two reactions are inhibited at the same level. This low influence of substrate on inhibition was confirmed by an experiment carried out on spinach AHAS (*tab.* 2). These results suggest that no relationship exists between AHAS inhibition and the fixation of the second substrate. The change of pyruvate into 2-ketobutyrate in the first step of condensation has a great effect on the inhibition in the case of the bacterial AHAS II. Schloss (1989) observed a lower sensitivity of the enzyme to sulfometuron methyl when carrying out the homologous condensation of two molecules of 2-ketobutyrate.

CONCLUSION

A reliable, simple and quantitative method for simultaneous assay of acetolactate and 2-aceto-2-hydroxybutyrate formation catalyzed by purified plant AHAS in reaction mixtures containing pyruvate and 2-ketobutyrate was developed. This method allows determination of a preference of plant AHAS for 2-ketobutyrate as second substrate. The two reactions of the enzyme are inhibited at the same extent by the sulfometuron methyl herbicide which suggests that the inhibition of AHAS by sulfonylureas occurs at the step of fixation of the first molecule of substrate (pyruvate).

METHODS

All chemicals were purchased from Aldrich Chemical Co and used as received. Sulfometuron methyl (methyl-[[[[(4,6-

dimethyl-2-pyrimidinyl) amino] carbonyl] amino] sulfonyl] benzoate) was purchased from Dr Ehrenstorfer Gmbh. ^{1}H NMR spectra were recorded an a JEOL EX 400 spectrometer (400 MHz). Absorbance measurements were carried out on a UV-visible HP8452A spectrometer. HPLC studies were effected on a Merck chromatographic system.

Synthesis of 3-hydroxy-2-pentanone. A solution of 3-chloroperoxybenzoic acid (10 mmol) in dry hexane (35 ml) was cooled in an ice methanol bath and treated with a solution containing 2-pentanone trimethyl-silyl enol ether (10 mmol) in hexane (15 ml), obtained as previously described (Walshe *et al.*, 1987). After addition was completed (*ca* 5 min), the reaction mixture was stirred for 45 min. The resulting slurry was then filtered to remove *m*-chlorobenzoic acid and the filtrate was concentrated. The crude product was partitioned between ether and 1.5 N hydrochloric acid solution (2 h) to affect hydrolysis. The organic layer was recovered and concentrated. Purification on a silicagel chromatography column (HCCl₃/ether, 1/1, v/v) afforded the product as a colourless liquid - ^{1}H NMR (CDCl₃) 0.9 (t, 3H), 1.6 (m,1H), 1.9 (m, 1H), 2.1 (s, 3H), 4.1 (m, 1H).

Synthesis of acetoin and 3-hydroxy-2-pentanone-2,4-dinitrophenyl hydrazone derivatives. 2,4-Dinitro-phenyl-hydrazine (0.5 mmol) was dissolved in concentrated sulfuric acid (5 ml). This solution was cautiously poured into a mixture of 95 % ethanol (25 ml) and water (7 ml), and cooled. The hydroxyketone (2.3 mmol) in a minimum volume of 95% ethanol was added. The solid was collected by suction filtration after 10 min, washed with a little volume of aqueous ethanol and recrystallized in 95% ethanol. DNP of acetoin: mp 102°C; ^{1}H NMR (CDCl₃). 1.45 (d, 3H), 2.1 (s, 3H), 4.5 (q, 1H), 7.9 (d, 1H), 8.3 (d, 1H), 9.15 (d, 1H), 11.1 (s, 1H). DNP of 3-hydroxy-2-pentanone: mp 143°C; ^{1}H NMR (CDCl₃). 0.95 (t, 3 H), 1.6 (qd, 2H), 2.0 (s, 3H), 4.35 (q, 1H), 7.9 (d, 1H), 8.25 (dd, 1H), 9.05 (d, 1H), 10.95 (s, 1H). Calculated analysis C: 46.80; H: 4.96; N: 19.86, found C: 46.94; H: 4.99; N: 19.50.

Enzymes. Seeds of barley (*Hordeum vulgare* L.) were placed on a filter paper wetted with distilled water and allowed to germinate in a dark chamber at 25°C. After 4 days, etiolated shoots were collected and stored at −20°C. Frozen shoots (200 g) were homogenized in 400 ml of standard buffer (KH₂PO₄/K₂HPO₄, 100 mM), pH 7.2, 80 μM FAD, 10 mM EDTA, 1 mM DTT and 20% (v/v) glycerol. Cell debris were removed by centrifugation (15 min at 25,000 x *g*). Solid ammonium sulfate was added to the supernatant (132 g l⁻¹). After centrifugation (5 min at 25,000 x *g*), the supernatant was applied to a column of phenylsepharose (2.5 x 12 cm). Elution was performed by a linear gradient consisting of 250 ml of standard buffer at a flow of 5 ml min⁻¹. Fractions containing ALS activity were pooled and concentrated by precipitation using 2.95 M ammonium sulfate.

Spinach (*Spinacia oleracea* L.) leaves (3 kg) were homogenized in a Waring blender for 1 s, four times, with 9 l of solution (0.33 M mannitol, 30 mM MOPS pH 7.5, 2 mM EDTA, Bovine Serum Albumine 0-15%, w/v). The homogenate was filtered through two layers of cheesecloth and the resulting filtrate was centrifuged (4 min at 3,000 x *g*). The pellet was resuspended in 4 ml of homogenizing solution, filtered on cheesecloth and applied to a discontinued gradient of Percoll (40 and 80%). After centrifugation (10 min at 3,700 x *g*), chloroplasts sedimented at the interface 40-80% were collected. Washing with solution and centrifuging (5 min at 1,500 x *g*) gave intact chloroplasts. These chloroplasts were broken in a hypotonic solution (1 μM β-mercaptoethanol, 5 mM MgCl₂, 10 μM sodium pyruvate, 500 μM thiamine pyrophosphate, 0.1 mM dithiothreitol, 20 mM buffer KH₂PO₄, pH 7.5). The suspension was centrifuged (15 min at 39,000 x *g*). The supernatant was filtered through a 0.22 μm filter (Millipore) and 20% (v/v) of glycerol was added. This solution was used as spinach ALS.

Enzymatic reaction conditions. A standard reaction mixture (125 μl) for the assay of AHAS contained 0.5 mM thiamine pyrophosphate, 2 mM MgCl₂, and 50 μM FAD in 100 mM potassium phosphate buffer (pH 7). A mixture of pyruvate (20-18 mM) and 2-ketobutyrate (0-2 mM) was added to make up 20 mM of total substrate. The reaction was initiated by addition of the enzyme extract. After incubation at 37°C for 1 h, the reaction was stopped with 6 N sulfuric acid (25 μl). The reaction media was assayed for acetolactate and 2-aceto-2-hydroxybutyrate by decarboxylation at 60°C for 10 min.

Determination of AHAS activity: colorimetric method (Westerfeld, 1945). 0.5% (w/v) creatine (300 μl) and 5% (w/v) of freshly prepared solution of α-naphthol in 2.5 N NaOH (250 μl) were added to the enzymic reaction mixture (150 μl) diluted with distilled water (70 μl). The reaction was kept at 60°C for 10 min, distilled water (450 μl). The absorbance was measured at 530 nm.

Determination of AHAS activity: HPLC method. A 35 mM solution of dinitro-2,4-phenylhydrazine in 1N H₂SO₄/CH₃CN (3/7, v/v) (125 μl) and the enzymatic reaction mixture (125 ml) were sonicated for 30 s and allowed to stand at room temperature for 1.25 h. The precipitate was removed by centrifugation and the supernatant diluted two-fold in CH₃CN. The HPLC analysis was carried out by using a C8 (5 μ) ultrabase silica gel column with CH₃CN/100 mM CH₃COONa (45/55, v/v) and CH₃COOH so as to have pH 6.3, as eluant. The dinitro-2,4-phenylhydrazones were detected at 348 nm.

Inhibition of AHAS. Sulfometuron-methyl was dissolved in water. Final concentrations in the enzyme assay mixture were respectively 10 nM for barley AHAS and 20 nM for

spinach AHAS. The inhibitor solution was added to the enzyme preparation containing sodium pyruvate (19.5 mM for spinach AHAS and 19 mM for barley AHAS) and sodium 2-ketobutyrate (0.5 mM for spinach AHAS and 1 mM for barley AHAS). The formation of acetolactate and acetohydroxybutyrate was measured at two different times: 20 and 70 min.

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