Volume 258, number 1, 113-115 FEB 07828 November 1989

Flavin adenine dinucleotide causes oligomerization of acetohydroxyacid synthase from Black Mexican Sweet corn cells

Bijay K. Singh and Gail K. Schmitt

American Cyanamid Company, P.O. Box 400, Princeton, NJ 08540, USA

Received 4 September 1989

Acetohydroxyacid synthase activity is stabilized and stimulated by flavin adenine dinucleotide. Flavin adenine dinucleotide was found to cause aggregation of acetohydroxyacid synthase from the dimeric to a tetrameric form. The different aggregation states of the enzyme have differential sensitivities to inhibition by branched chain amino acids as well as by imazapyr, an imidazolinone herbicide. These observations indicate that flavin adenine dinucleotide is of structural as well as of functional importance for the plant acetohydroxyacid synthase enzyme.

Acetohydroxyacid synthase; Flavin adenine dinucleotide; Imidazolinone; Sulfonylurea

1. INTRODUCTION

Inhibition of AHAS (EC 4.1.3.18) by 3 different classes of herbicides [1-5] has stimulated much interest in this enzyme. AHAS catalyzes the first step in the parallel biosynthetic pathway of the branched chain amino acids valine, leucine and isoleucine. These reactions involve the condensation of two moles of pyruvate to give rise to acetolactate, or condensation of pyruvate and 2-oxobutyrate to yield acetohydroxybutyrate. AHAS requires TPP, Mg^{2+} and FAD as co-factors to carry out these reactions. The requirement for FAD has been shown for AHAS from both bacteria and plants [6-9], an unusual feature because there is no oxidation-reduction involved in these reactions. Furthermore, the enzyme activity is unaffected by reduction of the endogenous FAD, or by its replacement with flavin analogues of higher or lower redox potentials [5]. Results presented here are the first demonstration of a role of FAD in the oligomerization of AHAS from plants. Furthermore, our data supports the involvement of FAD in the reaction catalyzed by AHAS.

2. MATERIALS AND METHODS

2.1. *Enzyme extraction*

Embryo-derived cell suspension cultures of *Zea mays* var. Black Mexican Sweet were used as the enzyme source. The culture conditions have been previously described [9]. The cells were powdered in liquid nitrogen and then homogenized in 50 mM potassium phosphate buffer (pH 7) containing 150 mM sodium chloride. The homogenate was filtered through a nylon cloth (53 μM mesh) and centrifuged at 25 000 *g* for 20 min. The supernatant fraction was used for gel permeation chromatography.

2.2. *AHAS assay*

The AHAS activity was measured by estimation of the product, acetolactate, after conversion by acid decarboxylation to acetoin according to the previously described procedures [9].

2.3. *Gel permeation chromatography*

Enzyme preparation (200 μl) was applied to a Waters Protein Pak 300 SW gel filtration HPLC column (30 cm × 7.5 mm i.d.) which had been pre-equilibrated either with 50 mM potassium phosphate buffer (pH 7) containing 150 mM sodium chloride or with a buffer described in section 3. Protein was eluted with the same buffer at a flow-rate of 0.5 ml/min. Fractions (0.25 ml) were collected and analyzed for AHAS activity. The proteins (and their molecular weights) used for calibration were α-amylase (mol.wt. 200 000), γ-globulin (158,000), aldolase (158 000), alcohol dehydrogenase (150 000), bovine serum albumin (67 000), ovalbumin (43 000), myoglobin (17 000) and ribonuclease A (13 700).

3. RESULTS AND DISCUSSION

Crude extracts of the enzyme were used to avoid any aggregation of enzyme caused by concentration of the enzyme due to ammonium sulfate. Another reason for using the crude extract was to minimize the effects of additional steps required for purification of the enzyme. In the first set of experiments, AHAS was extracted and chromatographed either in 50 mM phosphate, pH 7, containing 150 mM NaCl (PBS) or in PBS containing pyruvate, TPP, FAD, and $MgCl_2$ either singly or in all possible combinations (16 treatments). AHAS activity was eluted in two peaks of about equal area when extracted and chromatographed in PBS

Correspondence address: B.K. Singh, American Cyanamid Company, P.O. Box 400, Princeton, NJ 08540, USA

Abbreviations: AHAS, acetohydroxyacid synthase; FAD, flavin adenine dinucleotide; TPP, thiamine pyrophosphate; PBS, phosphate buffered saline

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

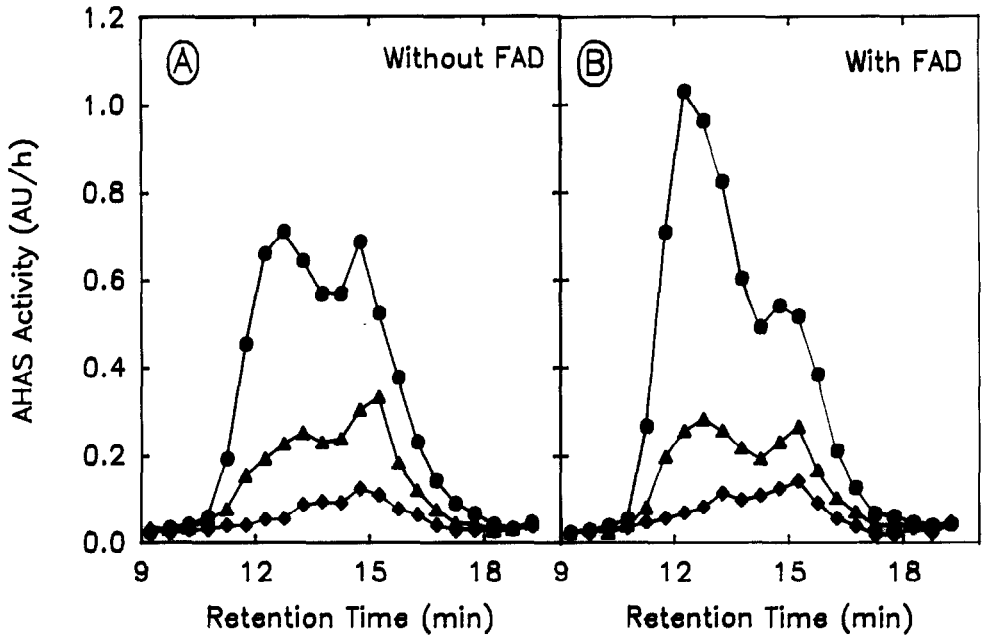

Fig.1. Gel permeation chromatography of AHAS on a Waters Protein Pak SW300 column in the absence or presence of FAD. AHAS was extracted from BMS cells in 50 mM phosphate (pH 7.0) containing 150 mM NaCl (PBS), centrifuged to remove particulate material and chromatographed on the column equilibrated with PBS (A) or PBS containing 10 μM FAD (B). AHAS activity was determined in different fractions in the absence of any inhibitor (●) and in the presence of 1 mM valine + leucine (▲) or 100 μM imazapyr (♦).

(fig.1A). The same elution pattern was observed in all treatments that included pyruvate, TPP and $MgCl_2$, either singly or in combination. However, inclusion of FAD in the extraction and chromatography buffer caused elution of AHAS predominantly in the higher molecular weight form (fig.1B). This elution pattern was unaffected by inclusion of substrate and other cofactors. Based on the deduced molecular weight of the mature protein from the AHAS gene sequence [12], AHAS in peak II is the dimeric form of the enzyme (MW = 150 000) whereas AHAS in peak I represents the tetrameric form of the enzyme (MW = 300 000).

AHAS activity in the column fractions showed significant differences in their sensitivity to inhibition by high concentrations of feedback inhibitors valine and leucine or imazapyr (fig.1). The tetrameric form of AHAS was more sensitive to inhibition by the feedback inhibitors as well as by imazapyr as compared to the dimeric form of AHAS. Further examination of the sensitivity of AHAS in the pooled fractions showed similar results (fig.2; data for valine + leucine not presented here). The differences in sensitivity to inhibition by imazapyr were not only seen in the I_{50} (12.3 μM for the dimer and 5.0 μM for the tetramer) but also in the level of maximum inhibition (fig.1 and 2).

Two explanations for the apparent high proportion of AHAS activity in the tetrameric form in the presence of FAD are: (1) FAD selectively stimulates the tetrameric form; and (2) dimeric form of AHAS aggregates to the tetrameric form in the presence of FAD. The stimulation of AHAS activity by FAD has been previously demonstrated [9]. To examine the first possibility, AHAS was extracted and chromatographed in PBS. AHAS activity was determined in the absence and presence of FAD. As expected, AHAS activity was eluted in two peaks with about equal proportion of the enzyme activity (fig.3). AHAS activity in both peaks was stimulated to similar extent when assayed in the presence of FAD. This observation rules out the first possibility of selective stimulation of tetrameric form of AHAS by FAD. To confirm the second possibility, enzyme extracted in PBS was chromatographed in PBS + FAD. AHAS activity was resolved in a profile similar to the one described in fig.1B. These results support the notion that FAD causes aggregation of the dimeric form of AHAS to the tetrameric form.

The conversion of dimers to tetramers in the presence of FAD is rapid but not complete. An experiment, where the enzyme extracted in the absence or presence of FAD was chromatographed either immediately or after 4 h of extraction, showed a constant ratio of dimers to tetramers at each time point. The rapid change in equilibrium of dimers and tetramers by FAD was also demonstrated by the reversal experiment. In this case, AHAS was extracted in PBS + FAD and chromatographed in the same buffer or in PBS alone. Chromatography in the presence of FAD gave predominantly tetrameric form of AHAS and the elution profile was similar to fig.1B. In contrast, elution of about equal amounts of dimeric and tetrameric form of AHAS was observed in the absence of FAD (similar elution profile as shown in fig.1A). Therefore, even though the enzyme was extracted in the presence of FAD, chromatography in the elution buffer probably

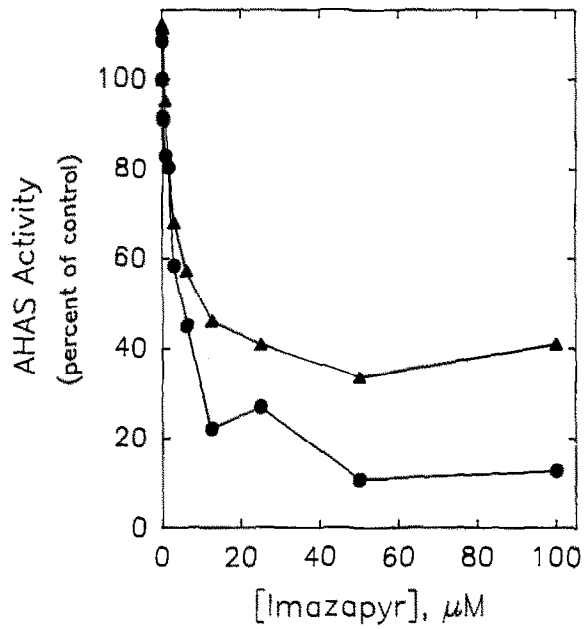

Fig.2. Inhibition of AHAS activity by imazapyr. AHAS activity was determined as described in section 2 except that FAD was not included in the assay buffer. The assay was performed in the presence of varying concentrations of imazapyr as described in the figure. Dimer (▲); tetramer (●).

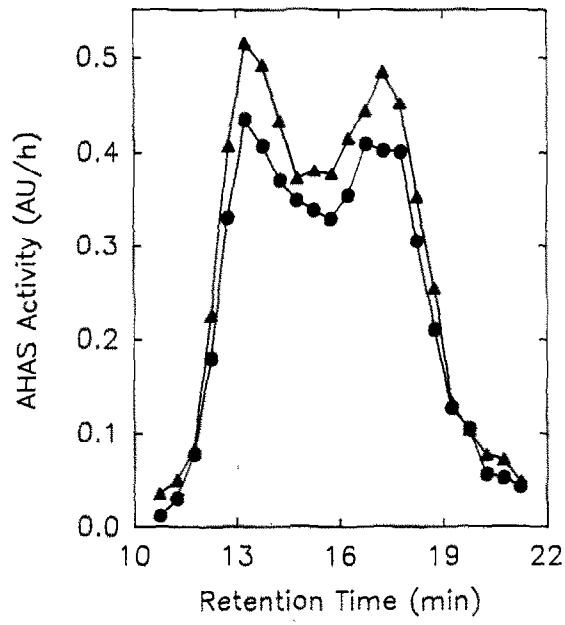

Fig.3. Gel permeation chromatography of AHAS on Waters Protein Pak SW300 column. AHAS was extracted from BMS cells in 50 mM phosphate (pH 7.0) containing 150 mM NaCl (PBS), centrifuged to remove particulate material and chromatographed on the column equilibrated with the extraction buffer. AHAS activity was measured in the absence (●) or presence of FAD (▲).

caused dissociation of FAD from the enzyme. These results suggest that the binding of FAD is loose with AHAS, an observation previously described for the bacterial enzyme [13].

FAD dependent aggregation of bacterial AHAS has been reported [13,15,16]. Reports of aggregation of AHAS from *Neurospora crassa* [17], maize [8] and barley [14] have been made, but the cause of the aggregation was unexplained. The experiments described here are the first systematic study demonstrating a role of FAD in the aggregation of plant AHAS. These results have some important implications: first, AHAS may be predominantly present in tetrameric form of the enzyme in vivo because FAD is present in the chloroplasts. Second, the dual role of FAD in stabilizing AHAS activity [11] and in converting the enzyme predominantly to the tetrameric form suggests that the tetrameric form of the enzyme is the more stable form of the enzyme. Finally, kinetic data show that the tetrameric form of the enzyme is more sensitive to inhibition by feedback inhibitors (valine and leucine) and by imazapyr. The work on bacterial AHAS represents two school of thoughts. Chang and Cronan [15] suggest that FAD requirement of AHAS plays a structural function. On the other hand, Schloss et al. [5] believe a functional role of AHAS that flavin may shield the hydroxyethylthiamine PP_i reaction intermediate from the solvent. Our results support both structural and functional roles of FAD in the reactions catalyzed by AHAS from plants.

REFERENCES

[1] Chaleff, R.S. and Mauvais, C.J. (1984) Science 224, 1443–1445.
[2] La Rossa, R.A. and Schloss, J.V. (1984) J. Biol. Chem. 259, 8753–8757.
[3] Ray, T.B. (1984) Plant Physiol. 75, 827–831.
[4] Shaner, D.L., Anderson, P.C. and Stidham, M.A. (1984) Plant Physiol. 76, 545–546.
[5] Schloss, J.V., Ciskanik, L.M. and Van Dyk, D.E. (1988) Nature 331, 360–362.
[6] Stormer, F.C. and Umbarger, H.E. (1964) Biochem. Biophys. Res. Commun. 17, 587–592.
[7] Schloss, J.V., Van Dyk, D.E., Vasta, J.F. and Kutny, R.M. (1985) Biochemistry 24, 4952–4959.
[8] Muhitch, M.J., Shaner, D.L. and Stidham, M.A. (1987) Plant Physiol. 83, 451–456.
[9] Singh, B.K., Stidham, M.A. and Shaner, D.L. (1988) Anal. Biochem. 171, 173–179.
[10] Wittenbach, V.A. and Erbes, D.L. (1986) Plant Physiol. (Suppl.) 80, 350.
[11] Singh, B.K., Newhouse, K.E., Stidham, M.A. and Shaner, D.L. (1989) in: Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry (Lopping, L.G., Dalziel, J. and Dodge, A.D., eds) BCPC Monograph 42, pp 87–95, British Crop Protection Council, Farnham, Surrey, U.K.
[12] Mazur, B.J., Chui, C.-F. and Smith, J.K. (1987) Plant Physiol. 85, 1110–1117.
[13] Grimminger, H. and Umbarger, H.E. (1979) J. Bact. 137, 846–853.
[14] Durner, J. and Boger, P. (1988) Z. Naturforsch. 43c, 850–856.
[15] Chang, Y.-Y. and Cronan Jr., J.E. (1988) J. Bact. 170, 3937–3945.
[16] Eoyang, L. and Silverman, P.M. (1984) J. Bact. 157, 184–189.
[17] Tanaka, H. and Kuwana, H. (1984) Biochem. Biophys. Res. Commun. 123, 418–423.