Plant Physiol. (1992) 99, 812–816
0032-0889/92/99/0812/05/$01.00/0

Received for publication October 7, 1991
Accepted January 22, 1992

Arabidopsis Acetohydroxyacid Synthase Expressed in *Escherichia coli* Is Insensitive to the Feedback Inhibitors

Bijay Singh*, Iwona Szamosi, J. Mark Hand, and Rajeev Misra

American Cyanamid Company, P.O. Box 400, Princeton, New Jersey 08543–0400 (B.S., I.S., J.M.H.); and Biology Department, Princeton University, Princeton, New Jersey 08544 (R.M.)

ABSTRACT

Acetohydroxyacid synthase (AHAS), the first enzyme unique to the biosynthesis of isoleucine, leucine, and valine, is the target enzyme for several classes of herbicides. The AHAS gene from *Arabidopsis thaliana*, including the chloroplast transit peptide, was cloned into the bacterial expression plasmid pKK233–2. The resulting plasmid was used to transform an AHAS-deficient *Escherichia coli* strain MF2000. The growth of the MF2000 strain of *E. coli* was complemented by the functional expression of the *Arabidopsis* AHAS. The AHAS protein was processed to a molecular mass of 65 kilodaltons that was similar to the mature protein isolated from *Arabidopsis* seedlings. The AHAS activity extracted from the transformed *E. coli* cells was inhibited by imidazolinone and sulfonylurea herbicides. AHAS activity extracted from *Arabidopsis* is inhibited by valine and leucine; however, this activity was insensitive to these feedback inhibitors when extracted from the transformed *E. coli*.

AHAS[1] (also known as acetolactate synthase; EC 4.1.3.18) catalyzes the first enzymic reaction leading to the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine. The enzyme catalyzes two parallel reactions: condensation of 2 mol pyruvate to give rise to acetolactate, and condensation of pyruvate and α-ketobutyrate to yield acetohydroxybutyrate. Biochemical and genetic studies have shown that AHAS is the target site of several classes of structurally unrelated herbicides, which include the imidazolinones, the sulfonylureas, and the triazolopyrimidines (1, 11, 15).

AHAS is a key controlling point for the levels of the branched chain amino acids in both prokaryotes and eukaryotes. In bacteria, this involves feedback inhibition of the enzyme activity by the end products of the pathway, or alteration of enzyme concentration, or both (21, 22). In plants, regulation of this enzyme by the feedback inhibitors valine, leucine, and isoleucine has been well characterized (9, 10, 15).

Escherichia coli and *Salmonella typhimurium* contain three different forms of AHAS. The enzymes from these enterobacteria exist as tetramer of two large and two small subunits (2, 4, 13). It has been demonstrated in *E. coli* that the small subunit of the enzyme is responsible for the feedback sensitivity of the enzyme (3). AHAS from plants also exists as high molecular mass aggregate (11, 16); however, it is not known whether AHAS from plants is composed of homologous or heterologous subunits. Because AHAS is highly conserved across bacteria, yeast, and plants (1, 8), it is possible that plants also contain a small subunit of AHAS.

Low abundance and the labile nature of eukaryotic AHAS have severely hampered the purification and biochemical characterization of this enzyme. Expression of enzymes in heterologous systems, such as in *E. coli*, has been successfully used in many cases to obtain large amounts of proteins for such studies. AHAS from *Arabidopsis* and *Brassica napus* have been expressed in *E. coli* and *S. typhimurium*, respectively (20, 23). In these cases, functional complementation of growth of the microbes and sensitivity of the enzyme and the microbes to AHAS inhibitors were examined. The present study was initiated to obtain large amounts of the plant enzyme for biochemical studies. During the biochemical characterization of the enzyme, it was observed that the plant enzyme expressed in *E. coli* was similar to the enzyme from *Arabidopsis* in all of the properties examined except the sensitivity of the enzyme to the feedback inhibitors. The implications of this observation are discussed.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana was grown in metromix in a greenhouse. Green shoots from 2- to 3-week-old seedlings were used for extraction of the enzyme.

Bacterial Strain and Construction of Recombinant Plasmid

MF2000 strain of *Escherichia coli* (*ilvB800::mu-1, Bgl32, ilv115, thi-1, argE3, rpsL31, #[ara,-leu,ilvHI]863, mtl-1, xyl-5, galK2, lacY1, recA1*) was obtained from M. Freundlich (State University of New York, Stony Brook). The expression vector pKK233–2 (Pharmacia, Piscataway, NJ) was used for the expression of the *Arabidopsis* AHAS in *E. coli*. This plasmid contains a strong chimeric promoter *trc* (*trp-lac*) and a *Nco*I site, which is part of the translational initiation codon for bacterial expression. The *Arabidopsis* AHAS gene, coincidently, contains *Nco*I site at the first codon (8). A 5.5-kilobase *Xba*I fragment, containing the *Arabidopsis* AHAS promoter region, the entire coding region including the chloroplast transit peptide, and 3′ noncoding region, was cloned into pBluescript SK- (Stratagene, La Jolla, CA). The multiple clon-

[1] Abbreviations: AHAS, acetohydroxyacid synthase; PA10, *E. coli* strain MF2000 transformed with plasmid pAC210.

ing site in this vector contains a *Pst*I site downstream from the *Xba*I site. Therefore, an *Nco*I/*Pst*I fragment containing the entire coding region of the *Arabidopsis* AHAS gene and the 3′ noncoding region was cloned into *Nco*I/*Pst*I-digested pKK233–2. The resulting plasmid, pAC210 (Fig. 1), was used to transform the *E. coli* strain MF2000. The cells were grown in M63 medium (14) containing the amino acids arginine, leucine, and histidine (0.1 mg/mL of each amino acid). Valine and isoleucine (0.1 mg/mL of each amino acid) were added as described. The bacterial cells transformed with the plasmid were grown in the presence of 100 μg/mL ampicillin. Plasmid preparation, transformation of *E. coli*, and other routine procedures were performed according to the protocols described by Maniatis *et al.* (7).

Extraction and Assay of AHAS

The procedure for the extraction of AHAS from plant material has been previously described (18). Bacterial extracts were prepared by sonication of lysozyme-treated cells in 50 mM Tris-HCl buffer (pH 7) containing 1 mM EDTA and 20% sucrose. Cell debris was discarded following centrifugation and the supernatant fraction was desalted on a Sephadex G-25 column equilibrated with 20 mM phosphate (pH 7) containing 10 μM flavin adenine dinucleotide. Desalted enzyme preparation was immediately used for the enzyme assay. The procedure for the *in vitro* assay of AHAS has been previously described (18). An *in vivo* AHAS assay for permeabilized *E. coli* cells was performed using previously described procedures (5).

Electrophoresis and Immunological Procedures

SDS-PAGE and western blotting procedures have been previously described (12, 17). Polyclonal antibodies raised against the *Arabidopsis* AHAS were used for immunological detection of AHAS protein on the western blots.

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.) that had been preequilibrated with 50 mM potassium phosphate buffer (pH 7) containing 150 mM sodium chloride and 10 μM flavin adenine dinucleotide. 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 mol wt) used for calibration were α-amylase (200,000), γ-globulin (158,000), aldolase (158,000), alcohol dehydrogenase (150,000), BSA (67,000), ovalbumin (43,000), myoglobin (17,000), and ribonuclease A (13,700).

RESULTS AND DISCUSSION

Complementation of Growth of the MF2000 Strain of *E. coli*

The coding regions of plant AHAS genes contain sequences representing the chloroplast transit peptide that are cleaved off during the protein import into chloroplast. Because the N-terminal sequence of the *Arabidopsis* AHAS protein is not known, it was not possible to construct an expression vector that would encode only the mature coding region of the AHAS protein. For this reason, the entire coding region of the *Arabidopsis* AHAS gene was cloned into plasmid pKK233–2 as described in "Materials and Methods" and in Figure 1. The resulting plasmid pAC210 and pKK233–2 (without an insert) were separately transformed into the *E. coli* strain MF2000. The cells were selected on medium supplemented with valine, isoleucine, and ampicillin. Cells from individual colonies were then streaked on medium lacking valine and isoleucine. MF2000 cells transformed with pKK233–2 did not grow in the absence of valine and isoleucine. On the other hand, cells transformed with pAC210 (designated PA10) grew normally in the absence of valine and isoleucine. These results are similar to functional complementation of a microbe lacking the endogenous AHAS activity using the plant enzyme demonstrated before (20, 23). To confirm our complementation observation, cells were grown in liquid culture in the presence or absence of valine and isoleucine. In the presence of valine and isoleucine, MF2000 cells transformed with either pKK233–2 or pAC210 grew at the same rate (Fig. 2). Once again, cells transformed with pKK233–2 did not grow in the absence of valine and leucine, whereas the cells transformed with pAC210 were able to grow under the same conditions. Interestingly, the cells transformed with pAC210 grew at the same rate in the absence or presence of valine and isoleucine. That expression of the *Arabidopsis* AHAS caused the growth of PA10 cells in the absence of valine and isoleucine was confirmed by retransformation of MF2000 cells with plasmids isolated from the primary transformants, restriction digest analysis of the isolated plasmid to demonstrate the presence of plant AHAS gene (not shown), and by enzyme assays as discussed below.

Expression of AHAS

There was no AHAS activity in the extracts prepared from MF2000 cells transformed with plasmid pKK233–2. On the

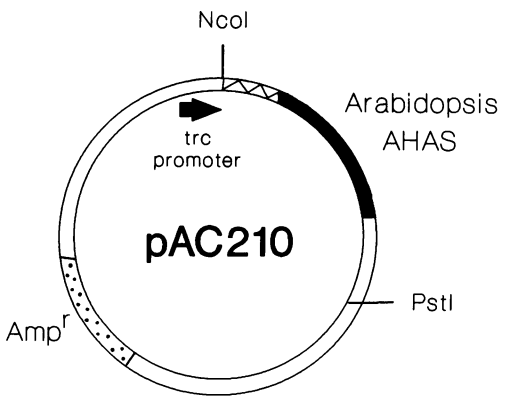

Figure 1. Construction of the plasmid pAC210. The entire coding region of the *Arabidopsis* AHAS that included the chloroplast transit peptide, the mature coding region, and the 3′ noncoding region of the DNA (the *Nco*I-*Pst*I fragment) was inserted into the vector pKK233–2. ⧄, Chloroplast transit peptide of AHAS; ■, mature coding region of AHAS; Amp^r = ampicillin resistance gene.

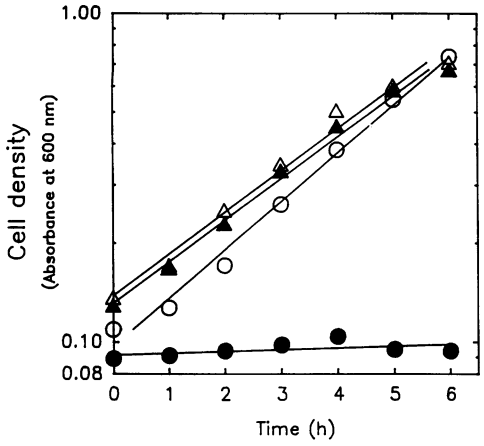

Figure 2. Growth of *E. coli* MF2000 and PA10 on M63 media in the presence or absence of valine and isoleucine. Cells were grown overnight in M63 minimal media in the presence of valine and isoleucine, washed twice in the minimal media, and then resuspended in the same media or in the media supplemented with valine and isoleucine to give an optical density at 600 nm of about 0.1. Cell growth was monitored by measuring absorbance at this wavelength. MF2000, without valine and isoleucine (●); MF2000, with valine and isoleucine (○); PA10, without valine and isoleucine (▲); PA10, with valine and isoleucine (△).

other hand, high levels of AHAS activity (5 μmol/mg protein · h) were observed in the crude extracts of PA10. The crude extracts prepared from PA10 and *Arabidopsis* seedlings were examined by western blotting for the expression of *Arabidopsis* AHAS. Polyclonal antibodies against *Arabidopsis* AHAS did not react with any protein in the extracts of MF2000 cells transformed with pKK233–2 (Fig. 3). On the other hand, a protein band with a molecular mass of 65 kD was detected by the antibodies in the extracts prepared from PA10. The mol wt of the expressed protein (65,000) is smaller than the mol wt of 72,593 predicted from the cloned gene sequence (8). Surprisingly, this protein band had the same molecular mass as the protein band detected in the extracts of *Arabidopsis* seedlings. Therefore, the *Arabidopsis* AHAS is processed in *E. coli* to a molecular mass that is apparently similar to the molecular mass of mature AHAS protein in *Arabidopsis* seedlings. Similar unpublished results were quoted by Smith *et al.* (20). However, it is not known whether the plant AHAS in *E. coli* is processed at the same site as in the *Arabidopsis* seedlings. Because the molecular mass of AHAS from the two sources are similar, the enzyme must be processed in *E. coli* at the same site or very close to the site where this protein is processed in the plant.

Pyruvate Saturation

AHAS from *Arabidopsis* seedlings and from PA10 showed very similar pyruvate saturation kinetics (Fig. 4). The saturation curves for pyruvate were hyperbolic for enzymes from the two sources. K_m for pyruvate for AHAS from *Arabidopsis* seedlings and from PA10 were 2.3 and 2.0 mM, respectively.

Inhibition of AHAS Activity

AHAS from *Arabidopsis* seedlings and from PA10 showed identical sensitivities to inhibition by two different classes of herbicides (Fig. 5, A and B). I_{50} values for imazethapyr, an imidazolinone, were 2 μM for AHAS from *Arabidopsis* seedlings and from PA10. Similarly, I_{50} values for chlorsulfuron, a sulfonylurea, were 2.1 nM and 2.0 nM for AHAS from *Arabidopsis* seedlings and from PA10, respectively.

AHAS from *Arabidopsis* seedlings was sensitive to inhibition by the feedback inhibitors valine and leucine (Fig. 5C). At the highest concentration of 1 mM for each amino acid, AHAS activity from *Arabidopsis* seedlings was inhibited by about 60%. In contrast, AHAS activity extracted from PA10 was insensitive to inhibition by valine + leucine (less than 10% inhibition). Assay of the *Arabidopsis* enzyme in the presence of *E. coli* strain MF2000 did not change the property of the native plant enzyme. Similarly, mixing of AHAS from PA10 with the *Arabidopsis* extract did not bring back valine + leucine sensitivity to the enzyme from PA10.

Feedback insensitivity of the *Arabidopsis* AHAS expressed in *E. coli* was an unexpected and intriguing result. A similar observation was noted in *Saccharomyces cerevisiae* where AHAS was always partly desensitized to valine inhibition in the crude extracts, but 100% inhibition by valine was reached when the enzyme was assayed *in vivo* in benzene-permeabilized cells (6). To examine this possibility, an *in vivo* AHAS assay protocol for *E. coli* cells (5) was used and compared with the *in vitro* AHAS assay procedure. The sensitivity of AHAS activity from PA10 to various inhibitors was similar in both assay systems, *i.e.* AHAS activity was inhibited by imazethapyr and chlorsulfuron but remained unihibited by valine and leucine (Fig. 6). Therefore, the enzyme activity is insensitive to valine and leucine in the *in vivo* environment also.

Our earlier results with AHAS from Black Mexican Sweet corn cells have demonstrated that a monomeric form of the enzyme is insensitive to valine and leucine, whereas the

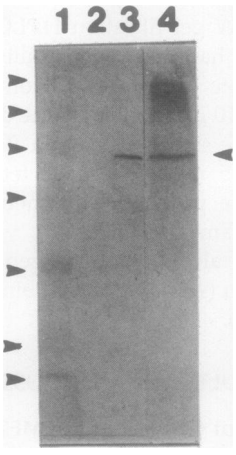

Figure 3. Detection of AHAS in extracts from various sources on a western blot probed with polyclonal antibodies against AHAS. Lane 1, mol wt standards; lane 2, *E. coli* strain MF2000; lane 3, PA10; lane 4, *Arabidopsis* seedlings.

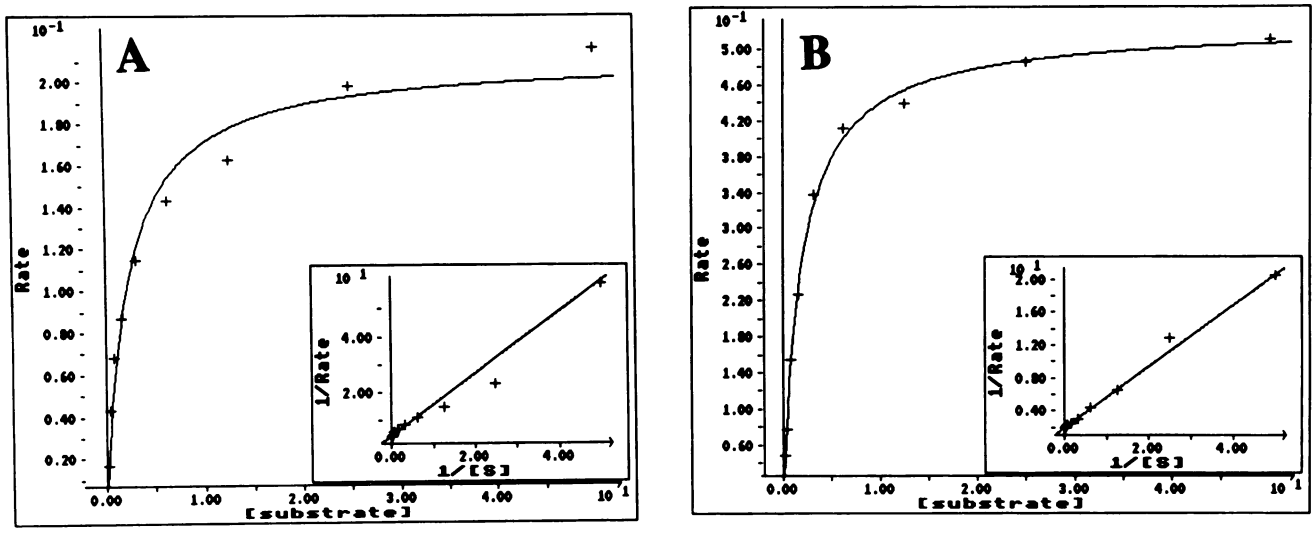

Figure 4. Pyruvate saturation of AHAS activity from *Arabidopsis* seedlings (A) and from PA10 (B). The inset shows the 1/S *versus* 1/V plot of the data.

dimeric and the tetrameric forms of the enzyme are inhibited by these inhibitors (16, 19). Therefore, insensitivity of the *Arabidopsis* AHAS extracted from PA10 cells to the feedback inhibitors could be due to inability of the enzyme to aggregate to the dimeric or the tetrameric form. However, gel permeation chromatography of the *Arabidopsis* AHAS from PA10 showed a dimeric form of the enzyme (112 kD). The activity following gel-permeation chromatography was also insensitive to the feedback inhibitors. Under the same conditions of chromatography, AHAS activity from *Arabidopsis* seedlings was inhibited by valine and leucine (data not shown).

Another possible reason for the insensitivity to feedback inhibitors of the *Arabidopsis* AHAS from PA10 could be inability of this enzyme to adopt the right conformation at 37°C, the growing temperature of PA10 in all of the experiments described thus far. Therefore, PA10 cells were grown at 22, 30, and 37°C for 8 h. Cells were harvested and AHAS activity was determined. The sensitivity of AHAS to imazethapyr or valine + leucine was identical for cells grown at the three different temperatures (results not shown). Therefore, a lower temperature for the growth of PA10 did not influence the sensitivity of the enzyme to the feedback inhibitors.

There are three additional possibilities for valine and leucine insensitivity of the *Arabidopsis* AHAS from *E. coli* cells. First, the plant enzyme is not folded in its native form in the heterologous system. Second, the transit peptide is not cleaved off at the appropriate processing site. The presence of extra amino acids or the deletion of a few amino acids at the N terminus might cause a change in the sensitivity of the enzyme to the feedback inhibitors. Third, a second subunit that is normally present in the plants is responsible for the feedback sensitivity of the enzyme. The *E. coli* AHAS I requires a second subunit for sensitivity to valine inhibition (3). It has been shown that the AHAS gene from plants (without the transit peptide) and from *E. coli* are of similar size as well as they have high sequence homology (1, 20). Therefore, it is possible that, similar to the *E. coli* enzyme, the plant enzyme also requires a second subunit for the feedback sensitivity. This possibility is supported by the observation made here that expression of the *Arabidopsis*

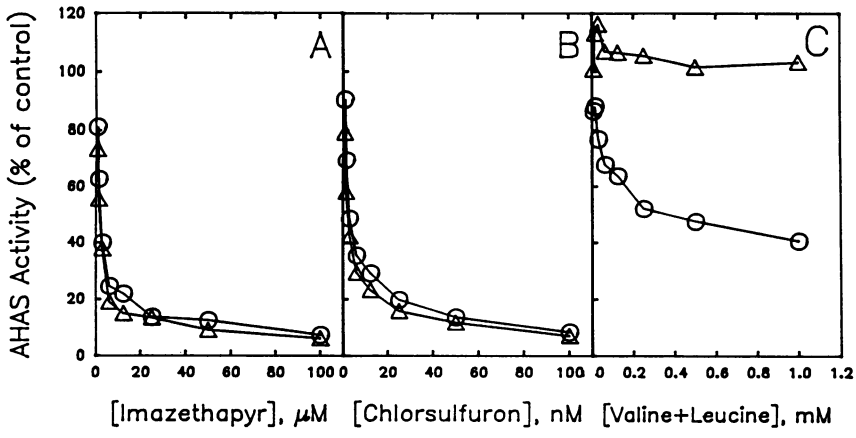

Figure 5. Inhibition of AHAS activity from *Arabidopsis* seedlings (O) and PA10 (Δ) by imazethapyr (A), chlorsulfuron (B), and valine + leucine (C).

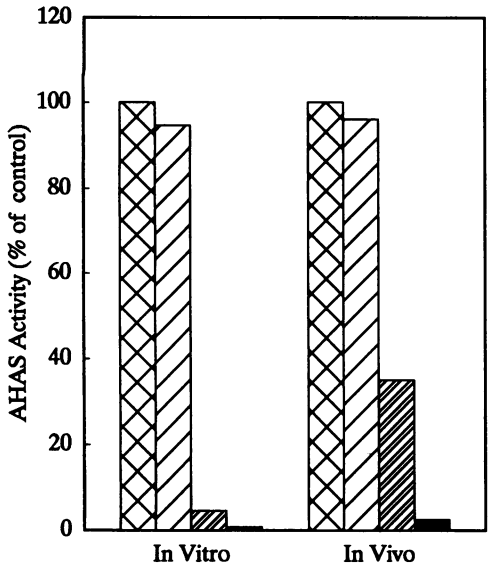

Figure 6. Inhibition of AHAS activity from PA10 in the *in vitro* and the *in vivo* assays of the enzyme. Control (▨); valine + leucine, 1 mM each (▨); imazethapyr, 100 μM (▨); chlorsulfuron, 100 nM (■).

AHAS gene alone, which is similar to the large subunit of AHAS in *E. coli*, yields an enzyme that is insensitive to inhibition by valine + leucine. Therefore, the present results make a strong case for a second subunit of AHAS in plants. Despite this compelling evidence, we cannot rule out the other two possibilities outlined above.

In conclusion, transformation of an AHAS-deficient *E. coli* strain MF2000 with the AHAS gene from *A. thaliana* resulted in growth of the cells in the absence of valine and isoleucine. The growth of this strain of *E. coli* was due to the functional expression of the *Arabidopsis* AHAS. Interestingly, the *Arabidopsis* AHAS protein in the transformed *E. coli* cells was processed to a molecular mass that was similar to that of the mature protein isolated from *Arabidopsis* seedlings. The AHAS activity extracted from the *E. coli* transformants was inhibited by imidazolinone and sulfonylurea herbicides. AHAS activity extracted from *Arabidopsis* is inhibited by valine and leucine; however, this activity was insensitive to these feedback inhibitors when extracted from the transformed *E. coli*.

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