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Imidazolinones and Acetohydroxyacid Synthase from Higher Plants

PROPERTIES OF THE ENZYME FROM MAIZE SUSPENSION CULTURE CELLS AND EVIDENCE FOR THE BINDING OF IMAZAPYR TO ACETOHYDROXYACID SYNTHASE *IN VIVO*

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ABSTRACT

Acetohydroxyacid synthase has been purified from maize (*Zea mays*, var Black Mexican Sweet) suspension culture cells 49-fold by a combination of ion exchange chromatography, gel filtration, and hydroxyapatite chromatography. Use of the nondenaturing, zwitterionic detergent 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propanesulfonate was necessary to dissociate the enzyme from the heterogeneous, high molecular weight aggregates in which it appears to reside *in vitro*. The solubilized maize acetohydroxyacid synthase had a relative molecular mass of 440,000. The purified enzyme was highly unstable. Acetohydroxyacid synthase activities in crude extracts of excised maize leaves and suspension cultured cells were reduced 85 and 58%, respectively, by incubation of the tissue with 100 micromolar (excised leaves) and 5 micromolar (suspension cultures) of the imidazolinone imazapyr prior to enzyme extraction, suggesting that the inhibitor binds tightly to the enzyme *in vivo*. Binding of imazapyr to maize acetohydroxyacid synthase could also be demonstrated *in vitro*. Evidence is presented which suggests that the interaction between imazapyr and the enzyme is reversible. Imazapyr also exhibited slow-binding properties when incubated with maize cell acetohydroxyacid synthase in extended time course experiments. Initial and final K_i values for the inhibition were 15 and 0.9 micromolar, respectively. The results suggest that imazapyr is a slow, tight-binding inhibitor of acetohydroxyacid synthase.

The imidazolinones are a new chemical class of herbicides discovered and under development at American Cyanamid Company. The mode of action of the imidazolinones appears to be via the inhibition of branched chain amino acid synthesis, since (a) the levels of leucine, valine, and isoleucine are drastically reduced as a result of imidazolinone treatment and (b) exogenous application of these three amino acids prevents the phytotoxic effects of imidazolinone treatment (22). The imidazolinones have also been shown to be potent inhibitors of acetohydroxyacid synthase (also known as acetolactate synthase, EC 4.1.3.18), the first enzyme in the biosynthetic pathway to valine, leucine, and isoleucine (23). Presumably this enzyme is the primary (if not the sole) site of action of the imidazolinone herbicides. A preliminary investigation of the inhibition of maize AHAS[2] by imazapyr, the active ingredient of the herbicide Arsenal®, suggested that these compounds were uncompetitive inhibitors of the enzyme (23). In this paper, we report on the *in vivo* binding of imazapyr to AHAS and present evidence which suggests that the biochemical basis for this *in vivo* effect is due to the tight binding nature of the inhibitor. In addition, conditions for tissue harvesting have been optimized for recovery of AHAS from maize suspension cells and the enzyme has been solubilized from the apparent heterogenous, high mol wt complexes in which it resides *in vitro*. The results of experiments to stabilize the maize enzyme in crude extracts and during purification are also presented and discussed. A preliminary account of this work has appeared (16).

MATERIALS AND METHODS

Chemicals. Tritiated and unlabeled imazapyr, 2-(4,5-dihydro-4-methyl-4-[1-methylethyl]-5-oxo-1*H*-imidazol-2-yl)-3-pyridinecarboxylic acid, were synthesized at the Agricultural Research Division of American Cyanamid Co., Princeton, NJ. DEAE Sephacel, Sephacryl S-300, Sephadex G-25 (PD-10) minicolumns and mol wt standards were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. High resolution hydroxyapatite was obtained from Calbiochem-Behring. Alpha-naphthol was purchased from Eastman Kodak Co.

Plant Material and Culture. Embryo-derived cell suspension cultures of maize (*Zea mays* var Black Mexican Sweet) were obtained from Molecular Genetics Inc., Minnetonka, MN and cultured on medium containing MS salts (17) with 2% (w/v) sucrose, 0.5 mg/ml thiamine, 0.15 mg/ml L-asparagine, and 2 mg/l 2, 4-dichlorophenoxyacetic acid. Cells were harvested on d 7, the age normally used for subculturing. Maize leaf material was obtained from 8 d old greenhouse grown maize (var Pioneer 3541).

Tissue Harvest and Enzyme Extraction. Maize tissue cultures to be used for AHAS extraction were harvested by collection on filter paper, washed with deionizedH_2O, and, except where otherwise noted (Table I) lyophilized and stored at room temperature under vacuum with desiccant. AHAS was extracted from the cell powder using the method described previously for maize roots and shoots (23). After centrifugation of the crude extract for 20 min at 20,000*g*, the supernatant was made up to 50% of satura-

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[2] Abbreviations: AHAS, acetohydroxyacid synthase; Bis-Tris, (bis[2-Hydroxyethyl]imino-tris[hydroxymethyl]methane); CHAPS, 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate; FAD, flavin adenine dinucleotide; TD, threonine deaminase (EC 4.2.1.16); NADP ME, NAPD malic enzyme (EC 1.1.1.40); Cyt$_c$ OX, Cyt *c* oxidase (EC 1.9.3.1); TTP, thiamine pyrophosphate; PMSF, phenylmethylsulfonyl fluoride.

tion with $(NH_4)_2SO_4$ (added as a saturated solution). The enzyme was precipitated by centrifugation as above, the supernatant was discarded, and the pellets were quickly frozen in liquid N_2 and stored at −20°C for later use.

Purification of Maize Cell AHAS. All procedures were performed at 0 to 4°C. The zero to 50% $(NH_4)SO_4$ fraction was collected as described above and in the legend of Table IV. The enzyme was desalted on a Sephadex G-25 PD-10 minicolumn equilibrated with 50 mM Bis-Tris (pH 6.5), containing 1 mM EDTA, 20% glycerin, 5 mM pyruvate, and 15 mM CHAPS and loaded onto a column of DEAE Sephacel (4.4 × 14 cm) equilibrated with the same buffer. After washing with 100 ml of starting buffer, AHAS was eluted using a 500 ml linear 0 to 0.5 M KCl gradient (in starting buffer) at a flow rate of 0.4 ml/min. Four ml fractions were collected. Fractions containing AHAS were pooled and ammonium sulfate was added as a saturated solution to 55% of saturation and the resultant protein precipitate was collected by centrifugation at 20,000*g* for 20 min. The enzyme was resuspended in about 10 ml of gel filtration media, consisting of 50 mM K-phosphate (pH 6.5), 1 mM EDTA, 100 mM KCl, 20% glycerin (v/v), 5 mM pyruvate, 10 μM FAD, and 15 mM CHAPS and concentrated by ultrafiltration. The concentrate was chromatographed on a Sephacryl S-300 column (2.2 × 53 cm) at a flow rate of 0.3 ml/min. Four ml fractions were collected and assayed for enzyme activity. Fractions containing AHAS were pooled, concentrated by ultrafiltration, diluted four times with hydroxyapatite starting buffer (10 mM K-phosphate [pH 7.2], with 5 mM pyruvate, 1 mM EDTA, 20% glycerin [v/v], and 15 mM CHAPS) and loaded onto a hydroxyapatite column (1.6 × 10 cm). After washing with 50 ml of starting buffer, AHAS was eluted with a 200 ml linear 0.01 to 0.5 M K-phosphate gradient (in starting buffer, all at pH 7.2) at a flow rate of 1 ml/min. Four ml fractions were collected and assayed for AHAS activity.

Molecular Weight Estimation. The Sephacryl S-300 column used for purification of maize cell AHAS was calibrated in the absence of CHAPS (25) with the following standards: aldolase (158,000), catalase (232,000), ferritin (440,000), and thyroglobulin (669,000).

Enzyme Assays. AHAS was assayed as described previously (23), with the modification that assay mixtures contained 34 μM FAD unless otherwise noted. TD was assayed according to the method of Sharma and Mazumder (24). NADP ME and Cyt_c OX were assayed as described in Winter *et al.* (30). The ability of fractions eluted from gel filtration to synthesize valine from pyruvate was tested using a modification of the "cofactor assay" of Leiter *et al.* (12). Aliquots of individual fractions were incubated for 2 h at 37°C in a 240 μl (final volume) assay mixture containing 40 mM glutamate, 250 mM pyruvate, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.8), 0.5 mM NADPH, 0.5 mM TPP, 0.2 mM FAD, and 0.8 mM pyridoxyl phosphate. Reactions were stopped by adding 60 μl of 7.5% TCA. Valine synthesis was monitered using a Beckman AA-3 Gradient HPLC amino acid system (Beckman Instruments, Inc., Berkeley, CA) which employs fluorescence detection of post ion exchange column *o*-phthaldialdehyde amino acid derivatives.

RESULTS

Tissue Harvest and Stabilization of Maize AHAS in Crude Preparations. Several different harvest procedures were tested in order to maximize the yield of the enxyme from corn cells. Of the methods tried, extraction of AHAS from lyophilized corn cells resulted in the highest yield of the enzyme, both on a specific activity basis and on a per gram fresh weight basis (Table I). This method was used in all of the subsequent studies and the enzyme was stored at −20°C as protein pellets as described in "Materials and Methods."

Table I. *Comparison of Harvest Procedures for Maize Suspension Cells with Regard to Recovery of AHAS Activity*

Harvest Procedure	AHAS Activity[a]	
	μmol acetoin/ h·mg protein	*μmol acetoin/ h·g fresh wt*
Fresh extraction	0.44	0.10
Liquid N_2, immediate extraction	0.42	0.31
Liquid N_2, −20°C storage	0.67	0.37
Liquid N_2, lyophilization, room temperature storage	1.30	0.79

[a] Enzyme assay mixtures did not contain FAD, otherwise the assays were performed as described in "Materials and Methods."

Table II. *Effects of Various Treatments on the Stability of Maize Cell AHAS in Resuspended Protein Pellets*

Maize suspension cell culture protein pellets (prepared as described in "Materials and Methods") were resuspended in 50 mM K-phosphate (pH 7, except where otherwise indicated), with or without 1 mM EDTA and desalted on a Sephadex G-25 PD-10 minicolumn equilibrated in the same solution. Treatment compounds were added as 10× concentrated solutions. AHAS assays were performed immediately after desalting (initial activity) and after a 16 h incubation at 4°C. Each incubation mixture contained 4 mg/ml of protein.

Treatment	Initial Activity[a]
	%
None (control)	26
−EDTA	0
pH 6	40
pH 5	24
40 mM pyruvate, 10 M $MgCl_2$, 0.6 mM TPP	16
5 mM leucine + 5 mM valine[b]	20
10 mM DTT	29
0.5 mM PMSF	28
1 mM FAD	70
25% (v/v) glycerin	47

[a] Initial activity = 0.82 μmol acetoin/h·mg protein. [b] Desalted before assay.

While AHAS activity was stable to storage as a protein pellet (data not shown), the resolubilized enzyme was quite labile, losing 74% of its activity after 16 h in 50 mM K-phosphate (pH 7.0) containing 1 mM EDTA at 4°C, (Table II, control). Various treatments were tested for their ability to preserve enzyme activity in resolubilized protein pellets (Table II). EDTA was essential for maintaining maize AHAS activity, whereas neither substrates plus cofactors nor the feedback inhibitors leucine and valine preserved activity over control levels. The thiol reagent DTT and the protease inhibitor PMSF had no effect. Treatments found to be effective were the addition of glycerin or FAD and the lowering of the pH of the resolubilizing buffer from 7 to 6.

The ability of FAD to preserve maize AHAS activity suggests that it is a cofactor of the plant enzyme. FAD stimulated enzyme activity by 70% when it was included in the assay mixture and it preserved AHAS activity (117% recovery) when it was included in the preincubation media (Table III). AHAS remaining after preincubating the enzyme in the absence of FAD was stimulated to the same degree by the inclusion of FAD in the assay mixture as were enzyme samples which were assayed immediately after resuspension (Table III).

Solubilization of AHAS. When extracts of maize suspension cells were chromatographed on a gel filtration column, AHAS eluted in the void volume. Also, binding of the enzyme to DEAE-

Table III. *Effects of FAD on Maize Cell AHAS*

Maize suspension cell culture protein pellets (prepared as described in "Materials and Methods") were resuspended in 50 mM K-phosphate (pH 6) containing 1 mM EDTA and 20% glycerol and desalted on a Sephadex G-25 PD-10 minicolumn equilibrated with the same media. FAD was added as a 10× concentrated solution as indicated. Incubation was for 16 h at 4°C. Each incubation mixture contained 3.5 mg/ml of protein.

Conditions	AHAS Activity		Stimulation[b]
	w/o FAD	with FAD[a]	
	μmol acetoin/h·mg protein		%
Immediate assay	1.07	1.82	70
Preincubation			
Without FAD	0.73 (68)[c]	1.23 (68)	69
With 1 mM FAD	ND[d]	2.13 (117)	

[a] Enzyme assayed in the presence of 1 mM FAD. [b] Percent stimulation of enzyme activity due to the presence of FAD in the assay mixture. [c] Numbers in parentheses indicate the AHAS activity as a percentage of enzyme activity in the corresponding immediately assayed samples. [d] Not determined.

Table IV. *Purification of AHAS from Maize Suspension Cells*

The 0 to 50% $(NH_4)_2SO$ fraction was prepared by extracting 32 g of lyophilized maize suspension cells as described in "Materials and Methods." Frozen protein pellets were resuspended in 50 mM Bis-Tris (pH 6.5), containing 1 mM EDTA, 20% (v/v) glycerin, 5 mM pyruvate, and 15 mM CHAPS at 4°C, desalted on Sephadex G-25 PD-10 minicolumns equilibrated in the same medium, assayed for AHAS activity and used as starting material for further purification.

Step	Protein	Specific Activity	Yield
	mg	*μmol acetoin/h·mg protein*	%
0–50% $(NH_4)_2SO_4$	720	0.82	(100)
DEAE sephacel	24	20.0	42
Sephacryl S-300	13.7	10.5	7
Hydroxyapatite	1.1	40.0	2.5

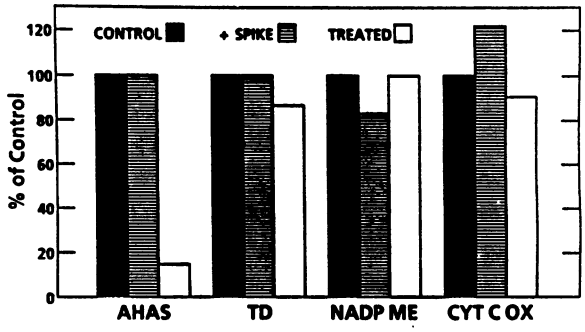

FIG. 1. Effects of pretreatment of excised maize leaves with imazapyr on AHAS, TD, NADP ME, and Cyt_c OX activities in subsequent extracts. Excised maize leaves from 8 d old greenhouse grown plants were incubated in a 1/10 strength Hoagland-type nutrient solution containing 100 μM imazapyr. Samples were harvested after 4 h. AHAS and TD were extracted in AHAS isolation media as described (23) and concentrated by 0 to 50% $(NH_4)_2SO_4$ precipitation. The resulting protein pellets were resuspended in 50 mM K-phosphate (pH 7.0), containing 1 mM EDTA and 10% glycerol and desalted on a Sephadex G-25 PD-10 minicolumn equilibrated with the same media. NADP ME and Cyt_c Ox were extracted in 50 mM Hepes (pH 7.0), centrifuged to remove cellular debris and used directly as enzyme sources. "+Spike" are control samples which were extracted as described, but with extraction media which contained 100 μM imazapyr. Control activities were 0.16 μmol/mg·h AHAS, 35.7 nmol/mg·h TD, 9.7 μmol/mg·min NADP-ME, and 4.6 μmol/mg·min Cyt_c OX.

Sephacel was not pH-dependent (data not shown). These results suggest that maize AHAS exists *in vitro* in large mol wt, heterogeneous protein aggregates. Based on the following criteria, CHAPS, a nondenaturing zwitterionic detergent, dissociated maize AHAS from these protein aggregates: (a) when CHAPS was included in the gel filtration medium the enzyme was able to enter into the internal space of the Sephacryl resin and (b) in the presence of the detergent the binding of the enzyme to DEAE Sephacel became pH dependent in a manner typical of free protein species (not shown).

In the presence of CHAPS, maize AHAS eluted from Sephacryl S-300 either as single peak corresponding to a M_r of 440,000 or as a 440,000 M_r peak with a 900,000 M_r shoulder (Fig. 3). The 900,000 shoulder most likely represents a dimer of the major eluting peak. The presence of pyruvate or cofactors in the column buffers did not influence the aggregation state of the maize enzyme as has been found for AHAS isozyme I from *E. coli* (8). Also, neither mol wt form of the enzyme posessed the ability to synthesize valine from pyruvate (data not shown), indicating that the solubilized maize enzyme was not a part of a multienzyme complex as is the case for one of the detergent-solubilized forms of AHAS from *Neurospora crassa* (1).

Purification of Maize AHAS. Attempts to purify maize AHAS by various chromatographic procedures were hampered by the poor recovery of the enzyme. The addition of FAD, which greatly stabilized maize AHAS in crude extract, did not preserve the enzyme during column chromatography. Recovery of the enzyme was greatly enhanced, however, by the inclusion of pyruvate in the chromatographic buffers. In spite of the stabilizing effects of pyruvate, large losses in enzyme activity still occurred during multicolumn purification (Table IV). The purified enzyme was not stable to storage at −20 or −80°C, either as frozen solution or as a protein pellet.

Effects of Imazapyr on Maize AHAS. We reported previously that the imidazolinones are uncompetitive inhibitors of maize AHAS with respect to pyruvate (23). Since one of the basic assumptions of kinetic theory is the reversible nature of the enzyme-inhibitor complex, we were surprised to find in subsequent experiments that the levels of extractable AHAS were drastically reduced by preincubating excised leaves in imazapyr prior to enzyme extraction (Fig. 1). Similar results were obtained using Black Mexican Sweet maize cell suspension cultures, in that treating 6 d old cultures with 5 μM imazapyr 24 h prior to enzyme extraction resulted in a 58% reduction in AHAS activity in subsequent extracts. The specificity of imazapyr action on AHAS is supported by its lack of effect on three other maize leaf enzymes, TD, NADP-ME, and Cyt_c OX *in vivo* (Fig. 1) or *in vitro* (not shown). Extraction of control leaves in isolation medium containing 100 μM imazapyr had no effect on AHAS activity, eliminating the possibility that the *in vivo* effects observed were merely due to the presence of imazapyr in the extraction buffer. These results suggest that imazapyr binds tightly, if not irreversibly, to AHAS *in vivo*.

Binding of imazapyr to the enzyme could also be demonstrated *in vitro* by preincubating resuspended protein pellets with 100 μM imazapyr for various lengths of time, desalting to remove any unbound inhibitor, and assaying for AHAS. The results (Fig. 2) revealed that about 40% of the enzyme activity was rapidly inactivated by 100 μM imazapyr, while up to 75% was lost after 4 h of preincubation.

Whether the inhibition of maize AHAS by imazapyr is reversible or not was determined by the criteria of Williams and Morrison (29). In one experiment, enzyme was preincubated with ^3H-imazapyr, desalted as before to remove unbound inhib-

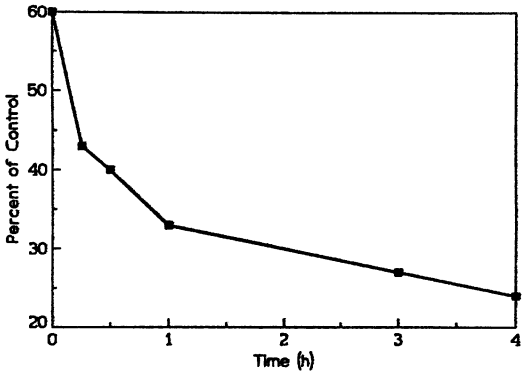

FIG. 2. Effect of preincubation of maize cell-free extracts with imazapyr on AHAS activity. Maize suspension cell protein pellets (prepared as described in "Materials and Methods") were resuspended in 50 mM K-phosphate (pH 7.0), containing 1 mM EDTA, 10% glycerol, and 5 mM pyruvate, desalted on a Sephadex G-25 PD-10 minicolumn equilibrated with the same medium (final protein concentration 0.3 mg/ml) and incubated at 4°C for various time periods, as indicated, in the presence or absence of 100 μM imazapyr. Treatment and control samples were desalted again before assay. Control activities expressed as μmol/h·mg protein were: 1.5 (0 h preincubation), 1.4 (0.25 h), 1.3 (0.5 h), 1.4 (1 h), 1.4 (1.5 h), 1.5 (3 h), 1.5 (4 h).

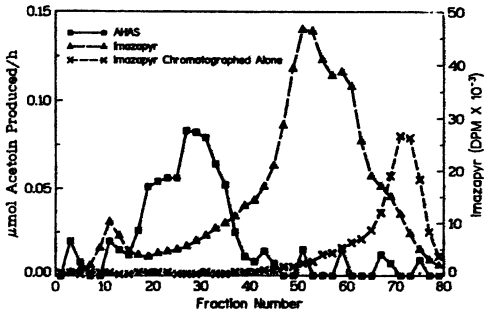

FIG. 3. Gel filtration of maize suspension cell AHAS after incubation with [^{3}H]imazapyr. Maize suspension cell protein pellets were resuspended in 50 mM K-phosphate (pH 7.0), containing 20% glycerol, 0.1 M KCl, 1 mM EDTA, 5 mM pyruvate, and 15 mM CHAPS and desalted on a Sephadex G-25 minicolumn equilibrated in the same medium (protein concentration 32.5 mg/ml). After a 3 h incubation at 4°C with 6.5 μmol of 20 Ci/mmol [^{3}H]imazapyr, an aliquot of this solution was desalted as before and 23 mg (1 ml) was chromatographed on a column of Sephacryl S-300 (2.2 × 53cm) equilibrated in the same buffer. Flow rate 0.85 ml/min. Fraction size 1.9 ml. In a separate experiment, [^{3}H]imazapyr was chromatographed alone using the same conditions as above.

itor, and then chromatographed over a gel filtration column (Fig. 3). Maize AHAS eluted as a 440,000 M_r peak with a 900,000 M_r shoulder, exactly as it does in the absence of imazapyr. The tritiated imazapyr, at least some of which was presumably initially enzyme associated, eluted much later as a double peak, but not in the same position as when the radiolabeled compound was chromatographed alone (Fig. 3). The elution pattern of the tritiated imazapyr from the gel filtration column after preincubation with the maize enzyme preparation could be the result of both its dissociation from AHAS during chromatography and its binding to other proteins in the enzyme preparation. That there appears to be no radioactivity specifically associated with AHAS itself, however, is consistent with the interaction between the herbicide and the enzyme being reversible. Imazapyr is similarly dissociated from the enzyme by gel filtration when excised leaves (incubated in the presence of tritiated imazapyr prior to extraction) were used. These results suggest that the binding of imazapyr to AHAS occurring *in vivo* is reversible as well. Whether there is a concomitant recovery of enzyme activity after the dissociation of the AHAS-imazapyr complex is difficult to determine because of the inherent variable recovery of maize AHAS from gel filtration. However, the reversible nature of the inhibition on enzyme activity is shown in two additional experiments. In one, enzyme was prepared, incubated briefly (1 to 2 min) with 100 μM imazapyr, desalted and assayed, all as described in Figure 2. Forty-two percent of the AHAS activity was inhibited in the imazapyr-containing samples while control samples were unchanged. When a parallel imazapyr-containing sample was 're-extracted' (precipitated with $[NH_4]_2SO_4$, resuspended and desalted as shown in Fig. 1), 91% of the original activity was restored. Similarly treated control samples retained all of their initial activity. In a second experiment, maize AHAS (23 mg/ml protein, prepared as described in Fig. 2) was incubated under assay conditions with 25 μM imazapyr for 3 h at 4°C, resulting in a 91% loss of enzyme activity. Enzyme activity was completely restored (92% of the original control activity) when parallel imazapyr-containing samples were diluted 100-fold into fresh assay media. Dilution of control samples had no effect on AHAS activity. Recovery of activity upon dilution of the enzyme-inhibitor complex is typical for tight-binding inhibitors (29).

The results of the previous experiments suggested that the inhibition of AHAS activity by imazapyr increases with time. When AHAS activity is measured over an extended (4 h) assay period in the presence of various imazapyr concentrations, inhibition was found to increase with time (Fig. 4). These results suggest that the equilibrium between imazapyr and the enzyme is reached slowly, a feature typical of many tight-binding inhibitors (29). Initial and final K_i values for the inhibition of maize AHAS by imazapyr are 15 and 0.9 μM, respectively.

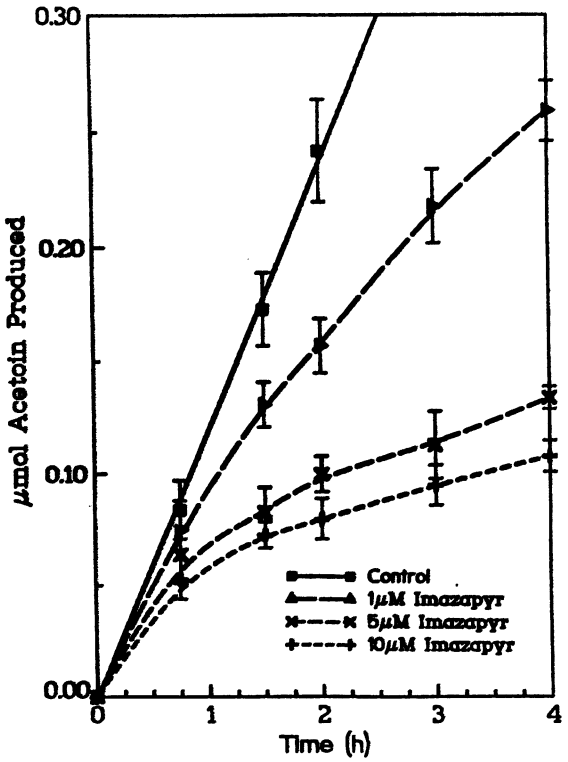

FIG. 4. Time course of maize suspension cell AHAS activity in the presence of various imazapyr concentrations. Each assay contained 19 μg of maize cell protein. Enzyme was prepared as described in Figure 2. Imazapyr concentration and incubation times were as indicated. $n = 6$. Control activity was linear through 4 h.

DISCUSSION

Maize AHAS. Several papers describing the properties of plant AHAS in concentrated crude extracts (2, 13, 15) or in partially purified form (14) have appeared. There have been no reports, however, of attempts to purify the enzyme from a higher plant source to homogeneity. While the genetics of AHAS from bacterial and fungal sources has been well studied (3, 4, 19) and several microbial AHAS genes have been sequenced (5, 6, 11, 26), the protein itself has proven difficult to purify due to its labile nature (8). Recently however Schloss *et al.* (21) have developed a new purification scheme for *Salmonella typhimurium* AHAS isozyme II based on their finding that FAD greatly enhances the stability of the isolated bacterial enzyme in the absence of TPP and $MgCl_2$. Our results reported here show that, while FAD stabilizes maize AHAS in resuspended protein pellets (Table II), pyruvate rather than FAD is required to recover active enzyme from chromatographic procedures. The ability of pyruvate to stabilize maize AHAS during purification procedures was surprising, since it had no effect on the enzyme in resuspended protein pellets (Table II). Pyruvate similarly stabilizes *Neurospora crassa* AHAS during gel filtration (27). Even with pyruvate included in all of the buffers, however, recovery of maize AHAS after several purification steps is low (Table IV) and inclusion of FAD along with pyruvate in the chromatography buffers does not enhance the recovery of the maize enzyme over that afforded by pyruvate alone (data not shown). The recovery of *N. crassa* AHAS after a several step purification procedure in the presence of both FAD and pyruvate is similarly poor (7). While pyruvate is partially effective in stabilizing maize and *N. crassa* (27) AHAS, additional stabilizing factors need to be elucidated in order to render AHAS from eukaryotic sources amenable to purification.

When subjected to gel filtration in the presence of CHAPS, maize AHAS elutes predominantly as a peak corresponding to a M_r of 440,000. Whether this form of the enzyme is monomeric or oligomeric has yet to be determined. However, its failure to synthesize valine from pyruvate suggests that it is not part of a multienzyme complex. This does not rule out the possibility that plant AHAS may exist in such a complex *in vivo* as does *N. crassa* AHAS (1). The fungal enzyme, when subjected to gel chromatography, elutes as a series of peaks (1, 27) indicating that it forms heterogeneous aggregates *in vitro*. The hydrophobic nature of the maize enzyme, based on its detergent requirement for solubilization and its late elution from leucine agarose (not shown) suggests that it too forms aggregates *in vitro*. The 440,000 maize AHAS most likely, therefore, represents an aggregated form of the enzyme, while the basal unit may be of a size similar (about 60–80,000) to the analogous enzymes of bacteria and yeast (21, 26, 27).

The ability of FAD to stimulate maize AHAS activity suggests that it is a cofactor of the plant enzyme. While most microbial forms of AHAS exhibit a FAD requirement (7, 8, 10), FAD has not previously been recognized as a cofactor for plant AHAS. This finding suggests that the mechanism of catalysis (and presumably the active site) of the higher plant enzyme is similar to that of microbial forms of AHAS and dissimilar to the *Euglena gracilis* enzyme (18) which is dependent on ATP and shows no requirement for TPP, FAD, or Mg.

Effects of Imazapyr on Maize Cell AHAS. We have reported previously that the imidazolinones are potent inhibitors of maize seedling AHAS activity (23). Our present data is consistent with imazapyr being a slow, tight-binding inhibitor of maize AHAS. The tight-binding nature of the inhibition is suggested by the fact that pretreatment of tissue with imazapyr prior to extraction (or preincubation of the enzyme *in vitro* prior to desalting and assay) results in a dramatic reduction in AHAS activity. The lack of inhibitory effect on AHAS activity when 100 μM imazapyr was included in the enzyme extraction buffer of control leaves ('spike' treatment, Fig. 1) superficially appears to contrast with the rapid effects on AHAS activity when resuspended protein pellets were incubated with the herbicide at the same concentration (Fig. 2). While imazapyr presumably binds to the enzyme when AHAS is isolated in an imazapyr-containing extraction media, the strength of the initial enzyme-inhibitor complex is most likely insufficient for imazapyr to remain bound throughout the enzyme extraction procedure. This hypothesis was supported by the reversibility experiment where AHAS activity in resuspended protein pellets incubated in the presence of 100 μM imazapyr was completely restored by re-extraction. In contrast, the binding of imazapyr to the enzyme which has occurred *in vivo* has approached final equilibrium sufficiently such that at least a portion of the herbicide remains bound to AHAS throughout the enzyme extraction procedure.

The reversible nature of the inhibition was demonstrated by the criteria of Williams and Morrison (29), namely that the enzyme-inhibitor complex dissociates during gel filtration and that enzyme activity is restored by dilution-reactivation. However, in contrast to typical tight-binding inhibitors, the inhibition of steady state maize cell AHAS by imazapyr does not vary with enzyme concentration (29); rather, plots of steady state velocity *versus* enzyme concentration are linear (data not shown). This is most likely the result of both K_i and total inhibitor concentration being much greater than total enzyme concentration (29) under our assay conditions. Purified enzyme of high specific activity is a prerequisite for further characterization of the nature of the inhibition by imazapyr on maize AHAS. It is interesting to note that another class of chemical compounds which inhibit AHAS, the sulfonylureas, also exhibit slow tight-binding inhibition (10), yet have no obvious structural similarities.

The tight-binding nature of imazapyr inhibition on maize AHAS enabled us to show that it binds to the enzyme *in vivo*. Treatment of tissues with a tight-binding inhibitor prior to enzyme extraction results in the reduction of target enzyme activities in other systems as well. For example, Werkheiser (28) demonstrated that when rats are injected with amethopterin, tight-binding inhibitor of folic acid reductase, enzyme activity in subsequent extracts was specifically and dramatically reduced. In plant systems, itaconate, tight-binding inhibitor of isocitrate lyase (20) results in a specific and potent reduction in enzyme activity in extracts from a variety of species which had been pretreated with this compound (9). The lack of effect by imazapyr on enzymes other than AHAS, either *in vivo* or *in vitro*, coupled with the ability of exogenous leucine, valine, and isoleucine to reverse imazapyr's herbicidal effects (22) strongly suggests that AHAS is the primary if not the sole site of action of the imidazolinone herbicides.

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