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Herbicides that Inhibit Acetohydroxyacid Synthase[1]

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Abstract. **Acetohydroxyacid synthase was discovered as the site of action of imidazolinone and sulfonylurea herbicides over 6 yr ago. In recent years, advances have been made in the understanding of this enzyme as a herbicide target site. Derivatives of both imidazolinones and sulfonylureas have yielded new herbicide chemistry. All of the herbicides display unusual "slow-binding" behavior with the enzyme, and this behavior may help explain efficacy of the herbicides. Resistance to these herbicides has been developed through a number of different procedures, and the mechanism of resistance is through changes in sensitivity of the enzyme to the herbicides. The changes are either selective to only one class of chemistry, or broad to a number of classes of chemistry. These data support the idea that binding sites for the herbicides on the enzyme are only partially overlapping. Progress in purification of AHAS from corn includes discovery of the existence of the enzyme in monomer and oligomer aggregation states. The interaction of the enzyme with the herbicides is affected by enzyme aggregation state. Nomenclature: AHAS, acetohydroxyacid synthase or acetolactate synthase, E.C. 4.1.3.18; sulfometuron methyl, *N*-[(4,6-dimethylpyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonyl-benzenesulfonamide; imazapyr, (±)-2-[4,5′-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-pyridinecarboxylic acid; imazethapyr, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid; 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-imazaquin, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-imazaquin, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-quinolinecarboxylic acid.**

Additional index words. **Herbicide, acetohydroxyacid synthase, imidazolinone, sulfonylurea.**

INTRODUCTION

In the last few years, we have witnessed unprecedented progress in the area of herbicide mode of action. Nowhere has the progress been as rapid as with herbicides that inhibit branched-chain amino acid biosynthesis. For the first time in the history of commercial herbicides, a new mode of action of new herbicide chemistry was known before the herbicides were widely commercialized. Moreover, the new classes of herbicide chemistry, sulfonylureas and imidazolinones, came from two independent programs. Two representatives of these herbicides, sulfometuron methyl and imazapyr, are shown in Figure 1. Chemically, sulfonylureas and imidazolinones have little in common. They have each spawned a second generation of herbicides, all retaining the same mode of action. In this article I focus on recent advances in the field of AHAS-inhibiting herbicides.

NEW AHAS-INHIBITING HERBICIDE CHEMISTRY

Sulfonylureas. Structure of a typical sulfonylurea is characterized by presence of a sulfonylurea "bridge" connecting two rings (Figure 2). The ring attached to the sulfur atom has an *ortho* substituent, but otherwise can consist of benzene, pyridine, or nonaromatic rings. At the other end of the bridge is a *meta*-substituted pyrimidine or triazine ring. Two rather new herbicide classes have evolved from the sulfonylureas. A rearrangement of the sulfonylurea bridge resulted in the discovery and patenting of the triazolopyrimidine sulfoanilide herbicides (9, 44). This class differs structurally from the sulfonylureas in that the pyrimidine ring has been joined into the bridge region of the sulfonylurea structure, the sulfonyl moiety is moved adjacent to the triazolopyrimidine rings, and the benzyl ring has two *ortho* substituents. An even more dramatic departure from the general sulfonylurea structure is the pyrimidyl-oxybenzoic acid (13). Here only the *meta*-substituted pyrimidine ring remains of the general sulfonylurea structure. The sulfonylurea bridge between aromatic rings has been replaced by an ether linkage, and the second ring has an ortho carboxylic acid rather than an ester function.

Imidazolinones. The second "original" class of AHAS-inhibiting herbicide chemistry, the imidazolinones, has also given rise to new herbicide classes. This class of chemistry is characterized by an imidazolinone ring bonded to an aromatic ring at the 2 position. The aromatic ring contains a carboxylic acid group *ortho* to the imidazolinone ring. Imidazolinones containing three different aromatic rings have been commercialized. Two new herbicide classes derived from imidazolinones are shown in Figure 3. A nonaromatic imidazolinone derivative differs from the general imidazolinone only in that the aromatic ring has been replaced by a 2-butenoic acid (47). Sulfonylcarboxamides are also imidazolinone derivatives (1).

MODE OF HERBICIDE ACTION

Two lines of investigation came together to prove that AHAS is the site of action of sulfonylurea herbicides. Experiments by LaRossa and Schloss showed an inhibitory effect of sulfonylureas on growth of bacteria (15). Nutritional and biochemical studies pinpointed the site of action in

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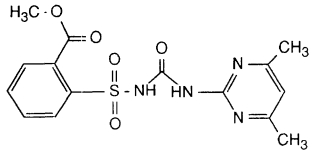
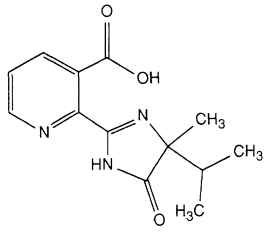
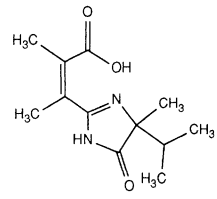


SULFOMETURON METHYL

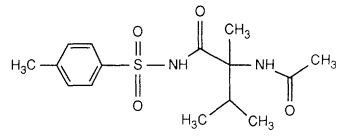


IMAZAPYR

Figure 1. Chemical structures of representative herbicides from the sulfonylurea and imidazolinone chemical class.



NON-AROMATIC IMIDAZOLINONE



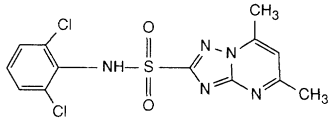
SULFONYLCARBOXIMIDE

Figure 3. Recent herbicides derived from imidazolinones.

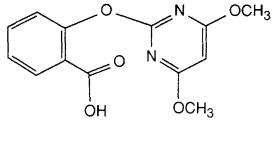
bacteria as AHAS. Concurrently, herbicide-resistant mutants of tobacco were selected in culture by Chaleff and Ray (4). After the work of LaRossa and Schloss, others performed biochemical studies showing AHAS inhibition by sulfonylureas in susceptible plants but not in selected resistant plants (5). Ultimate proof for this site of action came from studies on regenerated herbicide resistant tobacco, in which breeding experiments showed cosegregation of the herbicide resistance trait and herbicide insensitive enzyme (4, 5).

Paul Anderson and Dale Shaner performed key experiments that led to discovery of the mode of action of imidazolinones. Anderson and Hibberd (2) found a decline in levels of valine, leucine, and isoleucine in corn cell cultures treated with imazapyr. Supplementation of these amino acids reversed the growth inhibitory effects of imazapyr. Similar experiments were performed by Dale Shaner using corn root tips [Figure 4; (35)]. In the absence of any supplement to roots, the herbicide caused a 70% reduction in both growth and rate of DNA synthesis. This inhibition was reversed when valine, leucine, and isoleucine supplements were included with the herbicide. These three amino acids are linked by a common biosynthetic pathway (Figure 5). Imazapyr strongly inhibited the first enzyme common to biosynthesis of all three amino acids, acetohydroxyacid synthase, also called acetolactate synthase (2, 34).

In the last 5 yr the tools of biochemistry, chemistry, and genetics have expanded our knowledge considerably. In the usual manner of science, this progress has led to still more questions that challenge our current understanding about the way these herbicides act.



TRIAZOLOPYRIMIDINE SULFOANILIDE



PYRIMIDYL-OXY-BENZOIC ACID

Figure 2. Recent herbicides derived from sulfonylureas.

BRANCHED-CHAIN AMINO ACID BIOSYNTHESIS

Valine, isoleucine, and leucine are synthesized in plants and microbes by a common pathway (Figure 5). Four enzymes are common to all three pathways: acetolactate synthase, ketol-acid reductoisomerase, dihydroxyacid dehydratase, and branched-chain amino acid transaminase. In addition, isoleucine requires one additional enzyme, threonine dehydratase, and leucine requires three additional enzymes, 2-isopropylmalate synthase, 3-isopropylmalate dehydratase, and 3-isopropylmalate dehydrogenase. The pathways and enzymes are apparently restricted to the plastid (14, 19).

Regulation of the pathway in plants occurs by inhibition of threonine dehydratase by isoleucine (37), inhibition of 2-isopropylmalate synthase by leucine (22), and inhibition of AHAS by all three amino acids (18, 20). In studies with isolated chloroplasts, 1 mM valine completely inhibited valine, leucine, and isoleucine biosynthesis from pyruvate, and isoleucine strongly inhibited (isoleucine + leucine) biosynthesis from pyruvate (32). These studies prove: a) the enzymes of branched-chain amino acid biosynthesis reside in the chloroplast, and b) complete regulation of the pathway can occur through feedback inhibition from end products of the pathway. This second point is noteworthy since AHAS

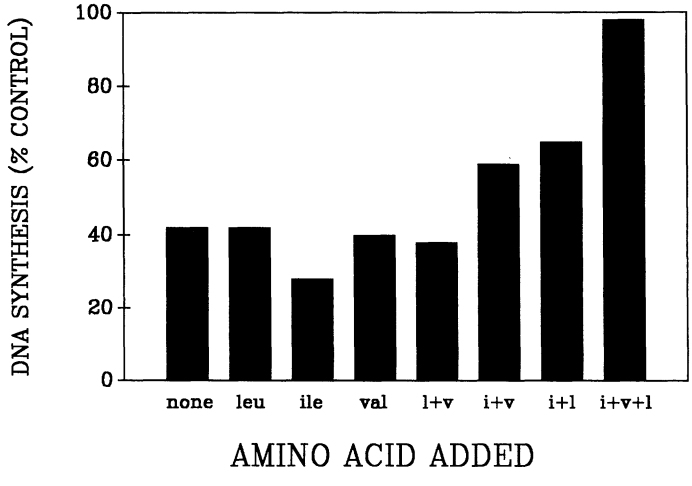


Figure 4. Reversal of imazapyr effects on DNA synthesis in corn roots. Seedlings were treated for 24 h with 15 μM imazapyr plus the various combinations of the amino acids at 1 mM each. Root tips were excised and incubated for 1 h with [^{14}C]thymidine (35).

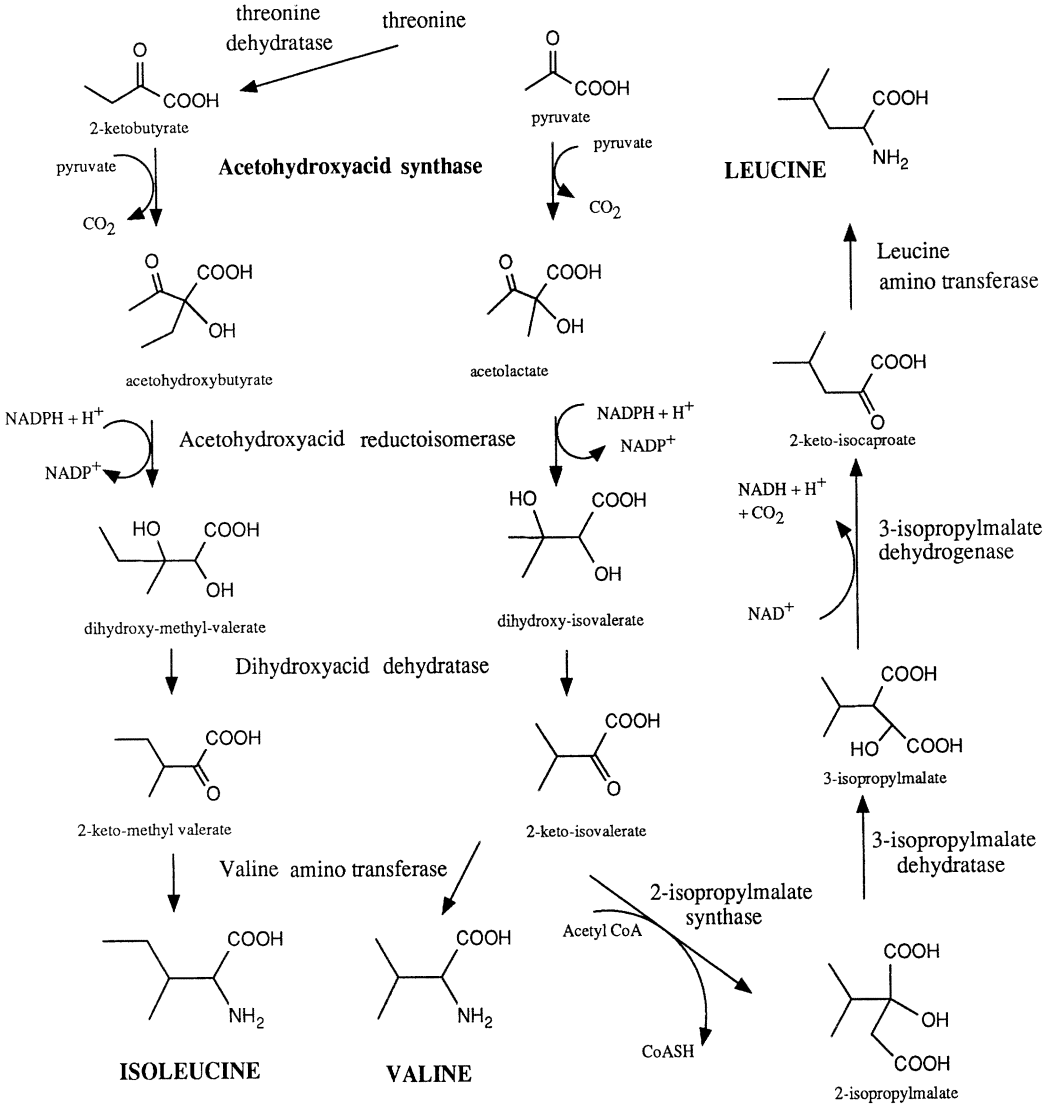


Figure 5. **Biosynthetic pathway of the branched-chain amino acids.**

inhibition by amino acids in vitro is notoriously weak, especially inhibition by valine (18). One explanation for this anomaly is that feedback regulation of AHAS in the chloroplast is complete, and partial feedback regulation observed in vitro only approximates properties of the enzyme in its native milieu. If this explanation is true, it follows that other properties of the enzyme may also be altered upon isolation of the enzyme from the chloroplast.

INHIBITION OF AHAS BY HERBICIDES

Following the observation of scientists studying sulfonylurea inhibition of the microbial enzyme (14, 27), Ray (23) found similar kinetics of sulfonylurea inhibition of the pea enzyme. Later, Muhitch et al. found that imidazolinones exhibited the same slow-binding inhibition with the maize enzyme (21). Figure 6 is a reaction progress diagram. In the absence of imazapyr, the reaction is linear over the 4-h time period. In the presence of imazapyr, inhibition of the enzyme increases over time. It is important to note that the reaction progress continues in the presence of the inhibitor even after prolonged incubation. Though inhibition is increasing, the interaction remains reversible. Studies on the bacterial enzyme have shown that the sulfonylurea binding site is proximal to the flavin adenine dinucleotide (27), and that bound sulfonylurea can be displaced by imidazolinone or triazolopyrimidinesulfonamide herbicides (30). The slow-binding inhibition is not restricted to herbicidal AHAS inhibitors (28).

When AHAS is extracted from corn tissue that has been treated with imazapyr, the level of extractable AHAS is drastically reduced (21, 36, 43). This effect occurs in excised leaf experiments when herbicide is fed into the transpiration stream or in whole plant experiments where the herbicide is

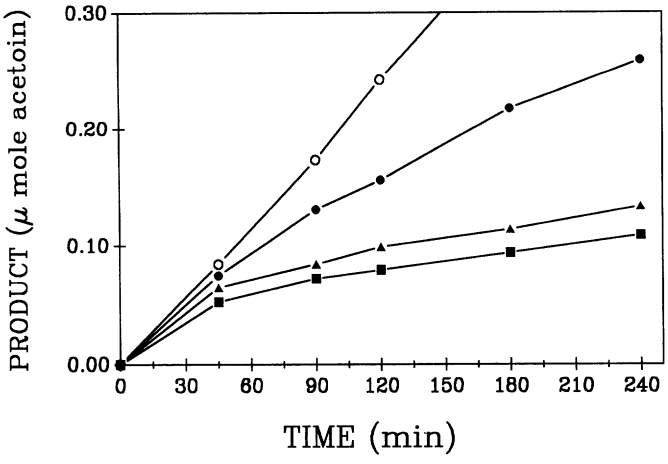


Figure 6. Reaction progress curve of corn cell AHAS in the absence and presence of imazapyr. AHAS from corn cells was prepared and assayed as in reference 21. The assay medium contained no additions (○), 1 μM (●), 5 μM (▲), or 10 μM (■) imazapyr. Time course in the control experiment was linear to 4 h (21).

applied through foliage or soil. The extent of loss of extractable AHAS activity is proportional to the log of the calculated internal herbicide concentration. Herbicides with different modes of action which are active on corn do not cause a similar effect on level of extractable AHAS.

One could argue that this effect of imidazolinones on extractable AHAS is simply a reflection of the slow-binding phenomena observed in vitro. However, treatment of corn with sulfonylurea sulfometuron methyl did not give the same decrease in extractable AHAS activity, though sulfometuron methyl is extremely toxic to corn and a more potent inhibitor of AHAS in vitro. Furthermore, sulfometuron methyl antagonizes the imidazolinone effect on extractable AHAS. Table 1 shows the extractable AHAS level 72 h after herbicide treatment. Imazaquin treatment alone resulted in a 65% reduction in the level of extractable AHAS, while either sulfometuron methyl treatment or imazaquin plus sulfometuron methyl treatment showed no difference in level of extractable AHAS. If plants were treated first by imazaquin followed by sulfometuron methyl 2 days later, extractable AHAS levels were reduced as in the treatment with imazaquin alone. If, however, sulfometuron treatment preceded imazaquin application, then imazaquin had no effect on level of extractable AHAS.

We have concluded from these studies that AHAS interaction with imidazolinones is somewhat different than the interaction of the enzyme with sulfometuron methyl. Though they both inhibit AHAS in vitro, only imidazolinone decreases the level of extractable AHAS. This paradox may be another difference of enzyme properties in vitro versus in vivo, as has been noted regarding amino acid regulation of AHAS.

MACROMOLECULAR ASPECTS OF IMIDAZOLINONE-AHAS INTERACTIONS

Though the herbicide-sensitive bacterial AHAS isozyme II has been purified and characterized (29), purification of plant AHAS has been hampered by enzyme lability and poor recoveries. Consequently, progress on biophysical characterization of imidazolinone-AHAS interactions has been limited (6, 40). Nonetheless, we have made some observations about different physical states of AHAS that reflect on the nature of imidazolinone binding interaction with AHAS.

When extracts from BMS cells are chromatographed on an isoelectric focusing column, two peaks of AHAS activity are observed (41). Subsequently, these two peaks were observable also on an anion exchange column and on gel filtration columns. These peaks have many different properties (Table 2). The difference most relevant for the present discussion is sensitivity to inhibitors. The smaller molecular weight minor form AHAS-II is more sensitive to imazapyr inhibition than is the larger molecular weight major form AHAS-I. Increased sensitivity is characterized by a small but measurably lower I_{50} and a greater maximal inhibition. Conversely, AHAS-II is virtually insensitive to inhibition by feedback regulators leucine and valine while AHAS-I is inhibited by these amino acids (41).

Table 1. Effect of imidazolinones and sulfonylureas on extractable AHAS activity 3 days after foliar spray at 10 g ha^{-1}. In combination treatments, the first herbicide was applied at day 0 followed by the second herbicide 2 days later (36).

Treatment	Extractable AHAS activity
	OD 520 mg^{-1} protein h^{-1}
Control	12.4
Imazaquin	4.4
Sulfometuron methyl	12.7
Imazaquin + sulfometuron methyl (same day)	11.1
Imazaquin followed by sulfometuron methyl	5.1
Sulfometuron methyl followed by imazaquin	10.3

Table 2. Summary of physical and kinetic properties of AHAS I and AHAS II (41).

Property	AHAS I	AHAS II
Molecular weight	195,000	55,000
pH optimum	6–7	7
K_m for pyruvate	5 mM	8 mM
Inhibitor sensitivity [Leucine + valine]:		
I_{50}	0.1 mM	>10 mM
% maximal inhibition	55%	5%
Imazapyr:		
I_{50}	2.0 μM	1.5 μM
% maximal inhibition	80%	>95%
Sulfometuron methyl:		
I_{50}	10 nM	10 nM
% maximal inhibition	80%	>95%

Table 3. Temperature effects on relative sensitivity of AHAS to inhibition by amino acids and herbicides. AHAS from corn cells was prepared as in reference 41. The enzyme was incubated for 1 h at the indicated temperature in assay medium containing no additions, 1 mM each [leucine + valine], or 100 μM imazapyr. Unpublished data from B. Singh.

		Inhibition	
Temperature	Control activity	[Leu + val]	Imazapyr
C	OD 520 mg^{-1} h^{-1}	%	
37	248	74	82
40	295	69	85
43	349	45	85
46	388	56	85
50	388	26	84

While we have not proven that these two forms of AHAS are not different gene products, the interpretation we currently favor is that these two forms of AHAS represent different aggregation states of the enzyme. In this interpretation, the site for feedback regulators requires an oligomeric configuration, and the binding site for imidazolinones would be altered in the oligomeric state. This hypothesis draws additional support from the cooperative feedback regulation long known for binding of valine and leucine, since cooperativity often involves interaction between different subunits.

There are two independent lines of evidence supporting a separate binding domain for imidazolinones and the feedback regulators. Mutant valine-resistant tobacco lines have been isolated and found to have imidazolinone-sensitive AHAS (24). Conversely, imidazolinone-resistant plants have normal sensitivity to feedback inhibition by leucine and valine (38).

Some additional lines of evidence come from the relative stability of the inhibitory activity of imidazolinones versus the inhibitory activity of amino acids. Table 3 shows the temperature dependence of AHAS from maize and the relative inhibition by valine plus leucine and by imazapyr. As temperature rises about 40 C, inhibition by (valine + leucine) begins to diminish. However, inhibition by imazapyr remains constant up to 50 C.

Similarly, a comparison of properties of freshly extracted AHAS and aged AHAS preparations shows this lability of the amino acid feedback regulation site relative to the imidazolinone binding site. Ammonium sulfate pellets prepared from maize BMS cells were assayed immediately after preparation and after 4 weeks storage at –20 C. The aged preparation was much diminished in its sensitivity to amino acids relative to the fresh preparation, while there was only a slight change in sensitivity to imazapyr (data not shown). Chromatography of the two preparations confirmed that most AHAS activity in fresh extracts was AHAS-I while most activity in aged preparations was monomeric AHAS-II (Figure 7).

At this point, a model of the herbicide binding site can be made from the macromolecular perspective (Figure 8). Both AHAS monomer and tetramer have a catalytic binding domain and an imidazolinone binding domain. Oligomerization of the enzyme results in formation of an amino acid binding domain which is distinct from the imidazolinone binding domain. Oligomerization also alters the imidazolinone binding domain such that the binding strength is diminished slightly.

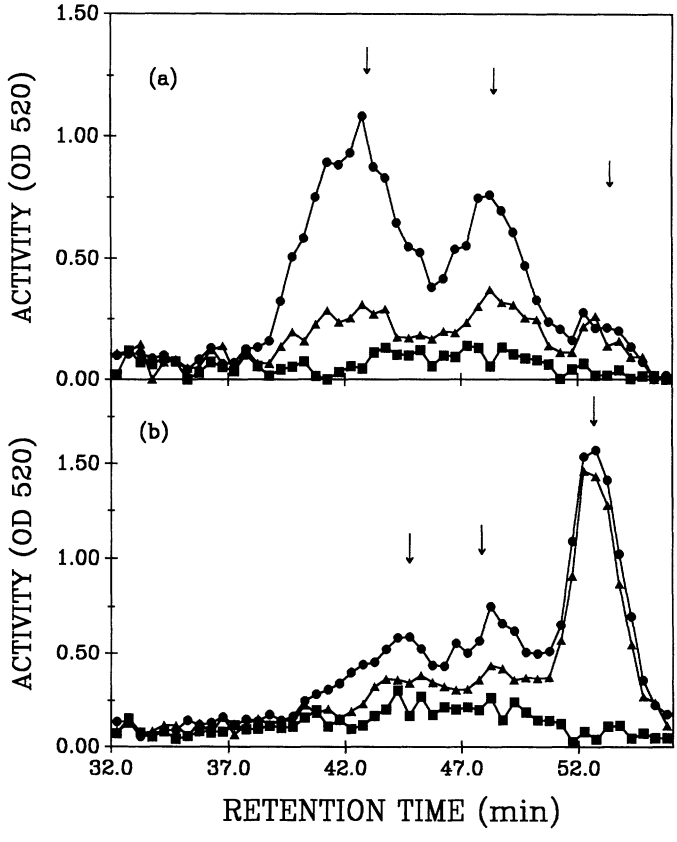


Figure 7. Effects of aging on AHAS oligomerization state and sensitivity to inhibitors. Ammonium sulfate pellets from corn cells were prepared and applied to a Waters Protein Pak 300 SW gel filtration HPLC column as in reference 41. The pellets were either freshly prepared or stored for 4 weeks at –20 C. Fractions from the chromatograph were split and assayed in standard medium (●), in medium plus 1 mM each [leucine + valine] (▲), or in medium plus 100 μM imazapyr (■). Arrows indicate presumed tetramer, dimer, and monomer aggregation states. Unpublished data from B. Singh.

PROPERTIES OF AHAS FROM HERBICIDE-RESISTANT MAIZE

Another area of our work that adds information about the binding site of the herbicides is analysis of AHAS from herbicide-resistant plants. Many such studies have appeared in the last few years (3, 7, 8, 10, 11, 17, 26, 33, 39, 45, 46). A broad generalization about these studies is that herbicide resistance can show high specificity to herbicide chemistry in some cases while in other cases show resistance to a broad range of chemistry. Analysis of imidazolinone-resistant maize is illustrative of the range of results.

Tissue culture selection, plant regeneration, and breeding have provided two isogenic lines of maize with different spectra of herbicide resistances. AHAS from these two lines, when compared with AHAS from sensitive maize, shows that

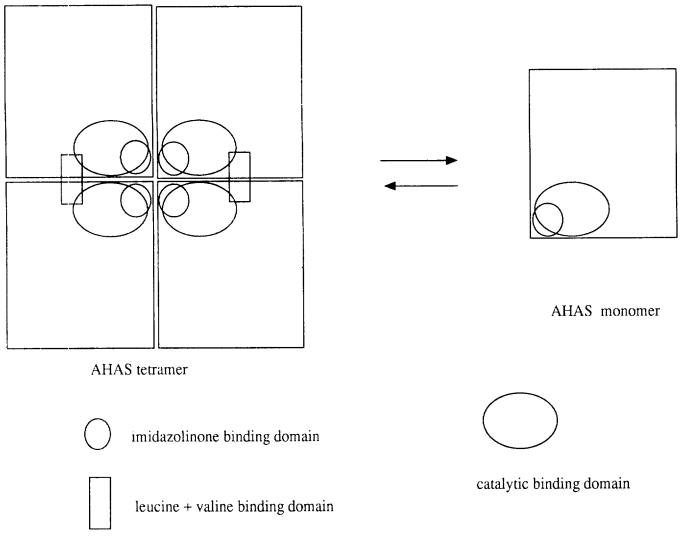


Figure 8. Model for effects of AHAS oligomerization on inhibitor sensitivity.

Table 4. Inhibitor sensitivity of AHAS extracted from herbicide-resistant maize lines (38).

Inhibitor	Sensitivity of maize line		
	B73	XA17	XI12
	I_{50} values		
Imazaquin	3 μM	>100 μM	80 μM
Imazethapyr	6 μM	>1 mM	>1 mM
Sulfometuron methyl	12 nM	>1 μM	120 nM
[Leucine + valine]	60 μM	60 μM	60 μM

some rather subtle interactions differentiate the binding of imidazolinones and sulfonylureas, and that some subtle interactions differentiate among individual imidazolinones (Table 4). AHAS from line XA17 is insensitive to imazaquin and imazethapyr, and to sulfometuron methyl. AHAS from line XI12 shows a sensitivity to sulfometuron methyl similar to the control B73 inbred while showing a high level of resistance to both imidazolinones. The AHAS from each of these lines has identical sensitivities to leucine plus valine.

Properties of these mutant enzymes suggest that single amino acid residues in AHAS can dramatically affect binding of the various inhibitors. Some mutations change binding interactions for two different herbicide classes (i.e., XA17 mutation), while others affect binding of imidazolinones more than binding of sulfonylureas (i.e., XI12). Further, the XI12 mutation affected binding of imazethapyr more than binding of imazaquin. Neither of these mutations affected binding of the feedback regulators leucine and valine.

OTHER PROGRESS

Space does not permit a full review of remaining areas in which progress has occurred in understanding mode of action AHAS-inhibiting herbicides. Tremendous strides have occurred in the area of molecular genetics. Complete gene sequences for plant AHAS have been published (3, 16, 17, 45) and amino acid substitutions conferring herbicide resistance have been patented (3). Twenty-four different amino acid substitutions at 10 different sites have been identified as conferring sulfonylurea resistance. Herbicide-resistant transgenic plants have been generated using these genes (3, 8, 12, 45). Plant AHAS genes have functionally complemented bacteria deficient in AHAS, thus opening a rapid microbial approach to understanding molecular details of plant AHAS structure and function (42). Recent progress in physiology of herbicide action has come from determining the nature of changes in free amino acid levels in response to sulfonylurea or imidazolinone application (25). Here, elevated levels of 2-aminobutyrate have been implicated in toxicity of these herbicides. Also, in the area of physiology is the recent discovery of herbicides acting at the next step in the pathway, at ketol-acid reductoisomerase (31). Though the target enzyme is not the same, similar phytotoxic symptoms occur, thus implying that it is inhibition of the pathway rather than inhibition at any particular step in the pathway that is important in eliciting herbicidal response.

Further refinements of the pictures proposed here are certain to emerge in the near future. Biophysical techniques applied to the purified enzyme will help define the binding site for the herbicides and the physical organic chemistry of the enzyme mechanism. With sufficient quantities of the purified enzyme it may also be possible to design better experiments to define the nature of the slow binding inhibition that has been observed with the two classes of herbicides. Amino acid sequence information of sensitive and resistant enzymes will help provide boundaries for interpretation of biophysical data. Purified enzyme will also be useful for performing rapid kinetics experiments and spectral experiments analogous to those already performed on the bacterial enzyme. At the macromolecular level, purified enzyme and antibody to the enzyme will be useful in clarifying questions about subunits, isozymes, and enzyme stability. The continuing emergence of new herbicidal AHAS inhibitors in the patent literature coupled with economic success of the older inhibitors ensure a widening interest in AHAS and more tools for elucidating the dynamics of this unusual enzyme.

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