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Communication

Acetolactate Synthase Inhibiting Herbicides Bind to the Regulatory Site

Mani V. Subramanian*, Vivian Loney-Gallant, Jennifer M. Dias, and Linda C. Mireles
DowElanco, Walnut Creek, California 94598

ABSTRACT

Acetolactate synthase from spontaneous mutants of tobacco (*Nicotiana tabacum*; KS-43 and SK-53) and cotton (*Gossypium hirsutum*; PS-3, PSH-91, and DO-2) selected in tissue culture for resistance to a triazolopyrimidine sulfonanilide showed varying degrees of insensitivity to feedback inhibitor(s) valine and/or leucine. A similar feature was evident in the enzyme isolated from chlorsulfuron-resistant weed biotypes, *Kochia scoparia* and *Stellaria media*. Dual inhibition analyses of triazolopyrimidine sulfonanilide, thifensulfuron, and imazethapyr *versus* feedback inhibitor leucine revealed that the three herbicides were competitive with the amino acid for binding to acetolactate synthase from wild-type cotton cultures. Acetolactate synthase inhibiting herbicides may bind to the regulatory site on the enzyme.

The three chemical families of highly active herbicides, namely, TPs[1] (3, 19), SUs chlorsulfuron and thifensulfuron (7, 12), and the IM imazethapyr (6, 9, 18), are potent inhibitors of ALS. This enzyme catalyzes the condensation of two molecules of pyruvate or one molecule of pyruvate and 2-ketobutyrate to form acetolactate or acetohydroxybutyrate, respectively. This reaction is the initial step in the biosynthetic pathway for the production of Val, Leu, and Ile in plants and microorganisms. The structures of these three families of herbicides are shown in Figure 1.

TP is a mixed-type inhibitor of ALS with respect to both pyruvate and TPP (19, 20). The compound has nearly equal affinity for free as well as ligand bound forms of the enzyme. The binding of SU (16) and IM (6, 9, 18) has also been shown to be unrelated to the substrate and cofactors of ALS. Schloss *et al.* (16) have demonstrated that SU, TP, and IM compete with each other for binding to ALS. Based on the cross-resistance pattern of a number of mutants, a model has emerged for the binding domain in which there are overlapping as well as nonoverlapping binding elements not only across inhibitor families, but also between analogs within each family (2, 4, 5, 14, 15, 19–21).

In the present article, we provide for the first time direct evidence that the three families of inhibitors compete with leucine for binding to ALS. These herbicides may bind to the regulatory site of the enzyme.

[1] Abbreviations: TP, triazolopyrimidine sulfonanilide; ALS, acetolactate synthase; SU, sulfonylurea; IM, imidazolinone; TPP, thiamine pyrophosphate.

MATERIALS AND METHODS

Chemicals

All ALS inhibitors used in the present study were either obtained commercially or synthesized by DowElanco chemists, Walnut Creek, CA. Pyruvate, DTT, TPP, DMSO, Val, Leu, Ile, $(NH_4)_2SO_4$, 2-naphthol, and creatine were purchased from Sigma Chemical Company, St. Louis, MO. The media components of tissue culture were purchased from Gibco Laboratories, Grand Island, NY.

Whole Plant and Tissue Culture Materials

Wild-type cultures of tobacco (*Nicotiana tabacum*) and cotton (*Gossypium hirsutum*) were routinely maintained in MS mineral medium (10) supplemented with 3% w/v sucrose, 0.5 mg/L thiamine hydrochloride, and 0.4 mg/L (tobacco) or 4.0 mg/L (cotton) 2,4-D. Mutants of tobacco and cotton were selected for resistance to TP as described previously (21). The mutants were maintained at the maximum tolerable concentration of TP (480 ppb for tobacco mutants KS-43 and SK-53; 80, 800, and 1000 ppb for cotton mutants DO-2, PSH-91, and PS-3, respectively). A chlorsulfuron-resistant biotype of *Stellaria media* was collected from Stony Plain, Alberta, Canada. *Kochia scoparia* resistant to chlorsulfuron was obtained from North Dakota by DuPont field research personnel and made available for testing by J. Saldini, E.I. DuPont De Nemours & Company. The same two plants collected from an adjacent area not exposed to chlorsulfuron were of insufficient viability for biochemical studies. Hence, commercially available *S. media* and *K. scoparia* purchased from Herbiseed, UK, and Seeds for Research, Plentywood, Montana, respectively, were used as wild-type controls. The plants were grown in 4-inch pots containing a sandy loam soil, under greenhouse conditions (20–22°C days and 15°C nights). Twelve- to 18-d-old shoots were excised from the pots and used as the source of ALS.

Enzyme Extraction and Assay

Crude extracts of suspension cultures at mid-log phase of growth were prepared in 20 mM potassium phosphate buffer, pH 7.15, containing 1 mM DTT and 5 mM $MgCl_2$ as described

previously (21). ALS was precipitated at 50% saturation of $(NH_4)_2SO_4$ from the crude extract and used for assays immediately or stored at −70°C until further use. Crude extracts of excised shoots of *Stellaria* and *Kochia* were prepared by homogenizing in a Waring Blendor with 20 mM potassium phosphate buffer, pH 7.15, containing 1 mM DTT and 5 mM $MgCl_2$ for 20 s. The extract was filtered through several layers of cheesecloth and centrifuged at 100,000*g* for 60 min. The clear supernatant was precipitated at 0 to 33% saturation of $(NH_4)_2SO_4$, and the pellet collected by centrifugation at 30,000*g* for 10 min and used for ALS assays. The enzyme was assayed colorimetrically as described previously (21) by quantitating the amount of acetoin formed from acetolactate, using creatine and 1-naphthol (22). Stock solutions of inhibitors were prepared in DMSO (10 mg/mL) and diluted in Tris-HCl buffer, pH 8.3, prior to addition to the reaction mixture. All kinetic constants were determined using Enzfitter software obtained from Elsevier-Biosoft, UK.

RESULTS AND DISCUSSION

Effect of Valine and/or Leucine on Mutant Enzymes

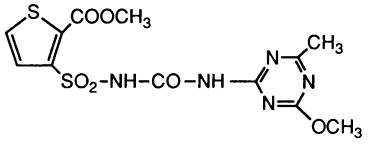
Both Val and Leu (end products of the ALS pathway) inhibited wild-type cotton ALS, but the enzyme from tobacco, *Kochia,* and *Stellaria* was inhibited only by Leu (Table I). None of the enzymes showed significant feedback inhibition by Ile at 2 mM (data not shown). In comparison, all mutant enzymes showed varying degrees of resistance to feedback inhibition by Val and/or Leu (Table I). ALS from DO-2 showed a very low level of resistance to inhibition by the end products. In contrast, PS-3 cotton and SK-53 tobacco enzymes were highly resistant, and the remaining mutant ALS showed moderate resistance to inhibition by the branched chain amino acids (Table I). A PS-3 culture was also shown earlier to accumulate higher levels of Val, Leu, and Ile compared with wild-type cotton (21). The differences in the sensitivity of ALS from resistant lines to Val and/or Leu (Table I) suggest that they are distinct mutants. This was also evident from the variation in the cross-resistance pattern to different inhibitors for the enzyme from KS-43 tobacco, DO-2, and PS-3 cotton (21). The remainder of the isolates (Table I) also varied in their cross-resistance pattern to different ALS-inhibiting herbicides (data not shown), suggesting different mutations. Rathinasabapathi *et al.* (11) have also noted loss of feedback sensitivity to Val, Leu, and Ile in four chlorsulfuron-resistant isolates of *Datura innoxia.* Resistance to inhibition by Val has also been observed in ALS from sulfometuron (a SU) resistant mutants of yeast (8). On the contrary, Creason and Chaleff (1) reported no alteration in the feedback sensitivity of mutant ALS from a SU-resistant tobacco plant. Conversely, a Val-resistant ALS from tobacco mutants was found to be not resistant to SU herbicides (13).

The alteration in the feedback inhibition characteristics of ALS resistant to herbicides appears to be a common feature. There is also a striking similarity in the kinetic mechanism of

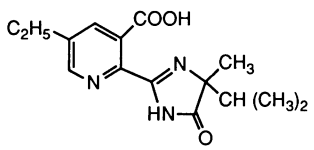
Table I. *Effect of Valine and/or Leucine on Wild Type and Mutant ALS*

Source of ALS	-Fold Resistance	
	Valine	Leucine
	I_{50} mutant enzyme/ I_{50} wild type enzyme	
Wild-type tobacco	NI[b]	1 (0.068)
KS-43 tobacco mutant	NI	2.9
SK-53 tobacco mutant	NI	30.0
Wild-type cotton	1 (0.56)	1 (0.35)
PS-3 cotton mutant	43–350	29–34
PSH-91 cotton mutant	3–4	3–4
DO-2 cotton mutant	2.0	1.5
K. scoparia (wild type)	NI	1 (0.29)
K. scoparia (chlorsulfuron resistant)	NI	7.0
S. media (wild type)	NI	1 (0.39)
S. media (chlorsulfuron resistant)	NI	2.0

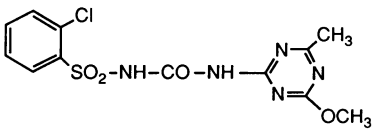
[a] I_{50}, concentration of the inhibitor required to produce 50% inhibition of enzyme activity. I_{50} values for wild type enzymes are given in parentheses (in mM). [b] NI, no significant inhibition at 0.5 to 1 mM.



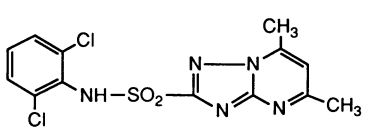
SU, THIFENSULFURON



IM



SU, CHLORSULFURON



TP

Figure 1. Three chemical families of herbicides known to inhibit acetolactate synthase. SU: chlorsulfuron and thifensulfuron; IM: imazethapyr; and TP. SU is proprietary chemistry of DuPont while IM and TP are proprietary to American Cyanamid and DowElanco, respectively.

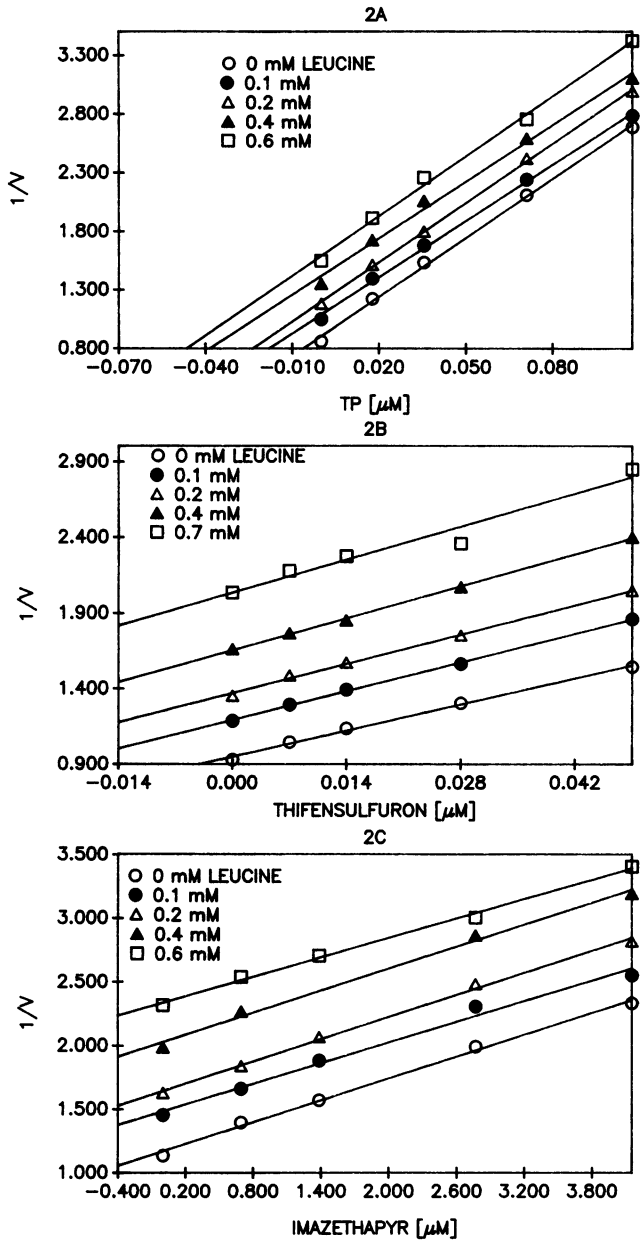


Figure 2. Dual inhibition analyses of cotton acetolactate synthase at different concentrations of TP (A), thifensulfuron (B), and imazethapyr (C) *versus* leucine at different fixed levels. Assay conditions are given under "Materials and Methods."

ALS inhibition by the branched chain amino acids (data not shown) and the herbicides. All of these compounds are linear mixed-type inhibitors with respect to both pyruvate and TPP. The relationship between the herbicides and Leu (and Val) was further examined by dual inhibition analyses of wild-type cotton ALS. The three inhibitors, TP (Fig. 2A), thifensulfuron (Fig. 2B), and imazethapyr (Fig. 2C), were varied at different fixed levels of Leu, under saturating substrate concentration. In all three cases, the kinetic pattern of inhibition best fit a family of parallel lines (Fig. 2). Similar results were obtained when Val was substituted for Leu (data not shown). This kinetic pattern, which is typical for two competitive linear mixed-type inhibitors (17), indicates that the binding of herbicides and Leu (or Val) to ALS is mutually exclusive. The herbicide binding site in the enzyme may actually overlap with that of Leu and/or Val, as is evident from the cross-resistance data presented in Table I. Alternately, the herbicides may bind to a remote site on the enzyme and still be competitive with Leu via allosteric effects. This is the first report of a direct relationship between the different families of ALS-inhibiting herbicides and its feedback inhibitor(s). Schloss *et al.* (16) have demonstrated that SU, TP, and IM are not only competitive with each other but also with quinone for binding to ALS. These herbicides have been proposed to bind to a vestigial quinone site on ALS that was derived during its evolution from pyruvate oxidase (16). The relationship between the ALS-inhibiting herbicides, Leu and quinone, remains to be explored further.

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