Properties of Mutant Acetolactate Synthases Resistant to Triazolopyrimidine Sulfonanilide

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ABSTRACT

Triazolopyrimidine sulfonanilides are a class of highly active herbicides whose primary target is acetolactate synthase. Spontaneous mutants of tobacco (Nicotiana tabacum) (KS-43) and cotton (Gossypium hirsutum) (PS-3 and DO-2) resistant to triazolopyrimidine sulfonanilide were selected in tissue culture. Acetolactate synthase partially purified from the three mutants were 80- to 1000-fold less sensitive to inhibition by the compound compared with the corresponding wild-type enzyme. The mutants also varied in the cross-resistance pattern to other acetolactate synthase inhibiting herbicides in the sulfonylurea, imidazolinone, and pyridymid-oxy-benzoate chemical families. Thus, acetolactate synthase from KS-43, PS-3, and DO-2 cultures have different mutations. The affinities for pyruvate, thiamine pyrophosphate, as well as the activity of the mutant enzymes were found to be comparable to the corresponding wild-type enzymes. However, the enzyme from PS-3 was highly resistant to feedback inhibition by valine and leucine. In contrast, acetolactate synthase from KS-43 and DO-2 were inhibited by valine and leucine to nearly the same extent as the wild-type enzymes. Also, PS-3 cultures accumulated much higher levels of the branched chain amino acids compared to the wild-type cotton culture. The mutation in the PS-3 enzyme has therefore rendered it insensitive to feedback regulation by valine and leucine.

Acetolactate synthase (EC 4.1.3.18) catalyzes the condensation of two molecules of pyruvate or one molecule of pyruvate and 2-ketoisobutyrate 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. During the last decade, several diverse chemical families of highly active, crop selective, herbicides have been shown to be potent inhibitors of ALS.1 These include TP (19; a new class of herbicides under development at DowElanco), SU (7, 13, 15; DuPont), IM (11, 17; American Cyanamid) and POB (2; recently patented by Kumiai Chemicals, Japan). The structures of the four classes of herbicides are shown in Figure 1. Growth inhibition caused by the above herbicides is completely reversed by branched chain amino acids (4, 7, 13, 15, 17). Also, mutants resistant to these herbicides have been shown to possess an altered form of ALS which is less sensitive to inhibition (1, 20). Therefore, inhibition of ALS is directly responsible for the herbicidal activity of these chemical families.

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), IM (6, 11, 17), and POB (6) have 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. Genetic evidence also supports the contention that all of the above herbicides have overlapping binding site(s) on the enzyme (14, 18-20). Mutants of soybean (Glycine max) and tobacco (Nicotiana tabacum) selected in tissue culture for resistance to TP, were cross-resistant to growth on SU and IM (19). ALS isolated from one of the TP selected tobacco mutants, KS-43 was cross-resistant to inhibition by IM and SU (20). Mutants

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1 Abbreviations: ALS, acetolactate synthase; TP, triazolopyrimidine sulfonanilide; SU, sulfonylurea; IM, imidazolinone; POB, pyrimidyl-oxy-benzoate; TPP, thiamine pyrophosphate; MS, Murashige and Skoog; PPB, parts per billion; EB, 20 mm potassium phosphate buffer pH 7.15 containing 1 mm DTT and 5 mm MgCl2; FAD, flavin adenine dinucleotide; PPM, parts per million; I50, concentration of the inhibitor required to produce 50% inhibition of enzyme activity.
displaying varying degrees of resistance to different ALS inhibitors (3, 14, 18, 20) as well as specific resistance to IM (14, 18) and some SU (5, 10) have also been reported. While the TP, SU, IM, and POB families of inhibitors do employ common elements of the inhibitory binding domain on ALS, these observations emphasize that there are additional non-overlapping structural elements which provide unique binding to individual families of inhibitors.

In the present paper, we have compared the cross-resistance pattern of three mutant ALS to compounds from different families of herbicides. In addition, the catalytic properties and feedback regulation of the mutant enzymes have also been examined.

MATERIALS AND METHODS

Chemicals

All the 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, (NH₄)₂SO₄, 2-naphthol, and creatine were purchased from Sigma Chemical Company. The media components of tissue culture were purchased from Gibco Laboratories, Grand Island, NY.

Plant Tissue Culture Material

All procedures were carried out using sterile materials and in a sterile environment. Suspension cultures of tobacco (Nicotiana tabacum) and cotton (Gossypium hirsutum) were derived from the shoots of the respective seedlings and optimized for uniform growth. The cultures were routinely maintained in MS mineral medium (12) 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.

Selection of Mutants Resistant to TP

Cultures of tobacco and cotton from mid-log phase of growth (about 3 × 10⁶ cells/mL) were plated in the respective media containing 30 to 50 PPB of TP and solidified with 0.7% w/v phytagar. Isolates of resistant calli were picked from plates after 4 to 8 weeks of selection pressure, at a frequency of about 0.1 to 0.5 × 10⁻⁶. Resistant calli were subcultured in the same media three times prior to transfer to appropriate liquid media containing 30 to 50 PPB of TP. After three generations in the liquid media, the mutants were grown at various doses of TP (at 15% inoculum, 50 mL) to establish a tolerance level. Growth of the cultures was measured after 25 to 30 d by transferring the contents in the flask into a graduated tube and noting the cell volume after 15 min settling time. Subsequently, each mutant was maintained at the maximum tolerable concentration of TP (480, 1000, and 80 PPB, respectively, for KS-43 tobacco, PS-3, and DO-2 cotton mutants).

Enzyme Extraction and Assay

Suspension cultures at midlog phase of growth were filtered, suspended in EB (2–3 mL/g), and lysed by French press at 10,000 p.s.i. The lysate was centrifuged at 100,000 g for 60 min and the clear supernatant precipitated at 50% saturation of (NH₄)₂SO₄. The pellet obtained by centrifugation at 30,000 g for 15 min was immediately dissolved in EB (7–10 mg/mL) and used for assay, or stored at -70°C until further use. The assay mixture in a final volume of 1.5 mL of 50 mM Tris-HCl buffer (pH 8.3) contained 26.66 mm (for tobacco enzyme) or 60 mm (for cotton enzyme) pyruvate, 0.26 mm TPP, 2 μM FAD, 0.5 mm MgCl₂, and 0.15 mL of enzyme (0.9–1.5 mg protein). Pyruvate was added after incubating the rest of the contents for 3 min at 37°C. At the end of 30 min, the reaction was terminated by the addition of 0.2 mL of 5% H₂SO₄. The acetoin formed from acetylactate was quantitated using creatine and 1-naphthol (23). For blank reading, pyruvate was

![Figure 2. Effect of triazolopyrimidine sulfonanilide on the growth of wild-type tobacco and KS-43 mutant cultures. Wild-type and mutant cultures were grown in MS mineral medium (12) pH 5.8 to 6.0, supplemented with 0.4 mg/L 2,4-D, 3% w/v sucrose, 0.5 mg/L thiamine hydrochloride, and various concentrations of the compound. The culture volume was 50 mL at 15% inoculum. Growth (settled cell volume) was measured at the end of 28 d as cell volume after 15 min settling time. Initial settled cell volume was 2.2 mL.](image)

![Figure 3. Effect of triazolopyrimidine sulfonanilide on the growth of wild-type cotton, PS-3, and DO-2 mutants. Experimental conditions were same as described in Figure 2 with the exception of the concentration of 2,4-D which was 4.0 mg/L. Initial settled cell volume was 4.5 mL.](image)
omitted from the reaction mixture. Inhibitors when used were added to the reaction mixture prior to addition of pyruvate. All the kinetic constants were determined using EnzFitter software obtained from Elsevier-Biosoft, United Kingdom. Protein concentration was determined according to the method of Lowry et al. (9), using BSA as the standard.

NMR Experiments

Extracts of cell cultures were prepared according to the procedure developed by Timothy Logan and David Lynn, University of Chicago (personal communication). Nine-d-old wild type and PS-3 cultures (in late log-phase of growth) were filtered, washed with water, frozen with liquid nitrogen, and ground to a fine powder in a pestle and mortar. The cells were then digested with 35 mL of 5% perchloric acid overnight at 4°C. The samples were then filtered, the supernatant neutralized to pH 7.0 with 3 m KOH and lyophilized. The dry solid was dissolved in 10 mL of water and the insoluble materials removed by centrifugation for 15 min at 30,000g. The supernatant was lyophilized, weights of dry matter normalized, and dissolved in about 2 mL D$_2$O containing 5.0 mm EDTA and microfuged. The clear solution was then transferred to a 5 mm NMR tube. Spectra were acquired on a Bruker AM 400 Spectrometer operating at 100 MHz at 25°C. Spectra were collected overnight using 60°C excitation pulses, a 5 s relaxation delay, and continuous proton decoupling. The resulting data were exponentially weighted with 2 Hz line broadening to enhance signal to noise and Fourier transformed. Chemical shifts were referenced to a similar sample containing dioxane which was assigned to 67.4 PPM. Amino acid peaks were assigned by comparison to spectra of each standard in D$_2$O/EDTA and, in some cases, by spiking the amino acid into the cell extract.

RESULTS

Growth of Mutants on TP

Growth of wild-type tobacco and KS-43 mutant at different concentrations of TP is compared in Figure 2. A similar comparison is shown in Figure 3 for wild-type cotton and the mutants PS-3 and DO-2. Both KS-43 and PS-3 cultures showed greater than 300-fold tolerance to growth on TP whereas DO-2 demonstrated 80-fold tolerance. The resistance mechanism for all three mutants was due to an altered ALS less sensitive to inhibition by the compound. This was shown earlier for KS-43 enzyme which was 350-fold resistant to inhibition by TP (20). Similarly, the enzyme from PS-3 and DO-2 were, respectively, 1000- and 80-fold less susceptible to inhibition by TP (Fig. 4). The magnitude of resistance to the compound at the enzyme and cellular level was comparable. The growth rates of KS-43 and PS-3 were equal to the corresponding wild-type cultures while that of DO-2 was slightly lower than wild-type cotton (data not shown).

Cross-Resistance Pattern of Mutant ALS to Different Inhibitors

The cross-resistance of ALS isolated from the three mutants to compounds representing different inhibitor families (Fig. 1), is compared in Table I. While each mutant enzyme did show cross-resistance to some or all of the inhibitors tested, the pattern of cross-resistance was different for each. For example, ALS from KS-43 had moderate to high resistance to inhibition by all the compounds (Table I). In contrast, the enzyme from DO-2 displayed low resistance to TP and chlorosulfuron (SU) but high resistance to other compounds (Table

Table I. Cross-Resistance Pattern of Acetolactate Synthase from KS-43 Tobacco Mutant, PS-3, and DO-2 Cotton Mutants to Compounds from Various Families of Inhibitors (Fig. 1)

<table>
<thead>
<tr>
<th>Compound</th>
<th>KS-43</th>
<th>PS-3</th>
<th>DO-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazolopyrimidine sulfonanilide</td>
<td>433 (0.009)</td>
<td>1000-2000</td>
<td>86 (0.0083)</td>
</tr>
<tr>
<td>Imazethapyr (imidazolinone)</td>
<td>118 (0.28)</td>
<td>1.2</td>
<td>&gt;2000 (0.51)</td>
</tr>
<tr>
<td>Chlorsulfuron (sulfonylurea)</td>
<td>ND*</td>
<td>1500</td>
<td>90 (0.03)</td>
</tr>
<tr>
<td>Accent (sulfonylurea)</td>
<td>529 (0.128)</td>
<td>19</td>
<td>715 (0.228)</td>
</tr>
<tr>
<td>Pyrimidyl-oxy-benzoate</td>
<td>188 (0.065)</td>
<td>2.9</td>
<td>4456 (0.158)</td>
</tr>
</tbody>
</table>

* Not determined.
1). PS-3 exhibited low level of resistance to Accent but very high to TP and chlorsulfuron. Virtually no resistance was observed with this enzyme to imazethapyr (IM) and POB. The variable cross-resistance pattern may indicate different mutations in the enzymes from KS-43, DO-2, and PS-3.

Comparison of Catalytic Properties of Wild-Type and Mutant Enzymes

The $K_m$ for pyruvate, TPP, and the specific activity of the wild-type and mutant enzymes are compared in Table II. No change in the activity or affinity for TPP was apparent in any of the mutant enzymes. Also, $K_m$ for pyruvate for PS-3 and KS-43 enzymes were unchanged. The only difference observed was in ALS from DO-2 which revealed a four- to ninefold higher $K_m$ for pyruvate compared to the cotton wild-type enzyme. Overall, no major deviation was found in the catalytic properties of the mutant enzymes when compared to the corresponding wild type ALS.

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 tobacco enzyme was inhibited only by Leu (Table III). None of the enzymes showed feedback inhibition by Ile (data not shown). DO-2 ALS was inhibited by Val and Leu to nearly the same extent as the wild-type cotton enzyme. Likewise, ALS from KS-43 showed only minimum variation from the wild-type tobacco enzyme with respect to inhibition by Leu (Table III). In contrast, ALS from PS-3 was resistant to inhibition by both Val and Leu (43- to 350-fold for Val and 29- to 34-fold for Leu, respectively). Clearly, the mutation in PS-3 enzyme has desensitized it to feedback regulation by branched chain amino acids.

Comparison of Valine, Leucine, and Isoleucine in Wild-Type and PS-3 Mutant by NMR

$^1$C NMR spectra of water soluble small metabolite extract of wild-type and PS-3 cotton mutants is shown in Figure 5. Peaks for Val ($^1$C chemical shifts of $\beta$, $\gamma$, and $\gamma'$ carbons at 29.8, 18.8, and 17.4 PPM, respectively) and Leu ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\delta'$ carbons at 54.2, 40.6, 25.0, 21.7, and 22.8 PPM, respectively) were much more prominent in PS-3 cells than wild type. Ile peaks ($\beta$, $\gamma$, $\gamma'$, and $\delta$ carbons at 36.6, 25.2, 15.5, and 11.9 PPM, respectively) were relatively small in the wild type (compared with Val and Leu), but again, much lower than in the PS-3. Although the actual concentration of the branched chain amino acids has not been established, it is clear that they are at much higher levels in the mutant. These results corroborate the earlier observation (Table III) that ALS from PS-3 is no longer feedback regulated by Val and Leu. A few other differences between the spectra of wild type and PS-3 may be evident in the peaks of Gly, Glu, and Lys. These may be attributed to transaminations to produce excess Val, Leu, and Ile in PS-3. Other aspects of the spectra were comparable to one another (Fig. 5).

**DISCUSSION**

The discovery of four diverse classes of ALS-inhibiting herbicides (TP, SU, IM, and POB) has established the enzyme as an effective target site. The lethal events following inhibition of ALS have remained obscure. Accumulation of 2-ketobutyrate has been implicated in the cause of death in microbes (21), but this has not been demonstrated in plants. None of the above class of compounds compete for the substrate or cofactor binding site; however, they are competitive with each other for binding to ALS (6, 16, 20). Mutations in ALS which show varying degrees of cross-resistance to different inhibitors (3, 14, 18–20), as well as specific resistance to some compounds (5, 10, 14, 18), suggest that there are overlapping as well as unique binding elements among the families of herbicides.

In the present report, we have compared properties of ALS from a mutant tobacco (KS-43) and two mutant cotton cultures (PS-3 and DO-2) that are resistant to TP. The mutant enzymes as well as the cultures differed in their magnitude of resistance to TP (Figs. 2–4). The cross-resistance pattern for the mutant enzymes toward imazethapyr (IM) chlorsulfuron (SU), Accent (SU), and POB was variable. In the case of ALS from PS3, very little cross-resistance was observed toward IM and POB (Table I). This enzyme also had low resistance to Accent (SU) but high to chlorsulfuron (SU). These results support a model for the inhibitory binding domain in which there are nonoverlapping binding elements, not only across inhibitor families, but also between analogs within each family. The variable cross-resistance also suggests that the mutations in KS-43, PS-3, and DO-2 are different.

**Table II. Comparison of Catalytic Properties of Acetolactate Synthase from Wild-Type and Mutant Cultures**

<table>
<thead>
<tr>
<th>Source of Acetolactate Synthase</th>
<th>Catalytic Property</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type tobacco</td>
<td>$K_m$ pyruvate</td>
<td>$K_m$ TPP</td>
</tr>
<tr>
<td></td>
<td>$\mu$mol/1h/mg</td>
<td></td>
</tr>
<tr>
<td>Wild-type tobacco</td>
<td>6.5–9.6</td>
<td>20–42</td>
</tr>
<tr>
<td>KS-43 tobacco mutant</td>
<td>5–11</td>
<td>22–38</td>
</tr>
<tr>
<td>Wild-type cotton</td>
<td>2.5–6.0</td>
<td>32–49</td>
</tr>
<tr>
<td>PS-3 cotton mutant</td>
<td>2.6–5.0</td>
<td>20–30</td>
</tr>
<tr>
<td>DO-2 cotton mutant</td>
<td>18.3–22</td>
<td>20–32</td>
</tr>
</tbody>
</table>

**Table III. Effect of Valine and Leucine on Wild-Type and Mutant Acetolactate Synthase**

<table>
<thead>
<tr>
<th>Source of Acetolactate Synthase</th>
<th>Fold Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type tobacco</td>
<td></td>
</tr>
<tr>
<td>KS-43 tobacco mutant</td>
<td></td>
</tr>
<tr>
<td>Wild-type cotton</td>
<td></td>
</tr>
<tr>
<td>PS-3 cotton mutant</td>
<td></td>
</tr>
<tr>
<td>DO-2 cotton mutant</td>
<td></td>
</tr>
</tbody>
</table>

| Wild-type tobacco               | VA: 1 (0.068) |
| KS-43 tobacco mutant            | LE: 2.9      |
| Wild-type cotton                | VA: 1 (0.56)  |
| PS-3 cotton mutant              | LE: 1 (0.35)  |
| DO-2 cotton mutant              | VA: 1.5      |

*No inhibition at 0.5 to 1.0 mM.*
A number of catalytic properties of the mutant enzymes were similar to that of the wild-type ALS. Virtually, no difference was apparent in their affinity for TPP or specific activity. ALS from KS-43 and PS-3 was unaltered with respect to $K_m$ for pyruvate but the DO-2 enzyme showed a 4- to 9-fold increase (Table II). Only the DO-2 mutant exhibited slower growth rate compared to corresponding wild-type culture. ALS from KS-43 and DO-2 were quite similar to the corresponding wild-type enzyme with regard to inhibition by Val and/or Leu. In contrast, the enzyme from PS-3 was highly resistant to feedback inhibition by both Val and Leu (Table III). This was confirmed by $^{13}$C-NMR analysis of a water-soluble extract of PS-3 cells which revealed high concentrations of the branched chain amino acids (Fig. 5). The actual concentration of Val, Leu, and Ile in PS-3 cultures was not determined. It is indeed possible that the herbicide binding site in ALS overlaps to a certain extent with the feedback inhibition site. Alternately, the loss of regulation of ALS in PS-3 may simply be due to a position effect of the mutation.

The frequency of spontaneous resistance to some of the ALS-inhibiting herbicides is high ($10^{-6}$-$10^{-7}$). This feature has also been evident in the fields where repeated applications of chlorosulfuron and sulfometuron (both long residue SU herbicides) has promoted the appearance of resistant weed biotypes; most notable among these are Kochia scoparia and Stellaria media (8, 22). The resistant populations evaluated to date have been shown to have an ALS that is less sensitive to inhibition by SU herbicides. The cross-resistance pattern of the above weeds to other families of ALS inhibiting herbicides (Fig. 1) is variable both at the whole plant and enzyme level (data not shown; also, M Devine, University of Saskatchewan, Canada, personal communication). The relative ecological fitness of naturally occuring resistant biotypes is a critical issue that remains to be vigorously analyzed. The results of this study indicate that some mutations in ALS can result in changes in enzyme properties such as loss of feedback regulation and lower affinity for pyruvate. Similar changes, if present in the enzyme from field-selected resistant biotypes could be expected to contribute negatively to the overall fitness profile.

ACKNOWLEDGMENTS

We thank Dr. David Lynn of the University of Chicago for his assistance in the interpretation of NMR spectra. We also thank Ms. Teri Lanzer for her assistance in typing this manuscript.

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