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Effect of mutagenesis at serine 653 of *Arabidopsis thaliana* acetohydroxyacid synthase on the sensitivity to imidazolinone and sulfonylurea herbicides

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Abstract Resistance to sulfonylurea and imidazolinone herbicides can occur by mutations in acetohydroxyacid synthase (EC 4.1.3.18). Changing serine 653 to asparagine is known to cause insensitivity to imidazolinones but not to sulfonylureas. Here, S-653 of the *Arabidopsis thaliana* enzyme was mutated to alanine, threonine and phenylalanine. The purified mutated enzymes resemble wild-type in their enzymatic properties. The threonine and phenylalanine mutants are imidazolinone-resistant and the latter is also slightly sulfonylurea-resistant. The alanine mutant remains sensitive to both herbicides. The results suggest that the β-hydroxyl group is not required for imidazolinone binding and that the size of the side-chain determines resistance.

Key words: Acetohydroxyacid synthase; Enzyme inhibition; Enzyme kinetic; Herbicide-resistance; Site-directed mutagenesis

1. Introduction

Herbicides act by affecting biochemical processes that are either unique to plants or are distinctly different between plants and other organisms. One such process is photosynthesis and inhibition of photosynthetic electron transport is the basis of the action of herbicides such as substituted ureas and triazines [1]. Another useful target in plants is the biosynthesis of amino acids [2] and these pathways are inhibited by glyphosate and phosphinothricin.

The enzymes involved in the biosynthesis of branched chain amino acids have attracted a great deal of interest as potential herbicide targets [3], since the demonstration that the first enzyme in this pathway, acetohydroxyacid synthase (AHAS, EC 4.1.3.18), is inhibited by imidazolinone [4] and sulfonylurea [5] herbicides. AHAS catalyzes the reaction shown as Eq. 1 while the structures of some of these herbicides are illustrated in Fig. 1.

$$\text{pyruvate} + \text{2-ketoacid} \rightarrow \text{2-aceto-2-hydroxyacid} + CO_2 \qquad (1)$$

The enzyme has a dual specificity. When the 2-ketoacid is pyruvate, the product is 2-acetolactate, the precursor of valine and leucine, while 2-ketobutyrate is converted to 2-aceto-2-hydroxybutyrate, the precursor of isoleucine. AHAS requires three cofactors for activity, thiamine diphosphate (ThDP), a divalent metal ion and FAD.

The herbicidal inhibitors are very potent, especially the sulfonylureas which are effective in the nM concentration range. However, their mode of action is yet to be established although they clearly are not analogs of the substrates, acting as uncompetitive (imidazolinones) or non-competitive (sulfonylureas) inhibitors [6,7]. Further, they do not compete with the cofactors [8] leading to the suggestion that they bind to an extraneous site [9] on the enzyme. Consistent with this hypothesis is the observation that several herbicide-resistant mutant forms of the enzyme show very large alterations in the sensitivity to these inhibitors with little change in the activity or kinetics towards substrate or cofactors [10].

Many herbicide-resistant variants of AHAS have been identified (summarized in [10]). These contain point mutations at sites that span almost the entire sequence of the protein but it is likely that these sites cluster together in the three-dimensional structure. Unfortunately, no experimental structure for AHAS has been determined although modeled structures [11,12] have been constructed and in these, the herbicide-resistance mutation sites do coalesce to form a putative herbicide binding site.

The various herbicide-resistance mutation sites are non-equivalent in two different senses. First, almost any substitution at some sites results in resistance (e.g. A-122: *Arabidopsis thaliana* numbering) while at others, such as G-121, only a limited range of substitutions are effective. This may indicate that herbicides have specific interactions with the amino acid side-chain at some sites while at others, a change in size is sufficient to weaken the herbicide binding. The second difference is that at some positions, mutation results in insensitivity to both imidazolinones and sulfonylureas (e.g. W-574) while at others (e.g. A-122), the binding of only one class of herbicide is affected. This observation is consistent with a theoretical model in which the binding sites for imidazolinones and sulfonylureas are overlapping but not coincident [13].

The present study concerns S-653 of *A. thaliana* AHAS. Only a single substitution (S-653-N) has been identified at this site [14] and this mutation results in resistance to imidazolinones with little or no effect on thesulfonylurea sensitivity [10,14]. It is not known if more conservative substitutions are also effective or whether more radical mutations would also result in sulfonylurea-resistance. Here, we investigate the effect of mutation of S-653 to alanine, threonine and phenylalanine. The mutations were introduced into the cloned gene that was expressed in *Escherichia coli*. Each mutant enzyme was purified and its enzymatic properties were determined. The specific activity and the kinetics towards cofactors and substrate of each mutant are similar to the properties of the wild-type. Each mutant was tested for inhibition by the four herbicides illustrated in Fig. 1. The threonine and phenylalanine mutants are each resistant to imazapyr and the phenyl-

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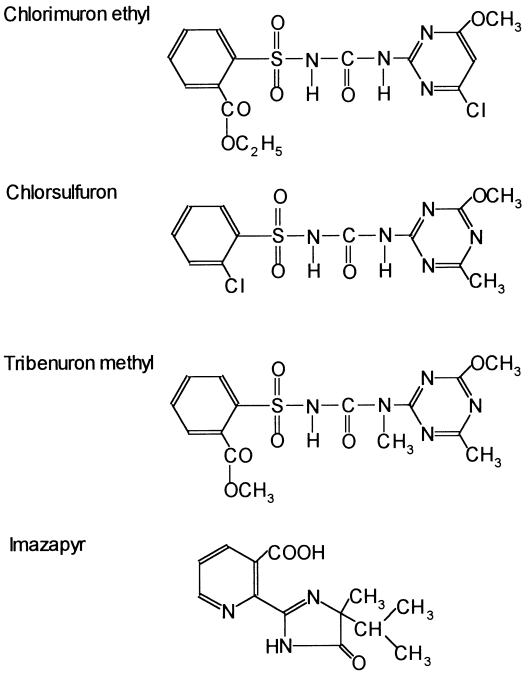

Fig. 1. Structures of the herbicides used in this study. Chlorimuron ethyl, chlorosulfuron and tribenuron methyl are members of the sulfonylurea class, while imazapyr is one of the imidazolinones.

alanine mutant shows a slightly decreased sensitivity to the three sulfonylureas. In contrast, the alanine mutant retains sensitivity to both classes of herbicide.

2. Materials and methods

All materials and experimental procedures were as described previously [10], except as noted below.

2.1. Construction of mutant plasmids

Mutants were prepared using the Altered Sites II (Promega) protocol using the pALTER-AHAS construct which contains the entire AHAS encoding sequence. Mutations were introduced using one of the three oligonucleotides shown below. Lowercase letters indicate a change from the wild-type and underlining shows the position of a restriction enzyme recognition sequence (*Pst*I for S-653-A or *Kpn*I for S-653-T and S-653-F) that was introduced to facilitate screening of mutants.

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                          653
                           ⌐¬
S653A  5′- GCCGATGATCCCtgcaGGTGGCACTTTC          -3′
S653T  5′-   CGATGATCCCGAcTGGTGGtACcTTCAACGATGTC -3′
S653F  5′-  CCGATGATCCCGttTGGTGGtACcTTCAACGATGTC -3′
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Expression vectors corresponding to the wild-type pT7-7-AHAS:T86 plasmid [10] but carrying the desired mutation were constructed by replacing a fragment of the wild-type DNA with the corresponding mutated fragment. pALTER-AHAS carrying the mutation was digested with *Eco*RI and *Age*I and this 1.1 kb fragment was used to replace the corresponding fragment in pT7-7-AHAS:T86. Mutations were confirmed by DNA sequencing using the Dye Terminator Cycle Sequencing Ready Reaction kit (ABI Prism) and DNA Sequencer 373A (Applied Biosystems).

2.2. Expression and purification of AHAS

Enzyme expression and purification followed a method adapted from those used previously for mutants of *A. thaliana* AHAS [10]. Briefly, the cell lysate was fractionated with ammonium sulfate (30–50% cut), then desalted and subjected to ion-exchange chromatography at pH 7.5 on DEAE-Sephacel. Active fractions were pooled and further purified by anion-exchange chromatography at pH 6.5 on Macro-Prep 50 Q. The purified enzyme was stored in small aliquots at −70°C.

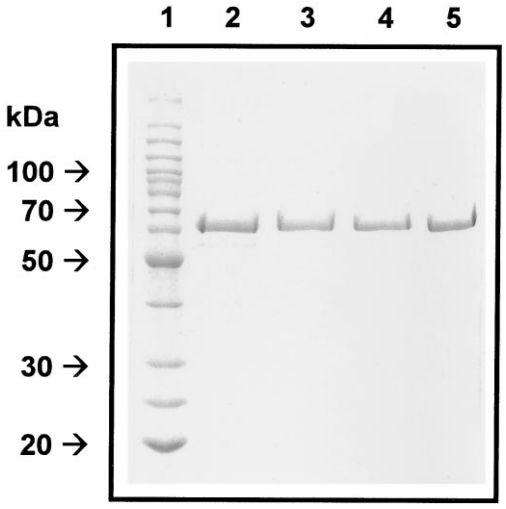

Fig. 2. SDS-PAGE analysis of purified wild-type and mutant *A. thaliana* AHAS. Lane 1: molecular weight standards, lane 2: wild-type, lane 3: S-653-T, lane 4: S-653-F, lane 5: S-653-A.

3. Results

3.1. Expression and purification

After induction of expression, similar levels of AHAS activity were detected in cell lysates for wild-type and all mutants indicating that none of the changes at S-653 destroys its catalytic ability. Each mutant was purified and subjected to SDS-PAGE, which revealed that only minor impurities are present (Fig. 2). The specific activity of each purified S-653 mutant is similar to that of wild-type (Table 1).

Table 1
Kinetic properties and herbicide inhibition of wild-type and S-653 mutants of *A. thaliana* AHAS

Enzyme	Specific activity (U/mg)	Michaelis constant (mM)	Cofactor activation constant			Apparent inhibition constant[a]			
			FAD (μM)	ThDP (μM)	Mg^{2+} (μM)	CE (nM)	CS (nM)	TM (μM)	IM (μM)
Wild-type	5.3	11.6 ± 0.7	1.06 ± 0.09	29 ± 1	77 ± 6	28 ± 4	20 ± 3	0.37 ± 0.03	25 ± 2
S-653-N[b]	8.5	6.7 ± 0.4	0.44 ± 0.04	22 ± 1	98 ± 5	18 ± 1	67 ± 4	1.30 ± 0.06	9610 ± 750
S-653-A	4.9	5.7 ± 0.5	0.82 ± 0.07	23 ± 2	72 ± 5	38 ± 8	17 ± 3	0.60 ± 0.04	30 ± 2
S-653-T	3.9	8.4 ± 0.6	0.72 ± 0.06	33 ± 2	92 ± 8	11 ± 4	37 ± 9	1.06 ± 0.07	2850 ± 290
S-653-F	3.5	19.0 ± 1.4	0.74 ± 0.07	26 ± 1	73 ± 6	118 ± 11	113 ± 12	4.01 ± 0.15	2950 ± 240

[a]CE, chlorimuron ethyl; CS, chlorosulfuron; TM, tribenuron methyl; IM, imazapyr.
[b]From Chang and Duggleby [10].

3.2. Kinetics towards substrate

The kinetics of AHAS towards pyruvate are complex exhibiting negative cooperativity that we have ascribed to interactions between the active sites of this dimeric enzyme [7]. Substrate binding at the second active site is quite weak ($K_{m2} \approx 100$ mM) and it is only possible to estimate binding at the first active site (K_{m1}) with any degree of reliability. Measurement of the substrate kinetics for each of the S-653 mutants investigated here gave a negatively cooperative saturation curve, as is illustrated for S-653-F by the curved scatchard plot shown in Fig. 3A. All mutants gave K_{m1} values that are similar to those of the wild-type (Table 1), in agreement with our previous study of S-653-N [10].

3.3. Cofactor activation

After removal of the cofactors, both wild-type and mutants of *A. thaliana* AHAS show little or no activity upon adding back any two of them. The only exception to this general observation is that some residual activity (2–7%) is observed in the absence of added FAD. Full activity is restored by the third cofactor with a hyperbolic dependence of the rate upon the cofactor concentration. These reactivation curves for S-653-F are illustrated in Fig. 3B (FAD), Fig. 3C (ThDP) and Fig. 3D (Mg^{2+}) with the cofactor concentrations plotted on a logarithmic scale because they spanned large (200–1000-fold) ranges. These data were analyzed to determine cofactor activation constants (Table 1). In each case, there is little difference between the mutants and wild-type apart from a possible small increase in affinity for FAD. We have observed previously that mutation of S-653 to asparagine also results in an increase in the affinity for FAD [10].

3.4. Herbicide inhibition

The sensitivity of each mutant towards herbicides (Fig. 1) was tested using three sulfonylureas and imazapyr, a representative of the imidazolinones. Chlorosulfuron and chlorimuron ethyl are very potent inhibitors with inhibition constants for wild-type *A. thaliana* AHAS of about 30 nM [10]. The concentrations that must be used to study these inhibitors are comparable to that of the enzyme so that tight binding effects must be allowed for in the data analyses. This makes it difficult to determine inhibition constants with a high degree of accuracy. Tribenuron methyl ($K_i \approx 300$ nM) and imazapyr ($K_i \approx 20$ μM) are much weaker inhibitors whose inhibition kinetics can be analyzed without allowance for tight binding effects, leading to more reliable estimates of K_i values. The measured apparent inhibition constants of each mutant for all four inhibitors are listed in Table 1.

S-653-A and S-653-T each had similar inhibition constants to wild-type for the three sulfonylureas. Although there is some variation, in no case, there is more than a 3-fold difference. S-653-F is somewhat different in that the K_i is noticeably higher, by factors ranging from four (chlorimuron ethyl) to 11 (tribenuron methyl). It is a question of semantics whether these elevated K_i values constitute resistance but it is clear that substitution with phenylalanine has a greater effect on the sulfonylurea sensitivity than the other S-653 substitutions.

Much more marked changes were observed in the K_i for

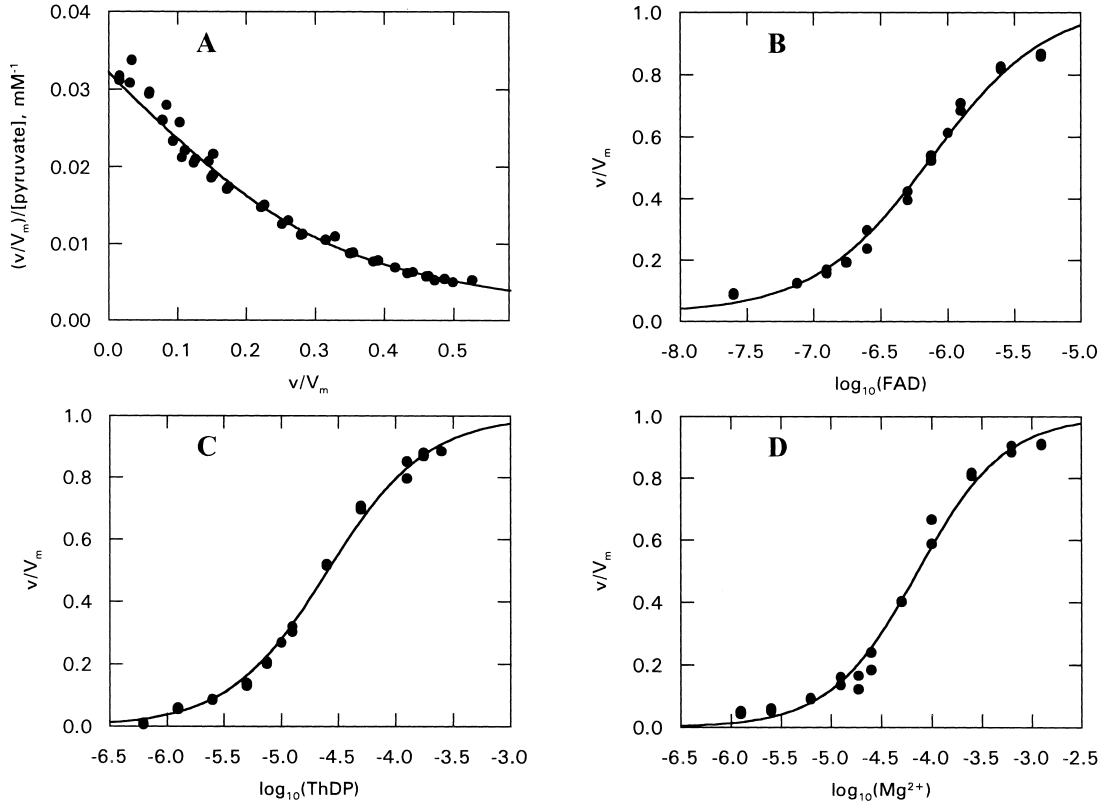

Fig. 3. Kinetic properties of the S-653-F mutant of *A. thaliana* AHAS. A: Substrate saturation curve, shown as a scatchard plot. Cofactor saturation curves for FAD (B), ThDP (C) and Mg^{2+} (D), with cofactor concentrations (*M*) shown on a logarithmic scale. In all cases, rates (*v*) are expressed as a fraction of the maximum velocity (V_m) and the curves shown represent the best fit to the data using the parameters shown in Table 1.

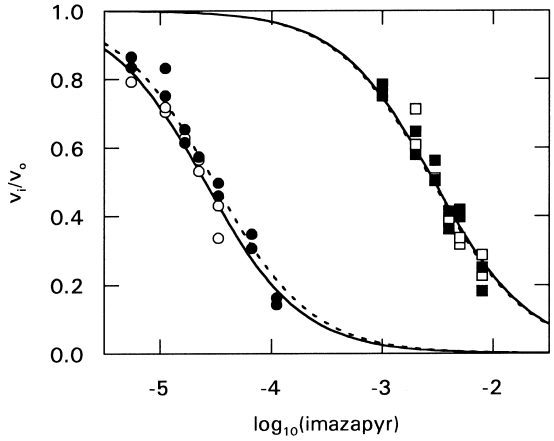

Fig. 4. Inhibition of wild-type and mutant *A. thaliana* AHAS by imazapyr. Wild-type: open circles, solid line; S-653-A: filled circles, dotted line; S-653-F: open squares, solid line; S-653-T: filled squares, dotted line. In all cases, imazapyr concentrations (M) are shown on a logarithmic scale, rates (v_i) are expressed as a fraction of the uninhibited rate (v_o) and the curves shown represent the best fit to the data using the K_i values shown in Table 1.

imazapyr (Fig. 4 and Table 1). S-653-T and S-653-F each show an over 100-fold lower affinity than wild-type having inhibition constants in the mM range and comparable to that of S-653-N. In contrast, S-653-A has a K_i for imazapyr that is indistinguishable from that of wild-type. Thus, substitution of S-653 with any amino acid with a larger side-chain (threonine, asparagine or phenylalanine) greatly impairs binding of this imidazolinone herbicide, while removal of the side-chain hydroxyl (S-653-A) results in an enzyme that retains full sensitivity to imazapyr.

4. Discussion

AHAS is inhibited by sulfonylurea and imidazolinone herbicides and it is through this inhibition that these compounds kill plants. The sulfonylureas are particularly potent, having inhibition constants in the nM range. Mutations of AHAS can reduce the sensitivity to inhibition and several herbicide-resistant forms of the enzyme have been found in this way.

An imidazolinone-resistant line of *A. thaliana* was first identified by Haughn and Somerville [15] and it was later shown [14] that this resistance was due to a single point mutation in AHAS that results in a serine to asparagine mutation at position 653 in the amino acid sequence. The mutant gene was subsequently used to transform tobacco [16] and resulted in 100-fold resistance, thus confirming that the mutation is the sole reason for its herbicide insensitivity.

Although many other mutations have been identified that confer herbicide insensitivity on AHAS (e.g. [17]) the S-653 site is unusual in that only the change to asparagine has been demonstrated to result in resistance. Moreover, the resistance is restricted to imidazolinones with little or no effect on the sulfonylurea sensitivity.

Here, we have investigated the effects of mutating S-653 to alanine, threonine and phenylalanine. The changes to be made were chosen for the following reasons. Phenylalanine is larger than asparagine and we wished to determine whether the additional size of the substitution might result in resistance to sulfonylureas. Asparagine is larger than serine and also lacks the β-hydroxyl group. In order to investigate which of these two differences is more important in the imidazolinone insensitivity, S-653 was mutated to threonine, which is intermediate in size between serine and asparagine but retains the β-hydroxyl group of serine, and alanine to test the effect of the loss of this hydroxyl group.

The three mutated cDNAs were constructed, then, the enzymes were expressed in *E. coli* and purified. All were active with specific activities similar to that of the wild-type enzyme. Thus, none of the changes can have any substantial effect on the active site. This was confirmed by measurements on the kinetics towards substrate and the cofactor activation constants. In all of these respects, the mutants were found to be similar to wild-type AHAS. Similar observations have been made previously for other mutants of *A. thaliana* [10] and *E. coli* [18] AHAS. These results demonstrate that mutations which result in herbicide-resistance occur at locations outside the active site and that this site is distinct from the herbicide binding site. This proposal is consistent with kinetic and physical measurements that have been made previously [6–8], as well as molecular models of AHAS [11,12].

The S-653-F and S-653-T mutants are resistant to imazapyr, one of the imidazolinone herbicides. Other members of this class of compounds were not tested because previous studies have shown that they all have similar inhibitory characteristics. The inhibition by three sulfonylureas was also tested although for two of them (chlorimuron ethyl and chlorosulfuron) it is difficult to measure accurate inhibition constants due to their tight binding properties. S-653-T showed little, if any, resistance to these compounds while the apparent K_i values of S-653-F seem to be somewhat higher than wild-type. A clearer picture emerged for the sulfonylurea tribenuron methyl, which does not show tight characteristics, a small increase in the apparent K_i for S-653-T and an 11-fold increase for S-653-F. As indicated earlier, the binding sites for imidazolinones and sulfonylureas are thought to be overlapping but not coincident [13] and a phenylalanine side-chain at position 653 may be large enough to partially intrude into the region where sulfonylureas bind.

The inhibition properties of the S-653-A mutant are markedly different from the other mutants and are barely distinguishable from those of wild-type AHAS. This result clearly demonstrates that the β-hydroxyl of serine is not required for imidazolinone binding. Further, taken together with the results presented here for S-653-T and S-653-F and the known properties of S-653-N, it appears that any mutation that increases the size of the side-chain at position S-653 will impede imidazolinone binding. This implies that herbicide-resistance has a purely steric origin but it should be noted that in the modeled *A. thaliana* AHAS structure of Ott et al. [12], S-653 appears somewhat distant from the site that is proposed for imidazolinone binding. It is difficult to imagine how any mutation at this site could affect directly imidazolinone binding although these authors concede that their model may be inaccurate in this region. This inconsistency can only be resolved by an experimental structure of AHAS, which we are actively pursuing.

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