Herbicide-resistant forms of *Arabidopsis thaliana* acetohydroxyacid synthase: characterization of the catalytic properties and sensitivity to inhibitors of four defined mutants

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Acetohydroxyacid synthase (AHAS) catalyses the first step in the synthesis of the branched-chain amino acids and is the target of several classes of herbicides. Four mutants (A122V, W574S, W574L and S653N) of the *AHAS* gene from *Arabidopsis thaliana* were constructed, expressed in *Escherichia coli*, and the enzymes were purified. Each mutant form and wild-type was characterized with respect to its catalytic properties and sensitivity to nine herbicides. Each enzyme had a pH optimum near 7.5. The specific activity varied from 13 % (A122V) to 131 % (W574L) of the wild-type and the K_m for pyruvate of the mutants was similar to the wild-type, except for W574L where it was five-fold higher. The activation by cofactors (FAD, Mg^{2+} and thiamine diphosphate) was examined. A122V showed reduced affinity for all three cofactors, whereas S653N bound FAD more strongly than wild-type AHAS. Six sulphonylurea herbicides inhibited A122V to a similar degree as the wild-type but S653N showed a somewhat greater reduction in sensitivity to these compounds. In contrast, the W574 mutants were insensitive to these sulphonylureas, with increases in the K_i^{app} (apparent inhibition constant) of several hundred fold. All four mutants were resistant to three imidazolinone herbicides with decreases in sensitivity ranging from 100-fold to more than 1000-fold.

INTRODUCTION

Valine and leucine are synthesized in plants and many micro-organisms by a common pathway that begins with the formation of 2-acetolactate from two molecules of pyruvate. Isoleucine, the third branched-chain amino acid, is synthesized in a parallel pathway starting with the formation of 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate. Both of these reactions are catalysed by acetohydroxyacid synthase (AHAS, EC 4.1.3.18; also known as acetolactate synthase), an enzyme that has attracted a great deal of interest because it is the target of several classes of herbicides including the sulphonylureas (SUs) [1], the imidazolinones (IMs) [2], the triazolopyrimidines [3] and pyrimidinyl oxybenzoate [4].

Detailed studies of AHAS from plants have been hampered by the difficulty in obtaining the enzyme in pure form, because of its instability and very low abundance. For example, specific activities of plant extracts are usually in the range 10^{-3} to 10^{-2} units/mg [5–7]. Only the maize [8], barley [9] and wheat [7] enzymes have been extensively purified from plants, although the latter has a relatively low specific activity. However, better understanding of the stability of AHAS, coupled with the development of heterologous expression systems, has allowed purification of the cocklebur [10], *Arabidopsis thaliana* [11,12] and tobacco [13] enzymes as fusion proteins, and the expressed *A. thaliana* AHAS has been purified in its native form [14].

Some plants are naturally resistant to particular AHAS inhibitors, allowing these compounds to be used as selective herbicides. For example, cereals are resistant to chlorsulfuron because of their ability to metabolize this compound to non-toxic derivatives [15]. In some cases, this detoxification can be enhanced by treatment with 'safeners' [16]. An alternative process by which resistance to herbicides can be acquired is by increased expression of the enzyme. This mechanism is not common for resistance to AHAS inhibitors although it has been demonstrated, at least in plant cell culture, for other herbicides such as the enolpyruvoylshikimate 3-phosphate synthase inhibitor glyphosate [17]. However, over-expression of mutant AHAS genes has been observed to confer strong resistance to SU and IM herbicides in tobacco [18] and to the SU herbicide chlorsulfuron in *A. thaliana* [19].

Mutation of AHAS to a herbicide-insensitive form appears to be a widespread mechanism for acquired resistance. Although many examples have been reported, there are relatively few cases where the actual mutation has been documented. By far the most extensive study is on yeast AHAS [20], for which a variety of mutations at ten different sites were reported to give rise to SU resistance. These mutations, and some of the others that are known to result in herbicide resistance for AHAS from various species, are summarized in Table 1.

One of the reasons for interest in herbicide-insensitive forms of AHAS is their possible use in construction of transgenic, herbicide-resistant crop plants and this has been achieved in several cases. Most commonly this has been done for tobacco [11,18–20,22,25,27,29,31–33] although transformed, herbicide-resistant crops of other species have also been described [34–36]. The ideal transgene for this purpose is one that encodes an AHAS with identical catalytic properties to the wild-type enzyme but with reduced sensitivity to one or more herbicides. However, the catalytic properties of most AHAS mutants have not been subjected to detailed scrutiny. Nevertheless, in some of the cases where these properties have been examined, changes have been reported.

Alterations in specific activity of cell extracts may arise from

Abbreviations used: AHAS, acetohydroxyacid synthase; DTT, dithiothreitol; IM, imidazolinone; SU, sulphonylurea; ThDP, thiamine diphosphate.

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Table 1 Some mutations in AHAS that confer herbicide resistance

The second column shows mutations that result in herbicide resistance. X indicates that almost any substitution gives rise to herbicide resistance; where several substitutions are effective, these are listed in parentheses. The third column shows the equivalent residue in the *A. thaliana* sequence. Where resistance to a particular group of herbicides is not shown in the fourth column, this may mean that either there is no resistance or, more commonly, that resistance has not been tested. TP, triazolopyrimidine; PB, pyrimidinyl oxybenzoate.

Species	Mutation	*A. thaliana*	Resistance	References
Yeast	G116(NS)	G121	SU	[20]
Yeast	A117X	A122	SU	[20,21]
E. coli	A26V	A122	SU	[21]
A. thaliana	M124E	M124	SU, IM	[11]
A. thaliana	M124I	M124	IM	[11]
A. thaliana	P197S	P197	SU, TP	[22,23]
Yeast	P192(AELQRSVWY)	P197	SU	[20,24]
Tobacco	P196(QS)	P197	SU, IM	[18,25]
A. thaliana	R199(AE)	R199	IM	[11]
Yeast	A200(CDERTVWY)	A205	SU	[20]
Yeast	K251(DENPT)	K256	SU	[20]
Yeast	M354(CKV)	M351	SU	[20]
Yeast	D379(EGNPSVW)	D376	SU	[20]
E. coli	M460(AN)	M570	SU	[26]
Yeast	V583(ACNY)	V571	SU	[20]
Yeast	W586X	W574	SU	[20]
Oilseed rape	W557L	W574	SU, IM, TP	[27]
Tobacco	W573L	W574	SU	[25]
Cocklebur	W552L	W574	SU, IM, TP, PB	[10]
Cotton	W563(CS)	W574	SU, IM	[28]
E. coli	W464(AFLQY)	W574	SU	[26]
Yeast	F590(CGLNR)	F578	SU	[20]
A. thaliana	S653N	S653	IM, PB	[23,29,30]

changes in either the level of expression or in the intrinsic catalytic capacity, and the only way to distinguish these alternatives is by estimating accurately the amount of AHAS protein. This has rarely been done, although Ott et al. [11] have reported an 11-fold reduction in catalytic capacity for the *A. thaliana* AHAS mutant M124E and Bernasconi et al. [10] observed a 5-fold decrease for the W552L mutant of the cocklebur enzyme.

Changes in the K_m for pyruvate have been described for a few AHAS mutants. Subramanian et al. [37] found a 4- to 9-fold increase for a cotton mutant while several variants of *Datura innoxia* show increases of 3- to 16-fold [38]. Changes in the K_m were also reported for *A. thaliana* AHAS mutants [39], although these authors suggested that the variations were not statistically significant. On the other hand, no difference in the K_m was observed for various other herbicide-resistant AHAS mutants [6,37,40,41].

AHAS requires three cofactors, thiamin diphosphate (ThDP), FAD and Mg^{2+} [14] or another divalent metal ion [42]. Herbicide-resistant mutants may show changes in the affinity for any of these cofactors and this seems particularly likely for ThDP and FAD, which appear to bind close to the site for herbicides [11,43]. Subramanian et al. [37] reported no change in the affinity for ThDP in one tobacco and two cotton mutants but data on cofactor affinity of AHAS mutants in the literature are scarce and changes may well occur that have not been described.

In the present study we have constructed four AHAS mutants, expressed them in *E. coli* and purified the enzymes. These mutants were chosen for the following reasons. The first mutation is A122V ($Ala^{122} \rightarrow Val$), which has been observed in yeast (*Saccharomyces cerevisiae*) and *E. coli* AHAS, but not in any plant enzyme (Table 1); it confers SU resistance, although the effects of other herbicides do not appear to have been tested. The most frequently encountered mutation is W574L ($Trp^{574} \rightarrow Leu$), which has been observed in yeast and several plants but not in *A. thaliana* to date (Table 1), and this was chosen as a second mutation. A variety of other mutations at W574, including W574S ($Trp^{574} \rightarrow Ser$), confer SU resistance on yeast AHAS [20] but not, apparently, on most plant enzymes. In fact, it has been suggested for the cocklebur enzyme that this mutation gives rise to an inactive protein [10]. Thus, W574S was chosen as a third mutant for examination. Finally, the fourth mutation S653N ($Ser^{653} \rightarrow Asn$) is the only known naturally occurring mutant giving resistance to IM but not to SU herbicides (Table 1). Each of these mutants, together with the wild-type enzyme, was characterized with respect to its catalytic properties and sensitivity to the six SU herbicides shown in Figure 1(A) and the three IM herbicides shown in Figure 1(B).

MATERIALS AND METHODS

Materials

FAD, ThDP, BSA, dithiothreitol (DTT), PMSF, α-naphthol, creatine hydrate, sodium pyruvate and Tris base were obtained from Sigma Chemical Co. SU herbicides were kindly provided by Dr. H. Brown (DuPont Agricultural Products, Newark, DE, U.S.A.) and IM herbicides were a gift from Dr. B. K. Singh (American Cyanamid, Princeton, NJ, U.S.A.). $(NH_4)_2SO_4$, KCl, $MgCl_2$ and potassium phosphate were purchased from Ajax. Phenyl-Sepharose CL-4B and DEAE-Sephacel were obtained from Pharmacia, and Macro-Prep high Q was obtained from Bio-Rad. Restriction enzymes, T4 DNA ligase and Vent DNA polymerase were purchased from New England Biolabs and oligonucleotides were obtained from the Centre for Molecular and Cellular Biology, The University of Queensland, Australia.

E. coli strain BL21(DE3) [*hsdS gal* (lc*I*ts857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene 1)] was obtained from Novagen, and strain CU1147 was kindly provided by Dr. H. E. Umbarger (Purdue University, West Lafayette, IN, U.S.A.). The expression vectors were obtained from United States Biochemical Corp. (pT7-7), Promega (pALTER-*Ex*1) and Pharmacia (pTrc99A).

Construction of mutant plasmids

The expression vector for wild-type *A. thaliana* AHAS (pT7-7-AHAS:T86) and the plasmid pTrc99A-AHAS were constructed as described previously [14]. Plasmid manipulation generally followed the methodology outlined by Sambrook et al. [44]. Mutants were prepared using the Altered Sites® II (Promega) protocol as follows. The construct pALTER–AHAS was made by cloning the entire AHAS coding sequence (excised from pTrc99A-AHAS), as a *Nco*I–*Xba*I fragment, into the *Nco*I–*Xba*I sites of pALTER-*Ex*1, and mutations were introduced using one of three oligonucleotides shown below (lowercase letters indicate a change from the wild-type):

(1) 5′-TACCCTGGAGGTGt*A*T*C*g*AT*GGAGATTCAC-3′;
(2) 5′-GGTTATGCAATyGGAAGATCGGT-3′;
(3) 5′-ATGATCCCGAaTGGTGGCACT-3′;

These oligonucleotides were phosphorylated using T4 polynucleotide kinase before use. For A122V, oligonucleotide (1), in which there is a mutation (C → T) in the A122 codon (underlined) plus a silent change (A → G) in the following codon, was used. The latter sequence alteration introduces a *Bsp*DI endonuclease recognition site (shown in italics), which was used to identify mutant clones. For the two W574 mutants, oligonucleotide (2) was used, where y is either C or T, yielding a mutation to serine

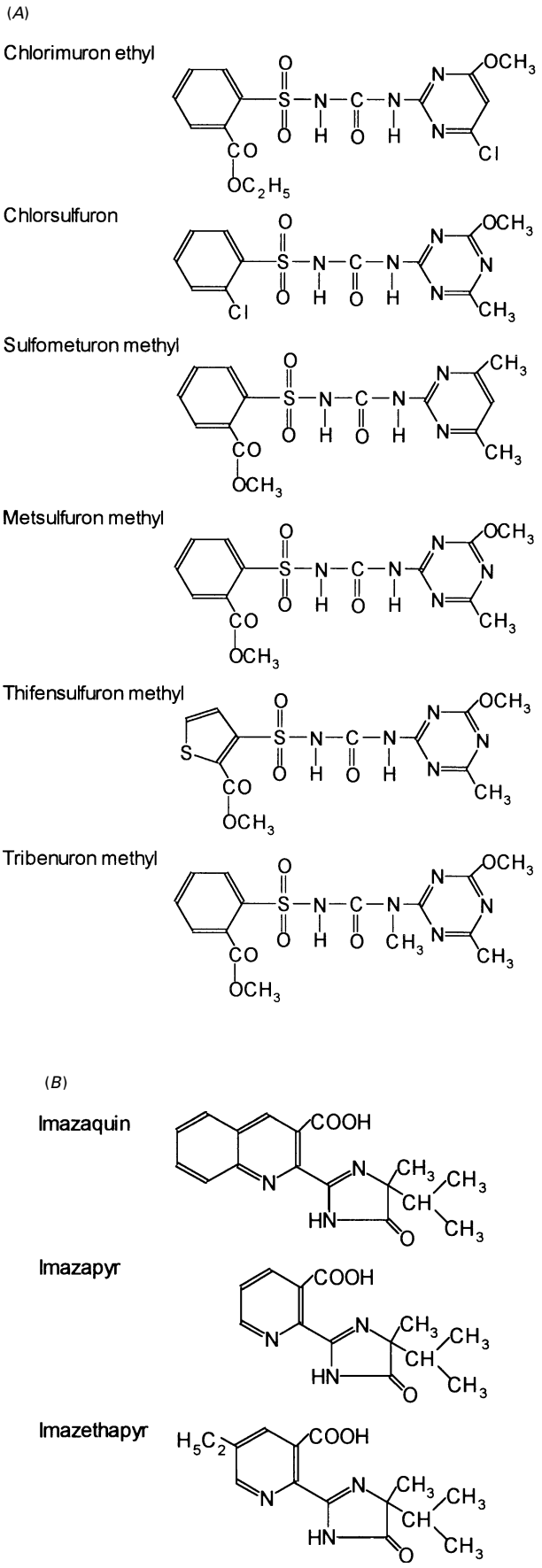

Figure 1 Structures of (A) SU and (B) IM herbicides used in this study

or leucine respectively. Finally, oligonucleotide (3) was used to create the S653N mutation. The mutagenesis reaction was used to transform *E. coli* BMH 71-18 *mutS* cells, which were grown under ampicillin selection at 37 °C, the plasmid was then isolated. This was used to transform *E. coli* JM109 which was grown on Luria-Bertani plates containing 100 μg/ml of ampicillin.

The presence of the A122V mutation was identified by restriction digestion using *Bsp*DI. The mutant gene was also tested for its ability to permit growth of the AHAS$^-$ *E. coli* strain CU1147 on minimal medium in the presence of one of the herbicides. Plasmid DNA was first cut with *Nco*I and *Eco*RI and the smaller *Nco*I–*Eco*RI fragment containing the mutation was used to replace the corresponding fragment of the wild-type gene in pTrc99A-AHAS. The recombinant plasmid obtained was then used to transform CU1147 and plated on to Luria-Bertani plates containing 100 μg/ml of ampicillin, followed by screening on a minimal plate containing 2 μM chlorimuron ethyl or 100 μM imazapyr.

Selection of the W574S, W574L and S653N mutants was based on growth of CU1147. Purified DNA from JM109 was cut with *Eco*RI and *Sma*I in the case of the W574 mutants or *Bgl*II and *Sma*I for S653N, the smaller fragment was then used to replace the corresponding fragment excised from pTrc99A-AHAS. The resulting DNA was transformed into CU1147 and the mutants were selected using herbicides as described above.

Expression vectors corresponding to pT7-7-AHAS:T86 but carrying the desired mutation were constructed by replacing a fragment of the wild-type DNA with the corresponding mutated fragment. pTrc99A-AHAS carrying the mutation was cut with *Eco*RI and *Sma*I (W574 mutants) or *Bgl*II and *Sma*I (S653N) and this fragment was used to replace the corresponding fragment in pT7-7–AHAS:T86. The construction of pT7-7–AHAS:T86 carrying the A122V mutation was as described previously for the construction of the wild-type [14] except that the mutant pTrc-99A–AHAS was used as the DNA template for the PCR step.

In each case, the mutation was confirmed by DNA sequencing using the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism) and DNA Sequencer 373A (Applied Biosystems).

Expression and purification of AHAS

For expression of AHAS, the appropriate plasmid was transformed into *E. coli* strain BL21(DE3) and cells were grown and induced as described previously [14]. Purification of the wild-type enzyme was as described previously [14]. Briefly, the cell lysate was fractionated with 30% $(NH_4)_2SO_4$ and the supernatant subjected to hydrophobic-interaction chromatography using Sepharose CL-4B. This was followed by ion-exchange chromatography at pH 7.5 and then at pH 6.5 on Macro-Prep 50 Q. The purified enzyme, which contained 22% of the initial activity present in the cell extract, was stored in small aliquots at -70 °C. The final product had a specific activity of 7.9 units/mg of protein.

Mutant AHAS was purified using similar procedures but with the following variations. For S653N, the anion-exchange matrix at pH 7.5 was replaced with DEAE–Sephacel and, for W574L, this step was omitted. DEAE–Sephacel at pH 7.5 was also used for A122V AHAS, but for this enzyme the phenyl-Sepharose chromatography was omitted and the enzyme was precipitated using $(NH_4)_2SO_4$ (30–50% cut) and desalted before anion-exchange chromatography.

For cofactor saturation studies of wild-type AHAS, the enzyme obtained from the final anion-exchange chromatography step was transferred into a buffer containing 50 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 15% (v/v) glycerol, 0.2 mM DTT and 1 mM

EDTA. For the study of Mg^{2+} activation, the Mg^{2+}-free enzyme was prepared using an alternative method by exchange into a buffer containing 50 mM Tris/HCl (pH 8.0), 15 % glycerol (v/v), 0.2 mM DTT and 1 mM EDTA followed by a second exchange into a buffer containing 25 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 5 % (v/v) glycerol and 0.2 mM DTT. Buffer exchange was performed by column chromatography using a Pharmacia PD10 column. All of the mutant enzymes were prepared for cofactor saturation studies using this second method.

AHAS assay

During purification of AHAS, activity was assayed using the colorimetric method of Singh et al. [45] exactly as described previously [14]. One unit of enzyme activity is defined as the production of 1 μmol acetolactate/min in this reaction. For all kinetic studies, AHAS activity was measured using a continuous assay, which monitored the consumption of pyruvate directly at 333 nm ($\epsilon_M = 17.5\ M^{-1}\cdot cm^{-1}$ [42]); assay conditions were identical to those used in the colorimetric assay except that the concentrations of pyruvate, cofactors or inhibitors were varied as appropriate for the particular experiment. For cofactor activation studies, the enzyme was equilibrated with saturating concentrations of two of the cofactors and the reaction was started by adding pyruvate plus the missing cofactor. For other kinetic experiments, the enzyme was equilibrated with all cofactors and the reaction was started by adding pyruvate plus, where appropriate, one of the herbicides. Samples were assayed in a 200 μl reaction mixture in a microtiter plate at 30 °C.

Protein determination and SDS/PAGE

Protein concentrations were determined by the dye-binding method of Sedmak and Grossberg [46] using BSA as standard. SDS/PAGE was performed using the method of Laemmli [47]. Proteins were separated on a 10 % (w/v) polyacrylamide gel using a Bio-Rad Minigel apparatus and were detected by staining with 0.1 % (w/v) Coomassie Blue.

Computer programs

Fitting of equations to experimental data was performed using either GraphPad Inplot® (GraphPad Software Inc.), GraFit® (Erithacus Software Ltd.) or programs based on DNRP53 [48].

RESULTS

Purification

The purification of the four *A. thaliana* mutants of AHAS is summarized in Table 2 and in each case the final product appeared to be close to purity by SDS/PAGE (Figure 2). All mutant forms were expressed quite well; taking the final specific activity of the purified product as indicative of that of the pure enzyme, the amount of each mutated enzyme in the cell extract ranged from 3.6 % of total soluble protein for A122V, to 14.3 % for W574S.

The initial cell extracts obtained from the expression of W574L and S653N had specific activities that were similar to those obtained previously (0.96 unit/mg of protein) for the wild-type enzyme [14] and the final specific activities and yields were also comparable to the wild-type values of 7.88 units/mg and 22 % respectively. The initial specific activity of W574S was somewhat lower and this appeared to be due mainly to the lower intrinsic activity of this mutant because the final specific activity was also lower than that of the wild-type by a similar fraction. However, the final yield of this mutant was also quite low, with substantial losses occurring during hydrophobic chromatography and the first anion-exchange chromatography step. The lowest initial specific activity, by an order of magnitude, was for A122V. Although this was due in part to a lower intrinsic activity, the expression level for this mutant was also low. Despite the low activity, the purification was quite good with an overall yield of 43 %, which was higher than for any of the other variants of AHAS.

Table 2 Purification of *A. thaliana* mutant AHAS

Purification step	Enzyme (units)	Protein (mg)	Specific activity (units/mg of protein)	Yield (%)
A122V				
Cell extract	29.3	783	0.04	100
$(NH_4)_2SO_4$	25.4	289	0.09	87
Anion exchange (pH 7.5)	21.8	53	0.41	74
Anion exchange (pH 6.5)	12.5	12	1.04	43
W574S				
Cell extract	277	763	0.36	100
$(NH_4)_2SO_4$	201	534	0.38	73
Phenyl-Sepharose	44	175	0.25	16
Anion exchange (pH 7.5)	15	10	1.53	5
Anion exchange (pH 6.5)	11	4.4	2.52	4
W574L				
Cell extract	450	688	0.66	100
$(NH_4)_2SO_4$	411	616	0.67	91
Phenyl-Sepharose	405	229	1.77	90
Anion exchange (pH 6.5)	116	11	10.29	26
S653N				
Cell extract	391	515	0.76	100
$(NH_4)_2SO_4$	356	424	0.84	94
Phenyl-Sepharose	285	193	1.48	73
Anion exchange (pH 7.5)	124	22	5.62	32
Anion exchange (pH 6.5)	65	7.7	8.51	17

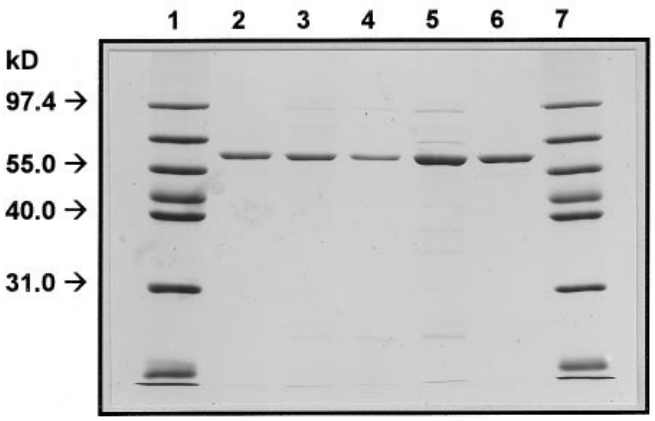

Figure 2 SDS/PAGE of purified *A. thaliana* AHAS and its mutants

Lanes 1 and 7, molecular-mass markers (kD, kDa); lane 2, wild-type; lane 3, A122V; lane 4, W574S; lane 5, W574L; lane 6, S653N.

Enzyme stability

We have shown previously [14] that purified wild-type AHAS was moderately stable, losing 10 % activity after three weeks,

Table 3 Stability of *A. thaliana* mutant AHAS

The enzymes obtained from the final step of the purification procedure were stored in the dark at the temperatures shown. The enzyme activity was measured after storage for the periods indicated, and the loss of activity was calculated as a percentage of the starting activity. Enzymes stored at −70 °C were subjected to one (7 days) or two (21 days) freeze–thaw cycles before assay. Protein concentrations for the enzymes were: 1.05 mg/ml (A122V); 1.42 mg/ml (W574S); 0.48 mg/ml (W574L); and 0.32 mg/ml (S653N).

	Loss of activity at 4 °C (%)		Loss of activity at −70 °C (%)	
Enzyme	7 days	21 days	7 days	21 days
A122V	4	30	< 1	15
W574S	< 1	6	< 1	< 1
W574L	5	20	< 1	3
S653N	< 1	18	< 1	< 1

and 23 % after five weeks of storage at 4 °C. At −70 °C, stability was better with losses of 4 % (three weeks, with three freeze-thaw cycles) and 8 % (five weeks, with five freeze-thaw cycles); with no intervening freeze-thaw cycles, only 9–10 % activity was lost in six months.

The stability of each of the mutants at 4 °C and at −70 °C is shown in Table 3. W574S was the most stable, with no detectable loss of activity at −70 °C and only 6 % after three weeks at 4 °C; stability was similar to that of the wild-type enzyme. This was somewhat unexpected because losses during purification were greatest for this mutant (Table 2), and suggests that the low yield was not due to enzyme inactivation. A122V was the least stable, losing 15 % activity in three weeks at −70 °C with one intervening freeze-thaw cycle. Nevertheless, the instability was not so severe that the enzyme was unusable but it was stored at −70 °C and used within a few weeks of preparation. The stability of the remaining two mutants was intermediate between that of W574S and A122V.

Effect of pH on activity

The pH activity profile of wild-type AHAS and each of the four mutants was determined over the pH range 5.5–9.5 (results not shown). While some variations were noted at extremes of pH, all

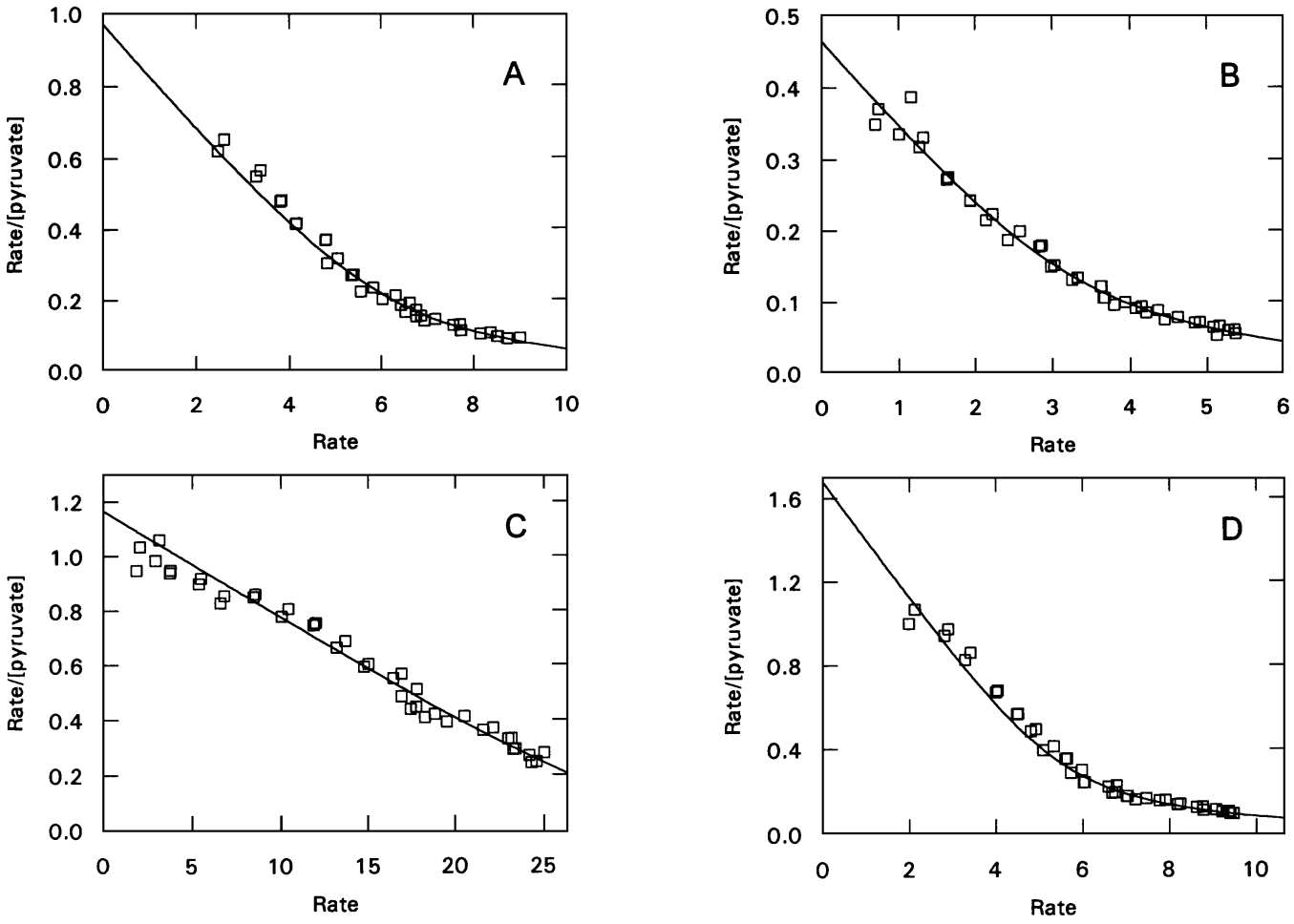

Figure 3 Pyruvate saturation curves of *A. thaliana* AHAS mutants

Rates (ΔmA_{333}/min) were determined as a function of pyruvate concentration (mM) and the data are shown in the form of a Scatchard plot. (**A**) A122V; (**B**) W574S; (**C**) W574L; (**D**) S653N. The lines represent the best fit to the data of eqn. (1).

Table 4 Substrate kinetics of *A. thaliana* and mutant AHAS

Data illustrated in Figure 3 were analysed by fitting eqn. (1), with K_{m2} fixed at 100 mM as described previously [14]; the results for the wild-type enzyme were taken from [14]. The data shown are the best-fit values ± S.E.

Enzyme	Kinetic parameter		
	k_{c2} (sec^{-1})	K_{m1} (mM)	ρ
A122V	6.9 ± 0.3	12.6 ± 1.0	0.812 ± 0.054
W574S	11.2 ± 0.3	14.8 ± 1.1	0.674 ± 0.042
W574L	47.9 ± 3.9	42.7 ± 4.3	1.406 ± 0.205
S653N	36.0 ± 0.8	6.7 ± 0.4	0.635 ± 0.028
Wild-type	27.8 ± 0.9	8.0 ± 0.7	0.935 ± 0.052

enzymes showed maximum activity in the pH range from 7.0 to 7.5, and the latter pH was chosen for all kinetic studies.

Kinetics towards pyruvate

We have shown previously that the substrate saturation curve of *A. thaliana* AHAS did not follow Michaelis–Menten kinetics, and have pointed out that this may be a general feature of plant AHAS [14]. The data exhibited negative co-operativity, which we have interpreted as arising from interactions between the subunits of this homodimeric enzyme. The dependence of rate (v) on the substrate concentration ([A]) conforms to eqn. (1):

$$v = V_{max}[A]([A]+\rho K_{m2})/([A]^2+2K_{m2}[A]+K_{m1}K_{m2}) \quad (1)$$

where V_{max} is the maximum velocity, K_{m1} and K_{m2} represent the binding of substrate to the first and second active site and $\rho = k_{c1}/k_{c2}$, the ratio of the catalytic-rate constants at the first and second active sites. V_{max} is equivalent to $k_{c2}[E]_o$, where $[E]_o$ is the concentration of enzyme subunits.

The substrate saturation curve for each of the mutants is shown in Figure 3 in the form of Scatchard plots. The upward curvature in these plots is characteristic of negatively co-operative kinetics; if the data followed hyperbolic saturation curves, the Scatchard plots would be linear. The data are described by eqn. (1), as shown by the lines in Figure 3; the values of the various kinetic parameters are summarized in Table 4.

The values of ρ vary somewhat from the wild-type value of 0.935, ranging from 0.635 for S653N to 1.406 for W574L. Similar variations have been observed from one experiment to another for the wild-type enzyme [14]. Thus none of the mutants appeared to be substantially different from the wild-type in this respect; ρ was close to unity, which meant that the catalytic rate constants at the first and second active sites were similar.

The values of K_{m1} showed differences from the wild-type enzyme, particularly for W574L where the value was elevated by more than five-fold. This was well outside the variation observed between experiments for the wild-type. The remaining three mutants showed rather small, but we believe real, differences from the wild-type enzyme.

The estimates of k_{c2} showed sizable differences from the wild-type; when these values were converted to specific activities they corresponded to 6.4, 10.7, 44.5, 33.8 and 26.1 units/mg for A122V, W574S, W574L, S653N and wild-type enzymes respectively. These values were all substantially higher than the specific activities measured using the purified enzyme (Table 2 and [14]). However, there are some important differences between the assay methods that contribute to the higher values obtained from the substrate saturation curves. First, the k_{c2} values were derived from extrapolated rates at saturating pyruvate, whereas in the assay used during the purification procedure a fixed concentration of 50 mM pyruvate was used. The specific activity at this substrate concentration can be predicted using the results

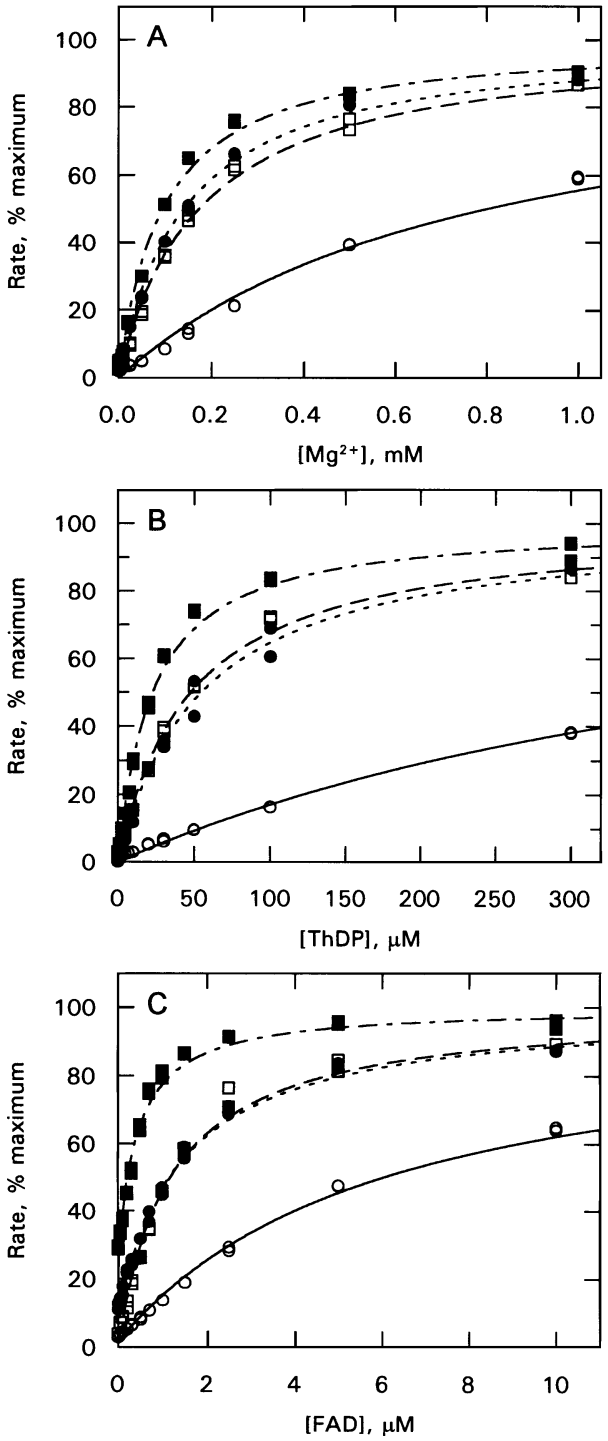

Figure 4 Cofactor saturation curves of *A. thaliana* AHAS mutants

Data are plotted as rate (as a percentage of the value at saturating cofactor) versus the cofactor concentration. (**A**) Mg^{2+}; (**B**) ThDP; (**C**) FAD. Some data, obtained at higher cofactor concentrations, have been omitted to avoid compression of the abscissa. The lines represent the best fit to the data of eqn. (2) for A122V (○, solid line); W574S (●, dotted line); W574L (□, broken line); S653N (■, broken–dotted line).

Table 5 Cofactor activation of *A. thaliana* and mutant AHAS

Data illustrated in Figure 4 were analysed by fitting eqn. (2), with v_o set at zero except for FAD activation of W574S and S653N. Values for activation of the wild-type enzyme by ThDP and by FAD are taken from Chang and Duggleby [14], and that for Mg^{2+} was re-determined in the present study. The value for Mg^{2+} (198 μM) reported previously was incorrect due to carry-over of EDTA into the assay mixture. The data shown are the best-fit values ± S.E.

	K_c		
Enzyme	Mg^{2+} (μM)	ThDP (μM)	FAD (μM)
A122V	797 ± 38	483 ± 27	5.55 ± 0.30
W574S	140 ± 6	54 ± 3	1.46 ± 0.10
W574L	172 ± 7	47 ± 2	1.16 ± 0.06
S653N	98 ± 5	22 ± 1	0.44 ± 0.04
Wild-type	64 ± 4	25 ± 1	1.46 ± 0.22

in Table 4; this calculation yields values of 3.1, 4.5, 25.3, 14.6 and 14.1 units/mg for A122V, W574S, W574L, S653N and wild-type enzymes respectively. Secondly, during purification a fixed incubation of 30 min was used for the colorimetric assay, followed by determination of the total acetolactate formed during this period. However, the rate of acetolactate formation may not be constant because of substrate depletion, inhibition by accumulating product and enzyme instability. The latter effect would be exacerbated by the lower enzyme concentrations that are used in the more sensitive colorimetric assay. Thus the most notable difference in specific activity between the continuous and colorimetric assay (a factor of 3.0) was for A122V, the least stable of the enzymes. The second highest difference (a factor of 2.5) was for W574L, the enzyme with the highest K_{m1} and for which substrate depletion is likely to have the most influence. For the remaining three enzymes, the difference was smaller (factors of 1.7 to 1.8) and might well be due to inhibition by accumulating product in the colorimetric assay.

Cofactor activation

AHAS requires FAD, ThDP and Mg^{2+} for activity and for the wild-type enzyme there was very little activity when any one cofactor was omitted from the assay, provided that the enzyme was first thoroughly exchanged against cofactor-free buffer. Supplying the missing cofactor in the assay resulted in restoration of total activity. It has been reported [49] that for barley and *E. coli* (isoenzyme II) AHAS, reactivation by ThDP was time-dependent and we have also observed lags of a few minutes before attainment of full activity after addition of this cofactor (results not shown). A detailed investigation of this phenomenon was not carried out and rates were measured after the lag had elapsed. Consequently, the activation constants reported here for *A. thaliana* AHAS correspond to the final steady-state phase of the time-dependent activation.

Each of the four mutant enzymes was also inactive when either ThDP or Mg^{2+} was omitted but W574S and S653N showed 10% and 25% of maximal activity respectively when FAD was omitted. Apart from these two anomalies, cofactor activation followed hyperbolic saturation curves (Figure 4) and was analysed using eqn. (2) to obtain values for K_c, the cofactor activation constants:

$$v = v_o + V_{max}[C]/([C] + K_c) \quad (2)$$

Except for the activation of W574S and S653N by FAD, the rate with no added cofactor [v_o in eqn. (2)] was set at zero. The values of K_c obtained for the wild-type and for each mutant enzyme, for each of the cofactors, are summarized in Table 5. It is of interest that the mutant that showed the greatest residual activity without added FAD is that which binds this cofactor most strongly. Thus it appears probable that the residual activity for these mutants was simply a reflection of the difficulty in removing FAD rather than any FAD-independent activity. Although an FAD-independent form of the enzyme does exist [50,51] we do not think that *A. thaliana* AHAS is capable of catalysis in the absence of FAD, despite the fact that the role of FAD in AHAS activity has not been established [52]. A122V bound all cofactors more weakly than did the wild-type enzyme, especially ThDP for which the activation constant was elevated by almost 20-fold. The other mutants had activation constants for Mg^{2+} and ThDP that were within a factor of 3 of the wild-type values.

Inhibition by SU herbicides

Quantitative characterization of the inhibition of AHAS was difficult for several reasons. First, as mentioned above, the kinetics with respect to the substrate are not straightforward so classical methods for the determination of inhibition constants cannot be applied. Secondly, as we [14,53] and others [7,52,54] have shown, SU herbicides are slow-binding inhibitors and the effects of these compounds on initial and final steady-state rates can differ substantially. It is only by using the continuous assay [42], where pyruvate consumption is measured directly, that true rates can be determined. Almost all of the data in the literature on herbicide inhibition of AHAS was obtained using the colorimetric assay after a prolonged incubation (usually 30 min to 2 h) and the measured rates reflect neither the initial nor final values. Consequently, any inhibition parameters derived from these data might be questionable and this may have contributed to the variation in reported estimates. For example, Southan and Copeland [7] observed that wheat AHAS was 50% inhibited by 600 nM chlorsulfuron while Ray [54] calculated a value of 22 nM. Thirdly, some of the SU herbicides are such potent inhibitors that they are used at concentrations which are comparable to the enzyme concentration and tight-binding effects, where combination with the enzyme significantly changes the concentration of inhibitor in solution, should be, but are not always, taken into account.

We have shown previously [14] that the apparent inhibition constant (K_i^{app}) of wild-type AHAS for chlorsulfuron can be determined accurately, provided that tight-binding effects are allowed for and that initial rates are measured. The data are well described by eqn. (3):

$$v_i^2[E]_o + v_i v_o([I]_o - [E]_o + K_i^{app}) - v_o^2 K_i^{app} = 0 \quad (3)$$

where v_i and v_o represent the respective rates with and without inhibitor, while $[E]_o$ and $[I]_o$ are the total molar concentrations of enzyme and inhibitor. It should be noted that for tight-binding inhibitors, K_i^{app} is not equivalent to IC_{50}, the concentration of inhibitor giving 50% inhibition. Rather, IC_{50} is equal to $[E]_o/2 + K_i^{app}$ [55]. In cases where inhibition was not so strong, allowance for tight-binding was unnecessary and the curves may be analysed using eqn. (4):

$$v_i = v_o/(1 + [I]/K_i^{app}) \quad (4)$$

Because our previous measurements showed that K_i^{app} was independent of the pyruvate concentration, valid comparisons can be made between mutants and wild-type, and between inhibitors, by measuring inhibition curves obtained at a single pyruvate concentration. The inhibition curves for each of the SU

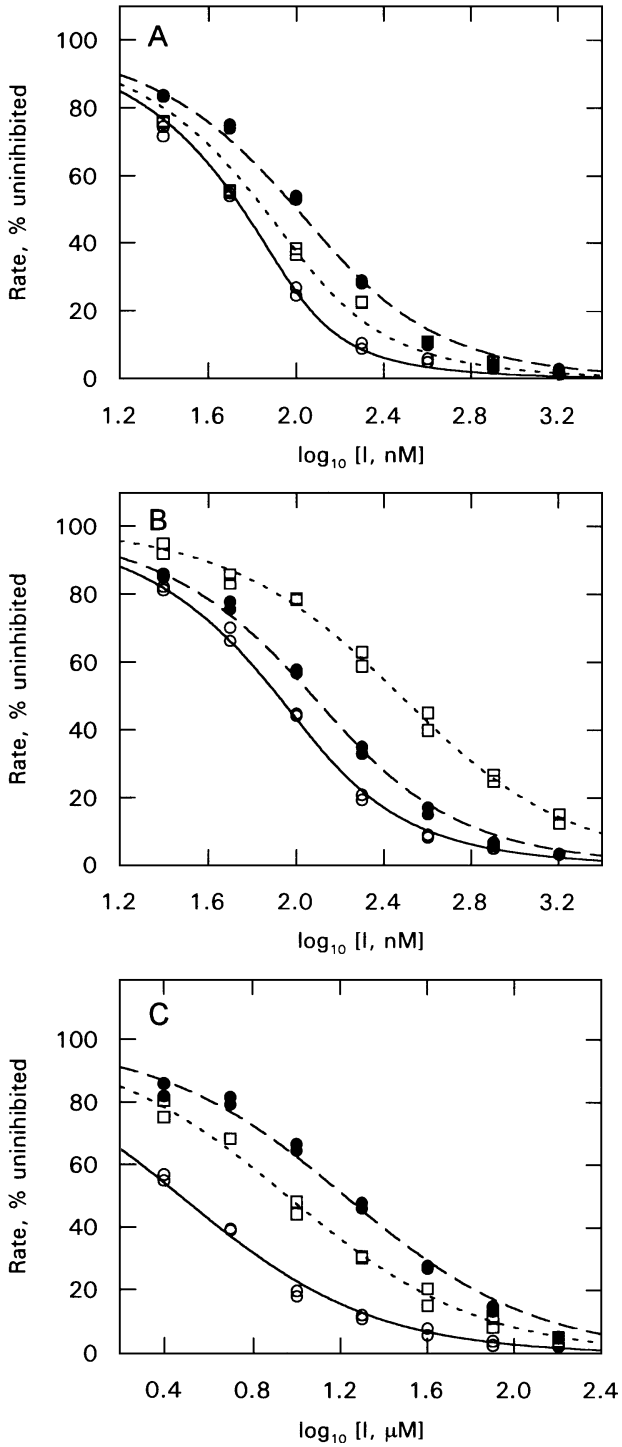

Figure 5 Inhibition of the wild-type *A. thaliana* AHAS by SU and IM herbicides

Data are plotted as rate (as a percentage of the uninhibited value) versus $\log_{10}$ of the inhibitor concentration. (**A**) Chlorimuron ethyl (○, solid line); chlorsulfuron (●, broken line); sulfometuron methyl (□, dotted line). (**B**) Metsulfuron methyl (○, solid line); thifensulfuron methyl (●, broken line); tribenuron methyl (□, dotted line). (**C**) Imazaquin (○, solid line); imazapyr (●, broken line); imazethapyr (□, dotted line). The lines in (**A**) and (**B**) represent the best fit to the data of eqn. (3) using $[E]_0 = 92.5$ nM and the K_i^{app} values reported in Table 6. In (**C**), the lines represent the best fit of eqn. (4) using the K_i^{app} values reported in Table 8.

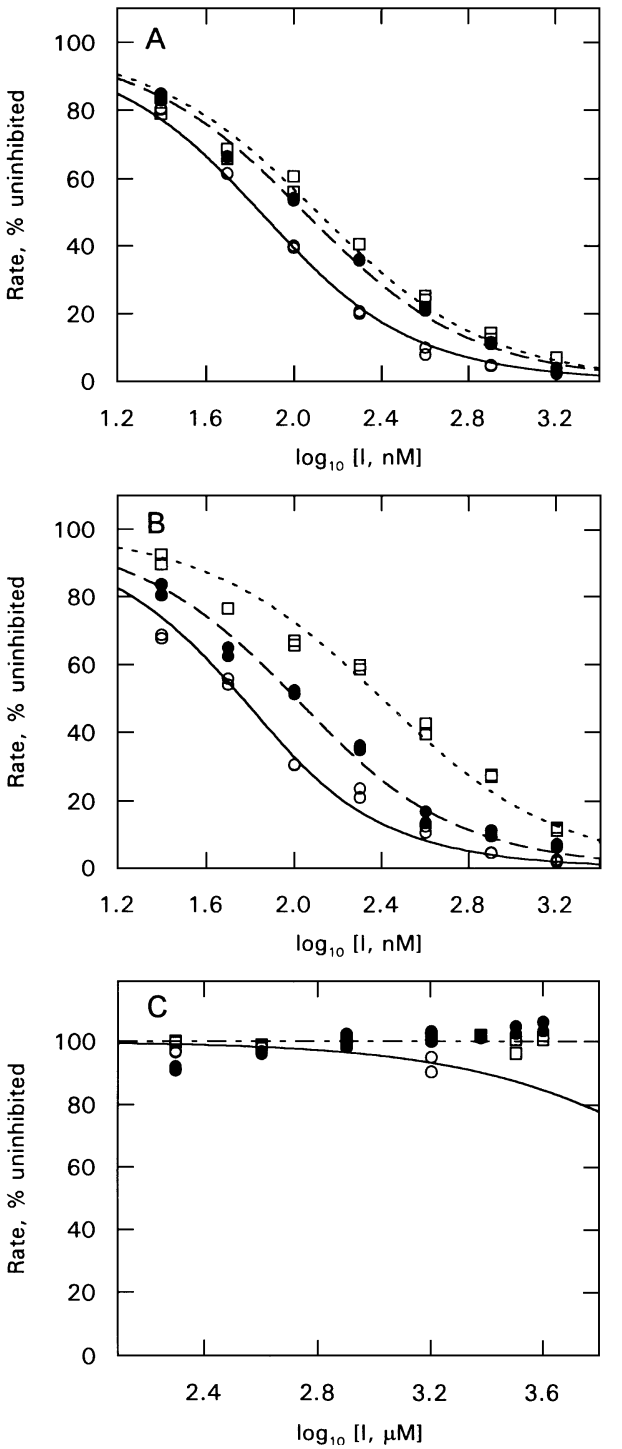

Figure 6 Inhibition of the A122V mutant of *A. thaliana* AHAS by SU and IM herbicides

As for Figure 5, except that $[E]_0 = 52.9$ nM was used.

inhibitors shown in Figure 1(A) are illustrated in Figures 5(A) and 5(B), wild-type; 6(A) and 6(B), A122V; 7(A) and 7(B), W574S; 8(A) and 8(B), W574L, and 9(A) and 9(B), S653N. The K_i^{app} derived from these curves are summarized in Table 6 and the resistance factors (mutant K_i^{app}/wild-type K_i^{app}) are shown in Table 7.

A122V was only weakly resistant to these herbicides; the K_i^{app} was elevated by, at most, a factor of 4.1 (for chlorimuron ethyl, Table 7) but this was a small difference when compared with the

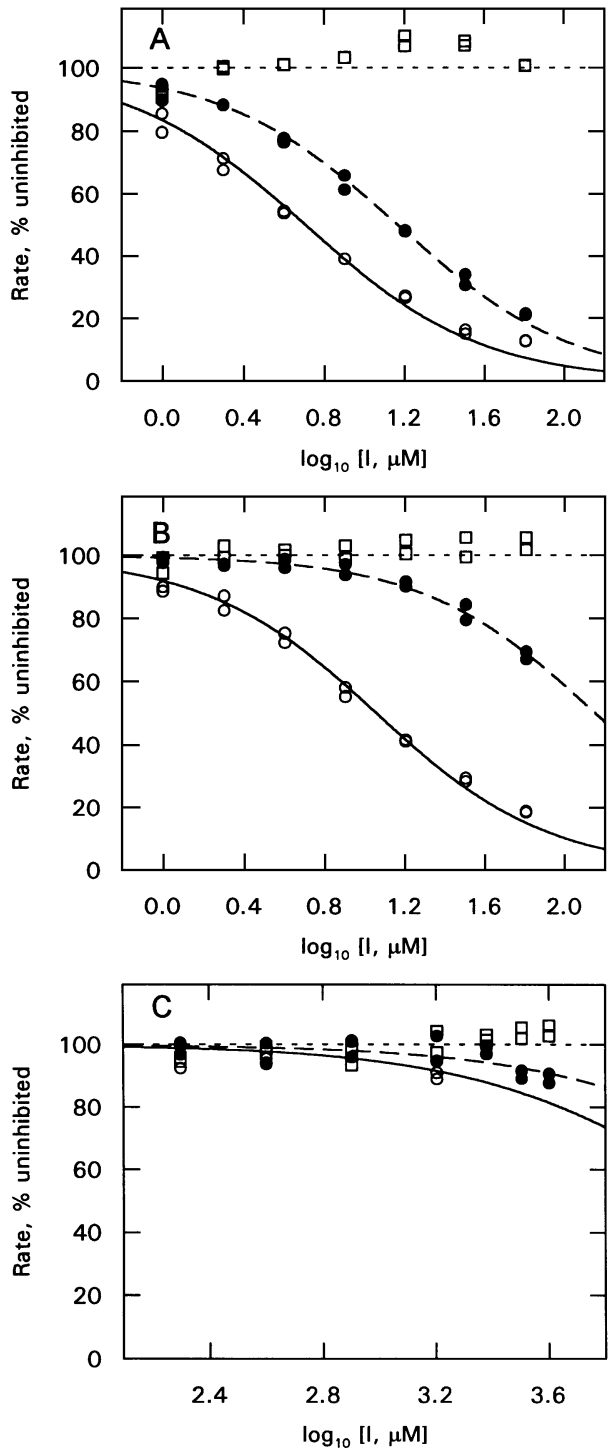

Figure 7 Inhibition of the W574S mutant of *A. thaliana* AHAS by SU and IM herbicides

As for Figure 5, except that all lines are best fits to the data of eqn. (4).

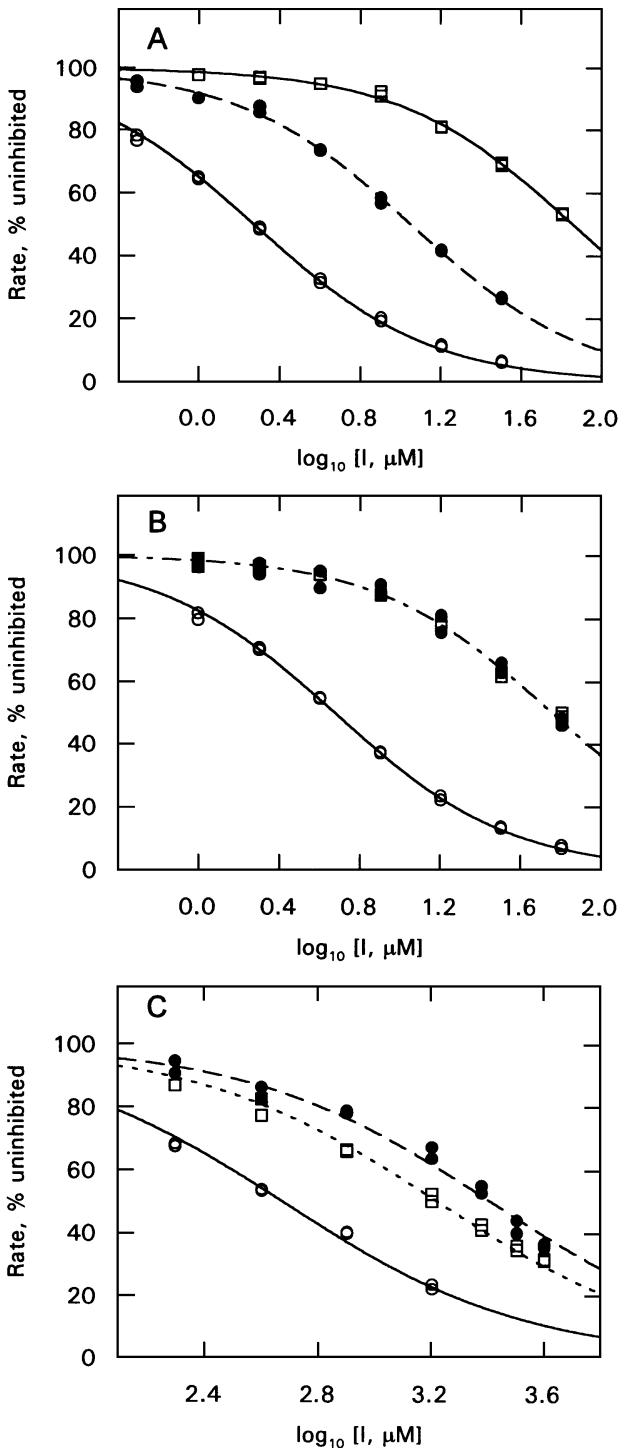

Figure 8 Inhibition of the W574L mutant of *A. thaliana* AHAS by SU and IM herbicides

As for Figure 5, except that all lines are best fits to the data of eqn. (4).

W574 mutant enzymes discussed below. This result was unexpected because the equivalent mutation in yeast [20] and *E. coli* [21] AHAS has been reported to confer SU resistance.

The S653N mutant also remained sensitive to SU herbicides although some reduction in sensitivity was observed, particularly with respect to sulfometuron methyl, thifensulfuron methyl and tribenuron methyl for which the K_i^{app} was increased by factors of more than 5 (Table 7).

The two W574 mutants were resistant to all of the SU herbicides, with increases in K_i^{app} by at least 130-fold and in several instances more than 1000-fold (Table 7). Indeed, in some cases, such as the effect of sulfometuron methyl on W574S

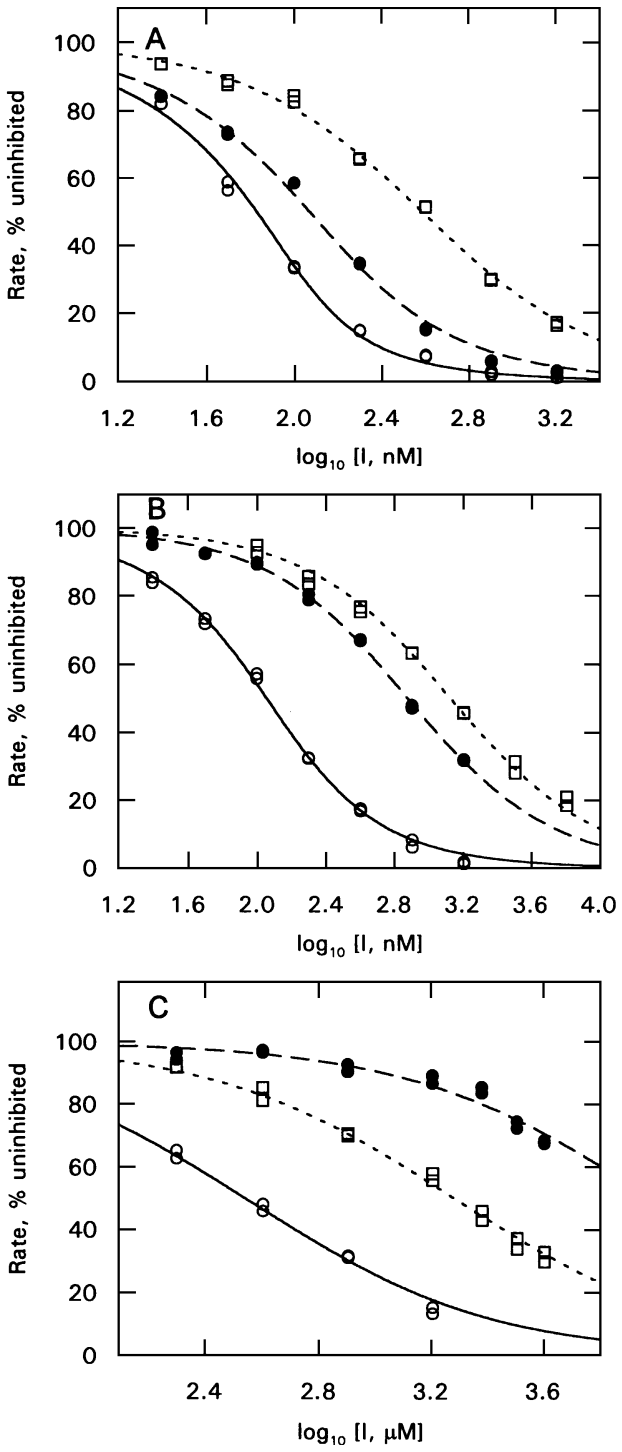

Figure 9 Inhibition of the S653N mutant of *A. thaliana* AHAS by SU and IM herbicides

As for Figure 5, except that $[E]_0 = 99.8$ nM was used.

(Figure 7B), inhibition was so weak that it was undetectable at the highest concentration tested (64 μM). In this situation, it was possible only to place a lower limit on the value of K_i^{app}, which we did by assuming that 20% inhibition would have been detected easily, and which implies that K_i^{app} must be at least four times the highest inhibitor concentration used in the experiment.

In all cases, the W574S mutation resulted in higher resistance to the SU herbicides than the W574L mutation (Table 7), possibly because replacing tryptophan with serine was a more radical substitution than replacement with leucine.

Inhibition by IM herbicides

The effect of IM herbicides on AHAS is complex for the same reasons that were outlined above for the SU inhibitors, except that none of these compounds is sufficiently potent to allow tight binding to occur. We have demonstrated previously [14] that the initial-rate inhibition of wild-type AHAS by imazapyr was of the uncompetitive type.

One of the characteristics of uncompetitive inhibition is that it becomes more pronounced at higher substrate concentrations and, as saturation is approached, K_i^{app} extrapolates to a true K_i. For this reason, together with the fact that all of the mutants are quite insensitive to IM herbicides, inhibition curves were measured at a single high pyruvate concentration of 100 mM. These are shown in Figures 5(C), wild-type; 6(C), A122V; 7(C), W574S; 8(C), W574L, and 9(C) S653N. The values of K_i^{app} obtained by fitting eqn. (4) to the data are summarized in Table 8 and the resistance factors are shown in Table 7.

Neither mutations A122V nor W574S have been reported previously to confer resistance to IM herbicides but these mutant enzymes were totally insensitive to imazaquin, imazethapyr and imazapyr (Table 8). In contrast, the mutation W574L is known to result in IM resistance for the oilseed rape [27] and cocklebur [10] enzymes and our results extend this to *A. thaliana* AHAS. Curiously, W574L was less resistant to IM herbicides than was W574S (Table 7). Finally, we have confirmed that the S653N mutation results in resistance to imazapyr [23,29,30] and have shown that it is cross-resistant to the other IM herbicides, imazaquin and imazethapyr.

DISCUSSION

In this paper we report the construction and expression in *E. coli* of four mutants of the *A. thaliana AHAS* gene. Each expressed enzyme was purified and characterized with respect to its stability, pH-activity profile, kinetics towards the substrate (pyruvate), activation by the three cofactors (Mg^{2+}, ThDP and FAD), and inhibition by six SU herbicides and three IM herbicides.

Purification

The specific activities of the mutant enzymes range from 1.04 units/mg of protein for A122V to 10.3 units/mg of protein for W574L (Table 2), whereas that of the wild-type was 7.9 units/mg of protein [14]. For comparison, the highest reported specific activity of purified barley AHAS was 3.1 units/mg [56], that of maize was 0.67 units/mg [8], and the specific activity of wheat enzyme was only 0.06 units/mg [7]. A further comparison can be made with plant AHAS that has been expressed as a fusion protein in *E. coli*. After removal by proteolysis of the fusion partner, or correcting for its mass, the specific activities were 0.61 (tobacco) [13], 3.0 (*A. thaliana*) [12] and 6.2 (cocklebur) [10]. Thus the upper limit for plant AHAS appears to be approximately 10 units/mg of protein, which is somewhat below that of most bacterial forms of AHAS for which reported specific activity values are 8.0 (*E. coli* isoenzyme I [57]), 25.3 (*Salmonella typhimurium* isoenzyme II [42]), 30.0 (*E. coli* isoenzyme III [57]); 39.3 (*Methanococcus aeolicus* [58]) and 52.7 (*E. coli* isoenzyme II [53]).

Table 6 Inhibition constants of *A. thaliana* and mutant AHAS by SU herbicides

Inhibition curves (Figures 5–9) were analysed using eqn. (3) (A122V, $[E]_0 = 52.9$ nM; S653N, $[E]_0 = 99.8$ nM; wild-type, $[E]_0 = 92.5$ nM) or eqn. (4) (W574S and W574L) to obtain best-fit values ± S.E. for K_i^{app}. Where lower limits only are given for W574S, there was no inhibition at the highest herbicide concentration used and K_i^{app} was taken to be at least 4 times this concentration.

	K_i^{app}				
Enzyme ...	A122V	W574S	W574L	S653N	Wild-type
Herbicide	(nM)	(μM)	(μM)	(nM)	(nM)
Chlorimuron ethyl	44.2 ± 2.2	5.1 ± 0.3	1.9 ± 0.1	17.5 ± 1.4	10.8 ± 1.3
Chlorsulfuron	85.6 ± 4.9	14.7 ± 0.7	11.2 ± 0.3	67.4 ± 4.0	54.6 ± 4.8
Sulfometuron methyl	102 ± 10	> 256	72.2 ± 1.7	334 ± 13	25.5 ± 4.1
Metsulfuron methyl	31.5 ± 3.2	11.4 ± 0.6	4.7 ± 0.1	64.5 ± 3.1	36.2 ± 1.9
Thifensulfuron methyl	75.1 ± 5.9	142 ± 8	57.8 ± 2.9	706 ± 19	72.2 ± 4.3
Tribenuron methyl	224 ± 26	> 256	57.4 ± 2.0	1295 ± 55	253 ± 12

Table 7 Resistance of *A. thaliana* and mutant AHAS to SU and IM herbicides

Resistance is calculated as the ratio of K_i^{app} for the mutant compared with that of the wild-type enzyme for the same herbicide.

	Resistance [K_i^{app} (mutant)/K_i^{app} (wild-type)]			
Enzyme ...	A122V	W574S	W574L	S653N
SU herbicides				
Chlorimuron ethyl	4.1	472	176	1.6
Chlorsulfuron	1.6	269	205	1.2
Sulfometuron methyl	4.0	> 10000	2830	13.1
Metsulfuron methyl	0.9	315	130	1.8
Thifensulfuron methyl	1.0	1960	801	9.8
Tribenuron methyl	0.9	> 1010	226	5.1
IM herbicides				
Imazaquin	> 2160	> 2160	161	117
Imazapyr	> 950	> 950	155	575
Imazethapyr	> 1770	> 1770	186	212

Table 8 Inhibition constants of *A. thaliana* and mutant AHAS by IM herbicides

Inhibition curves (Figures 5–9) were analysed using eqn. (4) to obtain best-fit values ± S.E. for K_i^{app}. Where lower limits only are given for A122V and W574S, there was no inhibition at the highest herbicide concentration used and K_i^{app} was taken to be at least 4 times this concentration.

	K_i^{app}				
Enzyme ...	A122V	W574S	W574L	S653N	Wild-type
Herbicide	(mM)	(mM)	(mM)	(mM)	(μM)
Imazaquin	> 6.4	> 6.4	0.47 ± 0.02	0.35 ± 0.02	3.0 ± 0.1
Imazapyr	> 16.0	> 16.0	2.58 ± 0.14	9.61 ± 0.75	16.7 ± 1.2
Imazethapyr	> 16.0	> 16.0	1.68 ± 0.07	1.91 ± 0.07	9.0 ± 0.5

It is of interest that W574S was active, albeit less so than the wild-type enzyme, because Bernasconi et al. [10] reported that this mutation resulted in inactivation of cocklebur AHAS. Moreover, it was the most stable of all the mutant enzyme (Table 2). Presumably, the reason that one was active while the other was not is that *A. thaliana* and cocklebur AHAS are different proteins so the same mutation can affect them in different ways. Consistent with this proposition is the observation that the W574L mutation increased the specific activity of *A. thaliana* AHAS by 30 % (Table 2), whereas the same mutation in the cocklebur enzyme resulted in a decrease of 80 % [10].

Kinetics towards pyruvate

The substrate saturation curve of A122V, W574S and S653N clearly showed negative co-operativity and that of W574L also showed a small deviation from Michaelis–Menten kinetics (Figure 3). This behaviour was consistent with the model that we have proposed previously [14], whereby binding of substrate to one active site of the homodimer makes binding of substrate to the second active site more difficult. The value of K_{m1} (Table 4) controls the first of these binding steps and none of the mutants was identical to the wild-type enzyme. However, by far the most pronounced difference was for W574L for which K_{m1} was elevated by a factor of more than 5. Because K_{m1} (43 mM) approaches K_{m2} (100 mM) for this mutant, the negative co-operativity was difficult to discern and the Scatchard plot (Figure 3C) was almost linear.

Herbicide resistance

The W574L mutation has been reported to result in resistance to SU herbicides for AHAS from yeast and several plants (Table 1), so it was not unexpected that it had a similar effect on *A. thaliana* AHAS. The extent of resistance varied somewhat from one SU to another (Table 7) ranging from 130-fold (metsulfuron methyl) to about 3000-fold (sulfometuron methyl). This mutation also resulted in resistance to IM herbicides and the degree of resistance was almost equal for those compounds tested.

The other W574 mutation, to serine, had a similar effect to that of W574L, except that the extent of resistance was uniformly greater for both SU and IM herbicides. Resistance as a result of this mutation is known only in yeast and cotton AHAS (Table 1) and we would not have been surprised if this mutant enzyme had been inactive, as reported for the cocklebur enzyme [10]. This proved not to be the case, although the specific activity was somewhat lower than that of the wild-type (Table 2).

S653N is the only known naturally-occurring mutation that is claimed to give resistance to IM, but not to SU, herbicides (Table 1) and our results confirm this in part. Certainly it is resistant to IM herbicides, although not to the same extent as W574S, which was unaffected by imazapyr and imazethapyr and barely affected by imazaquin at the highest concentrations tested (Figure 6C). It was also sensitive to some SU herbicides (chlorimuron ethyl, chlorsulfuron and metsulfuron methyl) with increases in K_i^{app} of

Table 9 Summary of the properties of *A. thaliana* mutant AHAS

Mutant ...	A122V	W574S	W574L	S653N
Specific activity	Low	Moderate	Normal	Normal
K_m for pyruvate	Normal	Normal	High	Normal
K_c for Mg^{2+}	Very high	Elevated	Normal	Normal
K_c for ThDP	Very high	Elevated	Elevated	Normal
K_c for FAD	High	Normal	Normal	Low
SU inhibition	Sensitive	Resistant	Resistant	Sensitive
IM inhibition	Very resistant	Very resistant	Resistant	Resistant

less than 2-fold (Table 7). Whether it was resistant to the remaining SU herbicides is rather a matter of definition. By how much does K_i^{app} need to be elevated to constitute resistance? If we arbitrarily nominate a 5-fold change as the threshold, then S653N was resistant to sulfometuron methyl, thifensulfuron methyl and tribenuron methyl.

We were surprised to find that the A122V mutation gave little or no resistance to SU herbicides, given that the corresponding mutation in yeast and *E. coli* AHAS was reported to result in resistance to these herbicides [20,21]. However, it should be noted that Falco et al. [20] remarked that this mutation resulted in only 5-fold resistance to unspecified SU herbicides and Yadav et al. [21] reported a 4-fold resistance to sulfometuron methyl for the equivalent *E. coli* mutant enzyme. These results are comparable with the 4-fold resistance (towards chlorimuron ethyl and sulfometuron methyl) that we observed.

There are no obvious correlations between the resistance to SUs (Table 7) and the structures of these herbicides (Figure 1A), which contain an aromatic substituent at one end and a heterocyclic moiety at the other. For example, metsulfuron methyl, sulfometuron methyl and tribenuron methyl each has the same aromatic substituent but W574S shows varying degrees of resistance ranging from 315 to more than 10000. This might suggest that W574 interacts with the heterocyclic substituent. However, chlorsulfuron, metsulfuron methyl and thifensulfuron methyl each has the same heterocyclic substituent yet W574S shows a 7-fold range in its resistance to these three herbicides.

Conclusions

In the past, commercial crops resistant to AHAS inhibitors have been obtained by selection of herbicide-insensitive plants without regard for the detailed properties of AHAS ([59] and references therein). Unfortunately, this same mechanism can also lead to the chance emergence of herbicide-resistant weeds [5,6,10]. Therefore it is of interest to speculate on how well each of the AHAS mutations might assist in the deliberate construction of herbicide-resistant crop plants, but there are some caveats that should be mentioned in this context. First, the herbicide sensitivities that we have characterized relate to the initial inhibition. However, plants will be exposed to herbicides for extended periods and the final inhibition, that we have not measured, may be more relevant to the biological effect. Secondly, in the enzymes that we have expressed part of the N-terminus has been removed in an attempt to mimic the native mature form. Since the exact cleavage site of the chloroplast transit peptide has not been determined experimentally, it is possible that the properties of the expressed enzyme may differ from the native form in some respects. Indeed, as we [14] and others [60] have shown, the expressed enzyme differs from the native form in being insensitive to inhibition by branched-chain amino acids. Thirdly, there is mounting evidence [61,62] that AHAS from eukaryotes, like its bacterial counterpart, may contain a small subunit that modulates its activity. The expressed enzyme studied in this report does not contain this subunit and further studies will be required to establish whether it exists and, if so, how it affects the properties of the enzyme. Subject to these reservations, it appears that the mutation W574S seems to show the most promise for use in plant transformation (Table 9). Although it possesses a somewhat lower specific activity than the wild-type enzyme, a moderate level of over-expression would compensate for this in a plant. This mutant exhibits strong resistance to both sulphonylurea and imidazolinone herbicides while showing no large changes in the affinity for substrate or cofactors that might otherwise have undesirable physiological side-effects.

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