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Systematic characterization of mutations in yeast acetohydroxyacid synthase

Interpretation of herbicide-resistance data

Ronald G. Duggleby, Siew Siew Pang, Hongqi Yu* and Luke W. Guddat

Department of Biochemistry and Molecular Biology, The University of Queensland, Brisbane, QLD 4072, Australia

Acetohydroxyacid synthase (AHAS, EC 4.1.3.18) catalyses the first step in branched-chain amino acid biosynthesis and is the target for sulfonylurea and imidazolinone herbicides, which act as potent and specific inhibitors. Mutants of the enzyme have been identified that are resistant to particular herbicides. However, the selectivity of these mutants towards various sulfonylureas and imidazolinones has not been determined systematically. Now that the structure of the yeast enzyme is known, both in the absence and presence of a bound herbicide, a detailed understanding of the molecular interactions between the enzyme and its inhibitors becomes possible. Here we construct 10 active mutants of yeast AHAS, purify the enzymes and determine their sensitivity to six sulfonylureas and three imidazolinones. An additional three active mutants were constructed with a view to increasing imidazolinone sensitivity. These three variants were purified and tested for their sensitivity to the imidazolinones only. Substantial differences are observed in the sensitivity of the 13 mutants to the various inhibitors and these differences are interpreted in terms of the structure of the herbicide-binding site on the enzyme.

Keywords: acetohydroxyacid synthase; herbicide inhibition; herbicide-resistance mutations; imidazolinone; sulfonylurea.

The inability of higher animals to synthesize branched-chain amino acids makes these compounds essential in the diet. In contrast, plants and many microorganisms do not require a supply of these amino acids and contain all of the enzymes for their biosynthesis (Fig. 1A). As a result, these enzymes have attracted attention as potential targets for inhibitors to be used as herbicides and as antibiotics [1,2].

To date, the only enzyme target for which practical inhibitors have been developed is acetohydroxyacid synthase (AHAS; EC 4.1.3.18). This enzyme is inhibited by several families of compounds [1] and two of these families (Fig. 2), the sulfonylureas [chlorimuron ethyl (CE), chlorsulfuron (CS), metsulfuron methyl (MM), sulfometuron methyl (SM), tribenuron methyl (TB) and thifensulfuron methyl (TF)] and the imidazolinones [imazapyr (IP), imazaquin (IQ) and imazethapyr (IT)], are used extensively as commercial herbicides.

The inhibitory herbicides do not resemble the substrates or products of the AHAS reaction (Fig. 1B). Neither do they resemble the organic cofactors required by AHAS, thiamine diphosphate (ThDP) and FAD. Thus, it has been suggested that these herbicides are 'extraneous site inhibitors' [3] that do not occupy the active site. Therefore, it could be anticipated that mutations could arise that diminish herbicide binding without abolishing the catalytic capacity of AHAS.

This expectation has been realized in field isolates and in mutants generated *in vitro*. The most extensive series of these are those reported for yeast AHAS [4] in which 10 sites were located where spontaneous mutation results in SM resistance. At some sites, almost any amino acid substitution results in resistance while at others only a few substitutions are tolerated. Studies of AHAS from other species have identified additional herbicide-resistance mutation sites [5–9] and have also confirmed some of those observed for the yeast enzyme. The natural amino acid at most of these sites is strongly conserved in AHAS across plant, fungal and bacterial species [1], suggesting that these residues play important roles. However, the fact that organisms carrying mutations at these sites are viable but herbicide resistant shows that the variants must be active. Indeed, detailed *in vitro* studies in this laboratory of selected variants [7–10] have shown that most have no major alterations in activity or the kinetic properties towards substrate or cofactors.

Until recent work from this laboratory [11,12], no three-dimensional structure of any AHAS had been determined

Correspondence to R. G. Duggleby, Department of Biochemistry and Molecular Biology, The University of Queensland, Brisbane, QLD 4072, Australia.
Fax: + 617 33654699, Tel.: + 617 33654615,
E-mail: Ronald.Duggleby@mailbox.uq.edu.au

Abbreviations: AHAS, acetohydroxyacid synthase; CE, chlorimuron ethyl; CS, chlorsulfuron; IP, imazapyr; IQ, imazaquin; IT, imazethapyr; MM, metsulfuron methyl; SM, sulfometuron methyl; TA, branched-chain amino acid transaminase; TB, tribenuron methyl; TF, thifensulfuron methyl; ThDP, thiamine diphosphate.

Enzymes: acetohydroxyacid synthase (EC 4.1.3.18); ketol-acid reductoisomerase (EC 1.1.1.86); dihydroxy-acid dehydratase (EC 4.2.1.9); branched-chain amino acid transaminase (EC 2.6.1.42); isopropylmalate synthase (EC 4.1.3.12); isopropylmalate isomerase (EC 4.2.1.33); isopropylmalate dehydrogenase (EC 1.1.1.85).

**Present address*: Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287-1604, USA.

Note: a web site is available at http://smms.uq.edu.au/~duggleby

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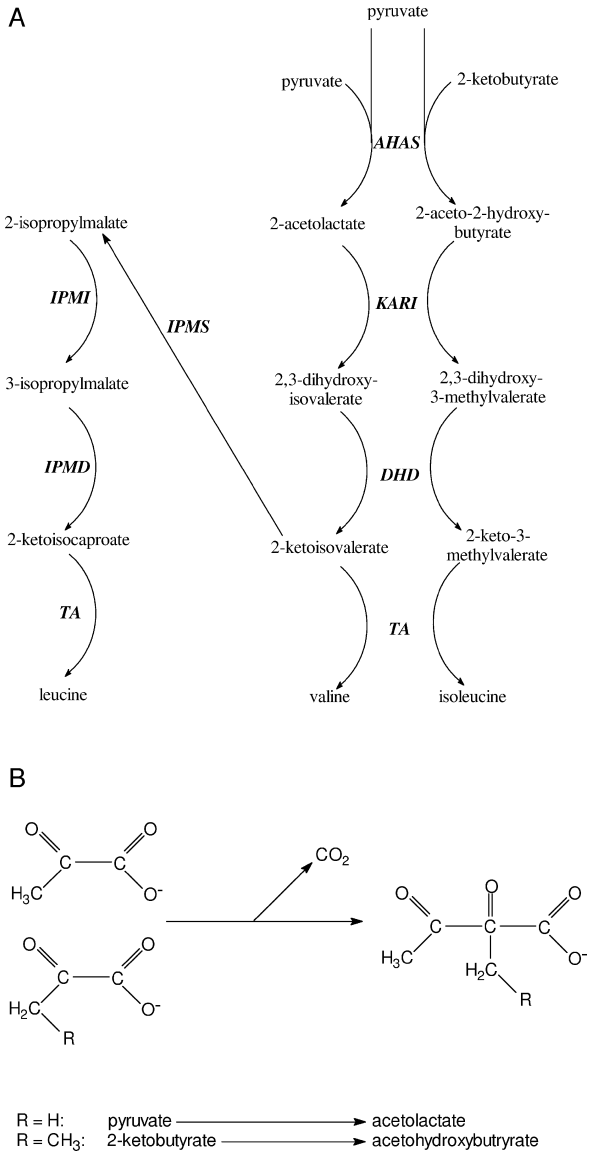

Fig. 1. Pathway of branched-chain amino acid biosynthesis (A) and the reaction catalysed by AHAS (B). Enzyme names are shown in bold italics, abbreviated as follows: AHAS, acetohydroxyacid synthase (EC 4.1.3.18); KARI, ketol-acid reductoisomerase (EC 1.1.1.86); DHD, dihydroxy-acid dehydratase (EC 4.2.1.9); TA, branched-chain amino acid transaminase (EC 2.6.1.42); IPMS, isopropylmalate synthase (EC 4.1.3.12); IPMI, isopropylmalate isomerase (EC 4.2.1.33); and IPMD, isopropylmalate dehydrogenase (EC 1.1.1.85). (B) The reaction catalysed by AHAS.

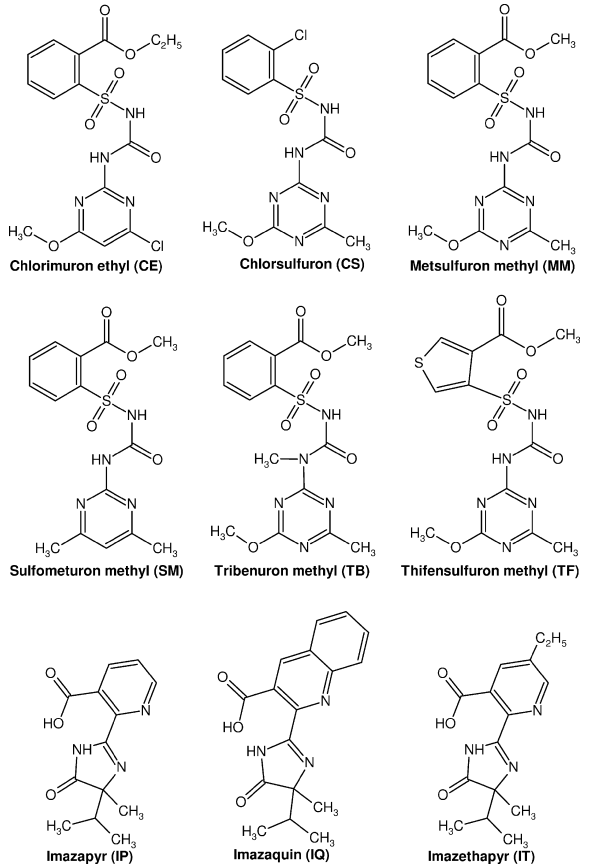

Fig. 2. Structures of the sulfonylurea and imidazolinone herbicides. The rings of the sulfonylureas that are connected to the sulphur and nitrogen atoms of the sulfonylurea bridge are referred to in the text as the 'S-ring' and 'N-ring' regions of the molecule, respectively.

and conjectures about the structure of the herbicide-binding site were based on homology models [5,6] or theoretical calculations [13,14]. Our structure of yeast AHAS with bound CE [12] has provided the first experimental basis for understanding the detailed molecular interactions between a sulfonylurea herbicide and the residues involved in herbicide resistance. Although it would be desirable to know the structure of a plant AHAS, diffraction quality crystals have not been produced thus far. However, the structure of yeast AHAS shows that every residue at which mutation in plant AHAS results in herbicide resistance is in close proximity to CE. Therefore, we believe that the yeast enzyme represents a very good model system for understanding plant AHAS.

The structure reveals that the enzyme is a homodimer with each subunit composed of three domains of similar size, designated α, β and γ. Herbicide-resistance mutations are spread through all three domains (Fig. 3A) but the protein folds so as to bring these together in a single region at the subunit interface and define a plausible herbicide-binding site (Fig. 3B) at the entrance of a channel leading to the active site. CE is inserted into this channel with the ring that is attached to the nitrogen atom of the sulfonylurea bridge (hereafter referred to as the 'N-ring') and the ethyl substituent projecting inwards, while the ring that is attached to the sulphur atom of the sulfonylurea bridge (the 'S-ring') lies across the top of the channel.

CE is unique amongst the sulfonylureas shown in Fig. 2 in that it is the only one with an ethylated carboxyl group attached to the S-ring. The other sulfonylureas have a smaller substituent, either a methylated carboxyl or chlorine atom in the case of CS. Since this side chain of CE makes important interactions with the protein, it is possible that the binding mode of other sulfonylureas differs from that of CE.

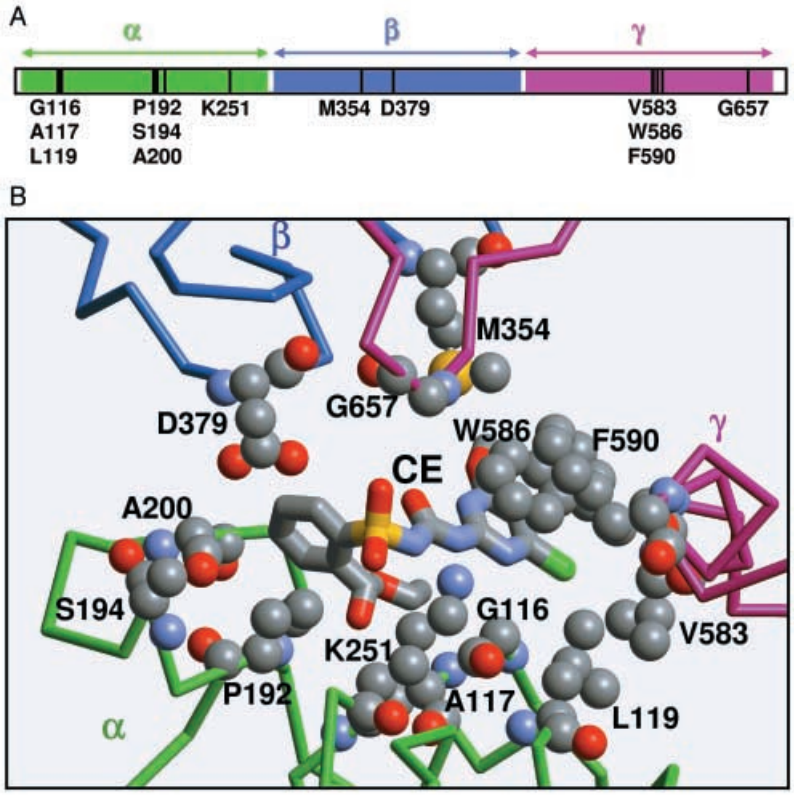

Fig. 3. Location of herbicide-resistance mutations in yeast AHAS. (A) Positions of mutation sites in the amino acid sequence, coloured green, blue and pink/purple for the α-, β- and γ-domains, respectively. (B) Positions of mutation sites in the three-dimensional structure. The location of CE was determined from the three-dimensional structure of the AHAS–CE complex [12]; PDB accession code 1N0H. The backbone Cα trace in the vicinity is coloured as in panel A. Atoms in residues that were mutated in the present study are shown as balls, while CE is shown as a stick model.

The binding orientation of imidazolinones is even more uncertain because they differ substantially in structure from the sulfonylureas. It should be recognized in this context that there is very little published information in the literature that would allow mapping of the amino acids with which imidazolinones interact. Indeed, the literature on the effects of mutation at each resistance site on sensitivity to all herbicides is very sparse and in only a few cases [7–10] has the specificity towards a range of different herbicides been determined. Therefore, we have now undertaken a systematic program of mutagenesis of residues in the proposed herbicide-binding site of yeast AHAS, and characterization of the inhibition by a series of six sulfonylurea and three imidazolinone herbicides.

Materials and methods

Mutagenesis

The expression plasmids for yeast AHAS catalytic and regulatory subunits were as described previously [15]. Methods for bacterial culture and DNA manipulation generally followed standard procedures [16]. Mutations were introduced by PCR using the megaprimer method [17]. Oligonucleotides were designed to introduce or remove a restriction enzyme recognition site to aid identification of mutated DNA. All constructs were tested by diagnostic restriction enzyme digestion and the mutations were confirmed by DNA sequencing using the Prism Ready Dye Terminator Cycle Sequencing Kit and DNA Sequencer 373A (Perkin-Elmer Applied Biosystems).

Protein purification and enzyme assays

The hexahistidine-tagged catalytic and regulatory subunits of AHAS were purified by immobilized metal affinity chromatography as described previously [15,18]. Mutant forms of the catalytic subunit were purified using an identical procedure. The purity of the proteins obtained was assessed by SDS/PAGE [19]. Protein concentrations were determined using the bicinchoninic acid method [20].

AHAS activity was measured at 30 °C in 1 M potassium phosphate buffer pH 7.0 containing 1 mM ThDP, 10 mM $MgCl_2$, 10 μM FAD and pyruvate concentrations as required, as described previously [15,18]. Stock solutions of inhibitors were prepared in 1 M Tris/H_2SO_4 pH 9.0 for the imidazolinones and in 0.1 M Tris/H_2SO_4 pH 9.0 for the sulfonylureas. These were added to reaction mixtures to give the desired final inhibitor concentrations, together with additional 1 M Tris/H_2SO_4 pH 9.0 to maintain a final concentration of 0.1 M for the imidazolinone inhibition assays. Inhibition experiments were conducted with a pyruvate concentration close to the K_m value of each individual mutant enzyme that was used. Where indicated, dimethylsulfoxide was added, usually to give a final concentration of 15% (v/v).

Preparation of FAD-free AHAS

When FAD-free AHAS was required for particular experiments, this cofactor was removed from the

preparation by adsorption using activated charcoal. Activated charcoal (ICI United States Inc.) was pretreated by washing several times in 0.2 M potassium phosphate pH 6.0 containing 1 mM dithiothreitol, and resuspended in the same buffer to give a concentration of 25% (w/v). The activated charcoal was added to the AHAS protein solution ($\approx$ 3 mg·mL^{-1}) to give a concentration of 0.5%, mixed and incubated on ice for $\approx$ 5 min. The activated charcoal was then removed by centrifugation. Usually the treatment had to be repeated several times to remove all FAD. The extent of cofactor removal was tested by measurement of the residual enzymatic activity with no added FAD.

Spectroscopy

Absorbance spectra of wild-type AHAS and the M354V mutant enzyme were collected using a SpectraMax 250 (Molecular Devices) microplate spectrophotometer at room temperature. The enzyme solutions were diluted to concentrations of 2.6–3.0 mg·mL^{-1}, using buffer containing 0.2 M potassium phosphate (pH 6.0), 10 μM FAD and 1 mM dithiothreitol. The diluted protein solutions were clarified by centrifugation. Absorbance spectra were recorded from 330 to 510 nm. The protein spectra were corrected for that of the buffer.

For MS of herbicides, they were diluted to a concentration of 0.1 or 1 mM in 0.1% acetic acid solution. The diluted samples were analysed using API 165 LC/MS System (PE SCIEX). Full scan data were acquired over the mass/charge range from 200 to 600, with a step size of 0.2 atomic mass units and a dwell time of 0.5 ms.

Kinetic data analysis

Kinetic data were analysed by nonlinear regression using the programs GRAPHPAD PRISM and INPLOT 4, SIGMAPLOT, or GRAFIT. The Michaelis–Menten equation (Eqn 1) was fitted to substrate and FAD saturation curves, while inhibition curves were analysed initially using a partial inhibition model (Eqn 2). If this initial analysis indicated that the residual activity (v_∞) at a saturating inhibitor concentration is not significantly greater than zero, the data were reanalysed with $v = 0$.

$$v = V_m[A]/(K_m + [A]) \quad (1)$$

$$v = v_\infty + (v_0 - v_\infty)/(1 + [I]/K_i) \quad (2)$$

Results

Choice of mutations

Falco *et al.* [4] described a series of spontaneous mutations of yeast AHAS that result in resistance to the sulfonylurea, SM. These mutations occur at 10 sites and further SM-resistant mutants were identified by deliberate mutagenesis at these 10 sites. We have reproduced one of the spontaneous substitutions at each of these 10 residues, as follows: G116S, A117V, P192S, A200V and K251T in the α-domain; M354V and D379N in the β-domain; and V583A, W586L and F590L in the γ-domain. Henceforth these variants will be designated the 'Falco mutations'. We expected all of these variants to be resistant to SM but their sensitivity to other sulfonylureas, and to imidazolinones, had not been determined.

Mutations at three additional sites (L119, S194 and G657), located in the herbicide-binding site (Fig. 3), were made. Ott *et al.* [5] described the mutations M124E and M124I of *Arabidopsis thaliana* AHAS that both result in resistance to the imidazolinone IT, as well as to the sulfonylurea SM for M124I only. In yeast AHAS, the equivalent residue is L119 and this was mutated to methionine as found in wild-type *A. thaliana* AHAS. It was hoped that this mutant would show increased sensitivity to imidazolinones and was tested with this family of herbicides only. For similar reasons we mutated S194 to arginine as found at the equivalent position (R199) in wild-type *A. thaliana* AHAS; Ott *et al.* [5] had found IT resistance when R199 in *A. thaliana* AHAS was mutated to alanine or glutamate. As well as these two α-domain variants, we made the G657A mutant in the γ-domain. In plant AHAS, the equivalent residue is a serine and mutation to larger residues (asparagine, threonine or phenylalanine) results in imidazolinone resistance [8,9].

Mutagenesis, enzyme purification and activity

Each mutation was introduced successfully by overlap extension PCR [17], cloned into the expression vector described previously [15], and the base changes confirmed by DNA sequencing (data not shown). Each protein was expressed and purified and in most cases was shown to be close to purity by SDS/PAGE (Fig. 4). The specific activity of each purified protein was determined (Tables 1–3); all proteins are active and in most cases the specific activity is

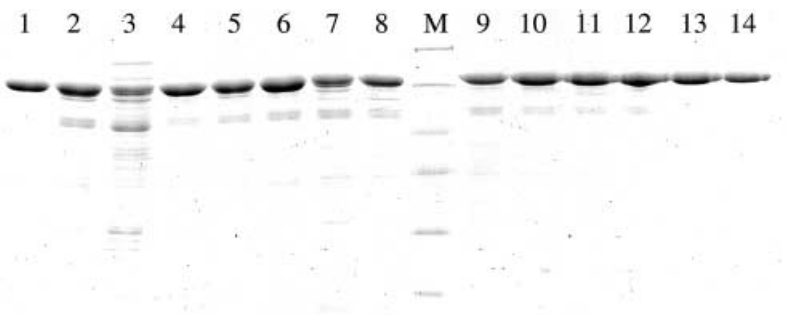

Fig. 4. SDS/PAGE of purified wild-type yeast AHAS and its mutants. The samples in the lanes are: 1, Wild-type; 2, G116A; 3, A117V; 4, M119L; 5, P192S; 6, S194R; 7, V200A; 8, K251T; M, molecular mass markers (116, 66, 45, 35, 25 and 18 kDa); 9, M354V; 10, D379N, 11, V583A; 12, W586L; 13, F590L; 14, G657A.

Table 1. Kinetic properties of wild-type yeast AHAS and the α-domain Falco mutants. Values shown in italics were obtained in the presence of 15% (v/v) dimethylsulfoxide, except for G116S with SM where 20% dimethylsulfoxide was used. K_i values of the wild-type in the absence of dimethylsulfoxide for CE and CS were reported previously [12].

	Wild-type	G116S	A117V	P192S	A200V	K251T
U·mg^{-1}	4.33	3.10	0.19	1.06	0.93	0.04
K_m, M	$4.71 \pm 0.17 \times 10^{-3}$	$1.13 \pm 0.11 \times 10^{-2}$	$3.17 \pm 0.43 \times 10^{-3}$	$3.92 \pm 0.23 \times 10^{-3}$	$9.60 \pm 0.93 \times 10^{-3}$	$3.77 \pm 0.61 \times 10^{-3}$
	$8.09 \pm 1.31 \times 10^{-3}$	*$1.50 \pm 0.14 \times 10^{-2}$*	*$1.82 \pm 0.27 \times 10^{-3}$*		*$4.93 \pm 0.95 \times 10^{-3}$*	*$4.65 \pm 0.48 \times 10^{-3}$*
K_i for CE (M)	$3.25 \pm 0.28 \times 10^{-9}$	$3.34 \pm 0.26 \times 10^{-6}$	$2.13 \pm 0.58 \times 10^{-8}$	$2.12 \pm 0.19 \times 10^{-7}$	$7.83 \pm 0.71 \times 10^{-8}$	$7.37 \pm 0.72 \times 10^{-8}$
	$3.66 \pm 0.61 \times 10^{-9}$					*$2.91 \pm 0.50 \times 10^{-7}$*
K_i for CS (M)	$1.27 \pm 0.17 \times 10^{-7}$	$5.59 \pm 0.45 \times 10^{-7}$	$1.79 \pm 0.27 \times 10^{-6}$	$3.18 \pm 0.28 \times 10^{-5}$	$2.33 \pm 0.24 \times 10^{-6}$	$1.20 \pm 0.06 \times 10^{-6}$
	$1.91 \pm 0.88 \times 10^{-7}$	*$1.11 \pm 0.01 \times 10^{-6}$*				*$1.76 \pm 0.36 \times 10^{-6}$*
K_i for MM (M)	$9.40 \pm 1.30 \times 10^{-9}$	$2.45 \pm 0.10 \times 10^{-6}$	$4.86 \pm 0.25 \times 10^{-8}$	$2.72 \pm 0.72 \times 10^{-6}$	$5.29 \pm 0.65 \times 10^{-7}$	$7.53 \pm 0.59 \times 10^{-8}$
	$1.15 \pm 0.35 \times 10^{-8}$	*$6.11 \pm 0.90 \times 10^{-6}$*		*$5.70 \pm 0.55 \times 10^{-6}$*		
K_i for SM (M)	$5.08 \pm 0.21 \times 10^{-8}$	Activated	Activated	$9.37 \pm 0.97 \times 10^{-5}$	$1.20 \pm 0.11 \times 10^{-5}$	$9.54 \pm 2.06 \times 10^{-6}$
	$6.39 \pm 0.41 \times 10^{-8}$	*$5.72 \pm 0.95 \times 10^{-4}$*	*$1.86 \pm 0.31 \times 10^{-4}$*			*$1.02 \pm 0.12 \times 10^{-5}$*
K_i for TB (M)	$1.14 \pm 0.07 \times 10^{-7}$	$8.54 \pm 0.31 \times 10^{-5}$	$3.04 \pm 0.35 \times 10^{-6}$	$4.37 \pm 0.24 \times 10^{-5}$	$6.14 \pm 0.32 \times 10^{-5}$	$1.74 \pm 0.41 \times 10^{-6}$
	$2.01 \pm 0.09 \times 10^{-7}$					$2.44 \pm 0.40 \times 10^{-6}$
K_i for TF (M)	$5.25 \pm 1.07 \times 10^{-8}$	$2.98 \pm 0.20 \times 10^{-6}$	$2.94 \pm 0.23 \times 10^{-7}$	$7.38 \pm 0.65 \times 10^{-6}$	$3.40 \pm 0.22 \times 10^{-6}$	$3.71 \pm 0.94 \times 10^{-6}$
	$1.04 \pm 0.24 \times 10^{-7}$	*$3.12 \pm 0.49 \times 10^{-6}$*				
K_i for IP (M)	$1.16 \pm 0.18 \times 10^{-2}$	Resistant	$4.16 \pm 1.01 \times 10^{-2}$	Activated	Activated	Resistant
	$1.33 \pm 0.10 \times 10^{-2}$			*$1.52 \pm 0.54 \times 10^{-2}$*	*Resistant*	*Resistant*
K_i for IQ (M)	$8.18 \pm 1.24 \times 10^{-4}$	$1.24 \pm 0.11 \times 10^{-2}$	$8.66 \pm 0.72 \times 10^{-4}$	Activated	Resistant	$1.89 \pm 0.30 \times 10^{-3}$
	$2.83 \pm 0.19 \times 10^{-3}$			*$5.39 \pm 0.65 \times 10^{-3}$*	*Resistant*	
K_i for IT (M)	$7.46 \pm 1.05 \times 10^{-4}$	Resistant	$6.69 \pm 1.32 \times 10^{-3}$	Activated	Activated	Resistant
	$1.18 \pm 0.58 \times 10^{-3}$			*$1.37 \pm 0.75 \times 10^{-2}$*	*Resistant*	*Resistant*

Table 2. Kinetic properties of wild-type yeast AHAS and the β- and γ-domain Falco mutants. Values shown in italics were obtained in the presence of 15% (*v*/*v*) dimethylsulfoxide.

	Wild-type	M354V	D379N	V583A	W586L	F590L
U·mg^{-1}	4.33	2.05	6.05	3.89	19.6	8.72
K_m, (M)	$4.71 \pm 0.17 \times 10^{-3}$	$3.73 \pm 0.10 \times 10^{-2}$	$7.85 \pm 0.31 \times 10^{-3}$	$2.53 \pm 0.25 \times 10^{-2}$	$4.17 \pm 0.39 \times 10^{-2}$	$1.71 \pm 0.08 \times 10^{-2}$
	$8.09 \pm 1.31 \times 10^{-3}$	*$4.88 \pm 0.18 \times 10^{-2}$*				
K_i for CE (M)	$3.25 \pm 0.28 \times 10^{-9}$	$1.48 \pm 0.10 \times 10^{-8}$	$2.19 \pm 0.14 \times 10^{-7}$	$1.25 \pm 0.10 \times 10^{-8}$	$2.03 \pm 0.18 \times 10^{-5}$	$6.44 \pm 0.61 \times 10^{-9}$
	$3.66 \pm 0.61 \times 10^{-9}$					
K_i for CS (M)	$1.27 \pm 0.17 \times 10^{-7}$	$1.07 \pm 0.05 \times 10^{-6}$	$3.43 \pm 0.27 \times 10^{-6}$	$6.54 \pm 0.47 \times 10^{-7}$	$1.68 \pm 0.08 \times 10^{-3}$	$1.05 \pm 0.13 \times 10^{-6}$
	$1.91 \pm 0.88 \times 10^{-7}$					
K_i for MM (M)	$9.40 \pm 1.30 \times 10^{-9}$	$3.16 \pm 0.16 \times 10^{-8}$	$1.45 \pm 0.08 \times 10^{-6}$	$4.06 \pm 0.32 \times 10^{-8}$	$2.82 \pm 0.19 \times 10^{-4}$	$2.44 \pm 0.22 \times 10^{-8}$
	$1.15 \pm 0.35 \times 10^{-8}$					
K_i for SM (M)	$5.08 \pm 0.21 \times 10^{-8}$	$3.08 \pm 0.12 \times 10^{-6}$	$4.30 \pm 0.28 \times 10^{-5}$	$5.04 \pm 0.31 \times 10^{-7}$	Activated	$1.15 \pm 0.04 \times 10^{-5}$
	$6.39 \pm 0.41 \times 10^{-8}$				*$9.92 \pm 2.66 \times 10^{-4}$*	
K_i for TB (M)	$1.14 \pm 0.07 \times 10^{-7}$	$1.06 \pm 0.19 \times 10^{-6}$	$4.48 \pm 0.47 \times 10^{-5}$	$1.42 \pm 0.14 \times 10^{-6}$	$1.01 \pm 0.12 \times 10^{-3}$	$2.77 \pm 0.24 \times 10^{-6}$
	$2.01 \pm 0.09 \times 10^{-7}$					
K_i for TF (M)	$5.25 \pm 1.07 \times 10^{-8}$	$1.18 \pm 0.05 \times 10^{-6}$	$7.84 \pm 0.51 \times 10^{-6}$	$3.20 \pm 0.16 \times 10^{-7}$	$3.91 \pm 0.32 \times 10^{-7}$	$2.87 \pm 0.30 \times 10^{-7}$
	$1.04 \pm 0.24 \times 10^{-7}$					
K_i for IP (M)	$1.16 \pm 0.18 \times 10^{-2}$	$1.22 \pm 0.11 \times 10^{-1}$	$2.38 \pm 0.16 \times 10^{-4}$	$1.12 \pm 0.11 \times 10^{-2}$	Activated	Activated
	$1.33 \pm 0.10 \times 10^{-2}$				*$8.34 \pm 1.04 \times 10^{-2}$*	*$6.48 \pm 0.68 \times 10^{-2}$*
K_i for IQ (M)	$8.18 \pm 1.24 \times 10^{-4}$	$4.57 \pm 0.51 \times 10^{-3}$	$3.19 \pm 0.14 \times 10^{-5}$	$1.37 \pm 0.06 \times 10^{-3}$	$7.67 \pm 1.47 \times 10^{-4}$	$5.21 \pm 0.43 \times 10^{-3}$
	$2.83 \pm 0.19 \times 10^{-3}$				$3.42 \pm 0.51 \times 10^{-3}$	
K_i for IT (M)	$7.46 \pm 1.05 \times 10^{-4}$	Activated	$4.64 \pm 0.35 \times 10^{-5}$	$2.23 \pm 0.21 \times 10^{-3}$	$2.41 \pm 0.24 \times 10^{-2}$	$2.95 \pm 0.38 \times 10^{-2}$
	$1.18 \pm 0.58 \times 10^{-3}$	*$8.05 \pm 0.52 \times 10^{-2}$*				

not substantially lower than that of the wild-type. Four of the variants (L119M, D379N, W586L and F590L) are at least 40% more active than wild-type while for others, particularly A117V and K251T, the specific activity is quite low. In the former case, the purity of the protein is noticeably lower (Fig. 4) and for this mutant at least, the measured specific activity must be regarded as a lower limit.

The K_m for the substrate (pyruvate) was measured (Tables 1–3) and, as with the specific activity, some differences from the wild-type value were observed. The most extreme value is for W586L with a K_m value nearly ninefold higher than that of wild-type AHAS. Despite these variations, in no case is the apparent affinity for pyruvate so impaired that one would conclude that the

Table 3. Kinetic properties of wild-type yeast AHAS and the imidazolinone mutants.

	Wild-type	L119M	S194R	G657A
U·mg^{-1}	4.33	6.25	3.63	4.74
K_m, (M)	$4.71 \pm 0.17 \times 10^{-3}$	$3.25 \pm 0.24 \times 10^{-3}$	$5.73 \pm 0.32 \times 10^{-3}$	$7.99 \pm 0.62 \times 10^{-3}$
K_i for IP (M)	$1.16 \pm 0.18 \times 10^{-2}$	$9.71 \pm 1.62 \times 10^{-3}$	$1.46 \pm 0.41 \times 10^{-2}$	$8.39 \pm 0.93 \times 10^{-4}$
K_i for IQ (M)	$8.18 \pm 1.24 \times 10^{-4}$	$1.07 \pm 0.04 \times 10^{-3}$	$1.59 \pm 0.11 \times 10^{-3}$	$1.04 \pm 0.05 \times 10^{-4}$
K_i for IT (M)	$7.46 \pm 1.05 \times 10^{-4}$	$4.50 \pm 0.51 \times 10^{-3}$	$3.99 \pm 0.78 \times 10^{-3}$	$1.33 \pm 0.10 \times 10^{-4}$

enzyme would be unable to support branched-chain amino acid biosynthesis *in vivo*. This agrees with the observation that yeast cells carrying these mutations are able to grow when these nutrients are not supplied [4]. For reasons that will be explained later, the K_m for pyruvate was also measured in the presence of 15% dimethylsulfoxide for wild-type AHAS and for selected mutants. Both increases and decreases in the K_m value were observed, with the largest change seen in wild-type AHAS, where it was nearly doubled.

AHAS contains FAD as an essential cofactor and its removal abolishes catalytic activity [7,21]. Mutations that prevent FAD binding [7,22] also yield an inactive protein. The wild-type enzyme is noticeably yellow due to tightly bound FAD and all of the mutants described here except one exhibit a strong yellow colour. The exception is M354V, which is quite pale in colour and this enzyme was investigated further. After removal of free FAD, both wild-type and M354V are inactive and the ability to bind FAD can be followed by measuring the regain of activity upon addition of this cofactor. The activation curves are similar (Fig. 5A) and the affinity for FAD measured in this manner differs by a factor of less than two (wild-type, 27.2 ± 3.3 nM; M354V, 46.8 ± 3.0 nM) so both enzymes would be fully saturated by 10 μM FAD that is present in the buffers used throughout the enzyme purification. However, when the spectra of bound FAD in the two enzymes were examined, substantial differences in absolute and relative peak heights were seen (Fig. 5B). We interpret the qualitative spectral difference to mean that changing M354 to valine alters the environment of the isoalloxazine ring of FAD.

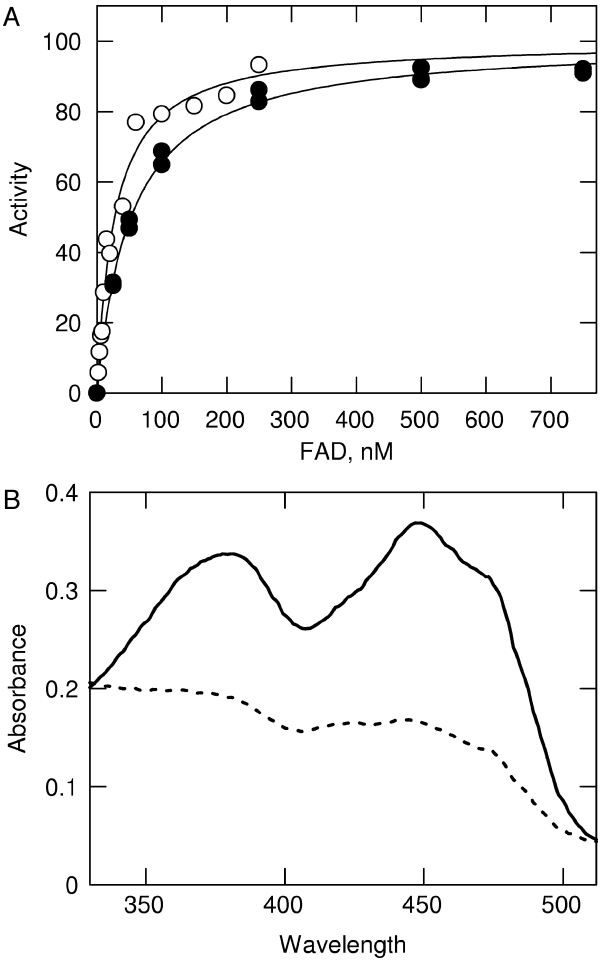

Fig. 5. Activation of wild-type yeast AHAS (○) and the M354V mutant (●) by FAD (A) and FAD spectra of wild-type yeast AHAS (solid line) and of the M354V mutant (dotted line) (B). (A) Data have been normalized to a common ordinate, set at 100% when FAD is saturating. (B) Spectra are corrected for the absorbance of the buffer, which contains 10 μM FAD.

Activation and inhibition

The inhibition of wild-type AHAS and 10 of the mutants was assessed using six sulfonylureas and three imidazolinones. The apparent K_i values obtained are shown in Table 1 (α-domain Falco mutations) and Table 2 (β- and γ-domain Falco mutations). The three remaining mutations (L119M, S194R and G657A) were tested with the three imidazolinones only (Table 3) because these mutations were directed specifically at altering sensitivity to this family of herbicides. To aid assessment of the alterations in the mutants, the properties of wild-type AHAS are reproduced in all three tables. Typical inhibition curves (wild-type with CE and IQ) are shown in Fig. 6A.

We were surprised to observe that some of the mutants, in combination with some of the herbicides, exhibit an unusual dependence of activity on herbicide concentration, with activation at low concentrations followed by inhibition at higher concentrations. An example is illustrated in Fig. 6B. This kinetic behaviour might mean that two herbicide molecules bind, with one activating the enzyme and the other inhibiting. Alternatively, there could be a contaminant in the herbicide samples that is responsible for the activation. To examine this second possibility, all of the herbicides were analysed by MS. No unexpected mass peaks were observed.

We had also observed, by chance, that wild-type yeast AHAS is activated by dimethylsulfoxide (Fig. 6C) with

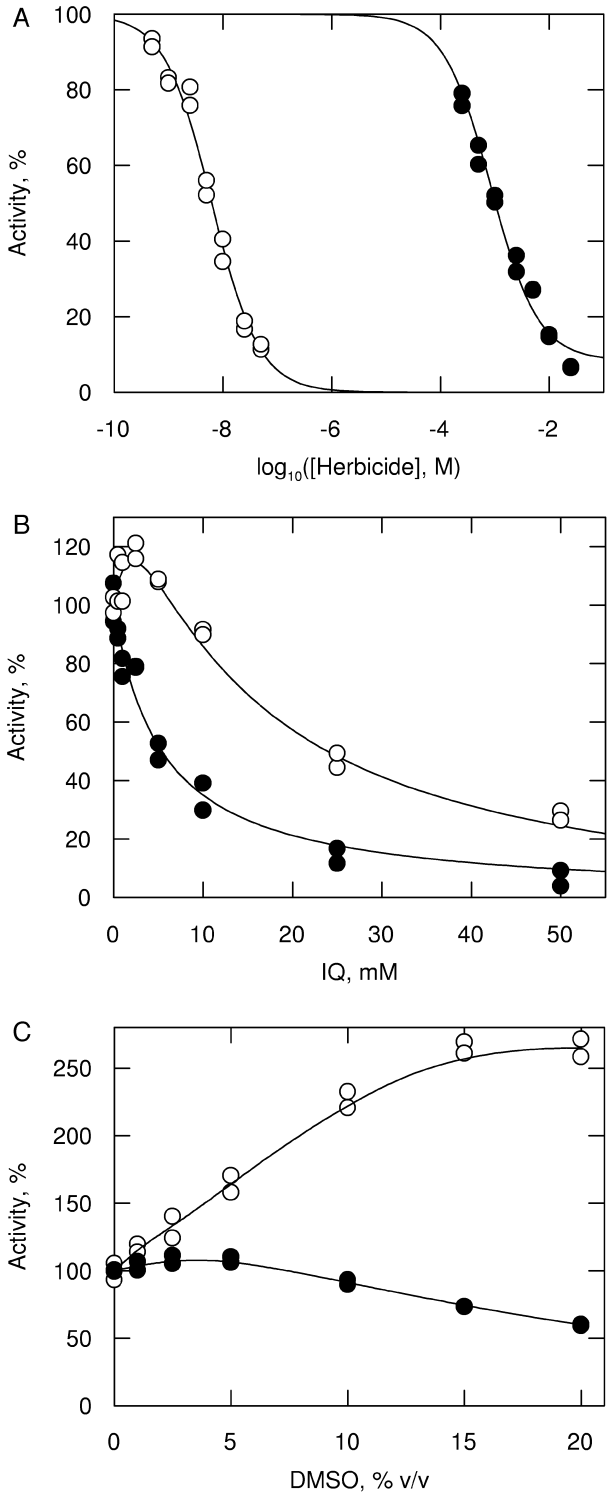

Fig. 6. Inhibition of wild-type yeast AHAS by CE (○) and IQ (●) (A), effect of IQ on P192S in the absence (○) and presence (●) of 15% dimethylsulfoxide (B) and effect of dimethylsulfoxide on the yeast catalytic subunit alone (○), and on the enzyme reconstituted with its regulatory subunit (●) (C). Data are expressed as a percentage of the activity observed in the absence of herbicides (panels A and B) or dimethylsulfoxide (panel C).

maximum activation (2.6-fold) occurring in the range 15–20% (v/v). AHAS is composed of catalytic and regulatory subunits with the latter stimulating activity by 7- to 10-fold [15] and conferring upon the catalytic subunit sensitivity to valine inhibition that is reversed by MgATP [18]. The activation of the catalytic subunit by dimethylsulfoxide is abolished by the regulatory subunit (Fig. 6C) and we conclude that dimethylsulfoxide is partially mimicking the effect of the regulatory subunit. In the same way, the activation by herbicides might also be mimicking the effect of the regulatory subunit and we reasoned that addition of dimethylsulfoxide might abolish activation by the herbicides without preventing inhibition. In all cases where activation was observed, dimethylsulfoxide greatly diminishes or totally abolishes the activation, such as in the example shown in Fig. 6B.

The activation observed in the absence of dimethylsulfoxide does not allow an accurate inhibition constant to be determined. Therefore, wherever activation was observed, the experiment was repeated in the presence of 15% dimethylsulfoxide. To compensate for any changes in the inhibition constant caused by dimethylsulfoxide itself, apparent K_i values were determined for wild-type AHAS in both the presence and absence of dimethylsulfoxide even though the wild-type enzyme did not show activation by any of the herbicides. For some of the mutants, apparent K_i values were measured both with and without 15% dimethylsulfoxide irrespective of whether herbicide activation was observed in the absence of dimethylsulfoxide. Although we suggest that dimethylsulfoxide might be mimicking the effect of the regulatory subunit, this was not our reason for adding this compound. The sole purpose of including dimethylsulfoxide was to permit evaluation of inhibition constants of the herbicides without the added complication of activation. Tables 1, 2 and 3 summarize all of these data.

Discussion

Expression and activity

All mutants were constructed and purified successfully, and all are active to varying degrees. One mutant, K251T, has a particularly low activity that cannot be explained by a lack of purity as is the case with A117V (Fig. 4). K251 is deep within the active site channel and mutation to threonine represents a rather large change in size and charge. Low activity, while not being predictable, is at least explicable by the nature of the change and its location.

We observed that the M354V mutant is noticeably less yellow than the wild-type enzyme and this was quantified by the decrease in the intensity and relative peak heights of the two absorbance bands that are due to the flavin (Fig. 5B). The spectral change, and in particular the change in relative peak heights, cannot be explained by the small decrease (twofold) in the affinity for FAD (Fig. 5A). M354 is closer to the isoalloxazine ring than any other side chain that we have mutated with the γ-carbon of M354 being less than 4 Å from one of the carbonyl oxygen atoms of FAD. We suggest that mutation to valine affects the environment of the flavin thereby altering its spectral properties.

Herbicide sensitivity of wild-type AHAS

Yeast AHAS is very sensitive to all sulfonylureas with apparent K_i values (Table 1) ranging from 3.25 (CE) to 127 (CS) nM. The K_i value for SM (51 nM) is comparable to the value of 120 nM reported by Yadav *et al.* [23] in permeabilized yeast cells. The potency is generally similar to that observed for wild-type *A. thaliana* AHAS [8] while for wild-type *Escherichia coli* AHAS II, the potency is approximately 10-fold lower [7]. In AHAS from all three species, CE is the strongest, MM is moderately strong, and TB is amongst the weakest, suggesting that the sulfonylurea binding site is largely conserved in bacterial, fungal and plant AHAS.

There is no obvious correlation between the potency of the inhibitor and its structure. MM, SM and TB all have the same methyl benzoyl group but vary in potency from the second to the fifth strongest. CS, MM, TB and TF each have the same substituted triazine but vary substantially in their effectiveness as inhibitors. The structure of the enzyme–CE complex provides a rational explanation for these observations. Bound CE, and presumably the other sulfonylureas, has a twisted structure with the side chain of the S-ring close to the N-ring. Thus, the two ends of the sulfonylureas act as an ensemble and it would be misleading to consider potential interactions of each alone.

The imidazolinones are much weaker inhibitors of wild-type yeast AHAS than the sulfonylureas (Table 1) with the strongest one (IT, $K_i = 746$ μM) being almost 6000-fold weaker than the weakest sulfonylurea (CS, $K_i = 127$ nM). In this respect, yeast AHAS is similar to wild-type *E. coli* AHAS II [7] but markedly different from *A. thaliana* AHAS [8], which has inhibition constants for the imidazolinones of around 10 μM.

AHAS activation

The field use of sulfonylureas and imidazolinones relies on their ability to inhibit AHAS (Fig. 6A). Therefore, it was unexpected to observe activation of some AHAS mutants, particularly for the imidazolinones although it was also observed for SM in three cases. The activation is superimposed upon inhibition, leading to an 'inhibition' curve showing a maximum (Fig. 6B). We had observed that dimethylsulfoxide would activate the enzyme (Fig. 6C) and that with 15% dimethylsulfoxide added, AHAS shows little or no activation by herbicides. However, it retained the ability to be inhibited by these compounds (Fig. 6B) so addition of dimethylsulfoxide allowed K_i values to be determined.

SM inhibition

The Falco mutations were originally identified in yeast cells showing spontaneous SM resistance. Our measurements on purified yeast AHAS confirm this resistance, with increases (Tables 1 and 2) in the K_i values ranging from 10-fold (V583A) to over 15 000-fold (W586L). The resistance factors, expressed as K_i(mutant)/K_i(wild-type), are illustrated in Fig. 7A for SM and other sulfonylureas, and for the imidazolinones in Fig. 7B. For seven of the 10 Falco mutations, resistance to SM is greatest and for the other three (A200V, W586L and V583A) it ranks second. Presumably this bias towards SM resistance reflects the fact that SM was used for selection and if a different herbicide had been used, a somewhat different set of mutations would have been identified.

Sulfonylurea cross-resistance

There is enormous variation in the cross-resistance to other sulfonylurea herbicides. At one extreme is A117V, which shows more than 100-fold greater resistance to SM than to any of the other sulfonylureas. At the other extreme are P192S and W586L, showing quite good resistance to all of the sulfonylureas tested.

The results with P192S and W586L suggest that these residues interact with molecular features that are common to all sulfonylureas and the structure of the AHAS–CE complex confirms this conclusion. P192 interacts with the S-ring itself and this is identical in all sulfonylureas except TF. Nonpolar interactions are likely to be similar in either case. W586 is stacked upon the N-ring system and replacement by leucine will eliminate π-orbital overlap. As observed, it should not matter whether the N-ring is a pyrimidine (CE and SM) or a triazine (CS, MM, TB and TF), and the substituents on this ring should have little influence.

In contrast, A117V exhibits rather specific resistance to SM only, suggesting an interaction with a molecular feature that is unique to this herbicide. However, comparison of the structures of the various sulfonylureas fails to identify such a feature. The structure of the AHAS–CE complex highlights the problem with drawing conclusions in the absence of structural data. The side chain of A117 points away from the herbicide so it is not immediately obvious how mutation of A117 would result in resistance to any sulfonylurea, let alone a specific resistance to SM. Possibly, the smaller substituent on the S-ring of SM compared to CE allows a reorientation of A117 so that the side chain can interact with the N-ring. Mutation of A117 could then result in a specific but adverse interaction with the pyrimidine ring. This speculative explanation can be assessed when the structure of AHAS with bound SM has been solved. We have crystals of this complex that are currently being investigated.

The results for G116S appear to be more straightforward because mutation has a markedly lesser effect on CS than on any of the other sulfonylurea herbicides. This mutation would result in overlap with the large *ortho* substituent on the S-ring; only CS, with a chlorine atom in this position would accommodate the G116 mutation easily.

V583A and F590L each have somewhat similar effects. V583 has only some rather distant (≈ 4 Å) van der Waals interactions with the chlorine atom of CE, while F590 has no direct interactions at all. W586 is sandwiched between F590 and the N-ring of CE and we think that the effects of mutation at F590 are mediated through W586. A200V and D379N have similar effects to one another and they are equidistant from the para position on the S-ring of CE. There is no obvious correlation with the herbicide structures but, as mentioned earlier, such considerations are overly simple in that they do not take account of the conformation of bound CE with the two ends of the molecule acting as an ensemble. It is not known whether all of the sulfonylureas

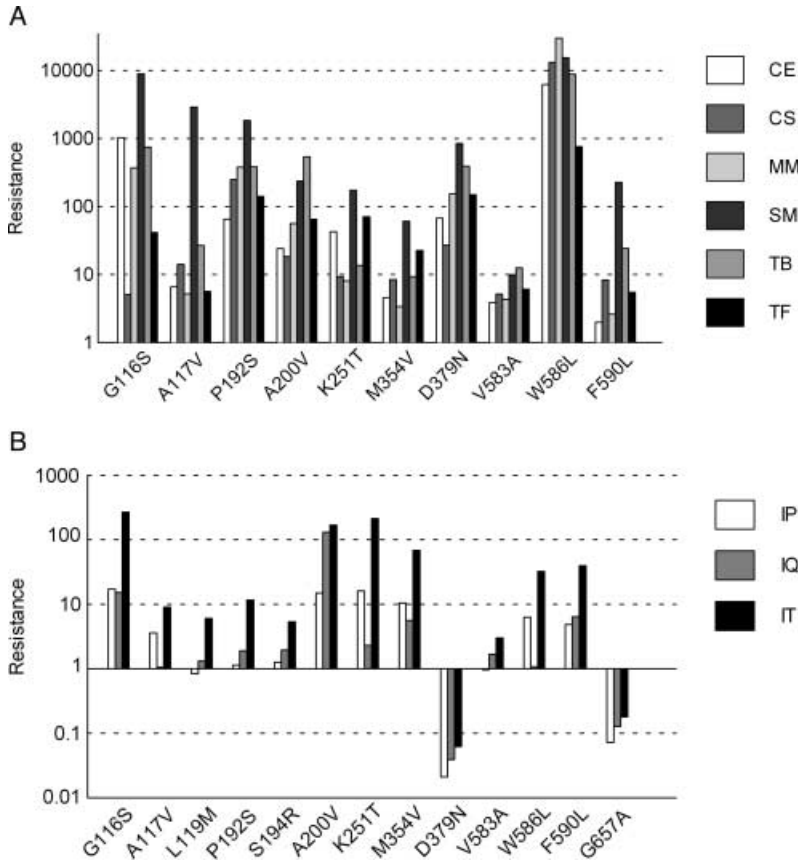

Fig. 7. Resistance of yeast AHAS mutants to (A) sulfonylureas and (B) imidazolinones. Resistance factors are expressed as the ratio K_i(mutant)/K_i(wild-type) and are plotted on a logarithmic scale. Where data were available in the absence and presence of dimethylsulfoxide, the geometric mean of the two resistance factors is shown. Resistance factors for CE with W586L, G116S, K251T, and P192S were reported previously [12].

adopt a similar conformation to that of CE when bound to AHAS. However, preliminary docking calculations have shown that an inverted position is possible with the S-ring, rather than the N-ring, inserted into the substrate access channel. Determining the structure of AHAS with other bound sulfonylureas will be of great interest. We have crystallized the enzyme in the presence of several other sulfonylureas and we aim to solve the structures of these complexes in the near future.

Imidazolinone resistance

Inhibition of yeast AHAS by imidazolinones is quite weak, with apparent K_i values in the millimolar range (Table 1) and solubility limitations allowed us to place a lower limit only on the extent of resistance. Subject to this caveat, every mutant shows the greatest resistance to IT (Tables 1–3). All 13 mutants showed an alteration in the K_i value for IT indicating that this herbicide interacts directly or indirectly with all of the mutation sites. Comparing resistance to IP and IQ, no obvious pattern emerges.

Microbial AHAS has low sensitivity to imidazolinones compared with the plant enzyme. This difference might suggest that the higher sensitivity of plant AHAS is due to residues that differ between the microbial and plant enzymes. The L119M, S194R and G657A mutants (Table 3) were constructed to test this hypothesis. Of these, only G657A is effective with increases in sensitivity ranging from 5.6-fold (IT) to 13.8-fold (IP). However, the most conspicuous increase in imidazolinone sensitivity is for D379N with increases of 16.1-fold (IT) to 48.8-fold (IP). This conservative mutation may remove adverse charge repulsion between D379 and the carboxyl group possessed by all of the imidazolinones tested. What remains puzzling is that plant AHAS, which also has an aspartate in this sequence position, is two- to three orders of magnitude more sensitive to imidazolinones than is yeast AHAS. In our view, these observations suggest some subtle difference in the way that imidazolinones bind to yeast and plant AHAS.

Conclusions

In summary, we have constructed and purified 10 mutants of yeast AHAS, and determined their resistance to a range of sulfonylurea and imidazolinone herbicides. Three further mutants were made with a view to decreasing imidazolinone resistance. Substantial differences are observed in the sensitivity of the mutant enzymes to these various inhibitors and these differences are generally consistent with the known structure of the herbicide-binding site.

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