

# Mutagenesis studies on the sensitivity of *Escherichia coli* acetohydroxyacid synthase II to herbicides and valine

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Acetohydroxyacid synthase (EC 4.1.3.18, also known as acetolactate synthase) isoenzyme II from *Escherichia coli* is inhibited by sulphonylurea and imidazolinone herbicides, although it is much less sensitive than the plant enzyme. This isoenzyme is also unusual in that it is not inhibited by valine. Mutating S100 (Ser<sup>100</sup> in one-letter amino acid notation) of the catalytic subunit to proline increases its sensitivity to sulphonylureas, but not to imidazolinones. Mutating P536 to serine, as found in the plant

enzyme, had little effect on the properties of the enzyme. Mutating E14 of the regulatory subunit to glycine, either alone or in combination with the H29N (His<sup>29</sup> → Asn) change, did not affect valine-sensitivity.

**Key words:** enzyme inhibition, herbicide resistance, site-directed mutagenesis, valine resistance.

## INTRODUCTION

The sulphonylurea and imidazolinone herbicides act by inhibiting acetohydroxyacid synthase (AHAS; also known as acetolactate synthase, EC 4.1.3.18), thereby interfering with the biosynthesis of the branched-chain amino acids. Although studies of the plant enzyme are more relevant to the action of these herbicides, the biochemical target of the sulphonylureas was first identified in bacteria [1]. Owing to their greater abundance and ease of purification, AHAS isoenzymes from the enteric bacteria have remained a useful model system for studying the inhibition by these herbicides.

*Escherichia coli* contains three major isoenzymes, each of which is a homotetramer of large ( $\approx 60$  kDa) catalytic subunits and small ( $\approx 9$ – $17$  kDa) regulatory subunits. The present work concerns isoenzyme II and its resistance, compared with plant AHAS, to inhibition by herbicides and its total insensitivity to the effector valine.

AHAS II is easily the most sensitive of the *E. coli* isoenzymes to inhibition by herbicides [2], although it is rarely acknowledged that this inhibition is much weaker than that of plant AHAS. For example, the apparent  $K_i$  for the sulphonylurea sulfometuron methyl of AHAS II is  $0.58 \mu\text{M}$  [3] compared with  $26 \text{ nM}$  for the *Arabidopsis thaliana* (thale-cress) enzyme [4]. Similarly, the apparent  $K_i$  for the imidazolinone imazethapyr of AHAS II is  $0.78 \text{ mM}$  [3] compared with  $9 \mu\text{M}$  for the *A. thaliana* enzyme [4].

It has been suggested [5] that the relative insensitivity of AHAS II to sulphonylureas is due to a serine residue at position 100 (S100 in one-letter amino acid notation) of the catalytic subunit. In plant and yeast AHAS there is a proline residue at the equivalent sequence position, and mutation to serine results in herbicide resistance [6–11]. Moreover, Mazur and Falco [5] reported that the S100P (Ser<sup>100</sup> → Pro) mutant of *E. coli* AHAS II has increased sensitivity to sulfometuron methyl. However, no quantitative data were reported, no effects on the catalytic properties of the enzyme were described, and no changes in imidazolinone-sensitivity were tested. We therefore decided to fully characterize this mutant.

An imidazolinone-resistant mutant of *A. thaliana* AHAS has been isolated [10,12,13] and shown to contain a mutation of S653

to asparagine. The apparent  $K_i$  for imazethapyr of this mutant is  $1.9 \text{ mM}$  [4], very similar to that of wild-type *E. coli* AHAS II ( $0.78 \text{ mM}$ ). The nature of the substitution does not appear to be important, and other amino acids with side chains larger than that of serine also results in imidazolinone insensitivity [14]. The equivalent residue in *E. coli* AHAS II is P536, suggesting that mutation to serine might increase the sensitivity to imidazolinones. Therefore this mutant was constructed and characterized.

AHAS from most species is subject to feedback inhibition by one, or by a combination, of the branched-chain amino acids. This is also true for isoenzymes I and III from *E. coli* (e.g. [15]), but AHAS II is unaffected by these compounds [16]. It has been shown that valine-sensitivity is conferred by the regulatory subunit of *E. coli* AHAS I and III [15] and of yeast AHAS [17]. Vyazmensky et al. [18] have demonstrated that the regulatory subunit of *E. coli* AHAS III binds valine and have noted that, in a spontaneous G14D mutant, this property is abolished. The equivalent mutation (G16D) has been discovered recently in a valine-resistant mutant of *Streptomyces cinnamonensis* AHAS [19]. Alignment of various AHAS regulatory subunit sequences (Figure 1A) shows that *E. coli* AHAS II is unique in that it contains glutamic acid rather than glycine at position 14. This suggests that mutation of E14 to glycine might cause it to acquire valine-sensitivity. However, we also noted that the *E. coli* AHAS II regulatory subunit sequence is unique in a second respect with a histidine residue at position 29 rather than the usual asparagine. We therefore tested the effect of the E14G mutation, both alone and in combination with the H29N change, on the sensitivity to inhibition by branched-chain amino acids.

## MATERIALS AND METHODS

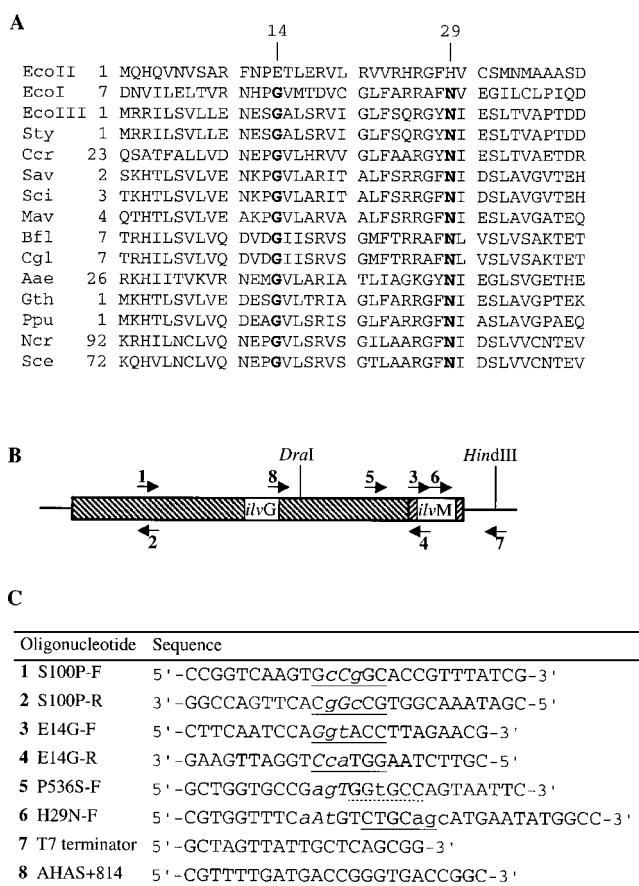
All materials, experimental procedures and data analyses were as described previously [3], except as noted below.

### Expression, purification and assay of AHAS

AHAS expression and purification were as described previously [16] using the plasmid pET-GM. This construct encodes both the catalytic (*ilvG*) and regulatory (*ilvM*) subunits (Figure 1B), with

Abbreviation used: AHAS, acetohydroxyacid synthase; one-letter amino codes are used (for example, S100 is Ser<sup>100</sup>, and H29N is a mutation of His<sup>29</sup> to Asn, and so on).

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**Figure 1** Mutagenesis of *E. coli* AHAS II

(A) Sequence alignment of various AHAS regulatory subunits with the first 40 residues of that of *E. coli* AHAS II. Residues conserved in all sequences except *E. coli* AHAS II are shown in **bold**. Species abbreviations are: Eco, *E. coli*; Sty, *Salmonella typhimurium*; Ccr, *Caulobacter crescentus*; Sav, *Streptomyces avermitilis*; Sci, *Streptomyces cinnamonensis*; Mav, *Mycobacterium avium*; Bfl, *Brevibacterium flavum* MJ233; Cgl, *Cornebacterium glutamicum*; Aae, *Aquifex aeolicus*; Gth, *Guillardia theta*; Ppu, *Porphyra purpurea*; Ncr, *Neurospora crassa*; Sce, *Saccharomyces cerevisiae*. (B) Diagrammatic representation of the catalytic (*ilvG*) and regulatory (*ilvM*) subunit genes of *E. coli* AHAS II. The numbered arrows represent oligonucleotides used for mutagenesis. (C) Oligonucleotides used for mutagenesis of *E. coli* AHAS II. All sequences are shown 5' to 3', except for the reverse oligonucleotides S100P-R and E14G-R. New restriction sites are shown with underlined bases, and the dotted underline in P536S-F shows where a restriction site for *NarI* (GGCGCC) is removed. In oligonucleotides 1–6, base differences from the wild-type sequence are shown in lower case and the mutated codon is shown in *italics*.

an N-terminal hexahistidine tag on the catalytic subunit. Purification was as described previously, except that elution during immobilized-metal-affinity chromatography used a gradient from 25 to 200 mM imidazole rather than batch elution with 100 mM imidazole.

AHAS assays were performed at 37 °C by monitoring the consumption of pyruvate at 333 nm [20]. Reactions were conducted in 50 mM potassium phosphate buffer, pH 7.8, containing 100 mM pyruvate, 1 mM thiamine diphosphate, 10 mM MgCl<sub>2</sub>, 10 μM FAD and various concentrations of herbicides or branched-chain amino acids. The concentration of pyruvate was varied over the range 0.5–50 mM in experiments to determine the  $K_m$  for the substrate.

In experiments to measure herbicide inhibition constants [ $K_i$  (app)], data were fitted initially using eqn. (1), where  $v_i$  is the

inhibited rate,  $v_o$  is the uninhibited rate, and  $[I]_T$  is the added concentration of inhibitor:

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

If this analysis indicated a value of  $K_i$  (app) that was comparable with the active-site concentration, then the data were reanalysed using eqn. (2) for tight-binding inhibition [21], where  $[E]_T$  is the enzyme active site concentration and other symbols are as defined for eqn. (1):

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

### Construction of mutant plasmids

Two of the mutations were made following the Stratagene QuikChange™ method, using the oligonucleotides shown in Figures 1(B) and 1(C); all procedures used were as described by Stratagene. The first of these mutations was S100P in the catalytic subunit using oligonucleotides 1 (S100P-F) and 2 (S100P-R), which introduce an additional *NaeI* site into the plasmid. This was used to identify mutant plasmids by the conversion of a 2534 bp fragment into two of 1944 and 590 bp upon *NaeI* digestion. The second mutation was E14G in the regulatory subunit, using oligonucleotides 3 (E14G-F) and 4 (E14G-R). These introduce an additional *KpnI* site into the plasmid, resulting in the conversion of a 6087 bp fragment into two of 5602 and 485 bp upon *KpnI* digestion.

The P536S (catalytic subunit) and the double E14G/H29N (regulatory subunit) mutants were made using the 'megaprimer' method described by Brøns-Poulsen et al. [22]. For the first of these mutations, pET-GM was used as the template in PCR with the primers 5 (P536S-F) and 7 (T7 terminator) (Figures 1B and 1C) to produce a 440 bp fragment. The PCR conditions used were: 1 min at 95 °C, 30 times (1 min at 95 °C, 1 min at 60 °C, 40 s at 72 °C) and 7 min at 72 °C. This fragment was then used as the megaprimer in a second PCR together with primer 8 (AHAS + 814) to yield a 1222 bp fragment. The PCR conditions used were 1 min at 95 °C, 30 times (1 min at 95 °C, 2 min at 72 °C) and 7 min at 72 °C. The PCR product was digested with *DraI* and *HindIII* (986 bp) and ligated to the pET-GM vector (6343 bp) digested with the same enzymes. The P536S-F primer removes an existing *NarI* site that was used for mutant identification by the appearance of a 6012 bp fragment upon *NarI* digestion, instead of the two of 4098 and 1914 bp found for the wild-type.

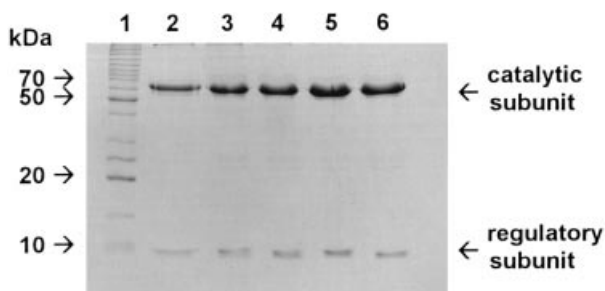
The E14G/H29N double mutant was prepared using a similar procedure as that used for P536S, except that the plasmid containing the E14G mutation was used as template, and primer 6 (H29N-F) (Figures 1B and 1C) replaced P536S-F to produce a 314 bp megaprimer from the first PCR. The mutagenic primer introduced a unique *PstI* site that was used for mutant identification.

All mutations were confirmed by DNA sequencing using the Prism Ready Dye Deoxy Terminator Cycle Sequencing Kit and DNA Sequencer 373A (Perkin-Elmer Applied Biosystems, Norwalk, CT, U.S.A.).

## RESULTS

### Expression, purification and assay

After induction of expression, wild-type and all mutants showed AHAS activity in cell extracts and when purified, indicating that none of the mutations destroys its catalytic ability. Each purified



**Figure 2** SDS/PAGE analysis of purified wild-type and mutant *E. coli* AHAS II

Lane 1, molecular-mass standards; lane 2: wild-type; lane 3: S100P; lane 4: P536S; lane 5: E14G; lane 6: E14G/H29N.

**Table 1** Catalytic properties of wild-type and mutants of *E. coli* AHAS II

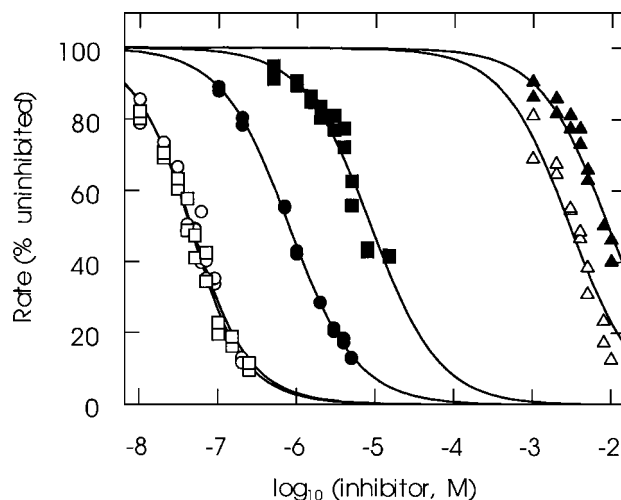
Enzyme	Specific activity (units/mg)	$K_m$ for pyruvate (mM)
Wild-type	73.0	$4.76 \pm 0.26$
S100P	25.2	$3.51 \pm 0.17$
P536S	15.4	$6.05 \pm 0.22$
E14G	60.9	$4.88 \pm 0.20$
E14G/H29N	25.1	$7.11 \pm 0.37$

**Table 2** Herbicide inhibition of wild-type and mutants of *E. coli* AHAS II

Enzyme	Herbicide ...	$K_i$ (app)			
		(nM)		(mM)	
	Chlorimuron ethyl	Chlorsulfuron	Tribenuron ethyl	Imazapyr	
Wild-type	$31.9 \pm 2.7$	$800 \pm 30$	$8800 \pm 900$	$9.2 \pm 0.8$	
S100P	$13.7 \pm 3.6$	$33.2 \pm 5.2$	$26.2 \pm 3.9$	$3.0 \pm 0.4$	
P536S	$37.9 \pm 3.1$	$1130 \pm 70$	$22300 \pm 1900$	$26.6 \pm 3.6$	

mutant AHAS was subjected to SDS/PAGE, which revealed that only minor impurities are present (Figure 2). The specific activity of each purified mutant is lower than that of wild-type (Table 1), but none showed less than 20% of the wild-type specific activity. The  $K_m$  for pyruvate also showed some small variations. Thus, none of the changes affects a residue that plays any crucial role in substrate binding or catalysis.

The activity assay used in the present studies is based on monitoring the consumption of pyruvate by its absorbance at 333 nm [20]. The advantage of this assay, compared with the widely used colorimetric assay [23], is that it is continuous, thereby allowing true initial rates to be observed. This is especially important for testing the effects of herbicides, which show a time-dependent increase in the potency of the inhibition [3]. The disadvantage of this assay is that it is about 1000-fold less sensitive than the colorimetric assay and requires the use of moderately large amounts of enzyme. For the present studies the active-site concentration used in assays for each enzyme, calculated using a heterodimer molecular mass of 74.3 kDa, were: wild-type, 26 nM; S100P, 33 nM; P536S, 49 nM; E14G, 67 nM;



**Figure 3** Inhibition of wild-type and the S100P mutant of *E. coli* AHAS II by herbicides

Closed symbols, wild-type; open symbols, S100P. The inhibition curves shown are for chlorsulfuron (circles), tribenuron methyl (squares) and imazapyr (triangles). In all cases, herbicide concentrations (M) are shown on a logarithmic scale, rates are expressed as a percentage of the uninhibited rate, and the curves shown represent the best fit to the data using the  $K_i$  (app) values shown in Table 2.

and E14G/H29N, 46 nM. These concentrations are comparable with those of the herbicides used in some experiments, and allowance for tight binding must be made when evaluating the data. The data analysis was described previously [3].

#### Herbicide-sensitivity of S100P

We have shown previously [3] that there is considerable variation in the sensitivity of wild-type *E. coli* AHAS II to various sulphonylurea herbicides. Three of these compounds were chosen (Table 2), representing a relatively strong [chlorimuron ethyl;  $K_i$  (app)  $\approx 3 \times 10^{-8}$  M], moderate [chlorsulfuron;  $K_i$  (app)  $\approx 8 \times 10^{-7}$  M] and weak [tribenuron methyl;  $K_i$  (app)  $\approx 9 \times 10^{-6}$  M] inhibitor. In contrast, the enzyme exhibits similar sensitivity to various imidazolinones [3] and imazapyr [ $K_i$  (app)  $\approx 9 \times 10^{-3}$  M] was chosen as a representative of these inhibitors.

Mutation of S100 to proline increases the sensitivity to all four herbicides (Figure 3; chlorimuron ethyl omitted for clarity), but the greatest effect (340-fold) is for tribenuron methyl. This mutation appears to abolish the capacity of the enzyme to discriminate between the various sulphonylureas, and all have  $K_i$  (app) values in the range  $(1-3) \times 10^{-8}$  M (Table 2). In contrast, the sensitivity to the imidazolinone imazapyr remained low, with a  $K_i$  (app) value in the millimolar concentration range.

#### Herbicide-sensitivity of P536S

By analogy with our previous results on mutagenesis of *A. thaliana* AHAS [4,14], we had hypothesized that mutation of P536 to serine would increase the sensitivity to imidazolinones without affecting the inhibition by sulphonylureas. The second part of this prediction was confirmed (Table 2), and there were only small changes (increases) in the  $K_i$  (app) values for the sulphonylureas. However, this mutant remained insensitive to imazapyr; if anything, the  $K_i$  (app) value increases somewhat.

### Sensitivity of E14G and E14G/H29N to branched-chain amino acids

Wild-type *E. coli* AHAS II is not inhibited by the branched-chain amino acids [16], unlike the other isoenzymes from *E. coli* [17], which are inhibited by valine. It has been suggested that a glycine residue at position 14 of the regulatory subunit of AHAS III [18] or the equivalent position of *S. cinnamonensis* AHAS [19] is associated with this valine-sensitivity. Since *E. coli* AHAS II has a glutamate residue at position 14, we mutated it to a glycine residue. The resulting mutant remained completely unaffected by valine at concentrations of up to 10 mM. Further, it was not inhibited by either leucine or isoleucine at 10 mM.

We also prepared a double mutant containing the E14G and H29N changes. This mutant was similar to the wild-type enzyme in that it was not inhibited by any of the branched-chain amino acids at 10 mM. To confirm that the expressed regulatory subunit contained both mutations, MS was performed, yielding a value of 9609 Da. This is 97 Da smaller than that obtained previously [24] for the wild-type regulatory subunit; the expected size decrease for the double mutant is 95 Da.

### DISCUSSION

Since the original discovery in bacteria [1] that sulphonylurea herbicides inhibit AHAS, the enzyme from the enteric bacteria has been studied extensively. Although these bacterial enzymes are useful models for investigating herbicide action, they differ from the plant enzymes in several respects. One of these differences concerns the herbicide concentrations needed to achieve significant inhibition, which can be more than two orders of magnitude higher.

Here we have attempted to increase the sensitivity of *E. coli* AHAS II to herbicides by mutation of S100 to proline and P536 to serine, each in the catalytic subunit. Each mutant enzyme was purified (Figure 2) and shown to have a similar  $K_m$  for pyruvate to that of the wild-type (Table 1). There is some reduction in the specific activity, but neither residue is essential for catalysis.

The S100P mutation resulted in increased sensitivity to sulphonylureas (Table 2), with the greatest effect (340-fold) on the weakest inhibitor (tribenuron methyl). The overall effect was to reduce the capacity of the enzyme to discriminate between the sulphonylureas tested. The mutant *E. coli* enzyme shows similar sensitivity to chlorimuron ethyl and chlorsulfuron as wild-type *A. thaliana* AHAS [4], which has  $K_i$  (app) values of 11 and 55 nM respectively (cf. Table 2). For tribenuron methyl, the  $K_i$  (app) value of wild-type *A. thaliana* AHAS is about 10-fold higher than the S100P mutant *E. coli* AHAS II. However, the  $K_i$  (app) for imazapyr is decreased by only a small amount and the mutant remains 180-fold less sensitive than *A. thaliana* AHAS. Thus this variant is a good model of plant AHAS compared with the wild-type with respect to inhibition by sulphonylureas, but not for imidazolinones. However, it does suffer from the deficiency that it does not discriminate between different sulphonylureas to the same extent as wild-type *A. thaliana* AHAS [4] or *E. coli* AHAS II [3]. We have observed previously that mutations that affect the herbicide-sensitivity of AHAS tend to reduce the ability to discriminate between different sulphonylureas [3,4]. The molecular explanation for this phenomenon will not become clear until a three-dimensional structure of AHAS has been determined.

In an attempt to increase sensitivity to imidazolinones, P536 in the catalytic subunit was mutated to serine. The basis for this choice was that the equivalent residue in imidazolinone-sensitive plant AHAS is serine, and mutation of *A. thaliana* AHAS to a larger residue (threonine, asparagine or phenylalanine) increases

the  $K_i$  (app) for imazapyr by more than 100-fold [14]. Thus we reasoned that replacing P536 with serine would decrease the  $K_i$  (app) value. This proved not to be the case; the mutant showed a small increase in the  $K_i$  (app) for imazapyr and also for sulphonylureas (Table 2).

To explore the roles of residues 14 and 29 in the regulatory subunit, two mutants were prepared. The mutated enzymes were purified (Figure 2), and it was found that E14G has very similar catalytic properties to those of the wild-type (Table 1), while the E14G/H29N double mutant has a slightly elevated  $K_m$  for pyruvate and a decreased specific activity. We have shown previously [16] that the regulatory subunit of *E. coli* AHAS II is absolutely required for catalytic activity, so we can conclude that these two mutant forms of the regulatory subunit remain capable of assembly into the active tetramer.

Neither mutant was responsive to valine, leucine or isoleucine. Although we have not tested the H29N mutation alone, we believe that it is unlikely to be different from the two mutants tested. Clearly, the lack of sensitivity of *E. coli* AHAS II to the branched-chain amino acids is more complex than just sequence changes at these two positions. Further regulatory subunit mutants are now being explored.

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