Mutagenesis of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and characterization of three herbicide-insensitive forms

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Sulphonylurea and imidazolinone herbicides act by inhibiting acetohydroxyacid synthase (AHAS; EC 4.1.3.18), the enzyme that catalyses the first step in the biosynthesis of branched-chain amino acids. AHAS requires as cofactors thiamin diphosphate, a bivalent metal ion and, usually, FAD. *Escherichia coli* contains three isoenzymes and this study concerns isoenzyme II, the most herbicide-sensitive of the *E. coli* forms. A plasmid containing the large and small subunit genes of AHAS II was mutagenized using hydroxylamine and clones resistant to the sulphonylurea chlorimuron ethyl were selected. Three mutants were isolated; A26V, V99M and A108V. A26V has been described previously whereas the equivalent mutation of A108V has been reported in a herbicide-insensitive variant of yeast AHAS. The V99M mutation has not been discovered previously in AHAS from any source. The mutants were each over-expressed in *E. coli*, and the enzymes were purified to homogeneity. Some differences from wild type in the kinetic properties (k_{cat}, K_m and cofactor affinities) were observed, most notably a 28-fold decrease in the affinity for thiamin diphosphate of V99M. None of the mutants shows marked changes from the wild type in sensitivity to three imidazolinones, with the largest increase in the apparent inhibition constant being a factor of approximately 5. The A26V mutant is weakly resistant (6- to 20-fold) to six sulphonylureas, whereas stronger resistance is seen in V99M (20- to 250-fold) and A108V (35- to 420-fold). Resistance as a result of these mutations is consistent with a molecular model of the herbicide-binding site, which predicts that mutation of G249 might also confer herbicide insensitivity. Three G249 mutants were constructed, expressed and purified but all are inactive, apparently because they cannot bind FAD.

INTRODUCTION

Unlike animals, plants are capable of synthesizing the branched-chain amino acids. This biochemical difference has been exploited to produce herbicidal compounds that act by inhibiting one of the enzymes in this pathway. Experimental herbicides have been created that are directed at several of these enzymes [1–4], but the only ones that have attained widespread field use are those that target acetohydroxyacid synthase (AHAS; EC 4.1.3.18). Such herbicides include the sulphonylureas [5] and the imidazolinones [6].

Although the primary interest in AHAS derives from its susceptibility to herbicides, much of the detailed enzymology has focused upon the enzyme from micro-organisms and principally the three isoenzymes (AHAS I, II and III) from the enteric bacteria. This is because it has proved difficult to obtain pure enzyme from plants owing to its low abundance and instability. Only the maize [7] and barley [8] enzymes have been isolated from their native sources and purified to a specific activity approaching that of AHAS from bacteria. The recent development of heterologous expression systems for plant AHAS genes by several groups [9–13] is likely to see a more detailed understanding of the properties of the enzyme emerging.

Herbicide-insensitive forms of AHAS have been described in many species and in several cases the nature of the mutation has been defined at the molecular level. It is unfortunate that, as a rule, these studies have not been accompanied by a thorough characterization of the mutant enzyme's enzymology. For example, the most extensive group of AHAS mutants are the sulphonylurea-resistant variants of the yeast enzyme described by Falco et al. [14]. Regrettably, they have presented little data on the enzymic properties of these variants; indeed, even the particular herbicide to which these mutants are insensitive was not clearly specified.

For the mutants that have been characterized to some degree, generally little more than an IC_{50} value for one or more herbicides has been determined. However, these mutants could display changes in several of the catalytic properties and, in some of the few cases where they have been examined, such changes have been identified. For example Yadav et al. [15] reported that the A26V substitution in *Escherichia coli* AHAS II results in a considerably reduced catalytic ability in the soluble cell extract, whereas Falco et al. [14] found that the same substitution in yeast AHAS results in a reduction to 10 % of the wild-type activity. It is not clear whether these reductions in activity represent true changes in the catalytic capacity of the enzymes or if they arise for other reasons, such as changes in the level of expression or inefficient folding of the mutants. However, Ott et al. [10] have reported an 11-fold reduction in catalytic capacity of purified recombinant enzyme for the *Arabidopsis thaliana* AHAS mutant M124E, and Bernasconi et al. [9] observed a 5-fold decrease for the W552L mutant of the recombinant cocklebur enzyme. Recently we [12] have shown both increases and decreases of k_{cat} in four mutants of recombinant *A. thaliana* AHAS.

Ibdah et al. [16] made nine substitutions in a proposed substrate-binding channel of *E. coli* AHAS II at three different sites, one of which (W464) is a herbicide-resistance site. They found that most substitutions cause a change in the K_m for the substrate pyruvate and the activation constants (K_c) for the cofactors thiamin diphosphate (ThDP) and FAD, the largest being a 53-fold increase in the K_c for ThDP. Similarly, Rathinasabapathi and King [17] found increases of 3- to 16-fold in the K_m for substrate in five herbicide-insensitive mutants of *Datura innoxia*. Subramanian et al. [18] observed no change in the K_m for

Abbreviations used: AHAS, acetohydroxyacid synthase; CE, chlorimuron ethyl; CS, chlorsulfuron; ThDP, thiamin diphosphate.

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pyruvate of one tobacco and one cotton mutant but a second cotton mutant displayed a 4- to 9-fold increase; there was no change in the K_c for ThDP of these three variants. Chang and Duggleby [19] observed changes in the K_m for pyruvate and the K_c for all three cofactors in four *A. thaliana* AHAS mutants. Changes in the K_m for pyruvate were also reported for *A. thaliana* AHAS mutants by Mourad et al. [20], although these authors suggest that the variations are not statistically significant. Similarly, no difference in the K_m for pyruvate was observed for various other herbicide-resistant plant AHAS mutants [18,21–23].

These studies of mutants have shown that several sites can lead to herbicide resistance. Easily the most frequent mutation is at W464 (*E. coli* AHAS II numbering), which has been found in yeast [14], oilseed rape [24], tobacco [25] and cocklebur [9] AHAS, and has been introduced into *E. coli* AHAS II [16]. A second resistance site that has been identified in several organisms is at position 100, having been found in *A. thaliana* [26,27], yeast [14,28] and tobacco [25,29].

Mutation at the second of these sites has not been shown to confer herbicide resistance on the isoenzymes of *E. coli* AHAS. However, there is indirect evidence that the amino acid at this position affects herbicide sensitivity. AHAS II naturally contains a serine at position 100 and mutation to a proline (as found in the wild-type plant enzymes) increases herbicide sensitivity [30]. The only other positions in *E. coli* AHAS where mutations have been shown to result in herbicide resistance are residues 26 and 460. In the former case, the change is from alanine to valine [15] and mutation of this alanine also confers herbicide insensitivity on yeast AHAS [14,15]. For M460, mutation to either alanine or asparagine, but not leucine, is effective [16].

In the present study, we have used random mutagenesis to isolate three herbicide-insensitive mutants of *E. coli* AHAS II; A26V, V99M and A108V. The first of these was mentioned above, whereas A108V is known to confer resistance on yeast AHAS but has not been described until now for any *E. coli* isoenzyme. V99M is a new mutation that has not been seen previously for AHAS from any source. Each of the mutant enzymes has been purified and characterized with respect to its kinetic parameters for substrate and cofactors, and susceptibility to inhibition by six sulphonylureas and three imidazolinones.

All three sites, including the newly-discovered V99, are within a putative herbicide-binding region derived from the model of *E. coli* AHAS II described by Ibdah et al. [16]. Another amino acid within this region is G249, which we have also mutated. Three mutants were isolated but none is active because the introduction of a side-chain at position 249 affects the ability of the proteins to bind FAD.

MATERIALS AND METHODS

Materials

The plasmid pAH29 [31], containing *ilv*GM from the *ilv*G2096 allele within a 4.6 kb *Hin*dIII fragment, was obtained from Dr. G. W. Hatfield of the University of California (Irvine, CA, U.S.A.). This was subcloned into pET-30a(+) (Novagen, Madison, WI, U.S.A.) to yield the expression vector pET-GM as described previously [32]. The *E. coli* strain CU1147 was kindly provided by Dr. H. E. Umbarger (Purdue University, W. Lafayette, IN, U.S.A.), while BL21(DE3) *E. coli* cells were purchased from Novagen.

Restriction endonucleases, DNA-modifying enzymes and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA, U.S.A.) or Progen Industries (Brisbane, QLD, Australia) and used in accordance with the suppliers' instructions. Most other molecular biological protocols followed the procedures outlined by Sambrook et al. [33]. Sulphonylurea herbicides were gifts from Dr. H. Brown (Du Pont Agricultural Products, Newark, DE, U.S.A.), whereas the imidazolinones were donated by Dr. B. K. Singh (American Cyanamid, Princeton, NJ, U.S.A.). The structures of the herbicides used in this study may be found in a previous report from this laboratory [19]. Other chemicals were from Ajax Chemicals (Auburn, NSW, Australia) or Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Random mutagenesis

The selection of herbicide-resistant mutants involves growth of the AHAS⁻ *E. coli* strain CU1147, which has been transformed with a plasmid carrying the mutated *ilv*GM genes, in minimal medium lacking the branched-chain amino acids but containing chlorimuron ethyl (CE). In previous work from this laboratory [32], we have described the plasmid pT7-GM for over-expression of the *ilv*GM genes and purification of the enzyme in its native form. A second plasmid, pET-GM, allows over-expression and purification of the enzyme with an N-terminal hexahistidine fusion peptide. Neither of these plasmids is suitable for selection in CU1147 because the *ilv*GM genes are under the control of the T7 promoter whereas this *E. coli* strain does not contain the gene for T7 RNA polymerase. Moreover, these plasmids give high levels of AHAS expression that are likely to overwhelm the inhibitory effects of added herbicides. Accordingly, it was necessary to transfer the genes to a plasmid capable of expressing them at moderate levels in CU1147. PCR was performed to amplify the *ilv*GM genes as described previously for pET-GM [32], and the PCR product was cloned into the *Bam*HI/*Eco*RI restriction sites of the plasmid pUC19. The genes were brought into frame by a T4 DNA polymerase fill-in of the overhangs created by an *Xba*I digest followed by blunt-end ligation to yield the construct pUC19E-GM. Transformation of CU1147 with wild-type pUC19E-GM allows the cells to grow on minimal medium and this growth is prevented by inclusion of 1 μg/ml (2.41 μM) CE.

Hydroxylamine-induced mutagenesis was performed following the method of Miller [34]. In a sterile glass tube, 0.4 ml of 500 mM potassium phosphate (pH 6.0) containing 5 mM EDTA was combined with 0.8 ml of 2.5 M NH_2OH (pH 6.0)/0.8 ml of water/20 μg of pUC19E-GM. The solution was incubated at 37 °C for 48 h and then diluted with 0.3 ml of water before it was applied to a PD10 (Pharmacia, Piscataway, NJ, U.S.A.) desalting column equilibrated with sterile water and eluted with 3.5 ml of water. The eluate was divided into seven 0.5 ml aliquots and the DNA precipitated by the addition of 0.625 ml of 96% ethanol and 0.125 ml of sodium acetate (pH 5.2). The DNA was collected by centrifugation (20800 ***g***) at 4 °C for 30 min and then dissolved in 20 μl of water and pooled.

Mutagenized pUC19E-GM was transformed into the *E. coli* strain CU1147. The transformation mixture was washed twice with minimal salts solution before plating on minimal medium containing 1 μg/ml CE. Cells expressing a functional and herbicide-insensitive AHAS II were allowed to grow during incubation of the plates at 37 °C for 48 h. Individual isolated colonies were inoculated into 3 ml of LB containing 100 μg/ml ampicillin, incubated at 37 °C overnight and then plasmid DNA was extracted and purified. The mutation in these clones was identified by completely sequencing the *ilv*GM genes. Unique mutant *ilv*GM genes were subcloned from the purified pUC19E-GM plasmid to yield mutant variants of the expression vector pET-GM [32] using the *Bam*HI and *Eco*RI restriction sites.

Table 1 Site-directed mutagenesis of G249 in *E. coli* AHAS II

The first line shows part of the wild-type DNA coding strand (5′ → 3′) and the corresponding amino acid sequence of the large subunit of *E. coli* AHAS II. Shown in bold is G249 and its corresponding codon. The second line shows the complementary synthetic oligonucleotide (3′ → 5′) that was used for mutagenesis; positions in italics are where partially degenerate bases were included with Y denoting C or T, and D indicating A, G or T. The remaining six lines show the possible variant DNA (5′ → 3′) and amino acid sequences that could be obtained, with differences from wild-type underlined.

	DNA sequence (bp 733–759)	Amino acids (245–253)
Wild-type coding strand	5′ CTG GGC ATG CTG **GGG** ATG CAC GGC ACC 3′	L G M L **G** M H G T
Mutagenic oligonucleotide	3′ GAC CCG TAC GAC *YD*C TAC GTG CCG TGG 5′	
Possible variants	5′ CTG GGC ATG CTG <u>GA</u>C ATG CAC GGC ACC 3′	L G M L <u>E</u> M H G T
	5′ CTG GGC ATG CTG <u>GC</u>C ATG CAC GGC ACC 3′	L G M L <u>A</u> M H G T
	5′ CTG GGC ATG CTG <u>GT</u>C ATG CAC GGC ACC 3′	L G M L <u>V</u> M H G T
	5′ CTG GGC ATG CTG <u>AA</u>C ATG CAC GGC ACC 3′	L G M L <u>N</u> M H G T
	5′ CTG GGC ATG CTG <u>AC</u>C ATG CAC GGC ACC 3′	L G M L <u>T</u> M H G T
	5′ CTG GGC ATG CTG <u>AT</u>C ATG CAC GGC ACC 3′	L G M L <u>I</u> M H G T

Site-directed mutagenesis

The *in vitro* mutagenesis system Altered Sites® II (Promega, Madison, WI, U.S.A.) was used to introduce mutations in the *ilv*G gene at the G249 codon. An oligonucleotide was designed against the region surrounding the G249 codon and contained two degenerate positions corresponding to the first and second bases of this codon (Table 1). After mutagenesis following the manufacturer's instructions, plasmid DNA was extracted and purified, and clones were screened for the presence of a mutation by direct sequencing. Mutations at G249 of *ilv*G were then subcloned into pET-GM as described in the previous section.

Bacterial growth, protein expression and purification

A single colony of the *E. coli* strain BL21(DE3), transformed with the plasmid pET-GM (wild-type or mutant), was grown, induced and harvested as described previously [32]. The protein expressed from pET-GM has an N-terminal oligohistidine tag that is used for purification by means of immobilized metal-affinity chromatography on a metal-chelating column. Cell lysis and chromatography were carried out following the procedure that we described earlier [32].

Gel-filtration chromatography and SDS/PAGE

Gel-filtration experiments were carried out on a Pharmacia Smart System fitted with a 3.2 mm × 300 mm Superose-12 column (Pharmacia). The column was calibrated with molecular-mass markers in the range 1.35–670 kDa (BioRad, Regents Park, NSW, Australia). Protein solutions (0.2–4.0 mg/ml) were applied at a flow rate of 40 μl/min to the column, which was equilibrated with 20 mM Tris/HCl buffer (pH 7.9) containing 500 mM NaCl. Samples eluting from the column were detected by monitoring the absorbance at 214 and 280 nm. SDS/PAGE was performed as described by Laemmli [35].

Sequence determinations

DNA sequencing was performed using the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism) and DNA Sequencer 373A (Applied Biosystems, Norwalk, CT, U.S.A.).

Enzyme and protein assays

For all enzyme assays and kinetic studies, initial rates were measured using a continuous assay [36], conducted at 37 °C, in which the disappearance of pyruvate was monitored at 333 nm ($\epsilon_M = 17.5\ M^{-1} \cdot cm^{-1}$). The standard assay contained 100 mM Tricine/NaOH (pH 7.8), 50 mM pyruvate, 1 mM ThDP, 10 mM $MgCl_2$ and 10 μM FAD. The assay was carried out on a Molecular Devices SPECTRAmax-250 microplate spectrophotometer. Enzyme (50 μl) was dispensed into the wells of a UV-transparent multi-well plate and complete assay buffer dispensed into the wells adjacent to the enzyme. Enzyme and assay buffers were pre-incubated at 37 °C for 5–15 min before the initiation of the reaction. The assay was started by the addition of 200 μl of the buffer to the enzyme using an EDOS 5221 electronic dispensing system (Eppendorf, Hamburg, Germany) fitted with a Multi-200 multichannel adapter. The pathlength of the 250 μl reaction was calculated using the absorption coefficient of pyruvate and was determined to be 0.82 cm. Activity (1 unit) is defined as that producing 1 μmol of acetolactate per min under the above conditions. Protein determination was performed using the bicinchoninic acid method [37].

CD

CD spectra were recorded over the range 185–350 nm on a Jasco J-710 spectropolarimeter set at 20 mdeg sensitivity, 1 nm resolution, 1 unit accumulation, 2 s response and a scanning speed of 200 nm/min. Protein solutions of 0.25–1.0 mg/ml were measured in a 300 μl cylindrical quartz cell of 1 mm pathlength.

FAD spectra

Absorbance spectra were measured on a GBC UV/VIS 920 spectrophotometer. Protein solutions (1.0 mg/ml) were dispensed into a 1 ml black-walled quartz cuvette and the absorbance measured over the range 300–550 nm at intervals of 0.1 nm. The baseline absorbance of the storage buffer was subtracted to obtain the spectra of bound FAD.

Cofactor-free enzyme and solutions

Cofactors were removed from AHAS II by treatment of the enzyme with activated charcoal followed by gel-filtration chromatography. The enzyme (200 μl of 9–20 mg/ml) in 20 mM Tris/HCl (pH 8.1), containing 10 μM FAD, 1 mM EDTA and 1 mM dithiothreitol was combined with 200 μl of 4 M KCl and 10–15 mg of activated charcoal (G-60 grade carbon, Darco). The mixture was incubated for 2 h at 37 °C then the charcoal was removed by two consecutive centrifugation steps (20800 ***g***) for 5 min at 4 °C. EDTA (20 μl of 100 mM) was mixed with the

sample which was then applied to a 5 ml Sephadex G-25 desalting column (HiTrap, Pharmacia) equilibrated with metal-free 100 mM Tris/HCl (pH 7.9).

The components of the assay buffer used in the cofactor saturation experiments were treated to remove traces of bivalent metal ions. The water (obtained from a Milli-Q PLUS Ultrapure water system), Tris/HCl buffer and ThDP solutions were passed through separate columns of Chelex 100 cation-exchange resin (BioRad), which was prepared before use according to the manufacturer's instructions. The pyruvate, FAD and dithiothreitol stock solutions were prepared using the metal-free water eluted from the Chelex column. The multi-well plate used in the assay was soaked in 20 % HNO_3 overnight and rinsed several times with water before use. The concentration of Mg^{2+} in stock solutions used in cofactor-saturation experiments was determined previously in this laboratory [38] by atomic absorption spectroscopy. Measurements were made at 285.2 nm with a slit width of 0.5 nm on a Varian Techtron model AA-6 equipped with an air–acetylene flame. Absorbance values were obtained by integration over 3 s. The concentration of ThDP stock solutions in 100 mM Tris/HCl (pH 7.9) were measured spectrophotometrically at 267 nm using an ϵ_{267} of 7800 $M^{-1} \cdot cm^{-1}$ [39].

Kinetic data analysis

The appropriate equations were fitted to the kinetic data by non-linear regression using the programs DNRPEASY (modified from DNRP53 [40]), GraFit (Erithacus Software Ltd) and InPlot4 (GraphPAD Software).

RESULTS

Random mutagenesis

Several herbicide-resistant clones were isolated following random mutagenesis and the entire DNA sequence of the *ilv*GM genes was determined in each case. All contained a C base at nucleotide 372, which differs from some of the published sequences (e.g. GenBank M37337 but not X04890) that contain a T base at this position. This difference cannot be responsible for the herbicide-resistant phenotype because it is silent (H124, CAT or CAC) and sequencing of the wild type confirmed a C base at nucleotide 372. Each of the clones contained only one difference from the wild-type; three variants were identified and the sequence differences are shown as mutants 1, 2 and 3 in Table 2. Hydroxylamine mutagenesis preferentially causes C → T transitions, or G → A if the mutagen reacts with the non-coding strand. All three mutations conform to the expected type of base change. In other experiments using a similar mutagenesis procedure, the mutation W464L was identified in several clones. This mutation was not investigated further as it has been characterized adequately by Ibdah et al. [16].

Table 2 Sequence changes in *E. coli* AHAS II mutants

Mutant	DNA	Protein
1	C77T	A26V
2	G295A	V99M
3	C323T	A108V
4	G746A	G249E
5	G746C	G249A
6	G746T	G249V

Expression and purification

Each of the mutants A26V, V99M and A108V was cloned into the expression vector pET-GM [32] and expressed. The resulting mutant protein was purified (Table 3) and in each case the product appeared to be close to purity, as judged by SDS/PAGE (Figure 1). This gel was overloaded in order to visualize the small subunit; at normal loadings, the impurities are barely detectable. The high-molecular-mass material represents photo-crosslinked large-subunit polymers that were described previously [32]. The final specific activity of each mutant is somewhat lower than the value of 44.8 units/mg obtained previously for the wild-type enzyme [32]. However, even the lowest of the values (A26V; 35 % of wild type) indicates that none of the mutations seriously compromises the catalytic capacity of AHAS II. The expression level of each mutant enzyme is high, ranging from 22 to 35 % of the soluble protein, as judged by comparison of the specific activities of the cleared lysate and the final product. These are similar values to the 32 % obtained in our earlier study of the wild-type enzyme [32]. The yields after purification differ to some extent from one mutant to another but the range (48–76 %) encompasses the value for the wild-type enzyme of 65 % [32].

Kinetic properties

The mutants and wild-type enzyme were characterized with respect to the saturation curves for substrate and for each of the cofactors. In all cases, hyperbolic saturation curves were observed with little or no detectable activity in the absence of substrate or any one cofactor (results not shown). From these data, k_{cat} and K_m for pyruvate, or the activation constant (K_c) for each of the cofactors, were determined (Table 4) by fitting equation 1 to the results:

$$v = k_{cat}[E]_o/(1+K/[X]) \quad (1)$$

In this equation, v is the rate, $[E]_o$ is the concentration of active sites (determined from the protein concentration), and K is K_m or K_c, depending on whether the varied component (X) is the substrate or a cofactor.

Each mutant shows a k_{cat} value somewhat lower than wild-type, in broad agreement with the relative specific activities measured during purification. The K_m of A26V is close to normal whereas that of the remaining two mutants is somewhat lower; however, none of the differences is large, indicating that the substrate-binding site is not seriously perturbed by the mutations.

The affinity for FAD of each mutant is affected to some degree, particularly for V99M, which shows a more than 4-fold increase in K_c. Changes were also observed in the K_c for Mg^{2+}, which is a little lower than normal in A26V but elevated in both V99M (1.7-fold) and A108V (2.9-fold). The most notable changes occur in the K_c for ThDP, which is elevated in all cases, particularly for V99M where it is 28-fold higher than wild-type. Even the smallest change in the K_c for ThDP (3.5-fold in A108V) is comparable with the largest change observed for the other two cofactors in these mutants. These results suggest either that the mutations are near the ThDP-binding site, or that their effects are transmitted to this site through the protein.

Herbicide inhibition

The mutants were selected by their resistance to the sulphonylurea CE. Thus it was of interest to determine how the affinity for this compound is altered, the extent of cross-resistance to other

Table 3 Purification of *E. coli* AHAS II mutants

IMAC, immobilized metal-affinity chromatography.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
A26V (5.75 g)				
Cleared lysate	899	264	3.4	100
IMAC	480	31	15.5	53
V99M (5.10 g)				
Cleared lysate	1389	300	4.6	100
IMAC	1055	50	21.1	76
A108V (8.16 g)				
Cleared lysate	4498	379	11.8	100
IMAC	2152	64	33.8	48
G249A (5.60 g)				
Cleared lysate	0	249	–	–
IMAC	0	29	–	–

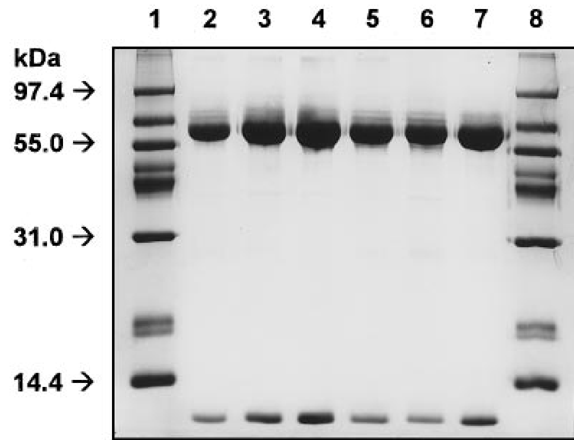

Figure 1 SDS/PAGE of purified *E. coli* AHAS II and its mutants

Lanes 1 and 8, molecular-mass markers; lane 2, A26V; lane 3, V99M; lane 4, A108V; lane 5, G249A; lane 6, G249V; lane 7, G249E. The gel was overloaded in order to allow the small subunit to be visualized.

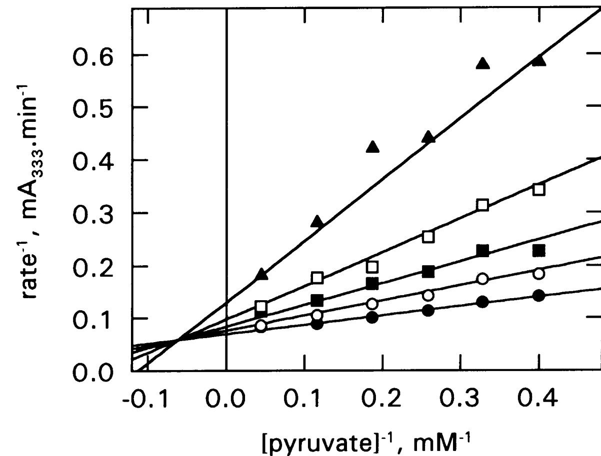

Figure 2 Non-competitive inhibition of the A26V mutant of *E. coli* AHAS II by CE

Rates (ΔmA_{333}/min) were determined as a function of pyruvate concentration (mM) at CE concentrations of 0 (●), 0.2 (○), 0.43 (■), 0.84 (□) and 1.80 (▲) μM, and the data are shown in the form of a double reciprocal plot. The lines represent the best fit to the data of equation 2.

Table 4 Kinetic properties of wild-type and mutant *E. coli* AHAS II

For each enzyme, the values shown are the best-fit estimate of the parameter ± S.E. obtained from regression analysis. CE, chlorimuron ethyl; CS, chlorsulfuron; ThDP, thiamin diphosphate; n.d., not determined.

Parameter	A26V	V99M	A108V	Wild type
k_{cat} (s^{-1})	25.7 ± 0.5	31.2 ± 0.6	41.1 ± 1.1	47.4 ± 0.9
K_m (mM)	2.77 ± 0.12	1.29 ± 0.08	1.68 ± 0.10	2.51 ± 0.08
k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	9.28 ± 0.25	24.2 ± 1.1	24.5 ± 1.1	18.9 ± 0.7
K_c (FAD, μM)	0.38 ± 0.03	0.85 ± 0.03	0.27 ± 0.02	0.20 ± 0.02
K_c (ThDP, μM)	15.7 ± 1.5	29.9 ± 4.4	3.78 ± 0.27	1.07 ± 0.05
K_c (Mg^{2+}, μM)	2.83 ± 0.24	6.64 ± 0.59	11.0 ± 0.6	3.84 ± 0.31
K_{is} (CE, μM)	0.33 ± 0.04	0.19 ± 0.04	1.68 ± 0.18	n.d.
K_{ii} (CE, μM)	2.08 ± 0.36	0.94 ± 0.11	9.29 ± 0.68	n.d.
K_{is} (CS, μM)	1.09 ± 0.22	17.3 ± 2.3	21.2 ± 2.3	0.29 ± 0.02
K_{ii} (CS, μM)	6.06 ± 2.14	285 ± 76	316 ± 50	0.76 ± 0.04

sulphonylurea herbicides, and whether there is any effect on the inhibition by imidazolinones.

Both CE and chlorsulfuron (CS) are non-competitive inhibitors of all three mutants, as is illustrated for CE on A26V in Figure 2. These data were fitted using equation 2 to obtain values for the two inhibition constants K_{is} and K_{ii}, which are shown in Table 4.

$$v = k_{cat}[E]_o/\{1 + [\text{inhibitor}]/K_{ii} + (K_m/[\text{pyruvate}])(1 + [\text{inhibitor}]/K_{is})\} \quad (2)$$

For all three mutants, both K_{is} and K_{ii} for CS are elevated in comparison with the values for wild-type AHAS II. The increase is relatively small in A26V, with K_{ii} affected to a greater extent than K_{is}. To put these values in perspective, we can use equation 3 [41] to calculate how the K_i^{app} (apparent K_i; that is, the concentration of inhibitor giving 50 % inhibition, which is also

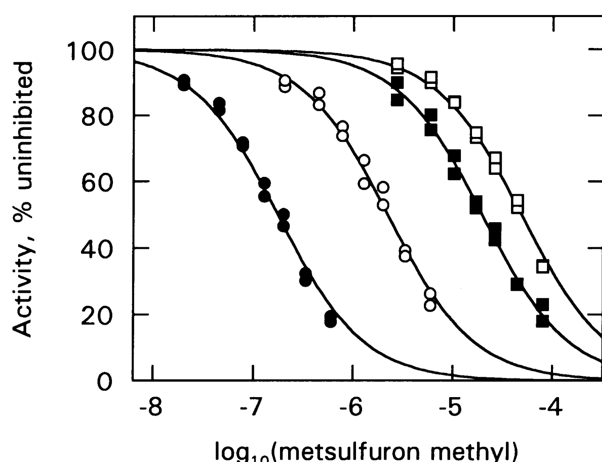

Figure 3 Inhibition of wild-type *E. coli* AHAS II and its mutants by metsulfuron methyl

Data are plotted as activity (as a percentage of the value in the absence of metsulfuron methyl) versus $\log_{10}$ of the molar inhibitor concentration. Wild-type (●), A26V (○), V99M (■) and A108V (□) are shown. The lines represent the best fits of equation 4 to the data.

Table 5 Inhibition properties of wild-type and mutant *E. coli* AHAS II

For each inhibitor, the first row shows the apparent K_i value ± S.E. obtained from regression analysis. Values in parentheses show the resistance factor, expressed as K_i^{app}(mutant)/K_i^{app}(wild-type). CE, chlorimuron ethyl; CS, chlorsulfuron; SM, sulfometuron methyl; MM, metsulfuron methyl; TF, thifensulfuron methyl; TB, tribenuron methyl; IQ, imazaquin; IP, imazapyr; IT, imazethapyr.

K_i^{app}	A26V	V99M	A108V	Wild type
CE (μM)	0.60 ± 0.02 (19.4)	0.61 ± 0.04 (19.7)	4.36 ± 0.12 (140)	0.031 ± 0.005
CS (μM)	1.09 ± 0.07 (6.1)	44.5 ± 1.4 (247)	75.5 ± 2.6 (420)	0.18 ± 0.01
SM (μM)	5.41 ± 0.28 (9.3)	51.9 ± 3.2 (90)	72.9 ± 3.6 (126)	0.58 ± 0.05
MM (μM)	2.21 ± 0.14 (13.0)	19.6 ± 0.9 (115)	48.5 ± 2.1 (285)	0.17 ± 0.01
TF (μM)	60.8 ± 3.0 (8.2)	219 ± 22 (30)	260 ± 27 (35)	7.41 ± 0.41
TB (μM)	44.5 ± 1.5 (12.6)	102 ± 6 (29)	283 ± 53 (80)	3.54 ± 0.15
IQ (mM)	0.98 ± 0.22 (2.4)	2.16 ± 0.54 (5.3)	1.62 ± 0.27 (4.0)	0.41 ± 0.03
IP (mM)	1.10 ± 0.23 (1.5)	2.35 ± 0.52 (3.1)	2.17 ± 0.48 (2.9)	0.75 ± 0.11
IT (mM)	1.08 ± 0.20 (1.4)	2.67 ± 0.79 (3.4)	2.22 ± 0.43 (2.8)	0.78 ± 0.13

known as the IC_{50}) would be affected at a substrate concentration equal to the K_m of the wild-type. These calculations show that K_i^{app} is elevated by a factor of 4.3 in A26V, compared with the wild-type.

$$K_i^{app} = K_{is}\ K_{ii}(K_m + [\text{pyruvate}])/(K_m\ K_{ii} + K_{is}[\text{pyruvate}]) \quad (3)$$

V99M and A108V show much larger changes in K_{is} and K_{ii} for CS than does A26V, although in all cases K_{ii} is affected to a greater degree than K_{is}. These changes in the inhibition constants of V99M and A108V would result in increases in K_i^{app} of more than 100-fold in each case.

The effect of the mutations on CE inhibition is more difficult to assess in quantitative terms because comparative values for the wild-type have not been determined. This is because CE is a tight-binding inhibitor that is effective in the nanomolar concentration range. We have reported previously a value of 24 nM for K_i^{app} [32]. However, it is evident that the three mutants are substantially less sensitive to CE and the data could be analysed without allowance for tight-binding, using equation 2. From the resulting values of K_{is} and K_{ii}, the calculated K_i^{app} values are 0.55, 0.48 and 3.29 μM for A26V, V99M and A108V, respectively.

Cross-resistance to other sulphonylureas and to the imidazolinones was assessed by measuring the effect on the rate of a range of concentrations of the inhibitor at a pyruvate concentration of 2.5 mM, close to the K_m of wild-type. Some examples of the inhibition curves obtained are shown in Figure 3. From these curves, K_i^{app} was determined by fitting equation 4 to the data. In this equation, v_i and v_o represent the rates with and without inhibitor, respectively:

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

Due to the tight-binding inhibition of the wild-type by CE, those data were fitted using equation 5:

$$v_i^2[\text{E}]_o + v_o\ v_i([\text{inhibitor}] - [\text{E}]_o + K_i^{app}) - v_o^2\ K_i^{app} = 0 \quad (5)$$

The values of K_i^{app} obtained and the calculated resistance factors (i.e. mutant K_i^{app}/wild-type K_i^{app}) are given in Table 5. A26V is resistant to all the sulphonylureas but the resistance is not very strong, ranging from 6.1-fold (CS) to 19.4-fold (CE). It is of interest that the strongest resistance is against CE, the herbicide that was used for selection. In direct contrast, CE is the sulphonylurea against which V99M shows the weakest resistance. However, the resistance factor for both A26V and V99M is about 20-fold, which may represent the lower limit of the selection method employed. Of course, we focused our attention on vigorously growing colonies and it may be that some of the slower growing ones contained mutations with lesser resistance to CE.

For the remaining sulphonylureas, V99M shows resistance factors ranging from 30-fold to 250-fold, with an inverse correlation between the resistance and the wild-type K_i^{app}. That is, the greatest effect is on the strongest inhibitors (CS and metsulfuron methyl) and the mutant K_i^{app} values cover a smaller range than those of the wild-type. A similar, but more marked, trend is evident for A108V and this mutant is uniformly more resistant than V99M to every sulphonylurea.

None of the mutants shows substantial resistance towards any of the imidazolinones. Perhaps this is to be expected, because wild-type AHAS II is already quite insensitive to these compounds with K_i^{app} values in the millimolar concentration range.

G249 mutants

The three-dimensional structure of AHAS has not been determined. However, by homology modelling based on the structure of pyruvate oxidase [42], Ott et al. [10] have constructed a theoretical structure of *A. thaliana* AHAS, whereas Ibdah et al. [16] have proposed the corresponding structure of *E. coli* AHAS II. Using the co-ordinates of the latter model (kindly provided by Dr. D. M. Chipman, Ben-Gurion University, Beer Sheva, Israel), we have mapped the residues that confer herbicide resistance upon yeast AHAS [14] to obtain the structure shown in Figure 4. The herbicide-resistance sites define a pocket at the subunit interface and it is likely that this is where the herbicides bind. It is significant that V99 (shown in light blue, see Figure 4), the new site identified in the present study, is also in this region, approximately 6 Å from both A108 (dark green) and A26 (light green).

At one end of this cleft is G249 (yellow) and we speculated that any substitution at this position would introduce a side-chain that could partially occlude the pocket, leading to a herbicide-insensitive AHAS. This site was subjected to mutagenesis and, of

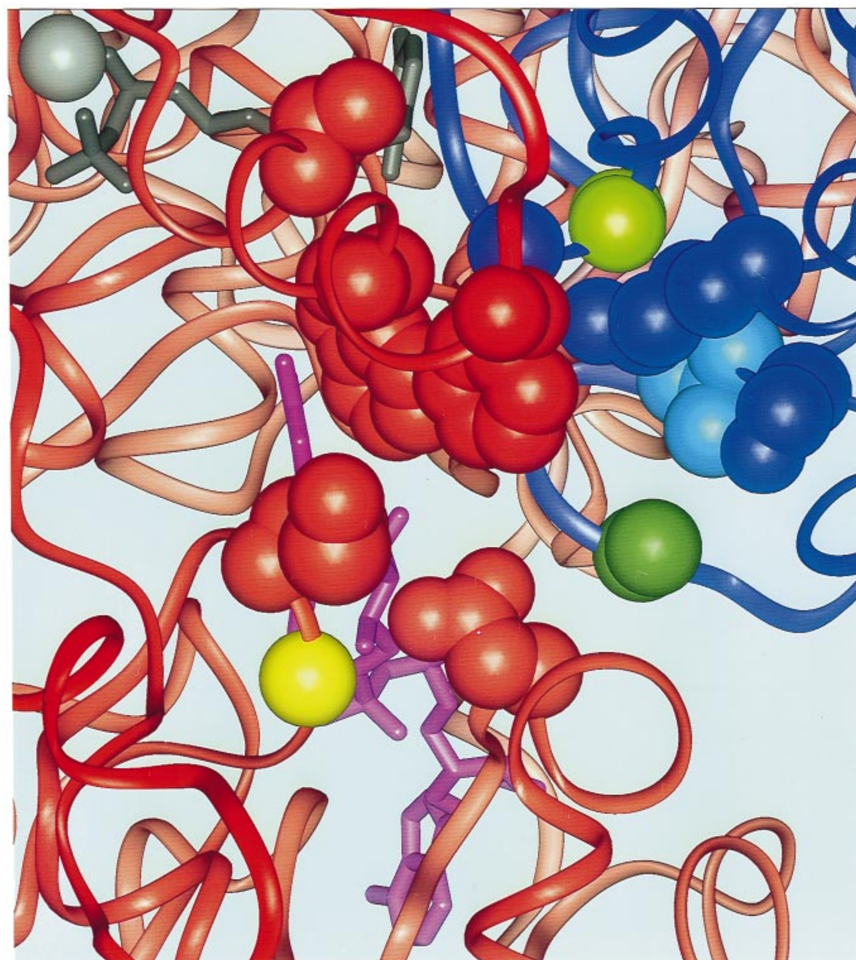

Figure 4 Molecular model of the herbicide-binding site of *E. coli* AHAS II

The backbones of the two subunits (denoted as A and B) are shown in red and dark blue, respectively. The side-chain and Cα of M250, D275, V461, W464 and F468 from subunit A are shown as red spheres, while dark blue spheres indicate G25, S100 and K159 of subunit B. Also shown are residues mutated in the present study: light green (A26, subunit B); light blue (V99, subunit B); dark green (A108, subunit B); and yellow (G249, subunit A). The backbones of FAD and ThDP are shown in magenta and grey, respectively, whereas the magnesium ion is shown as a grey sphere.

the six possible mutants that could be expected from the oligonucleotide used for mutagenesis (Table 1), only the three that preserve a G in the first coding position were found among 16 mutant clones that were sequenced. These three mutants (4–6; Table 2) were each expressed, purified and characterized. In each case, the presence of both large and small subunits was detectable by SDS/PAGE (Figure 1). Since each has similar properties, only the results for G249A will be described.

G249A is totally inactive, although it appears to express and purify normally as a soluble protein. The quantity of protein obtained after purification represents 12 % of the total soluble protein, which is similar to the result obtained for A26V, but somewhat lower than for wild-type and the other mutants (17–21 %). Since there is no activity, it was not possible to measure the affinity for substrate, cofactors or herbicides, as was done for active mutants and wild-type AHAS. However, it was observed during purification that G249A did not show the intense yellow colour that is typical of FAD bound to AHAS, and spectral measurements (Figure 5A) confirmed that this mutant binds very little FAD. The spectrum of wild-type AHAS shows peaks at 390 and 475 nm, similar to those reported previously [43]. In contrast, G249A shows only traces of these peaks, superimposed on a background that rises progressively at lower wavelengths. This background is characteristic of light-scattering, suggesting that the protein may be aggregating. This was confirmed by gel-exclusion chromatography (results not shown); wild-type AHAS II elutes as a single peak with an estimated molecular mass of 145 kDa (expected 149 kDa) whereas G249A shows two components. Approximately 62 % of the protein elutes at a position very close to that of the wild-type, whereas the remainder elutes in the void volume, indicating a molecular mass in excess of 1.4 MDa. This aggregation of G249A does not appear to be associated with gross structural changes, as CD spectroscopy (Figure 5B) indicates no substantial difference from the wild-type. Evidently, mutation of G249 causes a small change in the tertiary structure that impairs FAD binding, destroys catalytic activity, and slightly favours aggregation of the protein.

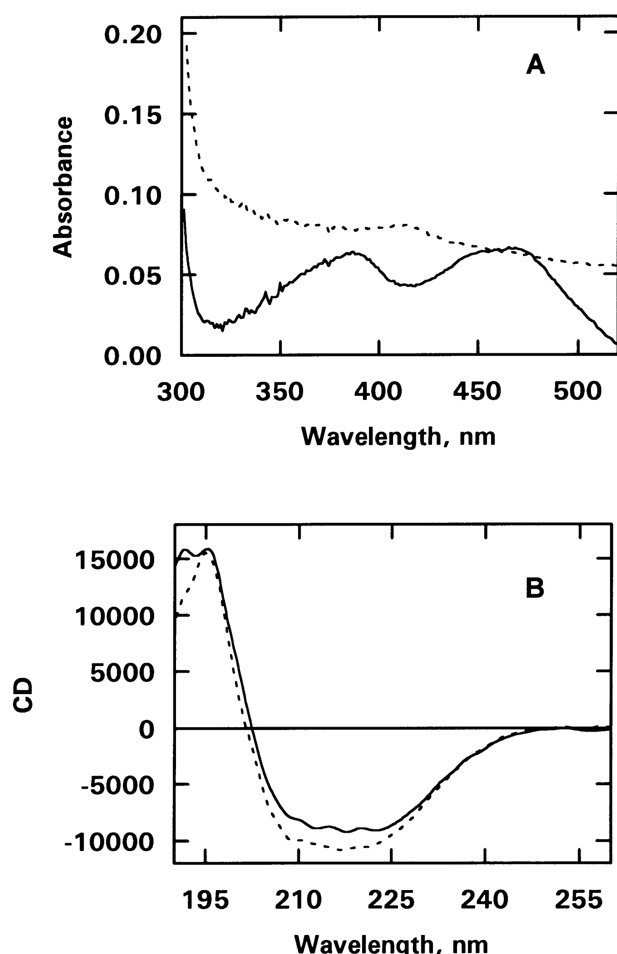

Figure 5 Absorption and CD spectra of wild-type *E. coli* AHAS II and the G249A mutant

(**A**) Absorption spectra. (**B**) CD spectra. Wild-type is indicated by the solid line whereas the G249A mutant is indicated by the broken line. Each protein was present at a concentration of 1 mg/ml.

DISCUSSION

Random mutagenesis of *E. coli* AHAS II and selection against an inhibitory concentration of CE has allowed the isolation of three herbicide-resistant mutants. A26V has been observed previously; it was the first such mutation to be described in AHAS from any organism [15] and, until the present work, was the only one identified by selection for herbicide resistance in *E. coli*. A second (A108V) has been found in yeast AHAS but not in any bacterial AHAS and the third (V99A) has not been described for AHAS from any source. Despite its success, we are conscious that the method of hydroxylamine-induced mutagenesis has some limitations because it preferentially causes C → T or G → A transitions, depending on the DNA strand with which it reacts. Thus, any codons composed solely of T and/or A cannot be affected. Even those amino acids with codons that do contain C and/or G will have available a limited range of possible alterations. Thus, a single hydroxylamine-induced base change of either the A26 and A108 codons (both GCG) could result in substitution by either threonine (ACG) or valine (GTG), but not by any other amino acid. Possible non-silent mutations of V99 (GTG) are even more limited, being restricted to methionine (ATG) only. Thus, it is probable that other methods for mutagenesis would reveal new sites where changes would result in herbicide resistance.

Each of the three mutants was characterized with respect to its kinetic properties and, in general, their properties were not substantially different from those of the wild-type. Each has a specific activity (and therefore k_{cat}) that is somewhat lower than the wild-type (Table 3) but the changes were not of such a magnitude that would suggest that any of these residues is involved in catalysis. Even the least-active mutant (A26V) has a specific activity that is 35 % of wild-type. However, this mutant has a normal K_m (Table 4), unlike V99M and A108V, for which the K_m values are somewhat lower than wild-type. Of course, for all but the simplest enzyme, K_m is a complex quantity involving the rate constants for most or all of the individual steps that constitute a complete catalytic cycle. It is somewhat easier to interpret k_{cat}/K_m, as this parameter depends upon rate constants for substrate binding and decarboxylation, and the value of k_{cat}/K_m differs substantially from normal only for A26V, where it is about one-half of the wild-type valve (Table 4).

Much larger effects of the mutations are seen on cofactor binding, particularly for ThDP, where K_c is elevated by a factor of 3.5 (A108V) to 27.9 (V99M). The K_c for FAD is also elevated, but to a much smaller extent. It is of interest that there appears to be a correlation between the effects on FAD and ThDP binding in the three mutants, with A108V least affected and V99M most affected. However, with only three examples it is unwise to place too much significance on this observation. The affinity for Mg^{2+} shows some changes, particularly for A108V where it is altered by a factor of 3.

Since the three mutants were selected by their ability to grow in a medium containing 1 μg/ml (2.41 μM) CE, it is not surprising that each is resistant to this herbicide. Based on the values of K_i^{app}, and assuming that the pyruvate concentration in *E. coli* cells is in the vicinity of 2.5 mM (i.e. equal to the K_m of wild-type AHAS), then the mutants would be inhibited by no more than 80 %, whereas the wild-type would be almost 99 % inhibited by this concentration of CE.

A26V is only weakly resistant to the other sulphonylureas (6- to 13-fold), which is somewhat surprising because this mutant, the first herbicide-resistant variant AHAS to be discovered [15], was originally described as being sulphonylurea-resistant. However, its resistance was rather low (about 4-fold against sulfometuron methyl) and is similar in magnitude to the 9-fold resistance that we observe. Much stronger resistance to sulphonylureas is observed for V99M, a mutation that has not been described previously for AHAS from any source. However, the model of the herbicide-binding site (Figure 4) shows that this residue is close to others that are known to confer herbicide resistance. Perhaps this is not too surprising given that it is immediately adjacent to S100, a site of known herbicide resistance in yeast AHAS [14].

We had expected that mutation of G249 would prevent herbicides from inhibiting but this turned out to be impossible to confirm because each of the mutants tested was inactive. We have shown previously that wild-type *E. coli* AHAS II is inactive without its small subunit [32] and we considered the possibility that these mutants were unable to associate to give the $\alpha_2\beta_2$ tetramer. Although the small subunit is difficult to visualize by SDS/PAGE, its presence is clearly detectable at high-protein loadings (Figure 1). Moreover, folding of the proteins as judged by their CD spectra (Figure 5B) appears normal. However, measurements of the absorbance spectra showed that these mutants do not contain FAD (Figure 5A), which accounts for their inactivity. Although the role of FAD is not yet clear, *E. coli*

AHAS II is inactive in its absence. Re-examination of the modelled structure provided an explanation for this observation. The α-carbon of G249 is within 4.3 Å of FAD and modelling the effect of substitutions at this position showed that the introduced side-chain would point towards FAD; in the case of G249A, the distance from the methyl carbon to FAD would be less than 3.5 Å. Thus it appears that the side-chain of G249 mutants intrudes into the FAD binding site and prevents this cofactor from binding, with a consequent loss of activity.

Despite the lack of success with mutating G249 to give a herbicide-resistant AHAS, our results clearly validate the model of Ibdah et al. [16] and suggest other sites where mutation might affect herbicide sensitivity. These sites include Q110 and G279, and it will be of interest to test the effect of changing these two amino acids.

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