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Crystallization of *Arabidopsis thaliana* acetohydroxyacid synthase in complex with the sulfonylurea herbicide chlorimuron ethyl

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Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) catalyses the formation of 2-acetolactate and 2-aceto-2-hydroxybutyrate as the first step in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine. The enzyme is inhibited by a wide range of substituted sulfonylureas and imidazolinones and many of these compounds are used as commercial herbicides. Here, the crystallization and preliminary X-ray diffraction analysis of the catalytic subunit of *Arabidopsis thaliana* AHAS in complex with the sulfonylurea herbicide chlorimuron ethyl are reported. This is the first report of the structure of any plant protein in complex with a commercial herbicide. Crystals diffract to 3.0 Å resolution, have unit-cell parameters $a = b = 179.92$, $c = 185.82$ Å and belong to space group $P6_422$. Preliminary analysis indicates that there is one monomer in the asymmetric unit and that these are arranged as pairs of dimers in the crystal. The dimers form a very open hexagonal lattice, with a high solvent content of 81%.

1. Introduction

Modern agricultural practice is heavily reliant upon the use of herbicides. Those that are used most extensively inhibit photosynthesis (*e.g.* triazines, ureas), block amino-acid biosynthesis (glyphosate, sulfonylureas, imidazolinones, phosphinothricin) or are plant-growth regulator mimics (2,4-D, dicamba). In view of their great importance to society, it is remarkable that the crystal structures of so few target proteins in complex with commercial herbicides have been reported. As far as we are aware, there are only three that have been published: the *Rhodopseudomonas viridis* photosynthesis reaction centre with a triazine (*e.g.* atrazine; Lancaster & Michel, 1999; PDB code 5prc), *Escherichia coli* enolpyruvylshikimate 3-phosphate synthase with glyphosate (Schönbrunn *et al.*, 2001; PDB code 1g6s) and *Saccharomyces cerevisiae* acetohydroxyacid synthase (AHAS) with chlorimuron ethyl (Pang *et al.*, 2003; PDB code 1n0h). It is curious that in each case the protein is derived from a microorganism rather than from a plant. Possibly, this reflects a more general underrepresentation of plant proteins in the Protein Data Bank. While each of these microbial proteins is undoubtedly similar to its plant homologue, our understanding of the protein–herbicide interaction would be better served by studying the protein from an organism that is the intended target.

AHAS (EC 2.2.1.6, formerly EC 4.1.3.18), also known as acetolactate synthase, catalyses the first step in the biosynthesis of the branched-chain amino acids (reviewed by Duggleby & Pang, 2000). The reaction involves the decarboxylation of pyruvate followed by condensation with a second molecule of pyruvate to give 2-acetolactate, the precursor of valine and leucine. The enzyme also catalyses a similar reaction in which the second substrate is 2-ketobutyrate, giving 2-aceto-2-hydroxybutyrate, the precursor for isoleucine synthesis. AHAS is inhibited by several classes of commercial herbicide including sulfonylureas, imidazolinones, triazolopyrimidines, sulfonylaminocarbonyltriazolinones and pyrimidinylthiobenzoates. These compounds are often selective for certain plants, with the selectivity arising from the differing abilities of various plants to convert the herbicides to non-toxic derivatives. Frequently, the inhibition of isolated AHAS is similar in sensitive and resistant species. However, an array of AHAS mutations have been reported that result in a herbicide-insensitive enzyme. In several cases, the same mutation has been described in plant and microbial enzymes, pointing to a similar structural organization of AHAS across diverse organisms. Nevertheless, there are also clear differences; for example, the plant enzyme is several orders of magnitude more sensitive to imidazolinones than is AHAS from *E. coli* and yeast.

Previously, we reported the crystal structures of yeast AHAS alone (Pang *et al.*, 2002) and in complex with chlorimuron ethyl (CE), a commercial sulfonylurea herbicide (Pang *et al.*, 2003). In view of the differences in herbicide sensitivity between the plant and yeast

enzymes alluded to above, we have now embarked upon structural studies of AHAS from the plant *Arabidopsis thaliana*. Here, we report the crystallization and preliminary X-ray diffraction analysis of the enzyme in complex with CE. These results represent the first report of structural data at atomic resolution (3.0 Å) for a plant protein with a bound commercial herbicide.

2. Materials and methods

2.1. Expression, purification and preparation

The cloning and expression in *E. coli* of the *A. thaliana* AHAS catalytic subunit was reported previously by this laboratory (Chang & Duggleby, 1997). The first 85 amino-acid residues from the N-terminus, which constitute the putative chloroplast transit peptide, were removed during the cloning procedure. The removal of the transit peptide is essential for a high level of expression of active plant AHAS in bacteria (Chang & Duggleby, 1997). The complex enzyme purification has been simplified by the introduction of a hexahistidine tag at the C-terminus of the protein. The *A. thaliana* AHAS gene was cloned into the pET30a(+) expression vector; the cloning procedure replaces the three C-terminal residues (IKY) with a hexahistidine tag (LEHHHHHH). The deduced molecular weight of the new recombinant *A. thaliana* AHAS construct is 64 641 Da.

The expression plasmid pET.At-AHAS(T86:C-His) was used to transform *E. coli* strain BL21(DE3) cells and the enzyme was expressed as described previously (Pang & Duggleby, 1999) for yeast AHAS. The hexahistidine-tagged AHAS was purified by immobilized metal-affinity chromatography using Novagen His-Bind metal-chelation resin as described previously (Pang & Duggleby, 1999), except that the elution buffer used was 50 m*M* Tris–HCl pH 7.0, 10 µ*M* FAD and 150 m*M* imidazole. The enzyme was purified further by size-exclusion chromatography (Superdex 200 HR 10/30) on a Pharmacia Biotech FPLC System. The elution buffer contained 50 m*M* Tris–HCl pH 7.0, 10 µ*M* FAD and 1 m*M* DTT. Fractions containing AHAS activity were collected and concentrated by ultrafiltration to 17 mg ml^{-1}. The purified enzyme was stored in small aliquots at 203 K.

2.2. Crystallization and data collection

Crystallization trials were carried out using the hanging-drop vapour-diffusion method at 290 K. Before each experiment, the cofactors, CE and DTT were added to the freshly thawed enzyme to give final concentrations of 1 m*M* ThDP, 1 m*M* FAD, 1 m*M* $MgCl_2$, 0.5 m*M* CE and 5 m*M* DTT. The search for suitable crystallization conditions was carried out using Wizard sparse-matrix macromolecule crystallization screens I and II (Emerald Biostructures, Inc.). Crystals, yellow in colour owing to protein-bound FAD, were observed in a number of conditions. Very fine needle clusters were typically obtained at acidic to neutral pH. Attempts to optimize these conditions to obtain single three-dimensional crystals were unsuccessful. A second, hexagonal crystal form could be grown at pH values between 9.5 and 10.5 in the presence of a range of PEGs with molecular weights ranging from 400 to 3000 or in the presence of potassium sodium tartrate. Optimal crystal-growth conditions were obtained by combining 1 µl of *A. thaliana* AHAS catalytic subunit with 0.5 µl reservoir solution. The reservoir solution consisted of 0.1 *M* CHES buffer pH 9.0–9.8, 0.2 *M* lithium sulfate and 0.9–1.1 *M* potassium sodium tartrate. Protein crystals appeared after 1–2 d and took approximately one week to reach maximum size. Preliminary X-ray studies on these crystals at room temperature showed diffraction to about 3.2 Å. For cryocooling, the crystals were transferred to reservoir solution containing 35%(*v*/*v*) ethylene glycol. Crystals were stable under these conditions for at least 15 min, showing no signs of cracking or dissolving.

A complete data set for the catalytic subunit of *A. thaliana* AHAS in complex with CE was collected using an FR-E X-ray generator operated at 45 kV and 45 mA and an R-AXIS IV^{++} image-plate area detector. The images were scaled and merged using the Rigaku/MSC *CrystalClear* 1.3.5 software (Pflugrath, 1999).

3. Results and discussion

Hexagonal single crystals of the *A. thaliana* AHAS–CE complex of 0.2 mm in all three dimensions could be grown in one week (Fig. 1*a*). Preliminary analysis showed that these crystals belonged to the space group $P6_422$ or its enantiomorph $P6_222$ and had unit-cell parameters $a = b = 179.92$,

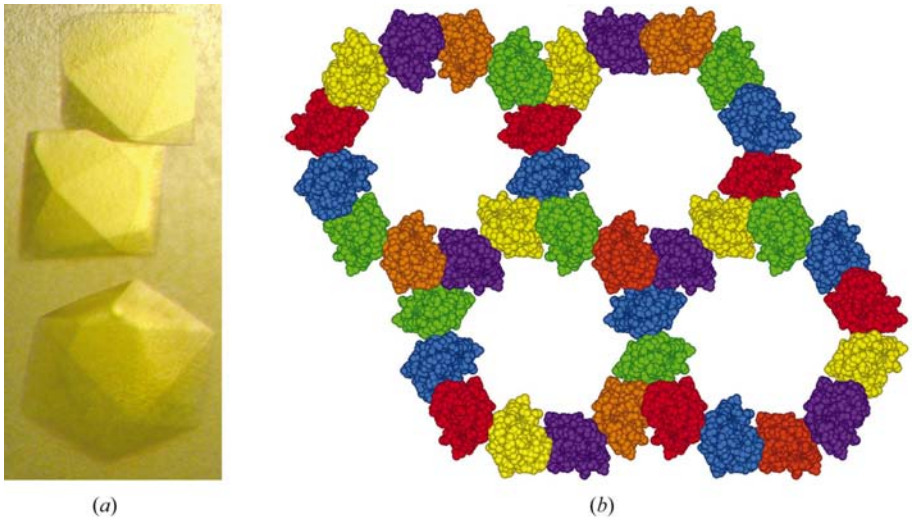

Figure 1
(*a*) Crystals of the catalytic subunit of *A. thaliana* AHAS in complex with sulfonylurea CE. The largest crystals have dimensions of 0.2 × 0.2 × 0.2 mm. (*b*) The molecular packing in these crystals viewed down the sixfold axis. The solvent content is 81%.

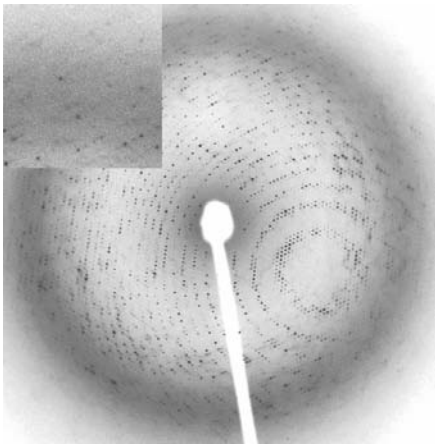

Figure 2
A 0.5° oscillation frame of a cryocooled crystal of the catalytic subunit of *A. thaliana* AHAS in complex with CE. Diffraction data is observed to 3.0 Å resolution (see Table 1). The inset is a magnification of a region from the outer diffraction shell.

Table 1
Data-collection statistics for *A. thaliana* AHAS–CE complex crystals.

Values in parentheses are for the outer resolution shell (3.11–3.00 Å).

Temperature (K)	100
Resolution range (Å)	71.85–3.00
Space group	$P6_422$
Unit-cell parameters (Å)	$a = b = 179.92$, $c = 185.82$
Mosaicity (°)	0.41
Crystal size (mm)	0.2 × 0.2 × 0.2
No. of observations [$I > 0\sigma(I)$]	190052
Unique reflections [$I > 0\sigma(I)$]	35750
R_{sym}†	0.117 (0.310)
Completeness (%)	99.3 (98.7)
$\langle I/\sigma(I)\rangle$	11.5 (4.8)

† $R_{sym} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$.

c = 185.82 Å. Soaking the crystals in reservoir solution with 35%(*v*/*v*) ethylene glycol added as a cryoprotectant yielded crystals with an excellent mosaicity of 0.41° that diffracted to 3.0 Å resolution (Fig. 2). Data-collection statistics for the enzyme–herbicide complex are presented in Table 1.

The *A. thaliana* AHAS–CE complex structure was solved by molecular replacement using *AMoRe* (Navaza, 1994). The search model was monomer *A* of the yeast AHAS catalytic subunit dimer in complex with CE (Pang *et al.*, 2003; PDB code 1n0h). Both the rotation function and translation search produced only one peak, with a maximum value for the space group $P6_422$. Based on one monomer per asymmetric unit, the solvent content is 81% with a Matthews coefficient (Matthews, 1968) of 6.7 Å^3 Da^{-1}; both values are well outside the normal range for proteins (∼50% for the solvent content and 1.7–3.5 Å^3 Da^{-1} for the Matthews coefficient). However, analysis of the crystal packing shows that the monomers are organized into pairs of dimers, similar to the structures observed for several related enzymes including acetolactate synthase (Pang *et al.*, 2004), pyruvate oxidase (Muller *et al.*, 1994), pyruvate decarboxylase (Dyda *et al.*, 1993; Dobritzsch *et al.*, 1998) and benzoylformate decarboxylase (Hasson *et al.*, 1998). No interpenetrating molecules were observed. Furthermore, analysis of the overall packing shows that the molecules form an extended three-dimensional lattice with enough molecular contact points to sustain a crystal (Fig. 1*b*). The high solvent content arises from the open arrangement of molecules in the *xy* plane. Inspection of the initial electron-density maps did not show any significant density in these holes. The initial *R* factor after rigid-body refinement was 0.420 using data in the resolution range 50.0–3.0 Å; the structure is currently being refined.

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