The crystal structure of plant acetohydroxy acid isomeroreductase complexed with NADPH, two magnesium ions and a herbicidal transition state analog determined at 1.65 Å resolution

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Acetohydroxy acid isomeroreductase catalyzes the conversion of acetohydroxy acids into dihydroxy valerates. This reaction is the second in the synthetic pathway of the essential branched side chain amino acids valine and isoleucine. Because this pathway is absent from animals, the enzymes involved in it are good targets for a systematic search for herbicides. The crystal structure of acetohydroxy acid isomeroreductase complexed with cofactor NADPH, Mg²⁺ ions and a competitive inhibitor with herbicidal activity, *N*-hydroxy-*N*-isopropylloxamate, was solved to 1.65 Å resolution and refined to an *R* factor of 18.7% and an *R* free of 22.9%. The asymmetric unit shows two functional dimers related by non-crystallographic symmetry. The active site, nested at the interface between the NADPH-binding domain and the all-helical C-terminus domain, shows a situation analogous to the transition state. It contains two Mg²⁺ ions interacting with the inhibitor molecule and bridged by the carboxylate moiety of an aspartate residue. The inhibitor-binding site is well adjusted to it, with a hydrophobic pocket and a polar region. Only 24 amino acids are conserved among known acetohydroxy acid isomeroreductase sequences and all of these are located around the active site. Finally, a 140 amino acid region, present in plants but absent from other species, was found to make up most of the dimerization domain.

Keywords: enzyme structure–function studies/herbicide target/isomeroreductase/three-dimensional structure/two cation-containing active site

Introduction

The branched chain amino acids valine, leucine and isoleucine belong to the group of nine essential amino acids that are not synthesized in mammals. There is, therefore, considerable agronomic interest in this metabolic pathway, owing to the role of these amino acids in animal and human diets. Furthermore, since the involved enzymes are absent in mammals, their characterization may provide the opportunity to find novel potential herbicide targets (Singh and Shaner, 1995). This point is well illustrated by the discovery of highly potent herbicides, the imidazoli-

nones and sulfonylureas, which act at very low dose rates, present low mammalian toxicity and which proved to inhibit acetohydroxy acid synthase (ALS), the first enzyme in the branched chain amino acid pathway (Schloss *et al.*, 1988). The unique mode of action of these herbicides and also the rather rapid emergence of weeds that become resistant to them due to mutations in the ALS target stimulated strong interest in a better knowledge of other enzymes in the branched chain amino acid pathway. Initial investigations showed that selective and highly potent inhibitors of acetohydroxy acid isomeroreductase, (EC 1.1.1.86), the second enzyme in the pathway, such as 2-dimethylphosphinoyl-2-hydroxy acetic acid (Hoe 704) and *N*-hydroxy-*N*-isopropylloxamate (IpOHA), exhibit herbicidal activity (Schultz *et al.*, 1988; Aulabaugh and Schloss, 1990). However, because these compounds bind very slowly to the enzyme and behave as competitive inhibitors with respect to the acetohydroxy acid isomeroreductase substrates, their herbicidal effectiveness is much smaller than that exhibited by non-competitive inhibitors targeting acetohydroxy acid synthase (Dumas *et al.*, 1994a). Therefore, biochemical and structural characterization of plant acetohydroxy acid isomeroreductase is needed for the design of new molecules inhibiting this enzyme.

Besides this potential agrochemical importance, acetohydroxy acid isomeroreductase from both plants and microorganisms presents several unique catalytic features. The enzyme catalyzes an unusual two-step reaction (Figure 1) consisting of an alkyl migration in which the substrate, either 2-acetolactate (AL) or 2-aceto-2-hydroxybutyrate (AHB), is converted to 3-hydroxy-3-methyl-2-oxobutyrate or 3-hydroxy-3-methyl-2-oxopentanoate, followed by a NADPH-dependent reduction to give 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate respectively. The enzyme-catalyzed reaction obeys an ordered mechanism in which NADPH and magnesium bind first and independently, followed by acetohydroxy acid substrate binding (Chunduru *et al.*, 1989; Dumas *et al.*, 1992). Also, acetohydroxy acid isomeroreductase has been shown to efficiently catalyze the reduction of ketopantoate (an intermediate in pantothenate biosynthesis) in the presence of a bivalent metal cofactor and NADPH (Primareno and Burns, 1993; Julliard, 1994; Dumas *et al.*, 1995), thus providing a means to investigate the reductive half-reaction independently of the isomerization reaction. The enzyme is selective towards stereoisomers, since studies from bacterial (Armstrong *et al.*, 1974, 1983; Hill *et al.*, 1979), fungi (Hawkes and Edwards, 1990) and plant enzymes (Dumas *et al.*, 1992) have demonstrated that the 2*S* isomers of AL and AHB are the true substrates for the reaction and that their diol products have the configurations 2*R* and 2*R*,3*R* respectively (Hill and Yan, 1971; Crout and White-House, 1972). Also,

Fig. 1. Reaction catalyzed by acetohydroxy acid isomeroreductase. The alkyl chain which undergoes migration is symbolized by R. R = CH₃ for AL, eventually giving valine; R = CH₃–CH₂ for AHB, eventually giving isoleucine. The inhibitor IpOHA is shown below the reaction scheme.

Arfin and Umbarger (1969) demonstrated that for the bacterial enzyme the reduction step requires transfer of the pro-*S* hydrogen atom from NADPH.

The two steps of the enzymatic reaction catalyzed by acetohydroxy acid isomeroreductase (rearrangement and hydride transfer; Figure 1) can be distinguished by their metal ion requirements (Chunduru *et al.*, 1989). The conversion of acetohydroxy acid substrate (AL or AHB) to the diol product shows a strong requirement for Mg²⁺. In contrast, reduction of the reaction intermediate is less specific, since catalysis can be performed with either Mg²⁺ or Mn²⁺. In addition, the plant enzyme displays an extremely high affinity for Mg²⁺, with a *K_m* value of the order of 5 µM (Dumas *et al.*, 1989), which corresponds to the strongest affinity ever reported between an enzyme and this metal ion. Furthermore, the plant enzyme is very selective towards NADPH compared with NADH and is competitively inhibited by NADP⁺ (Dumas *et al.*, 1992).

The spinach enzyme was shown to be a homodimer of 114 kDa (Dumas *et al.*, 1992), and its cDNA was used to overexpress the enzyme in *Escherichia coli* (Dumas *et al.*, 1992) and to produce mutants by site-directed mutagenesis (Dumas *et al.*, 1995). An alignment of all known amino acid sequences from acetohydroxy acid isomeroreductases suggested regions of the enzyme that might play a role in the reaction. This sequence analysis also showed that plant sequences have a unique feature in that they possess an extra sequence of 140 amino acids that is absent from fungi and most bacterial sequences. Furthermore, these biochemical data suggested the possibility that the plant enzymes may require the participation of two magnesium atoms for catalysis (Dumas *et al.*, 1995).

The aim of this study was to determine the folding of an enzyme performing such a complex reaction, to identify the role of the 140 amino acid extra sequence of the plant enzymes, to assess the role of the conserved regions suggested by sequence comparisons and site-directed mutagenesis and to understand the function of magnesium atoms in the active site. To this end, and as reported (Dumas *et al.*, 1994b), we have crystallized the over-expressed spinach enzyme as a complex with NADPH, magnesium and IpOHA. These crystals diffracted to beyond 1.6 Å resolution at a synchrotron source. We present here the structure of this complex, refined to 1.65 Å resolution.

Results

Present model

The present model for the asymmetric unit contains 17 830 non-hydrogen atoms, among which are 15 731 protein

Table I. Final refinement statistics for model from data collected at 100 K and 1.65 Å resolution

Crystal temperature	100 K
Resolution range	10–1.65 Å
Number of reflections	241 827
Completeness (%)	91.5 (92.5)
<i>R</i> factor (%)	18.8 (24.4)
Free <i>R</i> factor (%)	22.9 (25.5)
R.m.s. deviation bond lengths (Å)	0.014
R.m.s. deviation bond angles (°)	1.71
R.m.s. deviation dihedral angles (°)	20.91
R.m.s. deviation improper angles (°)	3.12
Average <i>B</i> factor (Å ²)	
For all atoms	14.4
For inhibitor IpOHA	8.8
For NADPH	8.9
For Mg ²⁺	8.8
Number of water molecules	1859

The numbers in parentheses indicate the values in the highest resolution shell.

Table II. Comparison between monomers

	Monomer 1	Monomer 2	Monomer 3	Monomer 4
Mean temperature factor (Å ²)	9.7	12.8	15.1	16.6
Crystal contacts	39	33	27	20
Cα atom r.m.s.d.				
Monomer 1		0.44 (0.33)	0.38 (0.32)	0.26 (0.25)
Monomer 2			0.26 (0.17)	0.41 (0.26)
Monomer 3				0.35 (0.26)

The mean temperature factor is calculated for all protein atoms of each monomer; the number of crystal contacts is the number of atoms in one monomer within 3.5 Å of a symmetry-related atom. r.m.s.d., root mean square deviation between the Cα atoms of monomers. The numbers in parentheses indicate the r.m.s.d. between residues 100 and 590, i.e. excluding the variable N-terminus region.

atoms, 240 ligand atoms and 1859 water oxygen atoms. In the main text, amino acid residues are numbered including the signal peptide. Therefore, the first residue of the mature protein corresponds to Met72 (Dumas *et al.*, 1991). The four monomers in the asymmetric unit can be assigned to two dimers and are referred to as monomers 1–4, the pairs of monomers 1 and 2 and 3 and 4 making up dimers 1 and 2 respectively. The N-terminus residues were disordered in the four monomers of the asymmetric unit so that the first visible residues are Ala83 for monomer 1, Thr85 for monomers 2 and 3 and Ser82 for monomer 4. However, all four monomers are ordered up to the C-terminus residue. The

Fig. 2. Ribbon representation of the spinach acetohydroxy acid isomeroreductase dimer. Each monomer is constituted of two domains: the α/β N-terminal domain with the NADPH-binding site and the α C-terminal domain binding the inhibitor via two Mg^{2+} ions. The inhibitor and the NADPH are stacked together at the domain junction. The N- and C-terminal domains are represented respectively in light and dark orange for monomer 1 and light and dark green for monomer 2. NADPH, the inhibitor IpOHA and the Mg ions are shown in pink, white and yellow respectively.

Ramachandran plot (Ramachandran and Sasisekharan, 1968) calculated with the program PROCHECK (Laskowski *et al.*, 1993) shows no residues in forbidden areas. Asp87 from monomer 3, situated at the N-terminal end, and Trp133 from monomer 1 fall in generously allowed areas. This latter residue is very well defined in the electron density map and its corresponding residues from other monomers have quite similar conformations, only within allowed ($\phi\psi$) angles. The results from the refinement are summarized in Table I. Geometric comparisons between monomers are given in Table II. The mean temperature factors for all atoms within monomers 1–4 are respectively 9.7, 12.8, 15.1 and 16.6 Å², with an average value for all atoms of 14.4 Å². This value is comparable with that of 13.1 Å² calculated from the Wilson plot in the program Truncate (Collaborative Computational Project no. 4, 1994). As shown in Table II, the temperature factors in the different monomers vary in inverse proportion to the number of packing interactions the monomers make in the crystal: monomers with a higher number of crystal contacts have a lower temperature factor. Inside the asymmetric unit the intradimer interfaces involve 53 contacts each and the interface between dimers involves 29 contacts. Contacts are defined as distances <3.5 Å between two atoms belonging to different molecules. The geometry of the protein model yielded an r.m.s. deviation of bond lengths of 0.014 Å and of bond angles of 1.71°.

During all of the refinement, the NADPH nicotinamide ring was kept planar by using improper angle restraints. The restraints were removed at the last positional run and replaced by a single planar restraint between atoms C2N, C3N, C5N and C6N. This brought the four independent nicotinamide rings into a boat conformation, which can

be described as *syn* antiperiplanar according to Almarsson and Bruice (1993).

Overall structure

The four monomers in the asymmetric unit clearly assemble into two dimers, presenting extensive intradimer contacts and much weaker interdimer contacts. This structural organization is in agreement with previous results, obtained by gel filtration and sedimentation analyses, showing that the plant isomeroreductase is functional as a dimer (Dumas *et al.*, 1992). Like most nucleotide-binding enzymes (Branden and Tooze, 1991), acetohydroxy acid isomeroreductase has a domain structure: the heart-shaped dimer is formed by two identical monomers and each monomer consists of two structural domains (Figure 2). The α -helical C-terminal domain (residues 308–595) is responsible for dimer formation and contains half of the active site, while the α/β N-terminal domain (residues 82–307) hosts the NADPH-binding fold. The topology of the N-terminal domain is illustrated in Figure 3A. It is built around a 10-stranded, open twisted mixed β -sheet, the N-terminal part of the sheet being antiparallel with four strands, followed by sets of two and five parallel strands respectively. The connecting helices are above and below the sheet plane. The five outer strands (strands B5–B4–B6–B7–B8) form the dinucleotide binding site, with a $\beta\alpha\beta$ motif (B4–A2–B5) corresponding to the canonical diphosphate-binding motif (Branden and Tooze, 1991).

The topology of the C-terminal domain is schematized in Figure 3B. The domain can be described as a six-helix core in which helices coil like cable threads around each other, thus forming a bundle. Helices involved in formation of this domain are A11–12, A13–14–15, A17, A20,

Fig. 3. Schematic representation of the domain secondary structures. Helices are drawn as cylinders and β -strands as arrows. Secondary structure assignment was made using option yasspa in O. (A) N-terminus domain. Secondary structure elements are named A1–A10 for the α -helices and B1–B11 for the β -strands. (B) C-terminus domain.

A21–22–23 and A25–26–27. To simplify the topological description, consecutive helices that had interruptions of only one or two residues and similar axis directions were grouped. In that way, A21–22–23 is a grouping of helices A21 (residues 486–492), A22 (residues 495–498) and A23 (residues 502–507). The other helices in the domain are at the surface of the monomer, being either in contact with the other monomer or accessible to the solvent. No similar topology could be found in known protein structures using the topology comparison programs DEJAVU (Kleywegt and Jones, 1994) and DALI (Holm and Sander, 1993).

Dimer interface

Stabilization of the dimer interface is mostly due to polar contacts involving hydrogen bonds, salt bridges and antiparallel helical dipole interactions. The dimer interface is symmetrical around the 2-fold dimer axis (Figure 2). The residues involved belong to four helices (A17–18, A20 and A26), plus one loop (residues 422–431) that extends out towards the other monomer (Figure 3B). One of the main building blocks of the interface is the antiparallel interaction between helices, with a well-known stabilizing contribution from the helical dipole. Helices A17 and A18 (residues 400–418) interact with correspond-

ing helices A18 and A17 from the other monomer. Similarly, helix A26 (residues 529–538) from one monomer interacts with its homolog from the other monomer. Though most contacts are polar, loop 422–431 from one monomer makes hydrophobic contacts with two helices from the other monomer: helix A17 around Met396 and helix A20 around Glu477. The contact between Leu428 and Ile478 seems to be the only shielding of hydrophobic side chains from the solvent. Also, six intermonomer hydrogen bonds and seven salt bridges can be found, rendering the interface highly polar. Interestingly, the 140 residue sequence (residues 331–471) present in the plant sequences and absent from most bacterial sequences analyzed so far contributes most to the dimer interface. Only helix A26 has a counterpart in bacteria, but there is hardly any similarity between plant and microorganism sequences in this region.

Conformation and binding of ligands

The active site region is at the interface between the N- and C-terminal domains. It is deeply buried inside the protein core and only the adenine moiety of NADPH is on the surface (Figure 2). The protein side of the active site includes acidic and polar residues, with a hydrophobic region where the aliphatic carbons of the inhibitor fit

Fig. 4. Schematic representation of inhibitor binding with the protein atoms and magnesium ions in the catalytic pocket. The figure shows the atomic contacts involved in inhibitor binding and the bond distances. Most of the contacts involve residues from the C-terminal domain.

(Figure 4). The whole active site is very well defined in the electron density map. The 1800 atoms located within 8 Å of an IpOHA molecule in the asymmetric unit have a mean temperature factor of 7.6 Å² and are therefore quite stable. The active site pocket is very sheltered from the outside: including the five water molecules directly bound to the cations (see Figure 4), this region hosts only seven water molecules.

In agreement with a previous site-directed mutagenesis analysis (Dumas *et al.*, 1995), the structure shows two magnesium ions located in close proximity within the active site. They are referred to as Mg1 and Mg2 in Figures 4 and 5A. As can be seen in these figures, these two cations play a major role in IpOHA binding to the protein. They bind the acidic residues from the active site, Asp315 playing a particularly important role by bridging both cation sites. Both metals have six oxygen ligands with a square based, bipyramid distribution, which is typical of magnesium binding (Bock *et al.*, 1995). Mg1 is bound to carboxylate oxygen O12 and carbonyl O2 from IpOHA, to Asp315 and Glu319 and to two water molecules. These water molecules are bound to His226 and oxygen O2'N of NADPH nicotinamide ribose. Mg2 is bound to carbonyl oxygen O2 and hydroxamate oxygen O3 from IpOHA, to Asp315 and to three water molecules.

These water molecules are bound to Glu492, Glu496 and to the main chain carbonyl of Pro251.

NADPH is in a folded conformation. The ribose rings have 2' *endo* pucker conformations (Saenger, 1984). The temperature factors are higher for the adenine than for the nicotinamide base, the mean temperature factor over the four NADPH molecules in the asymmetric unit being 9.6 Å² for the adenine base and 6.0 Å² for the nicotinamide base. The relatively higher temperature factor of the adenine base originates most probably from its rather exposed position at the surface of the N-terminus domain. The nicotinamide ring, on the other hand, is buried inside the active site and quite well ordered. Both average temperature factors are significantly lower than the overall average (14.4 Å²), indicating that the NADPH is quite rigidly bound to the enzyme. In the crystal structure, phosphate P2' oxygen atoms make hydrogen bonds with three water molecules and with the side chains of residues Arg162, Ser165 and Ser167. These residues are located on a loop between strand B5 and helix A3 at the protein surface.

The inhibitor-binding site is composed of loops connecting helices (residues 516–519), and of residues located in helical regions, such as Asp315 and Glu319 in helix A11 and Glu496 in helix A22. The carboxylate moiety of

Fig. 5. Views of the active site of spinach acetohydroxy acid isomeroreductase. (A) Stereo view of the active site, showing the relative positions of IpOHA, NADPH, the magnesium ions, water molecules contributing to Mg²⁺ binding and residues 315 and 319, which also bind Mg²⁺. The water oxygen atoms are represented in blue, the Mg ions in gray, the bonds for the protein residues in green and those for NADPH and IpOHA in gray. The atoms are colored using standard colors: C, black, N, blue, O, red, P, green. Dashed lines are drawn to show the Mg²⁺ ligands. (B) Electron density map in the active site area. Shown is a 2F_o-F_c map contoured at the 1 σ level.

the inhibitor is in contact with the N-terminal end of a long helix (A25), thus benefiting from the positive pole of the helical dipole. The binding pocket has a clear hydrophobic content, with an apolar region composed of Leu323, Leu324 and Leu501 side chains. This region accommodates the isopropyl moiety of IpOHA. Figure 5A shows the relative positions of NADPH and IpOHA. Within the active site, IpOHA defines a plane containing the two magnesium ions, the nicotinamide ring being stacked above this plane. Carbon C4, which is the proton donor from the nicotinamide ring, is positioned just above the potential acceptor oxygen in IpOHA. There is no direct interaction between NADPH and IpOHA. The only interaction between these two molecules is mediated by a water molecule in the active site.

Discussion

General features of the structure

Like other NAD(P)-dependent oxidoreductases, and as mentioned above, acetohydroxy acid isomeroreductase possesses two structurally different domains, one being involved in binding of the nucleotide cofactor and the other in specific binding of the acetohydroxy acid substrate analog (Figure 2). The N-terminal domain, which interacts

with NADPH, shares the typical structural characteristics of the Rossmann fold commonly found in NAD(P)-dependent oxidoreductases (Branden and Tooze, 1991). The C-terminal domain, composed almost entirely of α-helices, has two functions. Firstly, it contributes to formation of the enzyme active site and, in particular, to substrate/analog specific recognition. Secondly, it is responsible for monomer-monomer interactions, thus allowing formation of an active homodimeric enzyme. The N- and C-terminal domains interact together to form the active site, where the two magnesium atoms, IpOHA and the nicotinamide moiety of NADPH are completely buried. This observation raises the question of how IpOHA can reach the active site and implies that conformational modifications occur during the binding process. In agreement with this finding, previous studies showed that the fluorescence of enzyme-bound NADPH is greatly modified upon addition of magnesium and IpOHA (Dumas *et al.*, 1992, 1994a). It is worth noting that the N- and C-terminal domains are connected only by helix A11, which may thus act as a hinge. It should also be noted that crystallization of the native, unliganded enzyme has not yet been successful, despite an intensive search for suitable crystallization conditions. Thus, although the N- and C-terminal domains exhibit a large interaction area in the present structure,

Fig. 6. Location of the conserved amino acid residues among acetohydroxy acid isomeroreductase sequences. **(A)** Ribbon representation of one monomer. The ligands are represented by spheres, using the same color scheme as in Figure 2 with additional blue spheres for water molecules involved in the active site; the side chains of conserved residues are shown in ball-and-stick, using color coding corresponding to conserved regions as in **(B)**. This figure was made using programs Molscript (Kraulis, 1991) and Render (Merritt and Murphy, 1994). **(B)** Location of conserved regions (Dumas *et al.*, 1995) along the sequence. Color coding: region I, black; region I', blue; region II, dark green; region III, green; region IV, orange; region V, red. See Table III for the role of these regions.

they may not have such a well-defined relative position in the absence of ligands. Since prior fixation of NADPH and Mg^{2+} to the enzyme is required for substrate/inhibitor binding (Dumas *et al.*, 1992, 1994a), interaction with these two cofactors somehow triggers structuring of the active site. Because the inhibitor binding site is buried in the present structure, it seems likely that the enzyme 'breathes' in order for substrate/inhibitor binding to occur.

Conserved regions and their implication for structure and activity

Comparison between the acetohydroxy acid isomeroreductase amino acid sequences from plant, bacteria and fungi disclosed that out of a total of 523 amino acid residues of the spinach enzyme only 24 are totally conserved (Dumas *et al.*, 1995). These conserved residues are found in five 'regions' referred to as regions I–V. His226–Gly227–Phe228, as numbered in the *Spinacia oleracea* sequence, is conserved among all sequences except for that from *Rhizobacter meliloti*. Previous results showed

that mutagenesis of these conserved residues leads to partial or total inactivation of the spinach enzyme activity, suggesting that they are somehow involved in catalysis (Dumas *et al.*, 1995). Remarkably, Figure 6A and B shows that although the conserved residues are spread all along the sequence, they cluster closely around the active site in the three-dimensional structure, indicating that they are involved in enzyme function, either by structuring the active site (region II) or by directly binding the enzyme ligands (regions I, III, IV, V and His226–Gly227–Phe228; Figure 6A). The roles of these regions are listed in Table III.

Since the plant acetohydroxy acid isomeroreductase does not exhibit kinetic cooperativity (Dumas *et al.*, 1992), the significance of the existence of the enzyme as a homodimer remains an enigma. The present data disclosed, however, a structural role for the extra 140 residue sequence present in the plant sequence (Dumas *et al.*, 1995). These residues are specifically involved in the dimerization process and, therefore, contribute to establish-

Table III. Residues conserved among acetohydroxy acid isomeroreductase and their functions

Region	Residue list	Comments
I	Gly132	NADPH-binding loop
	Gly134	NADPH-binding loop
	Gln136	H bonds with NADPH diphosphate oxygens
I'	Asn143	interacts with region II
	Asp146	interacts with region II
	His226	interacts with Mg ²⁺ binding water
	Gly227	structural role
II	Phe228	H bonds with NADPH ribose
	Lys252	interacts with region III
	Gly255	structural role
	Val258	structural role
III (all region III residues are in helix A11)	Arg259	interacts with region I
	Gly265	structural role
	Glu311	interacts with regions I' and II
	Asp315	binds both Mg ²⁺
IV	Glu319	binds Mg ²⁺
	Leu323	hydrophobic contact with IpOHA
	Gly325	shifts helix pitch after ligand-binding site
	Glu488	structural role
V	Glu492	interacts with active site water bound to Mg ²⁺
	Met513	structural role
	Ser518	binds IpOHA carboxylate
	Thr520	structural role
	Ala521	structural role
	Gly524	structural role

Conserved residues are grouped in 'regions' as defined previously (Dumas *et al.*, 1995).

ment of the native structure of the plant enzyme. Site-directed mutagenesis experiments within this region, coupled with crystallographic studies, are in progress to better understand the function of this extra region in protein-protein interactions and to investigate whether the plant enzyme may show catalytic activity as a monomer.

Enzyme-ligand interactions

A site-directed mutagenesis analysis and a study of the reduction of ketopantoate suggested that regions III and IV are involved in binding two Mg²⁺ ions. One magnesium ion, in contact with region IV, is indispensable for the isomerization reaction and the other, in contact with region III, is needed for the reduction step (Dumas *et al.*, 1995). The present structure determination demonstrates that regions III and IV are indeed involved in binding two magnesium atoms, numbered Mg1 and Mg2 (Figure 5). However, this structure shows that interactions of magnesium with region III are more complex than previously assumed. Thus, Glu319 (belonging to region III; see Table III) binds Mg1, whereas Asp315 (also belonging to region III) bridges *both* Mg1 and Mg2. Interactions of magnesium with region IV are weaker, since Glu496 and Glu492 interact only indirectly with Mg2 via water molecules. In addition, the two magnesium ions interact tightly with IpOHA.

Since IpOHA is an analog of the transition state (Aulabaugh and Schloss, 1990), the present structure provides a view of a catalytic intermediate occurring by

the end of the isomerization reaction (Figure 1). The structure refined at 1.65 Å clearly shows the position of IpOHA interacting strongly with the two magnesium ions and residues from conserved regions, i.e. Glu319 (region III), Glu496 (region IV) and Ser518 (region V). In addition, hydrophobic interactions occur with Leu323 (region III), Leu324 and Leu501 (conserved amongst plant sequences) (Figure 4).

Our previous kinetic and equilibrium studies pointed out two characteristic features of the interaction between IpOHA and the plant enzyme. On the one hand, this compound behaved as a tight binding, nearly irreversible inhibitor of the enzyme. In agreement with this finding, titration data at equilibrium, obtained from fluorescence measurements of enzyme-bound NADPH, indicated a stoichiometric binding of IpOHA to the plant enzyme (Dumas *et al.*, 1994a). Such tightness of IpOHA binding can be well understood from the present structure. In particular, it is clear that the position of the two enzyme-bound magnesium ions perfectly matches three oxygen atoms of the inhibitor (Figure 5B). Also, as noted above, IpOHA establishes six additional hydrogen bonds and two hydrophobic interactions with the protein moiety. In summary, the binding of IpOHA to the active site is quite optimal. On the other hand, IpOHA proved to bind only very slowly to plant acetohydroxy acid isomeroreductase. Thus, although formation of the enzyme-inhibitor complex involved a single bimolecular step, binding was very slow, being characterized by an association rate constant of 1900 M⁻¹ s⁻¹ (Dumas *et al.*, 1994a). This rate value is four orders of magnitude lower than the diffusion-controlled limit for bimolecular reactions (Fersht, 1988). It is difficult to understand the slowness of IpOHA binding to the enzyme from the present data. However, since the present observations establish that IpOHA and magnesium interact within the enzyme active site, it is possible that IpOHA also forms a complex with this metal ion in solution. Furthermore, the two protein-bound magnesium ions, which constitute part of the IpOHA-binding site, are strongly bound to the protein ($K_d = 5 \mu\text{M}$; Dumas *et al.*, 1995). Therefore, in order for IpOHA to bind to the active site, magnesium atoms must first dissociate either from the incoming Mg²⁺-ligand complex or from the enzyme. Presumably this dissociation process would be a kinetically unfavorable event, thus explaining the slow binding process. Further work is needed to clarify this point.

Pioneer work from Arfin and Umbarger (1969) established that for *E.coli* acetohydroxy acid isomeroreductase the reduction step requires transfer of the pro-S hydrogen atom from NADPH. The present structural data are in total agreement with this previous biochemical investigation. It can be seen in Figure 5A that, according to Almarsson and Bruice (1993), the nicotinamide-binding site is of the B-type, which corresponds to involvement of the pro-S hydrogen in the reaction. The preference for NADPH versus NADH can be explained by a number of interactions between the enzyme and the NADPH P2' phosphate group. Interaction of the phosphate P2' oxygen atoms with a positively charged amino acid, such as Arg162 in the present structure, is usually found in NADP-dependent enzymes, rendering interactions with NADP tighter than with NAD (Branden and Tooze, 1991). In our case, additional interactions with Ser165 and Ser167 may

Table IV. Data collection

Data set	Native 100 K ^a	Native ^b	Uranyl nitrate ^c	Phenyl mercuriacetate ^d	Platinum terpyridine ^e	Potassium tetracyanoplatinate ^f
No. of measurements	969 140	245 870	125 008	314 783	137 663	244 393
Unique reflections	242 843	77 874	43 619	55 422	61 769	79 965
Completeness (%)	92 (96)	89	89	82	75	90
Resolution limit (Å)	1.65	2.4	2.9	2.9	2.6	2.4
R_{sym} (%)	6.4 (20.0)	6.2	8.3	9.4	6.4	8.2
R_{iso} (%)			17.7	21.1	20.0	8.1
Anomalous scattering used			yes	yes	no	yes
No. of heavy atom sites in asymmetric unit			6	9	14	7
Phasing power			1.21 (34 187)	1.66 (36 023)	1.13 (32 139)	0.62 (35 672)
R_{Cullis} (%)			0.81 (1992)	0.66 (2284)	0.81 (1869)	0.92 (2241)

Native crystals were grown as described in the text with an excess of Mg^{2+} (100 ions/monomer).

^aThe high resolution data were collected on station BM14 at the ESRF. The figures in parentheses correspond to the last resolution shell (1.74–1.65 Å).

^{b–f}Collected on an in-house diffractometer. The MIR phasing was done using the ^bnative data set. The following heavy atom derivatives were obtained: ^csoaked for 3.5 h in 10 mM uranyl nitrate at pH 6.2; ^dsoaked for 2.5 h in 0.6 mM phenyl mercuriacetate; ^esoaked for 4 h in 2.5 mM platinum terpyridine; ^fsoaked for 4 h in 20 mM potassium tetracyanoplatinate. All solutions contained 1.8 M $(\text{NH}_4)_2\text{SO}_4$ and 0.1 M Tris–HCl buffer, pH 7.2.

$$R_{\text{sym}} = \frac{\sum(|I| - \langle I \rangle)}{\sum |I|}, R_{\text{iso}} = \frac{\sum |F_{PH} - F_P|}{\sum F_P}, R_{\text{Cullis}} = \frac{\sum |F_{PH} \pm F_P| - |F_H|}{\sum |F_{PH} - F_P|} \quad \text{for centric reflections. } F_P, F_{PH} \text{ and } F_H, \text{ native derivative and}$$

heavy atom structure factors respectively. f_H/E , phasing power, where f_H is the r.m.s. heavy atom structure factor amplitude and E is the r.m.s. lack of closure error. The values for R_{Cullis} and phasing power are given between 15 and 3.2 Å resolution. For these two lines, the numbers in parentheses represent the number of reflections involved in the calculation.

The mean figure of merit in the range 15–3.2 Å is 0.73 over 36 073 reflections.

increase the strength of NADPH binding, making up four out of the 20 direct hydrogen bonds between NADPH and the enzyme. Therefore, all of these interactions account for the high selectivity of the plant enzyme for NADPH ($K_m = 5 \mu\text{M}$) compared with NADH ($K_m = 200 \mu\text{M}$) (Dumas *et al.*, 1992).

Structural and functional comparison with other enzymes: xylose isomerase

Regions III and IV function as two binding sites for magnesium. Thus, acetohydroxy acid isomeroreductase belongs to the small family of enzymes containing two catalytically active magnesium atoms, as also do xylose isomerase (Lavie *et al.*, 1994) and DNA polymerase (Wang *et al.*, 1996). Xylose isomerase catalyzes the interconversion of glucose and fructose (xylose and xylulose under physiological conditions) by utilizing two metal cofactors to promote a hydride shift. Although folding of the two enzymes is different (xylose isomerase is a β -barrel), there is a striking coincidence between the active sites of plant acetohydroxy acid isomeroreductase and xylose isomerase complexed with a hydroxamate inhibitor (Brookhaven Protein Databank entry 1GYI), notably concerning the position of the two metal ions with respect to the hydroxamate inhibitor, IpOHA and D-threonylhydroxamic acid.

Conclusion

In conclusion, the high resolution structure of acetohydroxy acid isomeroreductase complexed with an inhibitor, magnesium ions and NADPH reveals the assembly of two structural domains, the first with nucleotide-binding specificity, the second with an original fold, making up a binding site for two divalent cations and the reaction intermediate analog inhibitor. This structure gives us a very detailed snapshot of the reaction intermediate state.

However, because the interaction between the acetohydroxy acid substrate and the catalytic pocket is probably slightly different from that seen with IpOHA, it is not yet possible to extrapolate a precise reaction mechanism from the data obtained with IpOHA-containing crystals. In order to understand the reaction at the atomic level, more work is needed to obtain crystallographic data on the free enzyme and on different enzyme complexes obtained with the acetohydroxy acid substrate, the intermediate and the product of the reaction. Towards this goal, we have crystallized the enzyme complexed with AHB and Mn^{2+} (no reaction can occur in this case, since the isomerization step requires Mg^{2+} as the metal cofactor). Another tool to investigate the mechanism is to use enzyme mutants, such as those described recently (Dumas *et al.*, 1995), that are affected in various steps of the overall reaction. In this case we have obtained crystals from a His226→Glu mutant complexed with AHB and Mg^{2+} . Both types of crystals diffract to high resolution and the structures are currently being determined.

Materials and methods

Protein purification and crystallization

The enzyme was overexpressed in *E. coli* and purified as previously described (Dumas *et al.*, 1992). It was crystallized at 20°C from 1.8 M ammonium sulfate in 0.1 M Tris–HCl, pH 7.2 (Dumas *et al.*, 1994b) in the presence of IpOHA, NADPH (10 ligand molecules/enzyme monomer) and Mg^{2+} (100 ions/enzyme monomer). The crystals belong to space group $P2_1$ with the following cell parameters: $a = 111.56 \text{ Å}$, $b = 62.43 \text{ Å}$, $c = 162.89 \text{ Å}$, $\beta = 94.93^\circ$, four monomers per asymmetric unit arranged in two dimers and 54% solvent content. This unit cell is equivalent to that described in the crystallization note (Dumas *et al.*, 1994b), but corresponds to the conventional unit cell definition. The presence of magnesium is necessary for crystallization and no crystal could be obtained with less than two magnesium ions per protein monomer, but ratios of 2–100 cations per monomer gave crystals.

Data measurement, phasing and refinement

A first native data set, obtained at room temperature up to 2.5 Å resolution, was used to build and refine a first model. The crystal structure was solved by multiple isomorphous replacement (MIR method) using four heavy atom derivatives (uranyl nitrate, phenyl mercuriacetate, platinum terpyridine and potassium tetracyanoplatinate) and diffraction data up to 3.2 Å resolution. Data sets were collected on a MAR Research image plate detector system mounted on a Rigaku RU-200 HB X-ray generator, using a copper rotating anode and double mirror focusing optics. The data were reduced using the program XDS (Kabsch, 1988) and the CCP4 package (Collaborative Computational Project No. 4, 1994) was used for further analysis, phase calculations and density modification (Table IV). Crystals grown in the presence of Co^{2+} instead of Mg^{2+} ions and NADH instead of NADPH were used for location of the Mg^{2+} ions and of the NADPH phosphate. Diffraction data were collected and the results are summarized in Table IV. Later, a high resolution native data set was collected at 100 K on beam line BM14 at the European Synchrotron Radiation Facility (Grenoble, France) using a MAR Research detector and was processed using Denzo (Otwinowski, 1993) up to 1.65 Å resolution.

The model was built using the O graphics program (Jones et al., 1991). Two magnesium-binding sites per monomer were located from the difference map between the Co^{2+} - and the Mg^{2+} -containing crystals. The partial structure traced around the magnesium-binding sites allowed determination of the non-crystallographic symmetry relationships between the four monomers. A mask was calculated for one monomer and used for solvent flattening, non-crystallographic averaging and histogram matching with the program DM (Collaborative Computational Project No. 4, 1994). The phase extension to 2.5 Å resolution yielded an interpretable map. One NADPH molecule per monomer was located from the difference map between NADPH- and NADH-containing crystals, showing clearly the position of the phosphate group. Similarly, the inhibitor IpOHA was located from a difference map between this data set and one measured from a crystal containing Hoe 704. The model was refined using all reflections in the resolution range 6–2.5 Å with the program X-PLOR (Brünger, 1992) and performing model building with O. After releasing the non-crystallographic symmetry, the *R* factor was 20%, with a free *R* factor of 27.4% (5% of the data) ($R = (\sum |F_o| - k|F_c|) / \sum |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes for a given reflection).

High resolution refinement

The high resolution refinement using the synchrotron data measured at low temperature up to 1.65 Å resolution was performed in the following way. The monomer was positioned with molecular replacement (AMORE) and rigid body refinement (X-PLOR) using data between 10 and 2.5 Å resolution to fit the model into the low temperature unit cell and refine the non-crystallographic symmetry relationships. Resolution was increased progressively and the model was then refined using all data between 10 and 1.65 Å resolution, firstly keeping strict non-crystallographic constraints, then progressively loosening harmonic restraints. Water molecules were progressively added using the Peakmax program (Collaborative Computational Project no.4, 1994). Peaks higher than $3.5 \times \sigma$ in the $(F_o - F_c)$ electron density map were selected and screened for hydrogen bonding to the model. All the refinement calculations were done with conjugate gradient minimization and simulating annealing. The parameters used are those from Engh and Huber (1991). An overall anisotropic temperature factor matrix and individual isotropic atomic temperature factors were refined. The *R* factor was 18.8%, *R* free 22.9% (5% of the reflections).

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