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Crystallization and preliminary X-ray crystallographic analysis of acetohydroxy acid isomeroreductase from *Pseudomonas aeruginosa*

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Acetohydroxy acid isomeroreductase (AHIR) is involved in the biosynthetic pathway of branched-chain amino acids in microorganisms and plants. AHIR from *Pseudomonas aeruginosa* has been overexpressed in *Escherichia coli* and crystallized at 297 K using potassium/sodium tartrate as a precipitant. X-ray diffraction data have been collected to 2.0 Å resolution at 100 K using synchrotron radiation. The crystals belong to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 184.38$ Å, $\alpha = \beta = \gamma = 90^\circ$. Six monomers are present in the asymmetric unit, giving a V_M of 2.34 Å^3 Da^{-1} and a solvent content of 47.4%.

1. Introduction

Acetohydroxy acid isomeroreductase (AHIR; EC 1.1.1.86), also known as ketol-acid reductoisomerase, is one of the enzymes involved in the biosynthetic pathway of the branched-chain amino acids valine, isoleucine and leucine. AHIR catalyses an interesting two-step reaction of alkyl migration (isomerization) and reduction in the presence of NADPH and magnesium ions. It uses either (2*S*)-2-acetolactate or (2*S*)-2-aceto-2-hydroxybutyrate as substrates, giving (2*R*)-2,3-dihydroxy-3-isovalerate or (2*R*,3*R*)-2,3-dihydroxy-3-methylvalerate as products, respectively (Dumas *et al.*, 2001). It follows an ordered mechanism where NADPH and magnesium ions first bind independently, followed by substrate binding (Halgand *et al.*, 1999). AHIR acitivity has been observed in bacteria, fungi and plants, but not in animals (Dumas *et al.*, 2001). Plant AHIRs are potential targets for the development of herbicides. Structural information on AHIR is at present limited to the spinach enzyme, which is a homodimer of 524-residue subunits (Biou *et al.*, 1997; Thomazeau *et al.*, 2000). A new algorithm to characterize knots in proteins discovered a deep figure-of-eight knot in this structure (Taylor, 2000). The origin of this knot was suggested to be gene duplication within the C-terminal helical domain.

Many bacterial AHIRs are ∼330 to ∼340 residues long, while those from *E. coli* and *Haemophilus influenzae* are ∼490 residues long. In order to reveal structural differences between bacterial and plant AHIRs, we have initiated the structure determination of *P. aeuruginosa* AHIR. Its polypeptide chain comprises 338 amino-acid residues, with a calculated molecular mass of 36 424 Da. The amino-acid sequence of the N-terminal two-thirds (residues 1–228) of *P. aeuruginosa* AHIR is aligned with the corresponding region of the spinach enzyme with ∼31% identity and ∼49% similarity. Here, we report its overexpression, crystallization and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The *ilvC* gene encoding AHIR was amplified by the polymerase chain reaction using the genomic DNA of *P. aeruginosa* as a template. The amplified DNA was digested with *Nde*I and *Xho*I and was then inserted into the *Nde*I/*Xho*I-digested expression vector pET-21a (Novagen). This vector construction adds six histidine residues to the C-terminus of the gene product to facilitate protein purification. The protein was overexpressed in *E. coli* B834(DE3) cells. Cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin and the expression of the recombinant protein was induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). Cell growth continued at 303 K for 6 h after IPTG induction and cells were harvested by centrifugation at 4200*g* (6000 rev min^{-1}; Hanil Supra 21K rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer *A* (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl) containing 5 m*M* imidazole, 2 m*M* phenylmethylsulfonyl fluoride and 1 m*M* β-mercaptoethanol and was then homogenized by ultrasonication.

The crude lysate was centrifugated at 70 400*g* (30 000 rev min^{-1}; Beckman 45 Ti rotor) for 1 h at 277 K. The supernatant was loaded onto a Hi-Trap chelating HP column

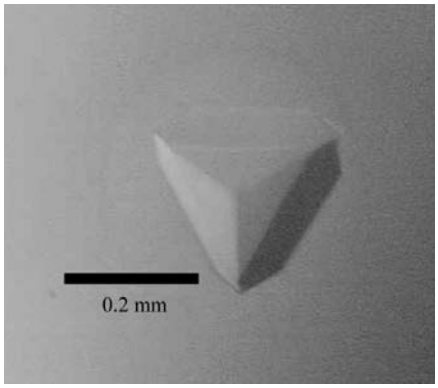

Figure 1
A cubic crystal of acetohydroxy acid isomeroreductase from *P. aeruginosa*. Its approximate dimensions are 0.2 × 0.2 × 0.1 mm.

(Amersham-Pharmacia), which was previously charged with Ni^{2+} and equilibrated with buffer *A*. After washing the column with buffer *A* containing 60 m*M* imidazole, 2 m*M* phenylmethylsulfonyl fluoride and 1 m*M* β-mercaptoethanol, the bound protein was eluted with a linear gradient of 0–1.0 *M* imidazole. Next, gel filtration was performed on a HiLoad XK 16 Superdex 200 prep-grade column (Amersham-Pharmacia), which was previously equilibrated with buffer *B* (50 m*M* Tris–HCl pH 7.0, 100 m*M* NaCl, 1 m*M* β-mercaptoethanol). The homogeneity of the purified protein was assessed by SDS–PAGE (Laemmli, 1970). Finally, the purified enzyme was concentrated to 22.2 mg ml^{-1} using an YM10 ultrafiltration membrane (Amicon) and stored at 203 K. The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 24 890 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

2.2. Dynamic light-scattering studies

The dynamic light-scattering experiment was performed on a Model DynaPro-801 instrument from Protein Solutions (Lakewood, New Jersey, USA). The data were measured at room temperature with ∼3 mg ml^{-1} of the protein in buffer *B*.

2.3. Crystallization and X-ray data collection

Crystallization was performed by the hanging-drop vapour-diffusion method at 297 K using 24-well tissue-culture plates. Each hanging drop was prepared by mixing 2 µl of the reservoir solution and 2 µl of the protein solution at 22.2 mg ml^{-1} concentration and was placed over 1 ml of the reservoir solution. Initial crystallization conditions were established using Screen I, Screen II and MembFac screening solutions (Hampton Research) and Wizard I and II screening kits (Emerald BioStructures, Inc.).

X-ray diffraction data were collected at 100 K using a DIP-2030 image-plate detector (MacScience Co.) at beamline BL-6B of Pohang Light Source, South Korea. The wavelength of the synchrotron radiation was 1.000 Å. A crystal was flash-frozen in liquid nitrogen using a solution of 0.1 *M* Tris–HCl pH 7.0, 0.2 *M* Li_2SO_4, 1.0 *M* potassium/sodium tartrate and 30%(*v*/*v*) glycerol as a cryoprotectant. The crystal was rotated through a total of 120°, with a 1.0° oscillation range per frame. The raw data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results

The recombinant *P. aeruginosa* AHIR with a C-terminal hexahistidine tag was highly overexpressed in soluble form in *E. coli*, with a yield of approximately 40 mg of homogeneous enzyme per litre of culture. The native molecular mass was estimated to be about 266 kDa, with a polydispersity of 8%, by dynamic light-scattering measurements. This suggests that *P. aeruginosa* AHIR is likely to exist as a homohexamer in solution. Well diffracting crystals were obtained using an optimized reservoir solution consisting of 0.1 *M* Tris–HCl pH 7.0, 0.2 *M* Li_2SO_4 and 1.0 *M* potassium/sodium tartrate. The crystals grew to approximate dimensions of 0.2 × 0.2 × 0.1 mm within 4 d (Fig. 1). The crystals diffracted to 2.0 Å resolution at 100 K using synchrotron radiation. The crystals belong to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 184.38$ (0.12) Å, $\alpha = \beta = \gamma = 90°$ (estimated standard deviations are given in parentheses). The asymmetric unit contains six subunits, giving a crystal volume per protein mass (V_M) of 2.34 Å^3 Da^{-1} and a solvent content of 47.4% (Matthews, 1968). Table 1 summarizes the statistics for X-ray data collection. Since molecular replacement was not possible owing to the low sequence identity between the amino-acid sequences of *P. aeruginosa* and spinach enzymes, the structure of *P. aeruginosa* AHIR will be solved by the multiwavelength anomalous diffraction method. Crystallization of *P. aeruginosa* AHIR in the presence of cofactor and metal ions is also being attempted.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.07–2.0 Å).

Space group	$P2_13$
Temperature (K)	100
$a = b = c$ (Å)	184.38
Resolution (Å)	30.0–2.00
Total/unique reflections	1090967/135868
R_{merge}† (%)	6.5 (28.9)
Data completeness (%)	97.1 (93.8)
$\langle I/\sigma(I)\rangle$	20.1 (4.7)
Redundancy	7.0 (5.8)

† $R_{merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

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