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Initiating a crystallographic analysis of recombinant (*S*)-2-hydroxypropylphosphonic acid epoxidase from *Streptomyces wedmorensis*

Scott Cameron, Karen McLuskey, Rachel Chamberlayne, Irene Hallyburton and William N. Hunter*

Division of Biological Chemistry and Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland

Correspondence e-mail: w.n.hunter@dundee.ac.uk

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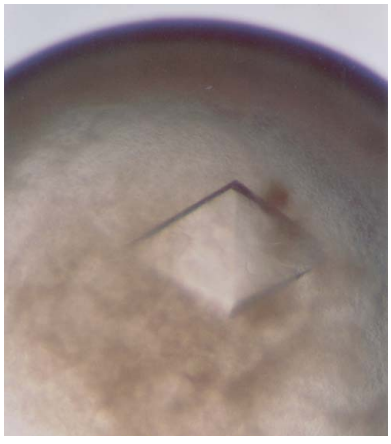
The oxirane (1*R*,2*S*)-1,2-epoxypropylphosphonic acid (fosfomycin) is a natural product antibiotic produced in *Streptomyces wedmorensis* by the metal-ion-dependent (*S*)-2-hydroxypropylphosphonic acid epoxidase. This epoxidase is highly unusual since it has no requirement for a haem prosthetic group. The gene encoding the enzyme, *fom4*, has been cloned and a highly efficient recombinant source of the enzyme established. Two different crystal forms, tetragonal and hexagonal, have been obtained. The hexagonal form displays symmetry consistent with space group $P6_{1/5}22$ and unit-cell parameters a = 86.44, c = 221.56 Å, γ = 120°. The Matthews coefficient, V_M, of 2.7 $Å^3\,Da^{-1}$ corresponds to two subunits, each of approximate weight 21.4 kDa, in the asymmetric unit with 55% solvent content. These crystals diffract to high resolution and experimental phases are being sought to determine the structure.

1. Introduction

Fosfomycin, (1*R*,2*S*)-1,2-epoxypropylphosphonic acid, is a broad-spectrum antimicrobial produced by *Streptomyces wedmorensis* and *Pseudomonas syringae* (Hendlin *et al.*, 1969; Shoji *et al.*, 1986). The antibiotic has mainly been used in the treatment of urinary-tract infections, where it exhibits low toxicity and few side effects (Patel *et al.*, 1997). More recently, efficacy against *P. aeruginosa* in cystic fibrosis patients has been demonstrated (Mirakhur *et al.*, 2003). Fosfomycin inhibits UDP-GlcNAc-3-*O*-enolpyruvyltransferase (MurA), an enzyme essential for bacterial cell-wall peptidoglycan synthesis (El Zoeiby *et al.*, 2003). Specifically, the drug alkylates an active-site cysteine in MurA (Kim *et al.*, 1996). One interest in the enzymes involved in the production of this antimicrobial drug is the application of protein-engineering methods to assist in the development of new antimicrobials based upon the fosfomycin structure.

Fosfomycin is a member of a growing family of natural products containing C—P bonds, such as the herbicides phosphinothricin and bialaphos, which are derived from phosphoenolpyruvate (PEP; Seto & Kuzuyama, 1999; Woschek *et al.*, 2002). An intermolecular rearrangement of PEP results in the formation of phosphonopyruvic acid and decarboxylation followed by methylation gives (*S*)-2-hydroxypropylphosphonic acid (HPP), the precursor to fosfomycin. The early steps of this pathway are well established; however, much less is understood about the remaining steps, especially the unusual mechanism of the oxiranyl ring formation (Liu *et al.*, 2003).This final step in the pathway is the epoxidation of HPP to produce fosfomycin, a reaction that is catalysed by the enzyme HPP epoxidase (Zhao *et al.*, 2002). HPP epoxidase is a 21.4 kDa protein consisting of 198 amino acids, reported to be iron(II)-dependent and to form a homotetramer (Liu *et al.*, 2003).

Here, we describe the cloning of the *S. wedmorensis fom4* gene, which encodes HPP epoxidase, the construction of a highly efficient protein-expression system and purification of the recombinant enzyme along with crystallization and preliminary diffraction experiments.



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2. Methods and results

2.1. Preparation of recombinant HPP epoxidase

Genomic DNA was extracted from *S. wedmorensis* (ATCC 21239) using cetyl trimethylammonium bromide, the so-called C-TAB method (Wilson, 1994). This provided the template for PCR with the following primers designed to amplify the *fom4* open reading frame with *Nde*I and *Bam*HI restriction sites (bold), respectively: 5′-**CAT-ATG**-AGC-AAC-ACC-AAG-ACA-GGA-G-3′, 5′-**GGA-TCC**-TCA-GAA-GTT-GAC-GGC-TTC-3′. The resultant single product was cloned into the pCR-BluntII-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Positive clones were sequenced to confirm the integrity of the product and then, following excision, the *fom4* gene was ligated into the expression vector pET-15b (Novagen). This ultimately produces a protein carrying an N-terminal hexahistidine tag (His-tag), which permits the use of metal-chelating chromatography for purification. The recombinant plasmid was then heat-shock transformed into *Escherichia coli* XL-1 blue cells (Invitrogen) for DNA amplification before being transformed into *E. coli* BL21 (DE3) for protein expression. The new plasmid was selected on Luria–Bertani agar plates containing 50 µg ml^{-1} carbenicillin (LB/carbenicillin). Bacteria were cultured in 1 l LB/carbenicillin at 310 K to an OD_{600} of 0.6–0.7 and then induced with 0.3 m*M* isopropyl-β-D-thiogalactopyranoside. The culture was then left overnight at room temperature with constant shaking. Cells were harvested by centrifugation (2500*g*, 20 min, 277 K) and resuspended in 50 m*M* Tris–HCl pH 7.9, 250 m*M* NaCl before being broken open by passage through a One-Shot cell disruptor (Constant Systems) and the insoluble cell debris separated by centrifugation (38 000*g*, 20 min, 277 K). The cell extract was then filtered and applied onto a 5 ml metal-chelating column (Hi-Trap; Pharmacia) charged with Ni^{2+}. The column was washed with 50 m*M* Tris–HCl pH 7.9, 250 m*M* NaCl and the His-tagged protein was eluted with a 0–600 m*M* imidazole gradient in the same buffer. The eluted protein was incubated at room temperature with 50 units of thrombin (Pharmacia) for 3 h at 293 K to remove the His tag. The protein was then dialysed overnight at 277 K into 50 m*M* Tris–HCl pH 7.9 and separated from the thrombin and cleaved N-terminal peptide by anion-exchange chromatography using a Porus HQ strong anion-exchange column (Applied Biosystems). Pooled fractions containing HPP epoxidase were dialysed into 20 m*M* Tris–HCl pH 7.9, 50 m*M* NaCl overnight at 277 K and concentrated to ~30 mg ml^{-1}. The purity of the enzyme sample was determined using both sodium dodecyl sulfate–polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (Voyager DE STR, Applied Biosystems) prior to crystallization experiments. The yield of purified protein was approximately 60 mg per litre of cell culture.

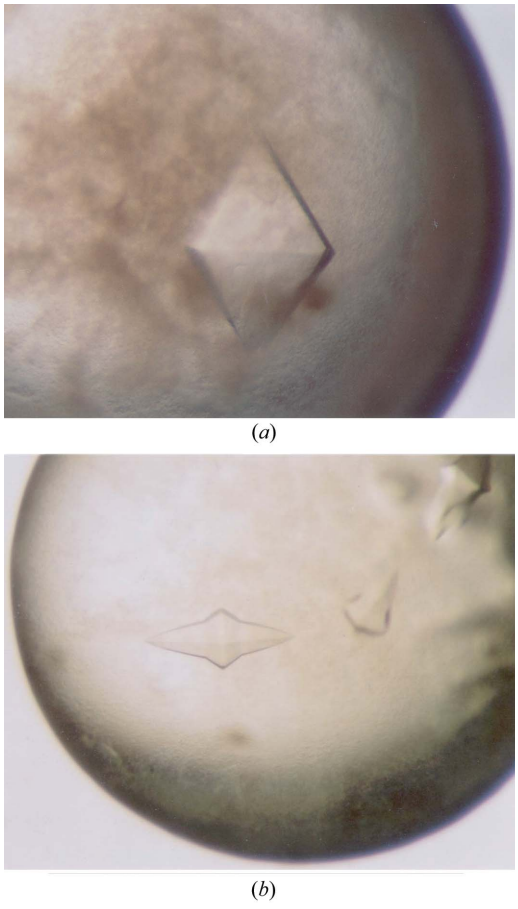

Figure 1
(*a*) Form I HPP epoxidase crystals, (*b*) a form II HPP epoxidase crystal.

2.2. Analytical ultracentrifugation

The quaternary structure of the protein was reported to be a tetramer based on molecular-sieve chromatography (Liu *et al.*, 2003). To confirm this result, HPP epoxidase was analysed by analytical ultracentrifugation. Samples were prepared in 20 m*M* Tris–HCl pH 7.9, 50 m*M* NaCl at concentrations that gave OD_{280} readings of 0.5, 1.0 and 2.0 (0.4, 0.8 and 1.7 mg ml^{-1}, respectively) in a 1.2 cm path-length cell. Sedimentation-equilibrium determinations were carried out using an Optima XL-1 analytical ultracentrifuge (Beckman Coulter) with absorbance optics. The sedimentation-velocity experiments were run at 35 000 rev min^{-1} at 293 K and the data were analysed with the program *SEDFIT* (Schuck, 2000). Two sedimentation coefficients of 3.2 and 5.1, consistent with molecular weights of approximately 40 and 80 kDa, were observed at each concentration. The weight ratio of the 80 kDa peak to the 40 kDa peak was ~20:1 for the two lower protein concentrations and ~70:1 for the highest, confirming that the major and most stable species of HPP epoxidase is likely to be a homotetramer.

2.3. Crystallization

Initial crystallization experiments were preformed with commercially available screens. Screening was carried out in 96-well trays using sitting drops containing 1 µl protein plus 1 µl of the reservoir set up for vapour diffusion against 500 µl reservoir at 293 K. Small crystals appeared within a couple of days using 2.0 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.5 as the reservoir. This condition was optimized using 2 µl purified HPP epoxidase plus 2 µl reservoir comprising 2.1 *M* $(NH_4)_2SO_4$ in 100 m*M* Tris–HCl pH 7.5. These conditions produced large (0.5 × 0.4 × 0.4 mm) tetragonal crystals (Fig. 1*a*), designated crystal form I. Although of good appearance and size, these crystals unfortunately displayed a high mosaic spread and diffracted poorly to approximately 4 Å resolution irrespective of whether they were placed in a capillary or a cryo-loop and tested in-house or at a synchrotron.

Consequently, further crystallization trials were carried out testing various additives and identified that $ZnCl_2$ produced a second crystal form. To obtain these crystals, HPP epoxidase was incubated with 1 m*M* $ZnCl_2$ for 30 min prior to setting up the drops and crystals were obtained under the same conditions as for form I. Hexagonal prisms, with a maximum size of 0.5 × 0.25 × 0.25 mm (Fig. 1*b*), grew in several days and are designated form II.

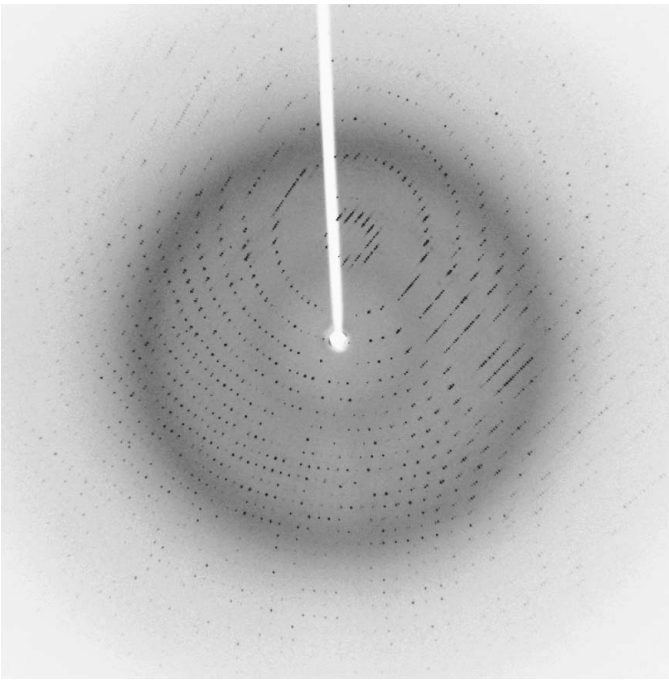

Figure 2
An example of diffraction obtained from the hexagonal crystal form II of HPP epoxidase. The crystal-to-detector distance is 200 cm, the oscillation angle is 0.5° and the exposure is 10 min with X-rays produced at 40 kV, 18 mA.

2.4. Cryoprotection and X-ray diffraction

Finding a cryoprotectant for HPP epoxidase crystals proved to be non-trivial. Initially, various organic solvents were tested as cryoprotectants by substituting 10–50% of the reservoir components with various organic solvents, *e.g.* glycerol, ethylene glycol, 2-methyl-2,4-pentanediol *etc*. In all cases damage to the crystal was observed, which resulted in poor diffraction qualities and low resolution (typically < 4 Å). This was believed to be a consequence of the crystals being grown under such high salt [2.1 *M* $(NH_4)_2SO_4$] concentrations and therefore other compounds were tested such as sucrose, sodium formate, ammonium sulfate/phosphate, sodium chloride, sodium malonate and various oils. Attempts were also made to grow the crystals in solutions already containing cryoprotectants. Eventually, HPP epoxidase crystals were soaked in 3 *M* Li_2SO_4 for up to 1 min without any signs of mechanical damage, before being flash-cooled to 103 K in a stream of nitrogen gas. Crystals treated in this manner gave well ordered diffraction extending to high resolution and a low mosaic spread (∼0.4°; Fig. 2).

Data were collected in-house on a Micromax 007 rotating-anode generator (Cu-anode X-ray source, λ = 1.5418 Å) with an R-AXIS IV^{++} dual image-plate detector system and processed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997) and the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The crystals displayed hexagonal Laue group 6/*mmm* symmetry, with unit-cell parameters a = 86.44, c = 221.56 Å. The data set is 99.6% complete to 1.9 Å and 39 459 unique reflections were obtained from 1 099 904 measurements. The data had an R_{merge} of 7.6% overall and 33.7% in the highest resolution bin (1.97–1.90 Å) and an overall $I/\sigma(I)$ of 28.0 with a value of 3.4 in the highest resolution bin. Inspection of potential systematic absences confirm a $P6_{1/5}$ axis along c, suggesting that the space group is $P6_122$ or the enantiomorph $P6_522$. HPP epoxidase is a homotetramer in solution with a subunit of 198 amino acids and a molecular weight of approximately 21.4 kDa. A Matthews coefficient, V_M (Matthews, 1968), of 2.7 Å Da^{-1} correlates with a dimer of approximately 43 kDa in the asymmetric unit with 55% solvent content.

These crystals are suitable for high-resolution crystallographic analysis and, since there is no obvious model for molecular replacement, work is under way to obtain experimental phases.

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