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A new crystal form of penicillin acylase from *Escherichia coli*

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Abstract

A new crystal form of penicillin acylase (penicillin amidohydrolase, E.C. 3.5.1.11) from *Escherichia coli* W (ATCC 11105) is reported. The crystals were grown using a combination of hanging-drop and streak-seeding methods. The crystals are in the monoclinic space group $P2_1$ with cell dimensions $a = 51.52$, $b = 131.95$, $c = 64.43$ Å, $\beta = 106.12^\circ$. There is one heterodimer in the asymmetric unit ($V_m = 2.45$ Å³ Da⁻¹) and the solvent content is 49%. Preliminary data have been collected to $d_{\min} = 2.7$ Å using a MAR Research image plate and a rotating-anode X-ray source. Subsequent experiments show diffraction beyond 1.3 Å at a synchrotron radiation source.

1. Introduction

Penicillin acylase (PA) is an enzyme of industrial importance for the production of 6-aminopenicillanic acid (6-APA) providing the penam nucleus for the synthesis of a range of penicillin antibiotics. 6-APA is derived from the enzymatic hydrolysis of the common antibiotic penicillin G. The enzyme from *E. coli* is a periplasmic 86 kDa heterodimer of A and B chains (209 and 557 amino acids, respectively). The physiological function of the protein is unknown. Expression of the enzyme *in vivo* is regulated by three major mechanisms: cAMP receptor protein (CRP), phenylacetic acid and thermoregulation giving rise to the hypothesis that the enzyme could be involved in the assimilation of aromatic compounds as carbon sources in the organism's free-living mode (Valle, Balbas, Merino & Bolivar, 1991). The crystal structure of PA has been determined by multiple isomorphous replacement and refined at 1.9 Å resolution (Duggleby *et al.*, 1995). The protein has a compact structure which is pyramidal in cross-section with a deep depression harbouring the active site. It has been proposed that the mechanism of PA catalysis is similar to that of the serine proteinases and involves nucleophilic attack by the N-terminal serine. The crystal structure shows no catalytic triad equivalent to that seen in the serine proteinases. Instead its nucleophilic capacity is enhanced by the seryl α -amino group mediated by a bridging water molecule. This is of particular interest in that PA uses a single amino acid to create its catalytic centre. Recently, PA has been assigned to a new structural superfamily of enzymes, the Ntn (N-terminal nucleophile) hydrolases (Brannigan *et al.*, 1995). They share a common fold in which the nucleophile and other catalytic groups occupy equivalent sites. The fold consists of a β -sheet sandwich flanked on either side by a pair of α -helices. This fold provides the catalytic framework in which the N-terminal serine acts as the nucleophile in the proposed acyl enzyme mechanism. There is a continued interest in probing the substrate specificity of PA and in engineering its specificity to expand its use not only in



Fig. 1. Batch crystals of penicillin acylase grown from 13% PEG 8K

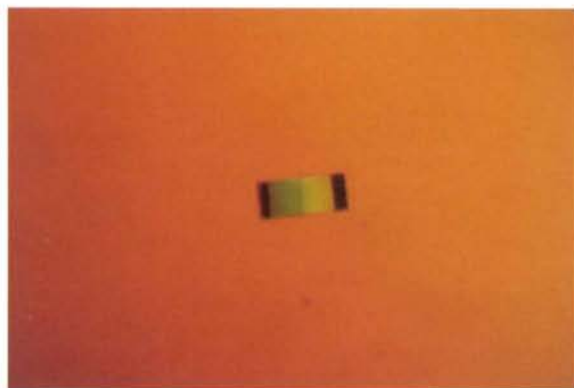


Fig. 2. A single monoclinic crystal grown from 11% MME PEG 2K, 25% ethylene glycol and MOPS buffer at pH 7.2 using streak-seeding methods.

the semi-synthesis of new β -lactam antibiotics but also in other synthetic and hydrolytic reactions. There is considerable information yet to be unveiled about this enzyme and it warrants further structural analysis.

Traditionally PA crystals were grown by batch methods which proved unreliable and required large amounts of protein (Hunt, Tolley, Ward, Hill & Dodson, 1990). Crystals were grown from protein (12 mg ml⁻¹) equilibrated in 50 mM MOPS pH 7.2 with 12–15% polyethylene glycol 8K as precipitant. Crystals appeared within two to three weeks, predominantly in the triclinic form (Fig. 1). Other methods of crystallization such as hanging drop and microdialysis were reported to be unsuccessful.

2. Experimental methods

2.1. Extraction and purification

Purification and characterization of recombinant PA will be described in more detail elsewhere (McVey & Brannigan, 1997). Briefly, the periplasmic protein was extracted by osmotic shock of cells which had been grown for longer than 46 h at 301 K. The first stage of purification involved ammonium sulfate precipitation in which PA precipitated in the 50–70% ammonium sulfate fraction. Using fast protein liquid chromatography the protein was purified to homogeneity by hydrophobic interaction chromatography (Phenyl-Sepharose; Pharmacia) followed by anion-exchange chromatography (Q-Sepharose; Pharmacia).

2.2. Crystallization

Monoclinic crystals were grown from a combination of hanging-drop vapour diffusion and streak-seeding techniques. A 4 μ l hanging drop [2 μ l protein (10 mg ml⁻¹ in 50 mM MOPS pH 7.2) + 2 μ l reservoir (50 mM MOPS pH 7.2, 10–12% MME PEG 2K, and 20–25% ethylene glycol)] was equilibrated at 291 K for 2 d. Using a stock of batch crystal seeds, 3–6 consecutive hanging drops were seeded using a mounted cat's whisker. This gave a smaller number of seeds in the latter drops. Crystals grew within 2–3 d, producing single crystals with dimensions in the order of 0.2 \times 0.2 \times 0.1 mm (Fig. 2). It was envisaged that the addition of ethylene glycol to the crystallization procedure would provide crystals in mother liquor that was mildly or fully cryogenic and thus minimize the need for a potentially damaging transfer to harvest buffer and serial transfer to cryoprotectant buffer (Gamblin & Rodgers, 1993; Rodgers, 1994).

3. Results

As a further development batch crystals were also grown using polyethylene glycol 2000 monomethyl ether (MME PEG 2K) as precipitant and crystals with a variety of morphologies were observed. MME PEG 2K is one of a number of novel precipitants now available (Brzozowski & Tolley, 1994) and it was hoped that it might lead to a better diffracting crystal form. Subsequent screening for crystal growth conditions using MME PEG 2K as precipitant (13%) resulted in crystals one of which produced X-ray data to 2.5 Å resolution at room temperature. These data were indexed in the monoclinic space group $P2_1$ with unit-cell dimensions $a = 117.95$, $b = 103.46$, $c = 139.13$ Å and $\beta = 95.58^\circ$ with an overall R_{merge} of 11% and the data were 96% complete. There were four molecules in the asymmetric unit. Further work on this crystal form was abandoned because of the non-reproducibility of the crystallization method and the fact that no significant improvement in diffraction quality was seen. However, the results prompted further investigation of crystal growth conditions with the use of MME PEG 2K and the development of an alternate crystallization protocol in the hope of producing crystals with better diffraction properties.

The crystallization protocol pursued was a combination of hanging-drop and streak-seeding methods using triclinic batch crystals as seeds. The results derived from streak seeding with MME PEG 2K as precipitant in the presence of cryoprotectant were much more encouraging and showed substantial improvement in crystal mosaicity and diffraction and in addition a single reproducible crystal form. X-ray diffraction data were collected at 120 K using a MAR Research image plate mounted

Table 1. Data-reduction statistics for the new $P2_1$ crystal form of penicillin acylase

	Overall	Outer resolution shell
Resolution (Å)	20–2.73	2.80–2.73
R_{merge} (%)	3.0	4.2
Completeness (%)	99.1	99.9
Multiplicity	2.5	2.4

on a Rigaku X-ray generator with Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å) generated at 50 kV and 100 mA against a rotating-anode target. Before cryocooling, crystals were transferred briefly to cryoprotectant (30% ethylene glycol, 12% MME PEG 2K, in 50 mM MOPS pH 7.2). The ethylene glycol concentration (25%) in the mother liquor was low enough so as not to form a protective glass upon cooling. Attempts to grow crystals in mother liquor containing 30% ethylene glycol produced crystals of lower quality. Crystals were mounted in rayon loops (Hampton Research) of similar dimensions to the crystal, embedded in a 0.2 mm glass capillary supported in a brass mounting pin, and quickly transferred to the liquid N₂ stream maintained at 120 K. Complete data were collected to 2.7 Å on a MAR Research detector (30 cm) using the oscillation method, employing a ϕ range of 1° and a total crystal rotation of 126°, Table 1. The crystal had a mosaic spread of 0.42° and diffracted to at least 1.9 Å but measurements were limited to a resolution of 2.7 Å because of the large unit-cell dimensions and time constraints. Data have recently been collected using synchrotron radiation at the EMBL Hamburg to 1.3 Å resolution. The space group from the 2.7 Å data set was determined to be $P2_1$ with cell dimensions of $a = 51.52$, $b = 131.95$, $c = 64.43$ Å and $\beta = 106.12^\circ$ with one molecule in the asymmetric unit ($V_m = 2.45$ Å³ Da⁻¹; 49% estimated solvent content) (Matthews, 1968). Data were indexed and integrated using *DENZO* (Otwinowski & Minor, 1996) and scaled using *ROTAVATA* and *AGROVATA* from the CCP4 suite (Collaborative Computational Project, Number 4, 1994) (see Table 1).

4. Discussion

Production of recombinant protein has provided a reliable supply for crystallization. Combining hanging-drop vapour-diffusion with streak-seeding techniques has also meant that crystals can be produced more quickly and reliably than previously and considerably less protein is needed. Inclusion of ethylene glycol (15–25%) in the crystallization procedure has proved to have a number of advantages, in particular reducing the mosaic spread upon freezing of the crystal. The $P1$ crystal form when frozen in 30% ethylene glycol had a mosaic spread of 0.6–0.8°, in contrast, the monoclinic crystal form had a typical mosaic spread of 0.4° and as low as 0.2°.

Ethylene glycol appears to serve two purposes, firstly, it is a stepping stone to adequate cryoprotectant conditions and secondly, as an additive in the crystallization conditions it produces a more ordered crystal form giving improved diffraction. The more ordered crystal packing may perhaps be attributed to improved crystal contacts rather than any significant reduction in solvent content, which was similar to the $P1$ crystal form. Absence of ethylene glycol in the mother liquor prior to seeding of the crystals does not yield monoclinic crystals but rather the triclinic form. The use of MME PEG 2K as precipitant is an important requirement in obtaining the monoclinic crystal form as shown from the evidence provided

when using MME PEG 2K in batch crystallization. However, the contribution of ethylene glycol is equally important and possibly stabilizes the crystal packing within the monoclinic form.

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