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Crystallization of a precursor penicillin acylase from *Escherichia coli*

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The crystallization of a mutant precursor penicillin acylase [penicillin amidohydrolase (amidase) E.C. 3.5.1.11] from *Escherichia coli* W (ATCC 11105) using the hanging-drop method is reported. The crystals are in space group $P1$ with unit-cell parameters $a = 51.04$, $b = 63.58$, $c = 71.17$ Å, $\alpha = 103.0$, $\beta = 110.6$, $\gamma = 105.3°$, with one molecule in the asymmetric unit, and diffract to 1.8 Å using synchrotron radiation.

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1. Introduction

Penicillin acylase (PA) has been isolated from a number of prokaryotic and eukaryotic microorganisms. It has been proposed that the enzyme's physiological activity could be involved in the metabolism of aromatic compounds as a carbon source (Valle *et al.*, 1991). Although the actual *in vivo* function of PA is not known, it has an industrially important role as the biocatalyst in the production of the penam nucleus used for the production of various semi-synthetic antibiotics. The β-lactam nucleus of penicillin antibiotics is generated by hydrolysis of the amido bond of penicillin G to yield 6-aminopenicillanic acid (6-APA) and phenylacetic acid. The active wild-type *E. coli* PA is a 86 kDa periplasmic protein comprising the *A* and *B* chains of the mature enzyme (209 and 557 residues, respectively). Refinement of the crystal structure of wild-type PA at 1.9 Å resolution (Duggleby *et al.*, 1995) revealed the heterodimer to be pyramid shaped with a cornet-shaped deep active-site cleft; the active-site serine is located at the base of the active-site cleft.

The gene for PA, *pac*, encodes the precursor protein and a signal sequence which directs translocation of the protein into the periplasm. The precursor protein comprises the *A* domain joined to the *B* domain by a linker peptide of 54 residues. The precursor remains inactive until the whole of the linker peptide has been proteolytically removed. Processing of the linker peptide is carried out in a complex pathway, with initiation of the enzyme maturation being the autocatalytic hydrolysis of the Thr263–Ser264 bond (Kasche *et al.*, 1999). The Ser264 residue has been shown to be critical for the processing pathway (Choi *et al.*, 1992) in addition to its role as the sole amino acid in the catalytic centre of the mature enzyme. Determination of the structure of an unprocessed precursor enzyme, therefore, required the autoproteolysis to be inhibited or at least delayed, and this was achieved by introducing a Thr263Gly mutation (Piotraschke, 1995).

The nucleophilic activity of the active-site serine together with a shared common fold has led to PA being assigned to the structural superfamily of N-terminal nucleophile hydrolases (Brannigan *et al.*, 1995). In recent years, it has become clear that there are post-translational modifications of proteins which involve similar processing pathways, such as the *Drosophila* sonic hedgehog gene which encodes for two proteins expressed as a large precursor and matured by inter- or intra-autoproteolysis of an internal region.

2. Experimental methods

2.1. Expression and purification

A Thr263Gly mutant precursor construct was prepared using PCR to generate the DNA used to transform the pHM12 plasmid (Piotraschke *et al.*, 1994) and the mutation was verified by DNA sequencing. The protein was expressed in *E. coli* K5 and purified using a monoclonal antibody liganded to CNBr-activated Sepharose 4B (Pharmacia) (Kasche *et al.*, 1994). The precursor was eluted with 50 m*M* KH_2PO_4 buffer pH 4 and immediately neutralized to pH 7 with 0.2 *M* KH_2PO_4. Protein purity of >95% was determined by SDS–PAGE and isoelectric focusing electrophoresis. The protein electrophoresed as a single band on SDS–PAGE rather than two fragments of 66 and 21 kDa equivalent to the *A* and *B* domains, respectively. This was taken as evidence that the protein was the complete precursor including the 54 amino-acid residue linker. Biological activity assays revealed that the Thr263Gly mutation slowed autoproteolytic processing of the linker considerably (Kasche *et al.*, 1999).

Table 1
Data-processing statistics for crystal structure of precursor PA.

	Overall	Outer resolution shell
Resolution (Å)	20.9–1.8	1.86–1.8
R_{merge}	0.063	0.216
Completeness (%)	96.9	89.1
Redundancy	1.9	1.5
Reflections $I > 3\sigma$	77.9	51.8

2.2. Crystallization

Crystals were grown by hanging-drop vapour diffusion. Nevertheless, it was essential that the protein was buffer exchanged into 50 m*M* MOPS pH 7.2 and concentrated to 7.5 mg ml^{-1} at 277 K as rapidly as possible to minimize the amount of active wild-type protein in crystallization experiments. Crystals were obtained with 2 µl protein mixed with 2 µl of a reservoir solution comprising 50 m*M* MOPS pH 7.2, 18–22% PEG–MME 5K or 15–20% PEG 4K and 10 m*M* $CaCl_2$, and appeared within 1–2 d. Individual crystals had dimensions 0.2 × 0.05 × 0.02 mm (Fig. 1) and were successfully transferred directly into the cryoprotectant comprising of 30% ethylene glycol, 50 m*M* MOPS pH 7.2, 18–22% PEG–MME 5K and 10 m*M* $CaCl_2$ for data collection.

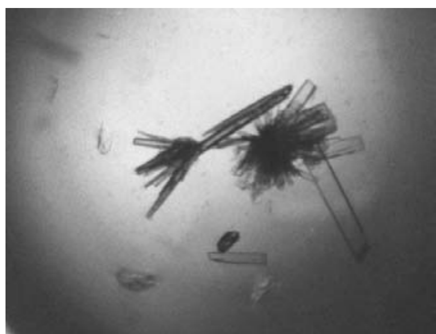

Figure 1
A photomicrograph of crystals of penicillin acylase Thr263Gly mutant precursor. The dimensions of the largest single crystal were 0.2 × 0.05 × 0.02 mm.

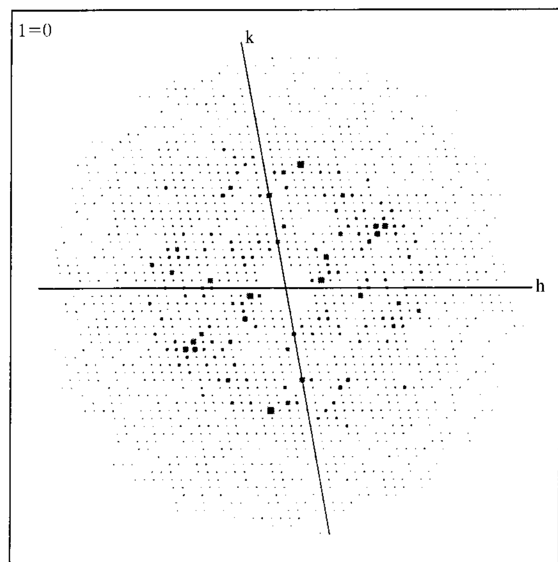

Figure 2
Simulated diffraction pattern showing the *hk*0 layer for penicillin acylase Thr263Gly mutant precursor.

2.3. Results

The mutant protein described here was processed more slowly than the wild-type enzyme, with the first hydrolysis being at the peptide bond between Gly263 and Ser264 (as is the case in the wild-type enzyme; Kasche *et al.*, 1999). This retarded processing is probably inhibited further by reduction of the pH; as revealed by a comparison of the fraction of free N-terminal (Ser264/Ser1 of the *B* chain) at pH 6 (10%) and pH 8 (50%) during an incubation of 100 h at 298 K (Kasche *et al.*, 1999). Unfortunately, crystallization experiments carried out with a buffer at pH 6 did not yield any crystals of sufficient quality for data collection.

Crystals were mounted in a rayon loop (Hampton Research) fixed into a steel pin and cap assembly (Oxford Cryosystems) prior to freezing and storage in liquid nitrogen. Data were collected to 1.86 Å resolution using a MAR Research 30 cm image plate from pre-frozen crystals which were transported in a dry shipper to beamline X11, EMBL, Hamburg. The unit-cell parameters of the crystal were a = 51.04, b = 63.58, c = 71.17 Å, α = 103.0, β = 110.6, γ = 105.3° and the space group was P1 (Fig. 2) with one molecule in the asymmetric unit (V_m = 2.17 Å^3 Da^{-1}, 43% estimated solvent content; Matthews, 1968); 133 678 measurements were recorded of 70 642 reflections. Data were indexed and integrated using *DENZO* (Otwinowski & Minor, 1997) and scaled using *SCALEPACK* (Otwinowski & Minor, 1997; see Table 1). The structure of the active wild-type *E. coli* PA has been determined and coordinates were available for determination of the precursor enzyme by molecular replacement (Duggleby *et al.*, 1995).

3. Discussion

Preliminary crystallization experiments on this protein were based on the conditions used previously to obtain PA crystals (Hunt *et al.*, 1990; McVey *et al.*, 1997) but these proved unsuccessful. Screening experiments were carried out and it was discovered that crystals only formed when between 5 and 10 m*M* $CaCl_2$ was included in the mother liquor.

Data processing revealed a unit cell of very similar dimensions to those of the wild-type PA crystals. It was thought that the precursor protein had carried out autoproteolytic processing and that the crystals were of active wild-type enzyme. This proved not to be true, as in the ongoing refinement the electron density for the majority of the linker region is well defined. Biochemical evidence suggested that autoproteolysis of the precursor mutant took days rather than hours (Kasche *et al.*, 1999) and crystals formed within 1–2 d. So it was interesting to observe that crystals harvested from a two-month-old drop analysed by SDS–PAGE showed three bands of 87, 66 and 21 kDa, confirming that some autoproteolytic processing had occurred during crystallization.

In addition to the reported precursor, further mutants of PA are currently being crystallized in an attempt to elucidate the mechanisms involved in this processing pathway.

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