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Purification, crystallization and preliminary X-ray diffraction of Cys103Ala acyl coenzyme A: isopenicillin N acyltransferase from *Penicillium chrysogenum*

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Penicillins and cephalosporins are an efficacious group of antibiotics produced by fungi such as *Penicillium chrysogenum* and *Acremonium chrysogenum*. The last step in their biosynthesis is catalyzed by acyl coenzyme A:isopenicillin N transferase (AT). This enzyme is produced as a single-chain proenzyme, which is activated by autocatalytic cleavage of the Gly102–Cys103 peptide bond, resulting in a heterodimeric protein with subunits of 11 and 29 kDa. The Cys103Ala mutant of the proenzyme, which does not undergo this cleavage, was purified and crystallized. Diffraction-quality crystals of the mutant and an L-SeMet-substituted mutant were obtained by vapour diffusion against solutions containing $(NH_4)_2SO_4$, NaCl and HEPES–NaOH pH 7.5. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a = 231.36$, $b = 68.27$, $c = 151.31$ Å and $\beta = 129.56°$. They diffract to 2.8 Å resolution with X-rays from a rotating-anode generator.

1. Introduction

Since the Second World War, major medical advances have been accomplished on account of the use of powerful antibiotics to combat otherwise deadly infections. Nevertheless, infectious diseases are still one of the major causes of mortality in the world. Fortunately, most infections can be treated successfully, despite the emergence of antibiotic resistance. β-Lactam antibiotics such as penicillins and cephalosporins are often still the first drugs of choice, because of their therapeutic effectiveness, low toxicity and abundant availability at cheaper prices.

Penicillins and cephalosporins are produced by organisms such as *Penicillium chrysogenum, Acremonium chrysogenum* (formerly known as *Cephalosporium acremonium*) and *Streptomyces clavilugerus*. The biosynthetic pathway of these compounds harbours interesting and mechanistically intriguing enzymes whose function at the atomic level has only been investigated recently (Burzlaff *et al.*, 1999; Roach *et al.*, 1995, 1997; Valegård *et al.*, 1998). The biosynthesis of these β-lactam antibiotics starts with the non-ribosomal peptide synthesis of the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) by ACV synthetase. This tripeptide is then converted by isopenicillin N synthase (IPNS) into the first functional penicillin, isopenicillin N (IPN). IPNS is a non-haem iron-dependent oxygenase and the only enzyme in the penicillin biosynthetic route that has so far been studied in atomic detail (Burzlaff *et al.*, 1999). The next step is the exchange of the α-aminoadipic acid side chain of isopenicillin N for a coenzyme A activated phenylacetic acid side chain, resulting in the formation of penicillin G. This reaction is catalyzed by acyl CoA:isopenicillin N acyltransferase (AT). *In vitro*, AT is able to perform a multitude of transferase reactions. It can not only convert isopenicillin N into penicillin G, but also exhibits penicillin transacylase activity, exchanging acyl side chains between various penicillins. It accepts a wide range of acyl-CoA derivatives, hydrophobic as well as hydrophilic. It even accepts various non-CoA thioesters. This demonstrates that AT is a very versatile enzyme that could well play an important role in the (semi-) synthetic production of future β-lactam antibiotics (Whiteman *et al.*, 1990; Alkema *et al.*, 2001).

AT is a heterodimeric enzyme with a 29 kDa and an 11 kDa subunit. It is produced as an inactive proenzyme of 40 kDa that is autocatalytically activated by cleavage of the Gly102–Cys103 peptide bond (Aplin, Baldwin, Roach *et al.*, 1993). We obtained a Cys103Ala mutant form of AT, which has lost its ability to be processed to the mature heterodimeric protein and thus represents the proenzyme form of AT. This study describes the purification and crystallization of this mutant AT and its L-Se-Met derivative. Apart from a few homologous ATs from other fungi, no significant sequence similarity to other proteins has been found.

2. Materials and methods

2.1. Protein preparation

The Cys103Ala mutant gene of AT was cloned into *Escherichia coli* JM109 as a host.

Cells were grown at 303 K in 2×YT medium (Sambrook *et al.*, 1989), supplemented with 30 µg ml^{-1} chloramphenicol. Cells were induced with 0.1 m*M* β-D-isopropylthiogalactopyranoside. After overnight growth, the cells were harvested (20 min, 277 K, 10 000*g*) and frozen at 253 K until further use.

The purification of the mutant AT is based on the method described by Aplin, Baldwin, Cole *et al.* (1993) with various modifications and extensions. Cell-free extract was prepared by thawing frozen cells in lysis buffer [50 m*M* Tris–HCl pH 7.5, 8.7%(*v*/*v*) glycerol, 0.1%(*v*/*v*) Triton X-100 and 0.1%(*v*/*v*) β-mercaptoethanol] and sonication (5 × 20 min), after which the lysed cell paste was centrifuged (20 min, 277 K, 48 000*g*). The cell-free extract was applied to a Fractogel TMAE column [EMD TMAE-650(M), 45–90 µm, 5 × 15 cm] equilibrated with 50 m*M* Tris–HCl pH 8.0. A gradient of 0–1 *M* NaCl in 50 m*M* Tris–HCl pH 8.0 was used to elute the protein. 1.5 *M* $(NH_4)_2SO_4$ was added to the AT-containing samples and the pool was loaded onto a MacroPrep Methyl-HIC column (2.6 × 15 cm; Biorad, USA). A gradient of 1.5–0.0 *M* $(NH_4)_2SO_4$ in 50 m*M* Tris–HCl pH 8.0 was used for elution. A final step was carried out on a Hiload Superdex 75 gel-permeation column (2.6 × 60 cm). This column was eluted with 50 m*M* Tris–HCl buffer pH 8.0 containing 150 m*M* NaCl. SDS–PAGE analysis of the samples was used to detect the presence of the mutant AT protein.

L-SeMet-containing AT C103A was prepared with the same host cells. The cells were grown in M9 minimal medium (Sambrook *et al.*, 1989) supplemented with a vitamin solution, trace elements and chloramphenicol (30 µg ml^{-1}). The cells were induced with 0.1 m*M* β-D-isopropylthiogalactopyranoside, after which amino acids repressing methionine biosynthesis (Van Duyne *et al.*, 1993) were added together with L-Se-Met. The cells were grown overnight at 303 K. Purification was the same as described above except that all buffers also contained 10 m*M* DTT (Doublié, 1997).

2.2. Crystallization

Crystallization screens were performed at 291 K with the sparse-matrix screen (Jancarik & Kim, 1991) and the Hampton Research Crystallization Kit II employing the hanging-drop vapour-diffusion method. The best crystallization conditions consisted of mixing 1.5 µl of protein solution (7.0 mg ml^{-1}) with 1.5 µl of reservoir solution containing 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* NaCl and 0.1 *M* HEPES–NaOH buffer pH 7.5. Initial crystals appeared within a week. Further crystals were grown using crushed initial crystals as seeds. With seeding, growth was much faster (1–2 d) and a large number of diffraction-quality crystals were obtained, with average dimensions of 300 × 200 × 1000 µm (Fig. 1).

Crystals of L-SeMet-containing protein were obtained using the same crystallization conditions except for the addition of 10 m*M* DTT to all solutions used. Non-L-SeMet-containing crystals were initially used as seeds and at least three rounds of seeding with the newly obtained crystals were performed to maximize the L-SeMet-containing protein in the crystals.

Crystals were frozen in a stream of nitrogen (100 K, Oxford Cryosystems). Before placing in the cold nitrogen stream, they were dipped into a cryosolution [1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* NaCl, 20%(*v*/*v*) glycerol in 0.1 *M* HEPES–NaOH buffer pH 7.5].

2.3. Mass spectrometry

The protein was mixed with an equal volume of 20 mg ml^{-1} sinapinic acid matrix in 40% acetonitrile, 0.1% trifluoroacetic acid. 2 µl of this mixture was applied to the target and allowed to dry in the air. MALDI-TOF mass spectra were recorded with a Micromass Tofspec E MALDI time-of-flight mass spectrometer operated in linear mode. The spectra were calibrated externally with bovine serum albumin.

3. Results and discussion

In *P. chrysogenum*, the last step of penicillin G biosynthesis is catalyzed by acyl CoA: isopenicillin N acyltransferase (AT). So far, this enzyme has not been studied by X-ray crystallography. A major reason for this has been the difficulty of obtaining pure homogeneous preparations of AT, as the mature AT enzyme is subject to severe aggregation. This problem is not present with the Cys103Ala mutant of AT, which could be purified as a monodisperse protein in large yields. Crystals of this mutant were readily obtained with the hanging-drop vapour-diffusion method employing 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* NaCl and 0.1 *M* HEPES–NaOH buffer pH 7.5 as precipitant. Using X-rays from a Cu $K\alpha$-emitting rotating-anode generator, frozen crystals showed diffraction to 2.8 Å resolution on a MacScience DIP2030 image-plate detector. Diffraction image data, indexed, integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), showed that AT crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 231.36$, $b = 68.27$, $c = 151.31$ Å, $\beta = 129.6°$ at 100 K.

Heavy-atom soaks were unsuccessful as the AT crystals completely lost their diffraction power upon soaking or showed severe anisotropy with an unacceptable reduction of the diffraction quality. Therefore, we prepared an L-SeMet derivative of the AT Cys103Ala mutant. The same host

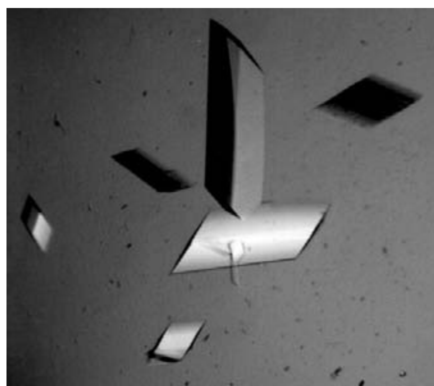

Figure 1
Microphotograph of crystals of the Cys103Ala mutant of AT.

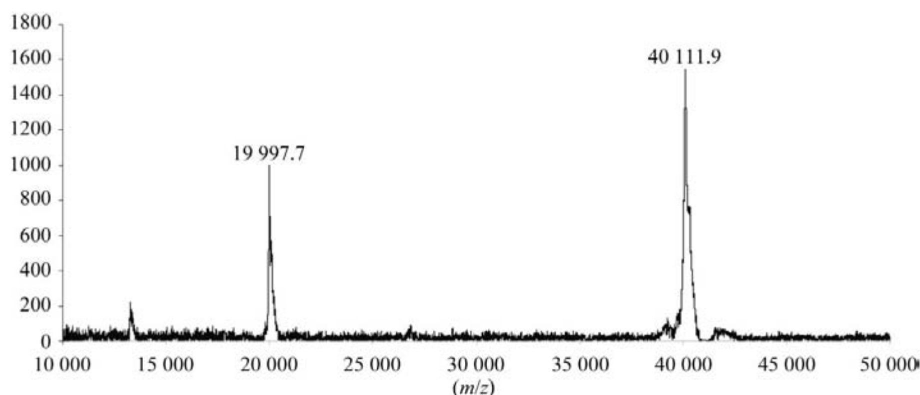

Figure 2
Mass spectrum of L-SeMet-substituted AT.

cells were used as for the wild-type enzyme and incorporation of L-SeMet was achieved by repressing Met biosynthesis. To establish the amount of L-SeMet incorporated, we determined the molecular masses of the non-substituted and substituted protein preparations with matrix-assisted laser desorption/ionization time of flight mass spectrometry. The mass spectrum of the L-SeMet-containing protein (Fig. 2) showed a mass increase of 205 Da, representing an L-SeMet substitution of approximately 55%. L-SeMet-containing crystals showed a similar diffraction quality to those of the Cys103Ala mutant. The successful crystallization of native and SeMet AT reported here is the first step in solving the X-ray crystal structure of AT and gaining a better understanding of the molecular details governing the activity of this enzyme.

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