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X-ray analysis of two antibiotic-synthesizing bacterial ester hydrolases: preliminary results

Thomas R. M. Barends,[a] Charles M. H. Hensgens,[a]† Jolanda J. Polderman-Tijmes,[b] Peter A. Jekel,[b] Erik de Vries,[b] Dick B. Janssen[b] and Bauke W. Dijkstra[a]*

[a]Laboratory of Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, and [b]Laboratory of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

† Current address: Mucovax BV, Niels Bohrweg 11–13, 2333 CA Leiden, The Netherlands.

Correspondence e-mail: bauke@chem.rug.nl

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α-Amino-acid ester hydrolases are multimeric enzymes of potential use in antibiotic production. Knowledge of their structure could help to engineer these enzymes into economically viable biocatalysts. The α-amino-acid ester hydrolases from *Xanthomonas citri* and *Acetobacter turbidans* have been crystallized. The *X. citri* enzyme crystallizes in a primitive monoclinic space group (unit-cell parameters a = 90.1, b = 125.8, c = 132.1 Å, β = 90.9°). The *A. turbidans* enzyme crystallizes in both a primitive orthorhombic (a = 99.1, b = 104.9, c = 284.9 Å) and a body-centred cubic space group with $a = b = c$ = 342.2 Å. From both enzymes, diffraction-quality crystals (resolution 3.0 Å or better) were obtained. Data-collection statistics are reported for data sets from both enzymes.

1. Introduction

A crucial step in the industrial production of semisynthetic β-lactam antibiotics is the coupling of an unnatural acyl group to a β-lactam nucleus. Conventional synthetic methods to achieve this involve hazardous and environmentally harmful reagents such as acid chlorides and anhydrides (Bruggink *et al.*, 1998). Enzymatic semisynthesis may provide a way around this problem. Enzymes can synthesize antibiotics in aqueous solution using a β-lactam molecule and a simple activated acyl compound such as an ester. A well known enzyme used for this purpose is the penicillin acylase from *Escherichia coli* (Bruggink & Roy, 2001).

The α-amino-acid ester hydrolases (AEHs) have also been considered for this application for some time (Ryu & Ryu, 1987). In contrast to penicillin acylase, these enzymes are capable of accepting positively charged acyl group donors and have a lower pH optimum, properties which are both advantageous in antibiotic production.

The coupling reaction catalyzed by AEHs is based on enzyme acylation by an ester, followed by cleavage of the acylated enzyme by a β-lactam compound such as 6-amino penicillanic acid (Takeshi *et al.*, 1974). During application, the AEHs can also catalyze the hydrolysis of the acyl donor and the antibiotic as unwanted side reactions (Fig. 1) (Fernandez-Lafuente *et al.*, 2001).

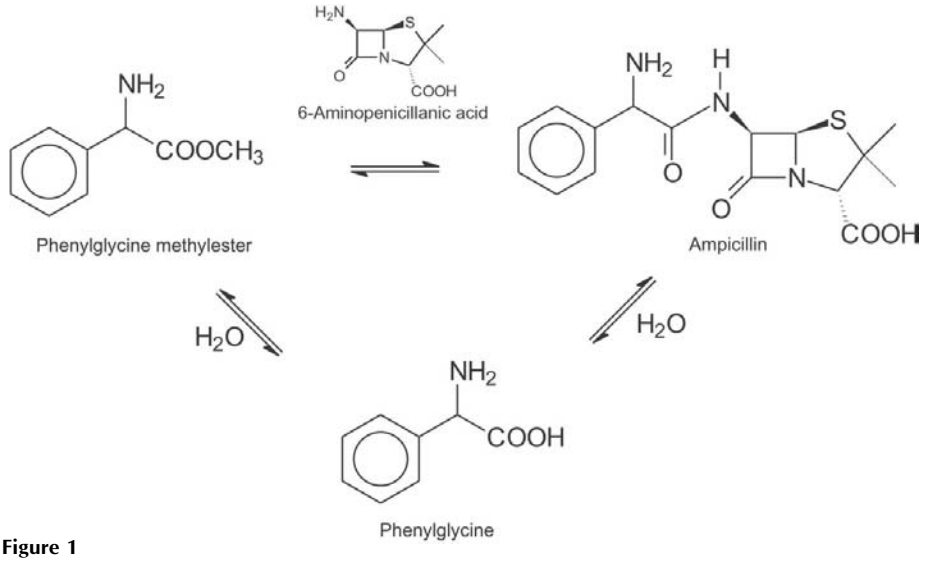

Figure 1
Reactions catalyzed by α-amino-acid ester hydrolases. As an example, the synthesis of ampicillin from phenylglycine methyl ester and 6-aminopenicillanic acid is shown. Hydrolysis of substrate and product are undesired side reactions.

The concomitant synthesis of the antibiotic and hydrolysis of the starting compound and product calls for kinetic control of the production process. In such a process, the antibiotic yield depends on the rates of antibiotic production, antibiotic hydrolysis and ester hydrolysis (Fernandez-Lafuente *et al.*, 2001). Thus, understanding and controlling the catalytic properties of the enzyme is crucial for the design of economically viable synthetic processes. Structural information on these enzymes is lacking, however, which hampers rational engineering of the catalyst.

We therefore set out to determine the structures of the well studied AEHs from *Xanthomonas citri* (Kato, 1980; Kato *et al.*, 1980*a*) and *Acetobacter turbidans* (Takeshi *et al.*, 1974). The *X. citri* enzyme occurs as a homotetramer in solution, with a monomer molecular weight of 72 kDa (Kato *et al.*, 1980*b*). The *A. turbidans* AEH was previously reported to be a heterotetramer ($\alpha_2\beta_2$), with molecular weights of the individual chains of 70 and 72 kDa (Ryu & Ryu, 1987). However, recent investigations show that it is most likely to be a homodimer consisting of two 70 kDa polypeptides (Polderman-Tijmes *et al.*, 2002).

2. Methods

2.1. Crystallization

Expression and purification of native and selenomethionine-labelled AEH from *X. citri* IFO3835 and His_6-tagged AEH from *A. turbidans* ATCC9325 will be described elsewhere. All crystallization experiments were performed at room temperature. *X. citri* AEH was concentrated to 5 mg ml^{-1} in 50 m*M* cacodylate buffer pH 6.5. Initial screening for crystallization conditions was performed in hanging drops with the Crystal Screen I matrix (Hampton Research, Laguna Niguel, USA). Each drop consisted of 3 µl protein solution and 3 µl reservoir solution and was suspended over 500 µl reservoir solution. Small rectangular crystals were found after 1 d in condition 18 (0.1 *M* sodium cacodylate buffer pH 6.5, 0.2 *M* magnesium acetate, 20% PEG 8000). This condition was optimized to 0.1 *M* sodium cacodylate pH 6.5 with 12–13% PEG 8000. Cacodylate was found to be indispensable for crystallization. The optimized condition gave crystals in 4 d with typical dimensions of 0.1 × 0.1 × 0.05 mm. Similar crystals of selenomethionine-labelled *X. citri* AEH could be grown under the same conditions including 5 m*M* dithiothreitol (DTT).

His_6-tagged *A. turbidans* AEH was purified in 50 m*M* sodium phosphate buffer pH 6.2 and concentrated to 5 mg ml^{-1}. An initial crystallization screen was performed with an ORYX-6 crystallization robot (from Douglas Instruments, Hungerford, England) using the modified microbatch method (D'Arcy *et al.*, 1996). Drops consisting of 0.5 µl protein solution and 0.5 µl precipitant solution taken from the Crystal Screen I matrix were mixed in a Terazaki 72-well plate. The plate was covered with 7.0 ml of a mixture of 50% silicone oil and 50% paraffin oil. Crystals grew in one week in two forms. Prism-shaped crystals (form I) were found in condition 10 (0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate and 30% PEG 4000). Rhombic dodecahedra (form II; see Fig. 2) were found in four conditions containing ammonium sulfate. Both crystal forms were optimized in hanging-drop setups. The best form I crystals were obtained by mixing 1.5 µl of protein solution with 1.5 µl of a reservoir solution containing 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate and 15% PEG 4000, followed by equilibration against 500 µl of reservoir solution. These crystals grew to dimensions of 0.4 × 0.15 × 0.1 mm in two weeks. Optimal form II crystals were grown from drops made by mixing 1.5 µl of protein solution with 1.5 µl of 1.75 *M* ammonium sulfate, 2%(*v*/*v*) PEG 400 and 0.1 *M* HEPES–NaOH pH 7.5 and equilibration against 500 µl of this solution. These crystals reached a maximum diameter of 0.2 mm in 4 d.

2.2. Data collection

All data were collected at 100 K and processed using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). Further analysis was performed with the *CCP*4 package (Collaborative Computational Project Number 4, 1994).

Crystals of native *X. citri* AEH were frozen in reservoir solution containing 25% glycerol. Selenomethionine-derivatized crystals were frozen in the same cryoprotectant after addition of 10 m*M* DTT. Data were collected from these crystals at the ID29 station at the ESRF, Grenoble, France.

A. turbidans AEH form I crystals were soaked for 30 s in a reservoir solution containing 25% glycerol and 0.75 *M* sodium iodide, frozen in liquid nitrogen and tested with Cu $K\alpha$ radiation from an Enraf–Nonius rotating-anode generator equipped with a MacScience DIP2030 image-plate detector. *A. turbidans* AEH form II crystals were frozen in a cryoprotectant consisting of 25% glycerol in mother liquor. Diffraction data were measured at the ESRF beamline ID14-2.

Table 1
Data-collection statistics for data collected from a crystal of AEH from *X. citri* and from a form II crystal of the *A. turbidans* AEH.

All data were collected at 100 K. Values for the highest resolution bin are indicated in parentheses.

Parameter	*X. citri* AEH crystal	*A. turbidans* AEH form II crystal
Unit-cell parameters		
a (Å)	90.2	342.2
b (Å)	126.1	342.2
c (Å)	132.1	342.2
α (°)	90.0	90.0
β (°)	91.0	90.0
γ (°)	90.0	90.0
Space group	$P2_1$	$I23$ or $I2_13$
Beamline	ID29 (ESRF)	ID14-2 (ESRF)
Wavelength (Å)	0.97940	0.933
No. observations	842292	391056
No. unique reflections	57221	132485
Resolution range (Å)	30.0–3.06 (3.12–3.06)	30.0–3.00 (3.11–3.00)
R_{merge}	0.103 (0.215)	0.097 (0.240)
Completeness (%)	100 (99)	99.3 (98.4)
$I/\sigma(I)$	25.7 (14.1)	25.8 (9.9)

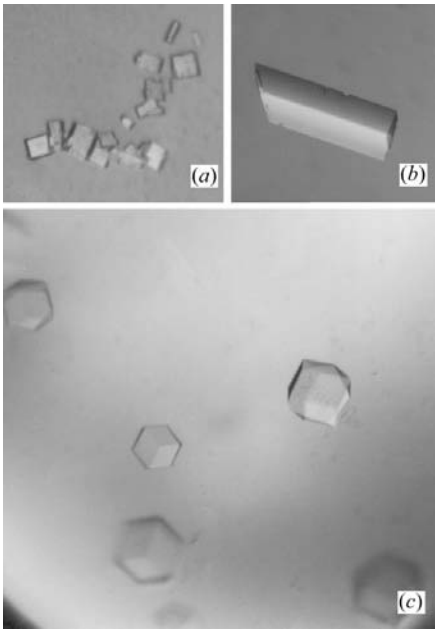

Figure 2
(*a*) Crystals of selenomethionine-labelled α-amino-acid ester hydrolase from *X. citri*. The average crystal dimensions are approximately 0.1 × 0.1 × 0.05 mm. (*b*) Form I crystal of the α-amino-acid ester hydrolase from *A. turbidans*. The dimensions of the crystal are 0.4 × 0.15 × 0.1 mm. (*c*) Form II crystals of the *A. turbidans* α-amino-acid ester hydrolase. The largest crystal has a maximum diameter of 0.1 mm.

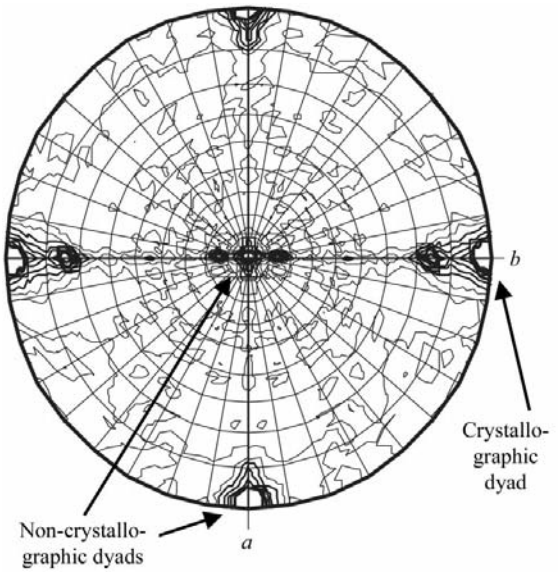

Figure 3
Projection of the $\kappa = 180^\circ$ section of the self-rotation function of a data set collected from a $P2_1$ crystal of the α-amino-acid ester hydrolase from *X. citri*. The crystallographic dyad parallel to *b* as well as two non-crystallographic dyads perpendicular to it have been indicated. This picture was generated using the program *MOLREP* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Upon autoindexing in *DENZO*, diffraction patterns from *X. citri* AEH crystals showed that this protein crystallizes in a primitive monoclinic lattice. A full data set was collected from selenomethionine-labelled crystals. Scaling and merging showed the space group to be $P2_1$, with unit-cell parameters $a = 90.1$, $b = 125.8$, $c = 132.1$ Å, $\beta = 90.9^\circ$. Assuming four monomers in the asymmetric unit, the solvent content is 54%, with a Matthews coefficient of 2.65 Å^3 Da^{-1}. Diffraction was observed to a maximum resolution of 1.6 Å. Statistics of this data set, collected from a selenium-labelled crystal at the selenium peak wavelength on the ID29 station at the ESRF, are shown in Table 1.

The self-rotation function of these data exhibits three mutually perpendicular dyad axes (Fig. 3). Given that the β angle is close to 90°, this may indicate pseudo-orthorhombic symmetry in the crystals.

A. turbidans AEH form I crystals diffracted to 3.0 Å resolution on a home X-ray source. The lattice was determined to be primitive orthorhombic, with unit-cell parameters $a = 99.1$, $b = 104.9$, $c = 284.9$ Å. With two 140 kDa dimers in the asymmetric unit, this corresponds to a solvent content of 54%, with a Matthews coefficient of 2.64 Å^3 Da^{-1}.

Form II crystals diffracted to 3.0 Å at the ID14-2 station of the ESRF. These crystals displayed *I*-centred cubic symmetry, their space group being either $I23$ or $I2_13$. Statistics of a native data set are reported in Table 1. Unit-cell parameters were $a = b = c = 342.2$ Å. This indicates four dimers in the asymmetric unit, with a solvent content of 59% and a Matthews coefficient of 2.97 Å^3 Da^{-1}. We expect the high sequence similarity between the *X. citri* and *A. turbidans* enzymes to allow the *A. turbidans* structure to be solved by molecular replacement using the *X. citri* structure as a model.

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