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Expression, purification, crystallization and preliminary X-ray diffraction studies of the *cmcI* component of *Streptomyces clavuligerus* 7α-cephem-methoxylase

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Cephamycins are broad-spectrum β-lactam antibiotics that show resistance to certain forms of β-lactamases. They differ from cephalosporins by the presence of a methoxyl group at the C-7α position. The gene products of *cmcI* and *cmcJ* are believed to control 7α-methoxylation of cephalosporins through successive steps of hydroxylation and methylation. Here, the expression, purification, crystallization and initial data-collection statistics of the 236-amino-acid protein product of *cmcI* from *Streptomyces clavuligerus* is reported. The crystals belong to space group $P2_1$, with unit-cell parameters a = 93.6, b = 182.6, c = 103.2 Å, β = 91.05°. Diffraction data were collected to 2.5 Å.

1. Introduction

Cephalosporins are derivatized with a 7α-methoxy group to form cephamycins by certain members of the actinomycetes. Cephamycins are of clinical interest because they are β-lactam antibiotics with increased antibacterial activity and resistance to β-lactamase degradation (Daoust *et al.*, 1973).

Methoxylation of the cephem nucleus at the 7α position occurred in the presence of molecular oxygen, *S*-adenosylmethionine, 2-oxoglutarate, Fe^{2+} and a reducing agent in studies on *Streptomyces clavuligerus* which employed *in vivo* methods and cell-free extracts (O'Sullivan *et al.*, 1979; O'Sullivan & Abraham, 1980). In the absence of *S*-adenosylmethionine, the hydroxylated intermediate was formed (Hood *et al.*, 1983). The successive steps of hydroxylation and methylation acted mainly on the substrates cephalosporin C and *O*-carbamoyldeacetylcephalosporin C (O'Sullivan & Abraham, 1980; Fig. 1).

Xiao *et al.* (1991) purified a cephalosporin 7α-hydroxylase from *S. clavuligerus* which had the properties of a 2-oxoglutarate-dependent dioxygenase (2ODD). From activity assays, it was concluded that this enzyme catalyzes the first step of cephamycin biosynthesis. More recent studies attributed cephem 7α-hydroxylase activity to two proteins encoded by the closely placed genes *cmcI* and *cmcJ* of *Amycolatopsis lactamdurans* (formerly *Nocardia lactamdurans*; Coque *et al.*, 1995). Methylase activity was also associated with the two genes and it was proposed that a two-protein component system dependent on 2-oxoglutarate performs the two steps of methoxylation in the actinomycetes (Coque *et al.*, 1995; Enguita *et al.*, 1996).

Sequence analyses of *cmcI* and *cmcJ* reveal little about the putative methoxylation complex of their protein products. *CmcI* encodes a 27.6 kDa protein with pockets of sequence characteristic of methyl transferases. The 32 kDa product of *cmcJ* shows low homology to a number of hypothetical proteins and to an oxidase with an unknown mechanism, gibberellin desaturase (Tudzynski *et al.*, 2003). Neither the product of *cmcJ* nor

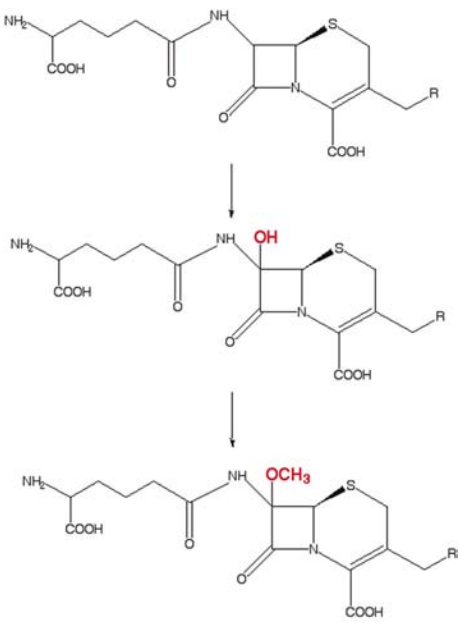

Figure 1
Biosynthesis of cephamycins from cephalosporins occurs *via* hydroxylation followed by methylation at the C-7α position. The main substrates for methoxylation are cephalosporin C (R = $-OCOCH_3$) and carbamoyldeacetylcephalosporin C (R = $-OCONH_2$). The two steps are assumed to be controlled by the genes *cmcI* and *cmcJ* in *S. clavuligerus* and *A. lactamdurans*.

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gibberellin desaturase have significant similarity with the 2ODDs, which catalyze earlier steps of cephalosporin synthesis. However, both contain a H*X*D motif that is generally characteristic of 2ODDs (Hegg & Que, 1997).

This report describes the first steps toward structural determination of the product of *cmcI* from *S. clavuligerus*.

2. Experimental

2.1. Protein expression and purification

The *cmcI* gene (Alexander & Jensen, 1998) of *S. clavuligerus* ATCC 27064 was amplified from genomic DNA by PCR with the forward primer 5′-GGTATTGAGGGTCGCATGAACGACTACTCTCGTCAG-3′ and the reverse primer 5′-AGAGGAGAGTTAGAGCCCTACTGCTTGGCCGCCACTCG-3′ using Advantage GC-Genomic Polymerase Mix (Clontech). The 740 bp PCR product was cloned into pET32 Xa/LIC (Novagen) and the expression plasmid (cmcIpET32) was transformed into *Escherichia coli* BL21 (DE3). Cells were grown at 310 K in 4 l LB medium with 75 µg ml^{-1} ampicillin with shaking at 220 rev min^{-1}; the circular orbit was 2.54 cm. When the OD_{600} of the culture reached 1.0, 1 m*M* IPTG was added and incubation continued for 5 h at 305 K. Cells were harvested by centrifugation, washed in 1× PBS and frozen at 253 K.

Cells (10 g) were resuspended in 1× PBS with 0.1 mg ml^{-1} lysozyme and lysed using a Vibra Cell sonicator at 65% amplitude for 60 s. The cell lysate was spun at 20 000*g* and the soluble fraction was passed over a HiTrap Chelating HP column (Amersham Biosciences) loaded with Ni^{2+}. A gradient of 0.0–0.4 *M* imidazole, 1× PBS was used to elute the poly-His-tagged thioredoxin cmcI fusion protein. Fractions representing the protein peak were pooled and dialyzed overnight against 100 m*M* NaCl, 1 m*M* $CaCl_2$, 50 m*M* Tris–HCl pH 8.0. The solution was incubated at room temperature overnight with factor Xa (Amersham Biosciences) at 5 U ml^{-1} and then dialyzed against 1× PBS. Ni–NTA beads (Qiagen) and benzamidine Sepharose (Amersham Biosciences) were used to remove poly-His-tagged thioredoxin and factor Xa from the protein solution. The remaining cmcI protein was concentrated and loaded onto a Superdex 200 column (Amersham Biosciences) in 1× PBS buffer. Gel-filtration fractions containing pure cmcI were pooled, dialysed against 10 m*M* HEPES and concentrated to 30 mg ml^{-1} using a VivaSpin 10 000 MWCO concentrator (Vivascience). Pure protein was analyzed by both SDS–PAGE (Fig. 2) and native PAGE (Fig. 3). The buffer used for native electrophoresis was 0.112 *M* Tris acetate pH 6.4.

The method of Budisa *et al.* (1995) was used to express cmcI protein with selenomethionine biosynthetically substituted for methionine. The cmcIpET32 plasmid was used to transform *E. coli* B834 (DE3). Cells were cultured at the same temperatures as those used for native protein expression and selenomethionine (Calbiochem) was added to the media to a final concentration of 0.5 m*M*. Selenomethionine-substituted cmcI protein was purified by the methods used for native protein with the following modifications. All buffers were degassed and contained 10 m*M* β-mercaptoethanol. A final desalting step on a Micro Bio-Spin 6 column (BioRad) was performed. The replacement of methionine with selenomethionine at the expected ten residues of cmcI was confirmed by mass-spectrometric analysis.

2.2. Crystallization

CmcI protein was screened for crystallization conditions using Crystal Screen I (Hampton Research) at 277 K. Droplets consisting of 1 µl protein and 1 µl mother liquor were suspended above 1 ml of well solution using the hanging-drop vapour-diffusion method. Secondary screens were conducted at 293 K in the presence and absence of 10 m*M* 2-oxoglutarate. Crystals of selenomethionine-substituted cmcI were grown at 293 K using a mother liquor consisting of 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, 12%(*w*/*v*) PEG 4000, 10 m*M* 2-oxoglutarate and 5 m*M* β-mercaptoethanol.

2.3. Data collection and analysis

Crystals were transferred to mother liquor containing 25%(*v*/*v*) ethylene glycol for cryoprotection and then immediately flash-cooled in liquid nitrogen. The crystals diffracted to 2.5 Å at beamline ID14-EH4 of the European Synchrotron Radiation Facility (Grenoble, France) using a Q4R4 CCD detector from ADSC at a wavelength of 0.939 Å. Diffraction data to 2.5 Å were collected from a single crystal at 100 K with the rotation method using 1° oscillations, 2 s exposure and a crystal-to-detector distance

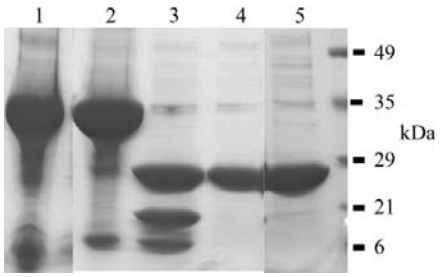

Figure 2
SDS–PAGE analysis of the expression and purification of recombinant *S. clavuligerus* cmcI from *E. coli* (DE3). Lane 1, soluble fraction of crude cell lysate after sonication; lane 2, thioredoxin-cmcI fusion protein after HiTrap Chelating HP column; lane 3, thioredoxin-cmcI fusion protein after cleavage with factor Xa; lane 4, cmcI protein after removal of thioredoxin; lane 5, pure cmcI after gel filtration on Superdex 200. The positions of molecular-weight markers (BioRad) are indicated on the right in kDa.

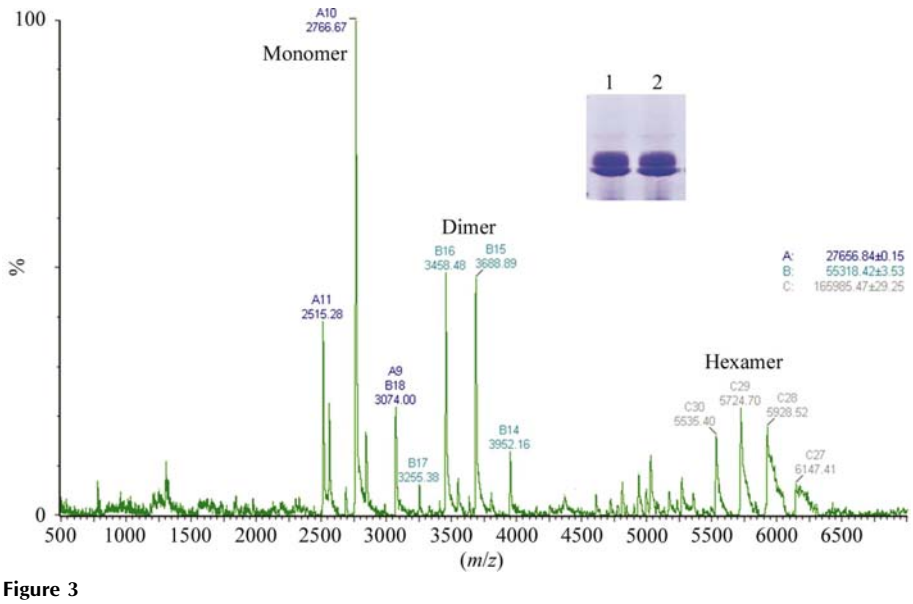

Figure 3
ESI–MS spectrum of 5 µ*M* cmcI in 50 m*M* ammonium acetate pH 7. The hexamer was not observed when the buffer concentration was lowered to 10 m*M*. The inset shows cmcI in Tris acetate pH 6.4 after native PAGE analysis in (1) the absence and (2) the presence of 10 m*M* 2-oxoglutarate.

of 220 mm. Processing and scaling were performed using *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997). Space-group determination was performed using *DENZO*.

Self-rotation functions were calculated with *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) using a radius of integration of 30 Å and data in the resolution range 7–5 Å (Fig. 4).

3. Results and discussion

3.1. Protein expression, purification and crystallization

As a thioredoxin fusion, cmcI accumulated to be the most abundant protein in the soluble cell fraction (Fig. 2). We employed a three-step purification protocol which produced cmcI protein with a purity of >90% at a yield of 5 mg per litre of culture. With a predicted molecular weight of 27.6 kDa, cmcI was expected to penetrate Superdex 75. However, it eluted in the void volume of this column. This result suggested that cmcI formed a multimeric complex in solution. Gel filtration on a calibrated Superdex 200 column confirmed that cmcI formed one complex that was at least a trimer; however, a more accurate estimation of molecular weight was not obtained. Analysis of cmcI in solution by electrospray ionization mass spectrometry (ESI–MS) gave evidence for hexamers as well as monomers and dimers (Fig. 3). By necessity, the salt concentration of the cmcI sample used in ESI–MS was lower than that of the sample used in gel filtration and this probably accounts for the difference in the results obtained by the two methods.

Preliminary screening of cmcI produced crystals overnight under several conditions. The largest crystals were box-shaped and appeared in 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5 and 12%(*w*/*v*) PEG 4000. When 10 m*M* 2-oxoglutarate was added to this mother liquor, a second crystal form appeared. Long needle-shaped crystals were present alongside the box-shaped ones in varying proportions at 293 K in 10 m*M* 2-oxoglutarate, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5 and 12%(*w*/*v*) PEG 4000. The average size of the needles was 0.1 × 0.03 × 0.04 mm.

The needle-shaped crystals grew only when 2-oxoglutarate was present. This result suggests that 2-oxoglutarate induces a conformational change in cmcI. A reported cofactor requirement of 7α-hydroxylation is 2-oxoglutarate (Xiao *et al.*, 1991). However, the sequence of cmcI does not show homology with previously reported 2ODDs and it contains pockets of sequence that are characteristic of methyl transferases. It cannot be excluded that 2-oxoglutarate influences crystallization of cmcI through a nonspecific effect.

Native gel electrophoresis of cmcI in the presence and absence of 2-oxoglutarate detected single bands with no obvious difference in mobility between samples (Fig. 3). The single band was identified as a multimer based on information provided by native gel molecular-weight standards combined with earlier gel-filtration experiments. ESI–MS analysis of cmcI suggests that this multimer is a hexamer (Fig. 3). The native gel results indicate that the tendency of cmcI to form a hexamer is not affected by 2-oxoglutarate.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	93.65
b (Å)	182.64
c (Å)	103.24
β (°)	91.05
Resolution range (Å)	95.3–2.5 (2.59–2.50)
Wavelength (Å)	0.939
No. observations	2198107
No. unique reflections	119750 (11574)
Redundancy	8
$R_{\rm merge}$ (%)	7.6 (33)
$I/\sigma(I)$	25.7 (3.4)
Completeness (%)	99.4 (96.3)

3.2. Data collection and processing

The two crystal forms of cmcI displayed quite different X-ray diffraction properties. The box-shaped crystals did not diffract to any appreciable extent. The needle-shaped crystals (grown in the presence of 2-oxoglutarate) diffracted to a resolution of ~2.5 Å. Data-collection and processing statistics for the needle-shaped crystals are presented in Table 1.

The crystals belong to space group $P2_1$, with unit-cell parameters a = 93.65, b = 182.64, c = 103.24 Å, β = 91.05°. The unit-cell volume is consistent with two cmcI hexamers in the asymmetric unit, with a Matthews coefficient of 2.7 Å^3 Da^{-1} (Matthews, 1968), corresponding to 55% solvent content. This is corroborated by analysis of the self-rotation function (Fig. 4).

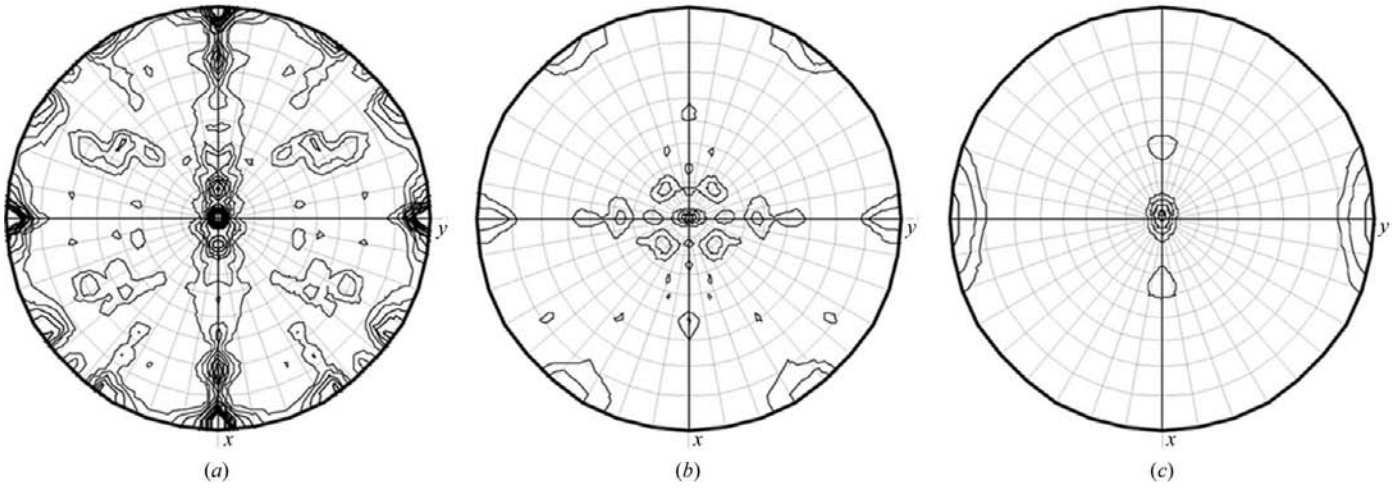

Figure 4
Stereographic projections of the self-rotation function calculated in the (*a*) χ = 180°, (*b*) χ = 90° and (*c*) χ = 60° sections. Two peaks are found in the χ = 60° section (*c*), which could either correspond to two sixfold axes, one parallel to the *z* axis and the other one parallel to the *y* axis, or two threefold axes parallel to two twofold axis. The expected peaks in (*a*) the χ = 180° and (*b*) the χ = 120° sections arising from both cases are readily observed, but there is no way to discriminate between the two options on the basis of the self-rotation alone.

An additional desalting step during protein purification was required to obtain crystals of selenomethionine-substituted cmcI. Diffraction data from these crystals are expected to enable the structural determination of the *cmcI* component of *S. clavuligerus* 7α-cephem-methoxylase. The X-ray crystal structure of cmcI will be invaluable in elucidating the mechanism of 7α-methoxylation of cephalosporins.

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