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Crystallization and preliminary X-ray analysis of the aromatic prenyltransferase CloQ from the clorobiocin biosynthetic cluster of *Streptomyces roseochromogenes*

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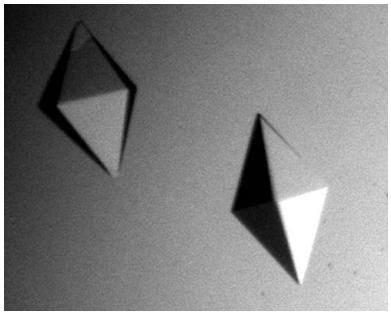
Crystals of recombinant CloQ (subunit MW = 35 626 Da; 324 amino acids), an aromatic prenyltransferase from *Streptomyces roseochromogenes*, were grown by vapour diffusion. The protein crystallizes in space group $I4_122$, with unit-cell parameters $a = b = 135.19$, $c = 98.13$ Å. Native data from a single crystal were recorded to a resolution of 2.2 Å in-house. Preliminary analysis of these data indicated that the asymmetric unit corresponds to a monomer, giving an estimated solvent content of 60.6%. CloQ is involved in the biosynthesis of the aminocoumarin antibiotic clorobiocin, which targets the essential bacterial enzyme DNA gyrase.

1. Introduction

Streptomycetes are responsible for over two-thirds of the naturally derived antibiotics in clinical use today (Bentley *et al.*, 2002). However, the widespread and often inappropriate use of these compounds has led to an alarming increase in the number of bacterial strains that have become resistant to them. Thus, there is a continual need to develop new antibiotics. To this end, we are studying the biosynthesis of a class of antibiotics termed the aminocoumarins, which are natural products of streptomycetes. There are three main compounds, novobiocin, clorobiocin and coumermycin A_1, that are synthesized by enzymes encoded in the *nov*, *clo* and *cou* gene clusters, respectively (Steffensky *et al.*, 2000; Wang *et al.*, 2000; Pojer *et al.*, 2002). They all share common structural features: a 3-amino-4,7-dihydroxycoumarin ring, an L-noviosyl sugar and an aromatic acyl component attached to the amino group of the aminocoumarin moiety. These compounds are potent inhibitors of DNA gyrase (Gellert *et al.*, 1976), an essential enzyme in bacteria and a validated drug target (Maxwell, 1997; Maxwell & Lawson, 2003). However, they have not seen widespread clinical application on account of their low solubility, poor uptake and eukaryotic cell toxicity. Using a combination of structural and mechanistic approaches, we aim to inform the rational redesign of these compounds through the manipulation of the individual enzymes in the pathways. We report here the crystallization and preliminary X-ray analysis of CloQ, an aromatic prenyltransferase from *Streptomyces roseochromogenes* that is responsible for attaching a dimethylallyl unit to the hydroxybenzoic acid moiety of clorobiocin (Pojer *et al.*, 2003).



2. Materials and methods

2.1. Protein expression and purification

The *cloQ* coding sequence (UniProtKB/TrEMBL entry Q8GHB2) was amplified by PCR using as a template the K1F2 cosmid (Pojer *et al.*, 2002) containing the complete clorobiocin biosynthetic gene cluster. The PCR product was cloned into pET28a (Novagen), resulting in an expression construct encoding for CloQ with a thrombin-cleavable N-terminal hexahistidine tag. This added a further 20 residues to the native protein (with sequence MGSSHHHHHHSSGLVPRGSH), giving a total deduced molecular weight of 37 790 Da. In order to maximize the yield of soluble protein, the plasmid containing the *cloQ* gene was co-transformed with plasmid pGroESL-911 encoding the GroES/GroEL chaperone

system (Ichetovkin *et al.*, 1997) into BL21(DE3) (Studier & Moffatt, 1986). For protein expression, 10 ml of an overnight culture of these cells was used to inoculate 1 l Luria–Bertani medium containing 50 mg kanamycin and 5 mg tetracycline. The cells were grown at 310 K to an OD_{600} of around 0.4. The culture was then cooled to 293 K, induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 m*M* and shaken for an additional 5 h at 293 K. The cells were harvested by centrifugation in a Sorvall Evolution centrifuge (10 min, 7000 rev min^{-1}, 277 K, SLC-6000 rotor) and stored at 253 K until further purification.

The cell pellet was subsequently resuspended in buffer *A* [50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 30 m*M* imidazole, 10%(*v*/*v*) glycerol, 10 m*M* β-mercaptoethanol] containing 1%(*v*/*v*) Tween 20 and lysed by two passes through a French press (6.9 MPa, 277 K). The cell debris was removed by centrifugation in a Sorvall RC5C centrifuge (30 min, 19 000 rev min^{-1}, 277 K, SS34 rotor) and the supernatant was applied onto a pre-equilibrated 1 ml Ni^{2+}-charged His-Trap Chelating HP column (GE Healthcare). The column was then washed with ten column volumes (CV) of buffer *A* and the bound protein was eluted over 10 CV in a linear gradient to 1 *M* imidazole in buffer *A*. The fractions containing the CloQ protein (confirmed by SDS–PAGE) were pooled and dialysed at 277 K overnight against 2 l buffer *B* [50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* dithiotreitol (DTT)]. Subsequently, the N-terminal His tag was cleaved using thrombin (Sigma). The cleavage site lies after the final Arg of the tag, leaving just three residues of the linker (*i.e.* GSH). The resulting 327-residue protein, which we shall refer to as GSH-CloQ, would have a calculated molecular weight of 35 908 Da. For cleavage, the CloQ protein was diluted to 100 μ*M* in buffer *B* and incubated for at least 5 h at 277 K in the presence of thrombin at a concentration of 0.3 U per milligram of CloQ protein. The completeness of the cleavage reaction was confirmed by SDS–PAGE. For final purification, the protein was applied onto a Superdex 75 HiLoad HP gel-filtration column (GE Healthcare) pre-equilibrated in buffer *B* without $CaCl_2$. The elution fractions containing CloQ were pooled and concentrated to around 10 mg ml^{-1} using an Amicon Ultra-4 10 kDa cutoff concentrator (Millipore).

Prior to crystallization, dynamic light scattering (DLS) was used to monitor the solution properties of the protein. For this purpose, approximately 30 µl sample was centrifuged through a 0.1 µm Ultrafree-MC filter (Millipore) to remove particulate material before introduction into the 12 µl microsampling cell of a Dynapro-MSTC molecular-sizing instrument (Protein Solutions Inc.). A minimum of 20 scattering measurements were taken at 277 and 293 K and the resulting data were analysed using the *DYNAMICS* software package (Protein Solutions Inc.).

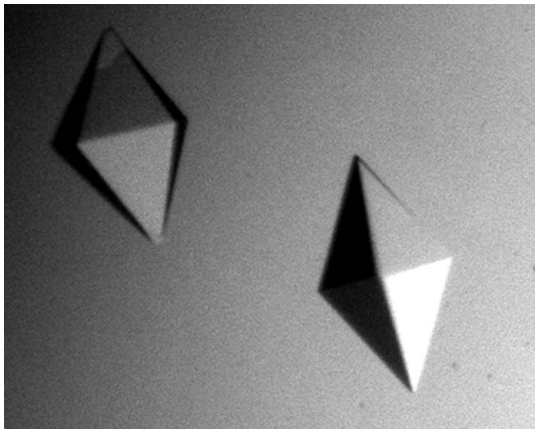


Figure 1
Single crystals of *S. roseochromogenes* CloQ approximately 300 × 200 × 50 µm in size.

2.2. Crystallization and X-ray data collection

Crystallization screening trials were carried out by vapour diffusion in a sitting-drop format in 96-well Greiner plates using a variety of in-house and commercially available screens (Hampton Research and Nextal) at constant temperatures of 293 and 277 K. Drops consisted of 1 µl protein solution mixed with 1 µl well solution and the well volume was 100 µl. The protein concentration was approximately 10 mg ml^{-1}. Trials were performed both with and without 4-hydroxyphenylpyruvate (HPP), a substrate of CloQ (Pojer *et al.*, 2003), at a concentration of 2 m*M*. Improved crystals were subsequently obtained by refining the successful conditions from the initial screens and adapting them to a hanging-drop format using 24-well VDX plates (Hampton Research). In this case, the well volume was increased to 1 ml.

Prior to cryogenic data collection, crystals were given a brief soak (<30 s) in cryoprotectant, which corresponded to the crystallization solution supplemented with 25%(*w*/*v*) xylitol in place of an equivalent volume of buffer. Crystals were routinely transferred from one solution to another and ultimately mounted for X-ray data collection using cryo-loops (Hampton Research). They were flash-cooled to 100 K in a stream of gaseous nitrogen produced by an X-Stream cryocooler (Rigaku-MSC). Diffraction data were collected in-house using a MAR 345 image-plate detector (X-ray Research) mounted on a Rigaku RU-H3RHB rotating-anode X-ray generator (operated at 50 kV and 100 mA) fitted with Osmic confocal optics and a copper target (Cu $K\alpha$; λ = 1.542 Å). The diffraction data were integrated using *MOSFLM* (Leslie, 2006) and scaled using *SCALA* (Evans, 2006). Further analysis of the data was performed using programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

CloQ was overexpressed and purified with a final yield of approximately 2 mg of protein from 1 l culture and was judged to be greater than 98% pure by SDS–PAGE analysis. The DLS analysis gave a monomodal distribution, with a polydispersity of 26%. From these results, the molecular size was estimated at 39.9 kDa, being close to the value expected for a GSH-CloQ monomer (*i.e.* 35.9 kDa). Although there was no significant difference in the DLS results obtained at 277 and 293 K, CloQ protein of this quality could only been obtained if the temperature was strictly maintained at 277 K during the purification process.

Preliminary crystals were obtained within 48 h at 293 K from several conditions containing at least 3.5 *M* sodium formate. Improved crystals were subsequently obtained by reducing the temperature of the crystallization to 277 K with a precipitant solution consisting of 3.0 *M* sodium formate in 100 m*M* HEPES pH 7.0. Further improvements were seen when 2 m*M* HPP was used in all buffer solutions during purification, resulting in large single crystals with dimensions up to 300 × 200 × 50 µm (Fig. 1). Simply adding HPP to the protein solution prior to crystallization did not have the same positive effect.

Native X-ray data were collected in-house from a single CloQ crystal: a total of 200 × 0.5° oscillation images were recorded in a continuous sweep to a maximum resolution of 2.2 Å. Indexing was consistent with an *I*-centred tetragonal lattice, with unit-cell para-

Table 1
Summary of X-ray data for CloQ.

Values in parentheses are for the outer resolution shell

Wavelength (Å)	1.542
Resolution range (Å)	31.86–2.21 (2.33–2.21)
Unique reflections	22556 (2718)
Completeness (%)	97.3 (81.6)
Redundancy	7.7 (7.3)
$R_{\rm merge}$†	0.084 (0.301)
$\langle I/\sigma(I)\rangle$	19.6 (7.3)
Wilson B factor (Å^2)	24.4

† $R_{\rm merge} = \sum_{\mathbf{h}}\sum_l |I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle| / \sum_{\mathbf{h}}\sum_l \langle I_{\mathbf{h}}\rangle$, where I_l is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**.

meters $a = b = 135.19$, $c = 98.13$ Å. After processing the data in space group $I4$, pseudo-precession plots were analysed using *HKLVIEW* (Collaborative Computational Project, Number 4, 1994). These strongly suggested that the symmetry was actually $I422$ and the systematic absences were indicative of space group $I4_122$. Reprocessing in this space group yielded a data set that was 97.3% complete to 2.2 Å resolution. Data-collection statistics are summarized in Table 1. Estimation of the content of the asymmetric unit based on a single GSH-CloQ molecule gave a crystal-packing parameter ($V_{\rm M}$) of 3.12 Å^3 Da^{-1}, with a corresponding solvent content of 60.6% (Matthews, 1968).

The Protein Data Bank (PDB; Berman *et al.*, 2000) was interrogated for potential molecular-replacement templates using the amino-acid sequence of CloQ. Only one suitable candidate was retrieved, namely Orf2, a prenyltransferase from *Streptomyces* sp. strain CL190 (PDB code 1zb6). Orf2 has a novel barrel fold, termed a PT barrel, which superficially resembles the canonical TIM barrel but has a distinctly different connectivity (Kuzuyama *et al.*, 2005). However, the *CLUSTALW* alignment server (Chenna *et al.*, 2003) aligned only two-thirds of the CloQ sequence with that of Orf2, with a relatively low sequence identity of 22%. A molecular-replacement search model was subsequently created using the program *CHAINSAW* (Collaborative Computational Project, Number 4, 1994) with reference to this alignment: the sequence of the Orf2 monomer structure was 'mutated' to that of CloQ and any non-aligned regions were deleted from the structure. In addition, all water molecules, metal ions and other ligands were stripped from the model. Molecular replacement was performed with the programs *AMoRe* (Navaza, 1994), *MOLREP* (Vagin & Teplyakov, 2000) and *Phaser* (McCoy *et al.*, 2005) using this monomer template. Unfortunately, no plausible solutions were obtained in any of the programs, although this is not too surprising given the low sequence identity between CloQ and Orf2. Therefore, we will need to solve the CloQ structure by isomorphous replacement methods. To this end, we are currently preparing selenomethionine-labelled protein: there are a total of six methionine residues present in the 324-amino-acid sequence of CloQ.

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