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Crystallization and preliminary X-ray diffraction studies of a monooxygenase from *Streptomyces coelicolor* A3(2) involved in the biosynthesis of the polyketide actinorhodin

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The aromatic monooxygenase ActVA-Orf6 from *Streptomyces coelicolor* A3(2) that catalyses an unusual oxidation on the actinorhodin biosynthetic pathway has been crystallized. The crystals diffract to 1.73 Å and belong to space group $P2_12_12_1$, with unit-cell parameters a = 46.95, b = 59.29, c = 71.67 Å. Solvent-content (44%) and self-rotation function calculations predict the presence of two molecules in the asymmetric unit. Structure determination should provide further insight into the enzyme mechanism and aid in the design of biosynthetic pathways to produce new polyketide natural products with novel functionality.

1. Introduction

Polyketide compounds such as tetracycline, erythromycin, daunorubicin and lovastatin produced by *Actinomycete* bacteria and fungi have enormous therapeutic and commercial importance. Consequently, there has been considerable research into the biosynthesis of these compounds in the hope that by combining a functional understanding of the enzymes of these pathways with the techniques of combinatorial biosynthesis, multiple new drug candidates can be isolated (Hutchinson, 1999). The potential of this methodology is illustrated by numerous reports in which genetically engineered *Streptomyces* strains have been made to produce novel natural products whose structures have been rationally designed (McDaniel *et al.*, 1995). This work has been aided by the cloning, sequence analysis and manipulation of the gene clusters responsible for the biosynthesis of aromatic and macrolide polyketides and has focused on the enzymes that catalyse the assembly and subsequent cyclization of the polyketide chain (for reviews, see Hopwood & Sherman, 1990; Hutchinson & Fujii, 1995; Hopwood, 1997; Hutchinson, 1997). This allowed many of the proteins responsible for the construction of the carbon skeleton to be identified, but has left the identity and function of the enzymes that perform the later steps on these pathways relatively unclear. Late-acting 'tailoring' enzymes catalyze a wide range of modifications to polyketide structures, such as hydroxylations, methylations or glycosylations; these subtle changes often cause step-change increases in desired activity or toxicological benefits for the modified compounds.

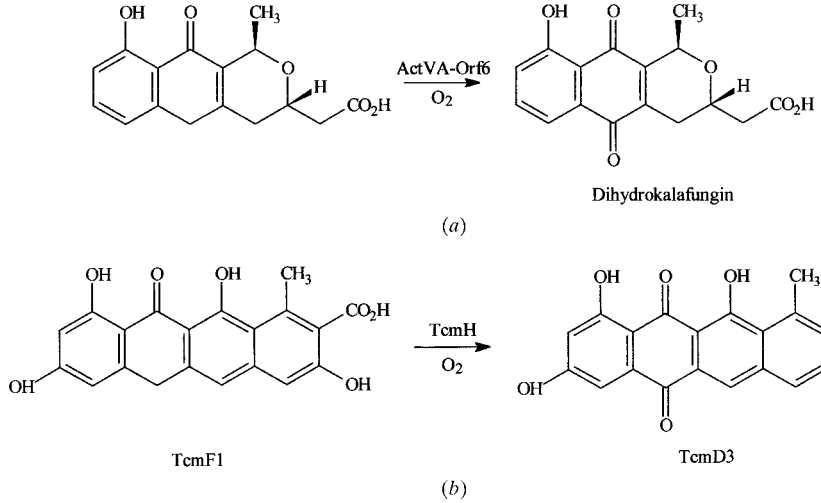

Figure 1
Formation of quinones in polyketide biosynthesis. (*a*) Proposed oxidation catalysed by ActVA-Orf6 monooxygenase on the actinorhodin biosynthetic pathway (Kendrew *et al.*, 1997). (*b*) The analogous oxidation catalysed by TcmH on the tetracenomycin pathway (Shen & Hutchinson, 1993).

Sequence similarities to genes from other polyketide-biosynthetic clusters have allowed enzyme activities to be tentatively assigned to many of the tailoring enzymes. However, the difficulty involved in purifying and assaying these enzymes, together with inadequate knowledge of the biosynthetic pathway and lack of substrates, has meant that few of these assignments have been confirmed with biochemical studies on the proteins. Still less is known about the structural aspects of these enzymes, although this would result in major advances towards understanding the events surrounding the reactions (Townsend, 1997) and in our ability to manipulate them in engineered biosynthesis.

Our work has focused on the tailoring enzymes of these pathways, particularly those catalysing the oxidations that introduce hydroxy or quinone functionality to the polyketide molecules (Kendrew *et al.*, 1995, 1997). We have reported the expression, purification and biochemical characterization of the enzyme ActVA-Orf6 (Kendrew *et al.*, 1997), a monooxygenase from the actinorhodin pathway that catalyses the oxidation of phenolic compounds (or their keto tautomers) to the corresponding quinone (Fig. 1). This was consistent with the results of a study by Shen & Hutchinson (1993) that proposed a role for the homologous protein TcmH, a monooxygenase from the tetracenomycin pathway. The ActVA-Orf6 monooxygenase is remarkable owing to its small size (subunit M_r = 12 000 Da) and the fact that it requires none of the prosthetic groups or cofactors usually associated with the activation of molecular oxygen towards oxygenation. The enzyme exhibits broad substrate specificity and is able to oxidize the corresponding substrates from the tetracenomycin pathway. Several homologs to the ActVA-Orf6 monooxygenase have been identified; however, there is no structural information for any of these proteins and no obvious similarity to proteins of known structure, raising the possibility that they possess a new protein fold. Here, we describe the next stage of our study of this enzyme; the crystallization and preliminary X-ray diffraction data of the ActVA-Orf6 monooxygenase. A structural understanding of this enzyme will allow a better mechanistic understanding of this unusual oxidation and potentially reveal some of the rules governing polyketide enzyme–substrate molecular recognition that will apply to many enzymes from this class of biochemical pathway.

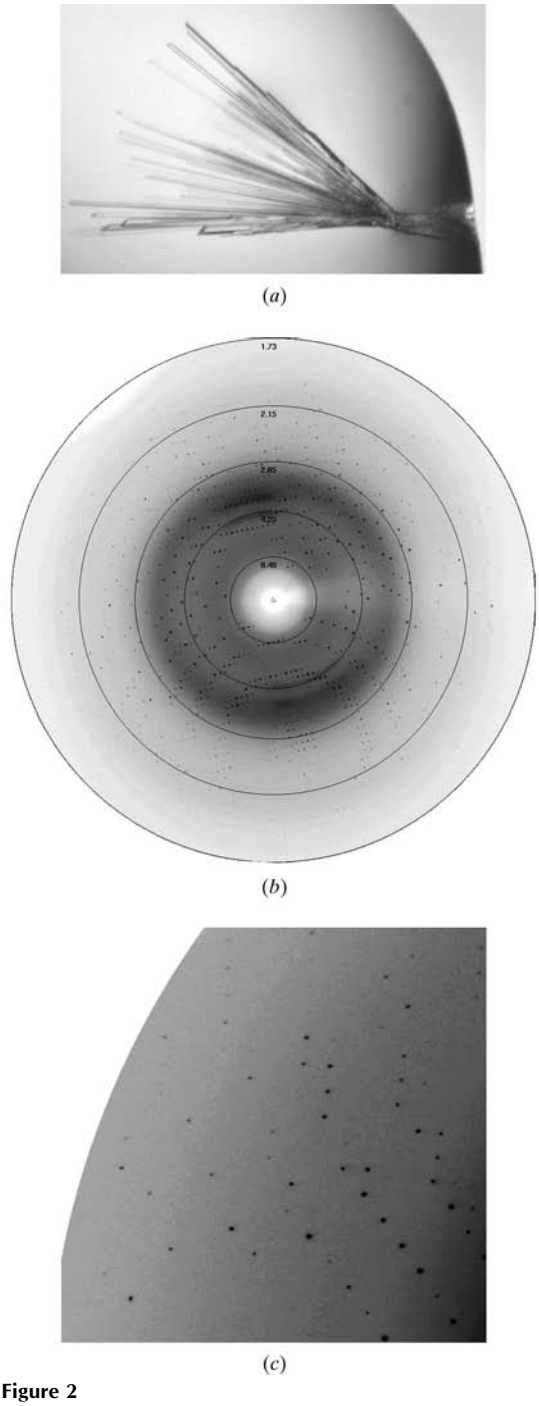

Figure 2
(*a*) Crystals of ActVA-Orf6 monooxygenase from a vapour-diffusion hanging-drop plate grown from 1.6 *M* ammonium sulfate, 100 m*M* HEPES pH 7.0. (*b*) A typical diffraction pattern of a crystal of ActVA-Orf6 monooxygenase. (*c*) Detail from the edge of frame of (*b*).

2. Materials and methods

2.1. Crystallization

Recombinant ActVA-Orf6 monooxygenase was expressed and purified from *Escherichia coli* following the methods previously described (Kendrew *et al.*, 1997) with minor modifications. Purified protein (approximately 98% pure as judged by Coomassie blue staining of an SDS–PAGE gel) was extensively dialysed against 10 m*M* Tris–HCl pH 8.0 and the concentration (8.2 mg ml^{-1}) determined spectroscopically using a calculated extinction coefficient (15 220 M^{-1} cm^{-1} at 280 nm). Initial crystallization trials were carried out using the hanging-drop vapour-diffusion technique (McPherson, 1990) with crystal screening reagents supplied by Hampton Research (Laguna Hills, California). Crystals were grown by mixing equal volumes (1 µl) of protein solution and reservoir solution on a silconized cover slip, placing it over a 0.7 ml reservoir and incubating at 293 K. In initial trials, diffraction-quality crystals grew in 1.6 *M* ammonium sulfate at pH 7, 8 and 9 (100 m*M* HEPES/Tris–HCl). Further trials showed crystal formation across a wide ammonium sulfate concentration range (1.2–1.7 *M*) in the pH range 7.0–9.0; crystals could also be grown from 1.5 *M* lithium sulfate but were of poorer quality. The crystals grew as bunches of small needles (Fig. 2*a*) over a period of approximately four weeks, the overall size and shape of these crystals being relatively independent of the crystallization conditions. The size of the crystals did not increase with time (four months), temperature (277 K), drop size (2–6 µl), protein dilution (4 mg ml^{-1}) or with the addition of commonly used additives (PEG 200, glycerol, alcohol, non-ionic detergents, salts). Microseeding techniques did result in better quality crystals. Optimal conditions (for size *versus* quality) for growth are 1.5 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.0 at 293 K, which results in crystals of average dimensions 0.2 × 0.02 × 0.02 mm. A single large crystal (0.5 × 0.1 × 0.1 mm) was obtained from 1.5 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.0, 9% PEG 200 at 293 K and this was used for native data collection at the synchrotron. Crystals were collected, washed with mother liquor and the N-

Table 1
Statistical data-collection parameters for ActVA-Orf6 monooxygenase crystals as a function of resolution.

Resolution (Å)	$I > 3\sigma$ (%)	χ^2†	$R_{\rm merge}$‡	Completeness (%)
15.00–2.95	98.7	0.683	0.023	87.3
2.95–2.35	98.1	1.290	0.038	97.0
2.35–2.05	97.4	1.876	0.060	98.5
2.05–1.86	93.3	2.318	0.094	98.6
1.86–1.73	88.5	2.467	0.146	91.7
Overall	95.3	1.703	0.037	94.5

† $\chi^2 = \sum_h \sum_i (I_{hi} - \langle I_{hi}\rangle)^2/[\sigma_h^2 N/(N-1)]$, where N is the number of observations. ‡ $R_{\rm merge} = \sum_h \sum_i |I_{hi} - \langle I_h\rangle|/\sum_h \sum_i I_{hi}$, where I_{hi} is the ith observation of the reflection h and $\langle I_h\rangle$ is the mean intensity of the hth reflection.

terminal sequence determined to confirm their identity.

2.2. Data collection

Data were collected using a Rigaku R-AXIS IIC imaging-plate detector mounted on a Rigaku RU-200 rotating-anode X-ray (Cu $K\alpha$) generator equipped with focusing mirrors and operating at 50 kV and 100 mA. For initial tests, we collected data at cryogenic temperatures (120 K) using 25% glycerol in the mother liquor as cryoprotectant. Crystals were picked up using a cryoloop (Hampton Research) and flash-frozen in a nitrogen stream at 120 K (Oxford Cryosystems). The crystals typically diffracted to between 2.5 and 3.0 Å on our home source. The best native data set was collected at 100 K at the ELETTRA synchrotron-radiation source (Trieste, Italy), using a fragment of the large crystal grown from 1.5 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.0, 9% PEG 200 using 20% PEG 200 as cryoprotectant. A total of 135° of data to a resolution of 1.73 Å were collected with an oscillation range of 1.5° per image on a MAR 345 imaging plate. Data were indexed with *DENZO* and scaled and reduced with *SCALEPACK* (Otwinowski & Minor, 1997). Subsequent calculations were performed using the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

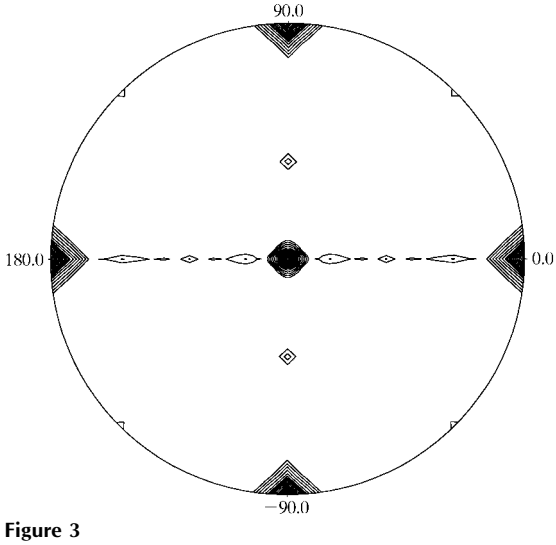

Figure 3
The $\kappa = 180°$ section of the self-rotation function calculated from the native diffraction data of ActVA-Orf6 monooxygenase. Integration radius is 30 Å using data in the resolution range 15–6 Å. The section is contoured at 0.5σ intervals from 5σ.

3. Results

ActVA-Orf6 monooxygenase crystals (Fig. 2) belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 46.95$, $b = 59.29$, $c = 71.67$ Å. Assuming the presence of a dimer in the asymmetric unit, as suggested by gel-filtration and ultracentrifugation evidence (Kendrew *et al.*, 1997), we obtained a value of 2.15 Å^3 Da^{-1} for the Matthews parameter (Matthews, 1968) and a solvent content of 44%. The unit-cell parameters determined at the synchrotron agree closely with those determined with the conventional rotating-anode source (with a maximum difference in cell dimension of 0.4%), indicating good isomorphism between different crystals. The final data are complete to 94.5%, with an overall $R_{\rm merge}$ of 3.7% and an average $\langle I/\sigma\rangle$ of 58.8. Data-collection statistics are summarized in Table 1. Figs. 2(*b*) and 2(*c*) show a diffraction image from the synchrotron source at ELETTRA.

As our initial calculations indicated the presence of a dimer in the asymmetric unit, we calculated the self-rotation function from the native data using *POLARRFN* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The $\kappa = 180°$ section of a map calculated with an integration radius of 30 Å using data in the resolution range 15–6 Å is presented in Fig. 3. There is a peak of height 5.6σ (28% of the origin peak) at polar angles $\omega = 45$, $\varphi = 90°$, corresponding to a non-crystallographic axis orientated at 45° to a plane defined by the two crystallographic axis, which should correspond to an axis relating two copies of the protein.

We are presently performing soaks of these crystals in heavy-atom solutions and mercurial reagents. Derivatives suitable for MIR and initial phase information have been obtained. A number of homologous sequences believed to catalyse similar reactions on the pathways towards their respective polyketides can be now identified in the databases using the *BLAST* and *PSI-BLAST* programs (Altschul *et al.*, 1990, 1997); these will aid our studies towards unravelling the mechanism of this protein and potentially developing rules governing polyketide recognition.

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