Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification and preliminary X-ray diffraction studies of RebC

Laura M. van Staalduinen,[a] Anupam Bhattacharya,[b] Katherine Groom,[b] David L. Zechel[b] and Zongchao Jia[a]*

[a]Department of Biochemistry, Queen's University, Kingston K7L 3N6, Canada, and [b]Department of Chemistry, Queen's University, Kingston K7L 3N6, Canada

Correspondence e-mail: jia@queensu.ca

Received 31 August 2007
Accepted 18 October 2007

The flavin-dependent monooxygenase RebC is a key enzyme in the biosynthesis of the indolocarbazole rebeccamycin. The synthesis of rebeccamycin is of great interest as it has been shown to be a natural antitumour agent. The enzyme has been recombinantly expressed in *Escherichia coli* and purified to homogeneity. Hanging-drop vapour diffusion in combination with microseeding was used to obtain suitable crystals for X-ray diffraction. Data were collected to 2.4 Å; the crystals belonged to space group $P2_1$, with unit-cell parameters a = 63.08, b = 77.85, c = 63.94 Å, $\alpha = \gamma = 90$, $\beta = 108.11^\circ$.

1. Introduction

The halogenated indolocarbazole rebeccamycin is a natural antibiotic/antitumour agent produced by *Lechevalieria aerocolonigenes* ATCC 39243 (formerly *Saccharothrix aerocolonigenes*). It acts as an antitumour agent through the stabilization of the DNA topoisomerase I cleavable complex, with an IC_{50} of 1.75 µ*M in vitro* (Bush *et al.*, 1987). A better understanding of how indolocarbazoles such as rebeccamycin are synthesized is of great interest for the production of new antitumour therapeutics.

The biosynthesis of rebeccamycin starts from the precursor L-tryptophan and involves seven enzymes that catalyze a series of halogenation, glycosylation and oxidation reactions (Onaka *et al.*, 2003). A pivotal step in the biosynthesis is the generation of the rebeccamycin aglycone through the oxidation of chlorinated chromopyrrolic acid by the enzymatic pair RebP and RebC (Sanchez *et al.*, 2005). It has been found that RebC dictates the oxidation state of the C7 site on the rebeccamycin aglycone and that the disruption of RebC results in the accumulation of multiple indolocarbazole products, indicating that RebC is required for the production of a single rebeccamycin aglycone (Howard-Jones & Walsh, 2006, 2007; Onaka *et al.*, 2003) RebC shows high sequence similarity to flavin-dependent monooxygenases and has been shown experimentally to bind flavin adenine dinucleotide (FAD; Howard-Jones & Walsh, 2006).

In order to better understand the mechanism of RebC, structural studies have been undertaken. Here, we present the expression, purification, crystallization and preliminary X-ray diffraction analysis of RebC.

2. Materials and methods

2.1. Cloning, expression and purification

L. aerocolonigenes was obtained from the ATCC (ATCC 39243) and grown overnight at 299 K in ISP Medium 2 (4 g l^{-1} yeast extract, 10 g l^{-1} malt extract, 4 g l^{-1} glucose). Genomic DNA was extracted from *L. aerocolonigenes* using a Qiagen DNeasy tissue kit. *Escherichia coli* XL-1 Blue and BL21(DE3) were obtained from Stratagene and Novagen, respectively. pET-28a was obtained from Novagen. Restriction enzymes were purchased from Fermentas. Nickel Sepharose and Sephacryl S-200 gel were obtained from GE Healthcare.

The *rebC* gene was amplified by polymerase chain reaction using the primers 5′-TATG**GCTAGC**ATGAACGCGCCCATCG-3′ and 5′-CCGC**AAGCTT**TCATTACGCGGCACCCCTCACC-3′ containing *Nhe*I and *Hin*dIII restriction-endonuclease sites (bold). The gene was ligated into the corresponding *Nhe*I–*Hin*dIII sites of pET-28a, which incorporates a hexahistadine tag at the N-terminus, and transformed into heat-shock competent XL1-Blue cells. Positive *rebC* clones were confirmed by DNA sequencing.

RebC was overexpressed in *E. coli* BL21(DE3) cells grown in Luria–Bertani medium supplemented with glucose (1%) and kanamycin (50 µg ml^{-1}). Cells were grown at 303 K to an OD_{600} of 0.6 and were then induced at 288 K with 1 m*M* isopropyl *β*-D-thiogalactoside (IPTG) and grown for an additional 24 h at 288 K.

Cells were harvested by centrifugation (20 min at 3000*g*) and resuspended in 50 m*M* Tris–HCl pH 7.2, 300 m*M* NaCl and 10 m*M* imidazole. Resuspended cells were lysed *via* two passes at 69–103 MPa through an Emulsiflex-C5 high-pressure homogenizer (Avestin) and the cell debris was removed by centrifugation (30 min at 40 000*g*). The supernatant was directly applied onto an Ni^{2+}-charged chelating column (Qiagen) previously equilibrated with lysis buffer (50 m*M* Tris–HCl pH 7.2, 300 m*M* NaCl and 10 m*M* imidazole). The protein was eluted with an increasing gradient of imidazole (0–500 m*M*). Fractions containing His-RebC were pooled for additional purification using a Sephacryl S200 size-exclusion column with 20 m*M* HEPES pH 7.5, 150 m*M* NaCl. Fractions from the size-exclusion column containing His-RebC were pooled. Approximately 10 mg pure RebC was obtained from 1 l culture. The purity of the protein was confirmed using SDS–PAGE stained with Coomassie Blue. The identity of the purified protein was confirmed using MALDI–TOF mass spectrometry. The predicted mass was 60 123.96 and the observed mass was 60 327.86, corresponding to a percentage error of 0.33%.

2.2. Crystallization

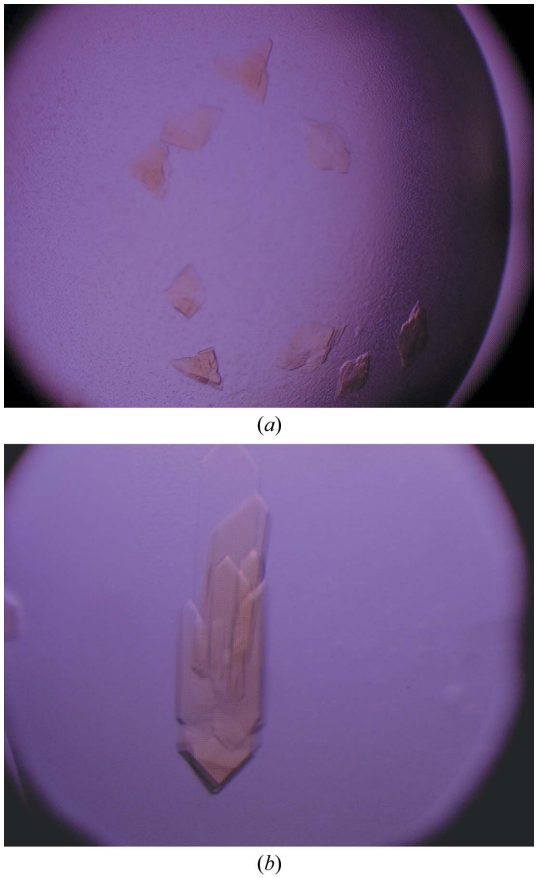
Purified RebC was concentrated using a Centricon (Amicon/Millipore; 10 kDa molecular-weight cutoff) at 977*g* to a final concentration of 10 mg ml^{-1}. Sitting-drop vapour-diffusion crystallization experiments were used to screen for initial crystallization conditions using the Classics Suite (Qiagen) at room temperature. The drops were set up using 1 µl protein solution mixed with an equal amount of reservoir solution and were equilibrated against 100 µl reservoir solution. The initial conditions were refined using the hanging-drop vapour-diffusion method, in which 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against a 1 ml reservoir volume. Crystals were further improved using standard microseeding techniques.

2.3. Data collection and diffraction measurements

X-ray data were collected from RebC crystals on the X3A beamline at Brookhaven National Laboratory National Synchrotron Light Source using a MAR 165 mm CCD detector. All data were collected under cryogenic conditions (100 K) using 28% ethylene glycol as a cryoprotectant. 1° oscillations with an exposure time of 30 s per image and a crystal-to-detector distance of 168 mm were used. The data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The recombinant RebC protein was purified to homogeneity using a two-step purification protocol. After size-exclusion chromatography,



(*a*)

(*b*)

Figure 1
(*a*) Initial crystals obtained from Classics Suite condition 64 (0.1 *M* HEPES pH 7.5, 10% PEG 8000) and (*b*) crystals obtained from the refined condition using a 1/100 dilution of microseed stock (0.1 *M* HEPES pH 7.3 and 10% PEG 8000).

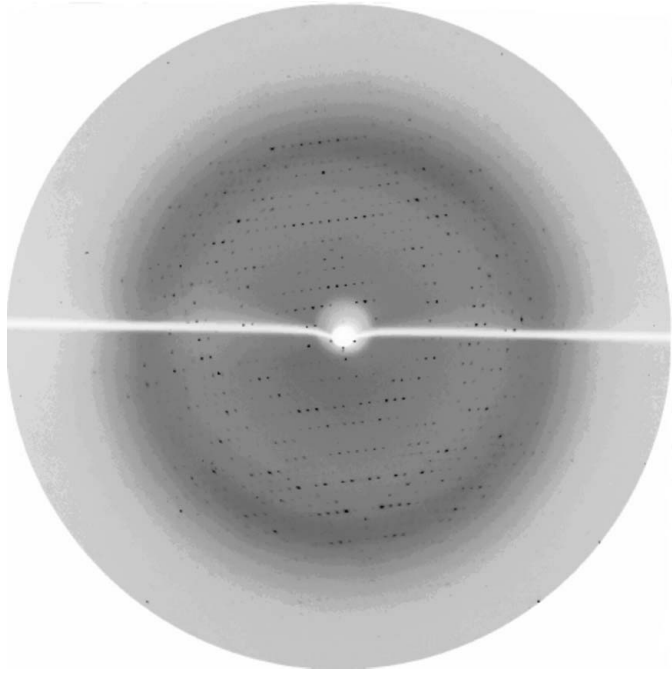


Figure 2
Diffraction of native RebC crystals. The edge of the detector is 2.18 Å; data were processed to 2.4 Å.

Table 1
Diffraction data and statistics for native RebC crystals.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 63.08, b = 77.85, c = 63.94, β = 108.11
Wavelength (Å)	0.97916
Temperature (K)	100
Resolution range (Å)	30.0–2.4 (2.49–2.40)
Observed reflections	84978
Unique reflections	22990
Data completeness (%)	99.6 (99.1)
Redundancy	3.7
R_{sym}† (%)	6.6 (37.6)
$\langle I/\sigma(I)\rangle$	18.6 (2.8)
Matthews coefficient (Å^3 Da^{-1})	2.59
Solvent content (%)	52.49

† $R_{sym} = \sum |I(k) - \langle I\rangle| / \sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values, respectively. The summation is over all measurements.

RebC was found to be approximately 95% pure as confirmed by SDS–PAGE. RebC initially crystallized in conditions 64, 67, 68, 95 and 96 of the Classics Suite (Qiagen). Optimization of all conditions resulted in tightly clustered layered crystals. The largest and least-clustered crystals, which were obtained from optimization of Classics Suite condition 64, were used for microseeding, which resulted in clustered plate crystals that were suitable for diffraction after manual separation (Fig. 1). The final crystallization condition was 0.1 *M* HEPES pH 7.3 and 10% polyethylene glycol (PEG) 8000 using a 1/100 dilution of the seed stock. The resulting crystals had approximate dimensions 0.2 × 0.1 × 0.05 mm.

The crystals belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 63.08, b = 77.85, c = 63.94 Å, β = 108.11°. Diffraction data for RebC were processed in the resolution range 30–2.4 Å (Fig. 2). A Matthews coefficient of 2.59 Å^3 Da^{-1} was obtained (Matthews, 1968) with a solvent content of 52.49%, representing the presence of a single monomer in the asymmetric unit. This is consistent with size-exclusion results, which indicate that the protein is monomeric in solution (data not shown). A summary of the crystal parameters and the statistics of diffraction data is presented in Table 1.

RebC shows moderate sequence homology (∼30% identity) to FAD-dependent monooxygenases, crystal structures of which have already been determined (3-hydroxybenzoate hydroxylase, PDB code 2dkh; phenol hydroxylase, PDB code 1pn0). Molecular-replacement searches were attempted using various full-length structures as well as using truncated models, but no solution has been obtained. Selenomethionine-labelled protein derivatives were therefore prepared. Currently, crystallization of the selenomethionine derivative is currently being optimized for MAD data collection and subsequent structure solution.

The authors would like to thank the organizers of RAPIDATA 2007 and Daniel Lee for data collection and initial processing. We would also like to thank Dr M. Adams for many helpful discussions. This research was funded by Queen's University, the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Institutes of Health Research (CIHR). ZJ is a Canada Research Chair in Structural Biology

References

Bush, J. A., Long, B. H., Catino, J. J., Bradner, W. T. & Tomita, K. (1987). *J. Antibiot. (Tokyo)*, **40**, 668–678.
Howard-Jones, A. R. & Walsh, C. T. (2006). *J. Am. Chem. Soc.* **128**, 12289–12298.
Howard-Jones, A. R. & Walsh, C. T. (2007). *J. Am. Chem. Soc.* **129**, 11016–11017.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Onaka, H., Taniguchi, S., Igarashi, Y. & Furumai, T. (2003). *Biosci. Biotechnol. Biochem.* **67**, 127–138.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Sanchez, C., Zhu, L., Brana, A. F., Salas, A. P., Rohr, J., Mendez, C. & Salas, J. A. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 461–466.