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Crystallization of the novel *S*-adenosyl-L-methionine-dependent C-methyltransferase CouO from *Streptomyces rishiriensis* and preliminary diffraction data analysis

Recombinant Q9F8T9 protein from *Streptomyces rishiriensis* (CouO), an *S*-adenosyl-L-methionine-dependent C-methyltransferase, has been successfully cloned, expressed and purified. CouO was crystallized from a single condition in the Morpheus crystallization screen. A vitrified crystal diffracted to 2.05 Å resolution and belonged to space group *P*2₁, with unit-cell parameters *a* = 33.02, *b* = 82.87, *c* = 76.77 Å, β = 96.93°.

1. Introduction

Methylation, which is a common transformation in all living cells, is catalysed by methyltransferases. *S*-Adenosyl-L-methionine (SAM or AdoMet) is the most important methyl donor for these cofactor-dependent enzymes (Cheng & Blumenthal, 1999). The substrates of these enzymes are diverse and include macromolecules such as DNA, RNA, proteins, lipids and polysaccharides as well as small molecules. The atoms targeted for methylation are nitrogen, oxygen, sulfur, selenium, carbon and halogens. Methyltransferases are highly selective toward the target atom. While all *O*-methyltransferases listed in the Brenda database (<http://www.brenda-enzymes.info/>) are SAM-dependent enzymes, a few *N*-methyltransferases have other methyl-donating cofactors (for example, betaine). The diversity of cofactors is highest for the known *S*-methyltransferases (for example, methyl-methionine, betaine and thetin). A small group of methyltransferases catalyse transfer of a methyl moiety to a C atom. Three cofactors have been described for C-methyltransferases: SAM, methylcob(III)alamin and methyltetrahydrofolate (Supplementary Fig. 1[†]).

Although overall sequence similarities among the SAM-dependent methyltransferases are not high, the enzymes exhibit a highly conserved structural fold (Martin & McMillan, 2003).

The catalytic mechanisms of some SAM-dependent methyltransferases have been elucidated based on structural biology investigations. X-ray structures of methyltransferases with or without the product of methyl transfer (*S*-adenosylhomocysteine) have been determined and used for mechanistic studies (Vidgren *et al.*, 1994; Cheng *et al.*, 1993). The mechanism of methylation using SAM as a methyl donor is known to be an S_N2 reaction (Ho *et al.*, 1991). Compared with O, N and S atoms, C atoms are not readily polarized. Some of the first mechanistic studies of C-methylation in RNA and DNA were performed by Kalman (1971). The proposed mechanism comprises the addition of the thiol moiety of a cysteine to the C-6 position of the aromatic ring in uridine in RNA or cytosine in DNA followed by methyl transfer to position C-5 and subsequent elimination of the thiol group (O'Gara *et al.*, 1996). Nothing is known about the reaction mechanism of C-methylation catalysed by SAM-dependent methyltransferases acting on small molecules.

The small-molecule C-methyltransferase CouO from *Streptomyces rishiriensis*, which is involved in the biosynthesis of the antibiotic coumermycin A1 (Pacholec *et al.*, 2005), was cloned and expressed in *Escherichia coli*. Biocatalytic characterization revealed the ability of this SAM-dependent enzyme to transfer other groups than methyl not only to the natural substrate but also to a diverse set of

non-natural substrates (Stecher *et al.*, 2009). In order to elucidate the structure–function relationship it is of great importance to solve the three-dimensional structure of this enzyme, which will provide mechanistic insights and pave the way for enzyme engineering. Here, we report the first results of crystallization of the SAM-dependent C-methyltransferase CouO.

2. Materials and methods

2.1. Protein cloning, expression and purification

Cloning, heterologous expression and purification of the methyltransferase CouO from *S. rishiriensis* (DSMZ 40489) was performed as described previously (Stecher *et al.*, 2009). In brief, PCR-amplified

couO was cloned into the expression vector pET26b(+). A Strep-tag II sequence was attached at the 3' end *via* site-directed mutagenesis (Supplementary Table 1; Braman *et al.*, 1996). The designated plasmid pET26b(+)-*couO*-Strep was transformed into electro-competent *E. coli* BL21 Gold (DE3) cells. In order to increase the amount of soluble Strep-tagged CouO protein, an auto-induction medium was used which consisted of LB Lennox medium supplemented with 1 mM MgSO₄, 0.5% glycerol, 0.05% glucose and 0.2% lactose. For protein expression, cells were grown for 20 h at 298 K. The cells were harvested by centrifugation (10 min at 6000g), resuspended in Strep-tag binding buffer W (100 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA) and lysed by sonication for 6 min. The resultant cell debris was removed by centrifugation (60 min at 50 000g) and the cleared lysate was immediately subjected to affinity purification using a Strep-Tactin Superflow High-Capacity column (IBA GmbH) following the supplier's protocol. Before crystallization, concentrated protein sample was applied onto a Superose 6 10/300 GL column (GE Healthcare) equilibrated with a multi-component buffer system (L-malic acid/MES/Tris pH 6.5; Newman, 2004) at 0.1× buffer concentration supplemented with 1.0 mM S-adenosyl homocysteine (Sigma–Aldrich). The sample was then concentrated to 10 mg ml^{−1} using 0.5 ml Amicon Ultra 10K centrifugal filters (Milipore) and used directly for crystallization.

2.2. Crystallization

The sitting-drop vapour-diffusion method was used for screening. Two drops with a 600 nl total volume (1:1 ratio of protein solution to reservoir solution) were dispensed using an Oryx8 protein crystallization robot (Douglas Instruments Ltd) at room temperature (293 K) into a SWISSCI three-well crystallization plate. The drops consisted of (i) concentrated CouO sample at 10 mg ml^{−1} or (ii) unconcentrated CouO sample at 2.3 mg ml^{−1} (the concentration of the combined peak fractions after gel filtration) and the Morpheus crystallization screen (Molecular Dimensions; Gorrec, 2009). After setup, the plate was stored at 290 K between inspections. Crystals appeared after two weeks of incubation only at the low protein concentration in condition B04: 12.5%(w/v) PEG 1000, 12.5%(w/v) PEG 3350, 12.5%(v/v) MPD, 0.03 M of each halide (sodium fluoride, sodium bromide and sodium iodide), 0.1 M MES/imidazole pH 6.5 (Fig. 1*a*).

Crystals were harvested from the original screening plate and vitrified without cryoprotection.

2.3. X-ray data collection

A native data set was collected at 100 K on the EMBL X12 beamline ($\lambda = 1.0507$ Å) at the DORIS storage ring, DESY, Hamburg using a MAR CCD 225 mm detector. 180 images (Fig. 1*b*) collected with 1.0° oscillation range were processed using *MOSFLM* (Leslie & Powell, 2007) and imported into the *CCP4* program suite. The initial space-group assignment (*P*₂₁) was verified with *POINTLESS* and the data were scaled with *SCALA* (Evans, 2006; Winn *et al.*, 2011). Data-processing statistics are presented in Table 1.

3. Results and discussion

Initial crystallization screening of the SAM-dependent C-methyltransferase CouO resulted in a well diffracting crystal. Interestingly, crystals only grew in drops set up using the low-concentration sample consisting of unconcentrated peak fractions from gel filtration. This might suggest that the concentration of purified CouO influences the homogeneity of the sample and interferes with crystallization at the

(a)

(b)

Figure 1
(a) CouO crystals from Morpheus screen condition B04, with an estimated crystal radius of between 0.1 and 0.2 mm. (b) X-ray diffraction image collected from a single CouO crystal on beamline X12 at the DORIS storage ring, DESY, Hamburg (the resolution at the detector edge is 2.05 Å).

Table 1Data processing and analysis (Winn *et al.*, 2011; Evans, 2006).

Values in parentheses are for the highest resolution shell. Data-quality and asymmetric unit analysis are as reported by *phenix.xtriage* from the *PHENIX* software suite (Adams *et al.*, 2010).

(a) Data processing.

Wavelength (Å)	1.0507
Focal spot (varies with energy) (mm)	2.0 × 0.4
Unit-cell parameters (Å, °)	$a = 33.02$, $b = 82.87$, $c = 76.77$, $\alpha = 90.00$, $\beta = 96.93$, $\gamma = 90.00$
Space group	$P2_1$
Resolution limits (Å)	41.44–2.05 (2.16–2.05)
R_{merge}	0.138 (0.532)
R_{meas} (all I^+ and I^-)	0.185 (0.733)
$R_{\text{p.i.m.}}$ (all I^+ and I^-)	0.097 (0.401)
Total No. of observations	87179 (10510)
No. of unique reflections	25206 (3498)
$\langle I/\sigma(I) \rangle$	6.0 (2.5)
Completeness (%)	97.9 (94.2)
Multiplicity	3.5 (3.0)
Wilson B factor (Å ²)	19.018

(b) Unit-cell analysis.

Molecules in asymmetric unit	Matthews coefficient (Å ³ Da ⁻¹)	Solvent content (%)
1	4.03	69.5
2†	2.01	39.0
3	1.34	8.5

(c) Ice-ring analysis. Ice rings in the data were detected by analyzing the completeness and the mean intensity. High Z scores and completeness in ice-ring areas might be a reason to reassess data processing if ice rings are present.

Resolution	Z score/completeness (%)	Relative ice intensity
3.897	0.15/1.00	1.000
3.669	2.09/0.99	0.750
3.441	0.28/1.00	0.530
2.671	1.59/1.00	0.170
2.249	3.55/0.96	0.390
2.072	3.12/0.89	0.300

† Most probable asymmetric unit composition.

higher protein concentrations. A native data set was collected to a maximum resolution of 2.05 Å at the DORIS storage ring, DESY, Hamburg and successfully processed. Analysis of the data quality and asymmetric unit content was performed with *phenix.xtriage* from the *PHENIX* software suite (Adams *et al.*, 2010). Gel-filtration chromatography of purified CouO indicated a dimeric state of the protein (data not shown), and two copies of CouO in the asymmetric unit were suggested as the most probable asymmetric unit composition. Only minor ice-ring effects were detected within the data set as reported by *phenix.xtriage*. Patterson analysis as well as twinning analysis revealed no significant problems.

The resulting processed data were used in initial structure-solution attempts. Unfortunately, neither manual molecular replacement with the programs *Phaser* (McCoy *et al.*, 2007) or *MOLREP* (Vagin & Teplyakov, 2010) using multiple and diverse search templates nor automated molecular-replacement pipelines produced satisfactory results. We believe that the main reason for the initial failure is the low sequence identity of the available templates as reported by the *SWISS-MODEL* template-identification tool [top gapped *BLAST* search result 36/113, 31% amino-acid identity (PDB entry 1ve3; Schäffer *et al.*, 2001); top profile *BLAST* search result 34/135, 25% amino-acid identity (PDB entry 1wzn; RIKEN Structural Genomics/Proteomics Initiative, unpublished work); Altschul *et al.*, 1997; Arnold *et al.*, 2006; Supplementary Fig. 2] as well as the relative

flexibility of methyltransferase domains (Tsao *et al.*, 2011; Akey *et al.*, 2011). In order to overcome the weak template problem, attempts to solve the structure with the recently described *Rosetta* molecular-replacement protocol (DiMaio *et al.*, 2011) are ongoing. We will also attempt to express and crystallize a selenomethionine derivative of CouO. The protein sequence contains a total of six methionines, corresponding to approximately 2.6 methionines per 100 amino acids. Thus, SeMet SAD/MAD appears to be the most promising strategy for solution of the phase problem in this case.

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