Purification, crystallization and preliminary crystallographic analysis of DR0248, an MNT–HEPN fused protein from *Deinococcus radiodurans*

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DR0248 is a protein identified in the *Deinococcus radiodurans* (DR) genome that is predicted to encompass two domains: an N-terminal minimal nucleotidyl transferase domain (MNT) and a C-terminal higher eukaryotes and prokaryotes nucleotide-binding domain (HEPN). These two domains, usually encoded in two ORFs, have been suggested to play the role of a toxin–antitoxin (TA) system in prokaryotes. Recombinant DR0248 was overexpressed and purified from *Escherichia coli* and diffraction-quality crystals were obtained in the presence of the detergent molecules dodecyltrimethylamine oxide (DDAO) and octaethylene glycol monododecyl ether (C12E8), which were used as crystallization additives. Crystals grown with DDAO diffracted to a resolution of 2.24 Å and belonged to space group $C22_1$, with unit-cell parameters $a = 98.4$, $b = 129.9$, $c = 59.2$ Å. Crystals grown with C12E8 diffracted to a resolution of 1.83 Å and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 51.6$, $b = 87.2$, $c = 108.2$ Å. The structure was solved by multiwavelength anomalous dispersion from zinc bound to the protein using a single crystal obtained in the presence of DDAO.

1. Introduction

Minimal nucleotidyl transferase domains (MNTs) have been identified as the minimal units of DNA polymerase β -like domains that are responsible for transferring nucleic acids to an acceptor hydroxyl group. MNTs can be involved in various molecular processes, including DNA replication and repair, transcription and RNA processing (Aravind & Koonin, 1999). MNTs are often associated with higher eukaryotes and prokaryotes nucleotide-binding domains (HEPNs) both in archaea and bacteria. HEPNs can possess an RNase motif that is typically lost, entirely or partly, when the HEPN is fused to an MNT (Anantharaman *et al.*, 2013).

MNT–HEPN systems have been proposed to represent a novel, nonconventional, type II toxin–antitoxin (TA) system (Makarova *et al.*, 2009). TA systems are found in plasmids and chromosomes and play a role in stress physiology and genetic element stabilization. Under normal conditions the toxin and antitoxin form a tight non-toxic complex, but under stress conditions the antitoxin is rapidly degraded by cellular proteases, releasing the toxin, which can then act on its cellular target and lead to growth arrest or cell death (Gerdes *et al.*, 2005). The molecular mechanisms and cellular targets of MNT–HEPN remain unknown; it has been proposed, however, that MNT might tag RNA for degradation by nucleotidylation, while HEPN could inhibit MNT (Makarova *et al.*, 2009; Grynberg *et al.*, 2003).

Deinococcus radiodurans, a Gram-positive bacterium that is highly resistant to environmental stresses such as oxidative stress or ionizing radiation (Cox & Battista, 2005), possesses three nonconventional type II TA systems belonging to the MNT–HEPN family. Two of these consist of two-component systems, while the third gene product fuses both toxin and antitoxin domains in a single protein, DR0248. Here, we report the purification, crystallization and preliminary X-ray analysis of DR0248.

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2. Materials and methods

2.1. Recombinant protein expression

The *dr0248* gene was amplified by PCR using the primers 5'-CACCATGTCCGCACCCCTTTTCC-3' (forward) and 5'-CTACGGACGGGGGCTGG-3' (reverse); the forward primer contains a 5'-CACC overhang for directional TOPO cloning. The PCR product was inserted into pET151/D-TOPO expression vector (Invitrogen) that contains an N-terminal His₆ tag and a recombinant *Tobacco etch virus* (TEV) protease-cleavage site. Transformation of competent *Escherichia coli* BL21(DE3) cells (Invitrogen) with the resulting expression vector was achieved by heat shock at 315 K. Transformed *E. coli* cells were grown at 310 K to an OD_{600 nm} of approximately 0.6 in Luria-Bertani growth medium supplemented with 0.1 g l⁻¹ ampicillin and induced at 310 K for 3 h by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside. The cells were harvested by centrifugation at 6000g for 20 min at 277 K and resuspended in lysis buffer consisting of 100 mM MES pH 6.5, 1 M NaCl, 20 mM imidazole supplemented with cOmplete mini EDTA-free protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 500 units of Benzonase nuclease (Sigma-Aldrich).

2.2. Protein purification

Cells were lysed with a Constant Systems cell disruptor at 150 MPa. After centrifugation at 20 000g for 20 min at 277 K, the supernatant was loaded onto a 5 ml HisTrap HP column (GE Healthcare) for initial purification. The protein was eluted with 100 mM MES pH 6.5, 1 M NaCl, 500 mM imidazole. DR0248 was identified by 12% SDS-PAGE. Fractions containing DR0248 were buffer-exchanged against 100 mM MES pH 6.5, 150 mM NaCl using a PD-10 desalting column (GE Healthcare). The His₆ tag was then cleaved with TEV protease [1:20(w:w) TEV:His₆-DR0248] by incubation overnight at 277 K. The cleaved protein was loaded onto a Superdex S200 16/600 column (GE Healthcare) pre-equilibrated with 100 mM MES pH 6.5, 150 mM NaCl. Fractions containing DR0248 were pooled and concentrated to 15 mg ml⁻¹ using an Amicon Ultra 10 kDa molecular-weight cutoff concentrator (Millipore).

2.3. Dynamic light scattering (DLS)

DR0248 samples were prepared in 100 mM MES pH 6.5, 150 mM NaCl at a concentration of 16 mg ml⁻¹. Samples were centrifuged

at 13 000g for 1 min at 277 K. Subsequently, 50 µl protein solution was dispensed into an Ultra-Micro cuvette (105.251-QS, Hellma). Measurements were performed at 293 K on a Zetasizer instrument (Malvern).

2.4. Thermal shift assay (TSA)

A fluorescence-based thermal shift assay was used to identify additives that could enhance protein stability. For this screening experiment, each of the 39 compounds at 1 mM was dispensed into wells containing 2 µl purified protein solution (1.4 mg ml⁻¹), 1 µl SYPRO Orange (5×), 150 mM NaCl, 100 mM MES pH 6.5 and water to 20 µl. The temperature was increased at a rate of 1 K min⁻¹ from 298 to 348 K using a Mx3005P Q-PCR system (Stratagene). Fluorescence from each well was recorded after each temperature increment.

2.5. Crystallization assays

DR0248 at a concentration of 10 mg ml⁻¹ was used for initial crystallization screening at 293 K in Greiner CrystalQuick sitting-drop vapour-diffusion plates. A Cartesian PixSys 4200 crystallization robot (Genomic Solutions, UK) facility [High Throughput Crystallization Laboratory (HTX lab) at EMBL Grenoble] was used to dispense 200 nl drops consisting of three different protein:precipitant ratios: 50:150, 100:100 and 150:50 nl. The following commercial screens were set up: Crystal Screen Lite, PEG/Ion, MembFac, Natrix, Quick Screen, Grid Screens (Ammonium Sulfate, Sodium Malonate, Sodium Formate, PEG 6K, PEG/LiCl and MPD) and Index (from Hampton Research), The Classics Suite (Qiagen/NeXtal; conditions equivalent to Crystal Screen and Crystal Screen 2 from Hampton Research) and the customized Screen Mme 5000 from HTX lab.

Manual crystallization screens were set up in 24-well sitting-drop plates (Hampton Research) using the commercial screens PEG/Ion and MDI-14 (Molecular Dimensions) with DR0248 at a concentration of 15 mg ml⁻¹. Two protein:precipitant ratios, 1:1 and 2:1, were tested in drops with volumes of 2 and 3 µl, respectively. Crystals appeared within a week in condition No. 38 of the PEG/Ion screen [0.2 M ammonium tartrate dibasic, 20%(w/v) PEG 3350] when the protein was pre-incubated with 5 mM zinc chloride.

In order to improve crystal quality, the zinc chloride concentration was reduced to 1 mM and crystallization trials were performed with

Figure 1

(a) Size-exclusion chromatography profile of DR0248. Absorbance at 280 nm is shown in blue. (b) Dynamic light-scattering analysis of DR0248, showing mass distribution as a function of the radius of gyration. (c) SDS-PAGE after size-exclusion chromatography of fractions collected at the beginning of elution, with the most intense band (marked with an asterisk) corresponding to the highest absorbing fraction.

Additive Screen and Detergent Screen 1 (Hampton Research). Large single crystals were obtained from drops comprised of 2 μ l protein solution mixed with 1 μ l precipitant solution consisting of 0.1 M ammonium tartrate, 14% (w/v) PEG 3350 and either 0.2% (v/v) DDAO or 0.24 mM C12E8.

2.6. Diffraction and data collection

2.6.1. Multiwavelength anomalous dispersion. X-ray anomalous diffraction experiments were performed on the ID29 Structural Biology beamline at ESRF (de Sanctis *et al.*, 2012) on a crystal grown using 0.1 M ammonium tartrate, 14% (w/v) PEG 3350, 0.2% (v/v) DDAO. The crystal was cryoprotected in the same solution as the mother liquor but containing 20% (w/v) PEG 3350. Two complete data sets were collected from different positions of the same crystal at the Zn *K*-edge absorption peak (9.668 keV) and inflection point (9.665 keV). A third data set was collected at high energy (12.7 keV) to achieve higher resolution. Data were collected to a maximum resolution of 2.24 Å using a PILATUS 6M-F detector on the ID29 beamline at ESRF.

Data were integrated using *XDS* (Kabsch, 2010b) and then scaled with *XSCALE* (Kabsch, 2010a). The *SHELX* package (Sheldrick, 2010) was used to estimate the amount of anomalous signal, find the positions of Zn atoms and calculate initial phases. *ARP/wARP* (Langer *et al.*, 2008) was used to build an initial model with the docked sequence. Manual model building and iteratively refinement

are currently under way using *Coot* (Emsley *et al.*, 2010) and *PHENIX* (Adams *et al.*, 2010), respectively.

2.6.2. Molecular replacement. The initial model obtained by MAD was then used as a model to solve the structure of DR0248 from a crystal grown using 0.1 M ammonium tartrate, 14% (w/v) PEG 3350, 0.24 mM C12E8 by molecular replacement (Rossmann, 1990). X-ray diffraction data were collected on the ID29 Structural Biology beamline at ESRF. Mother liquor containing 30% (w/v) PEG 3350 was used as a cryoprotectant.

Data were integrated using *XDS* (Kabsch, 2010). Molecular replacement was performed with *Phaser* (McCoy *et al.*, 2007). Structure refinement is currently under way with *PHENIX* (Adams *et al.*, 2010) and manual model building with *Coot* (Emsley *et al.*, 2010).

3. Results and discussion

Native DR0248 consists of 238 residues with a theoretical molecular weight of 26.6 kDa. It was successfully expressed in *E. coli* strain BL21(DE3) and was purified to homogeneity as assessed by size-exclusion chromatography (SEC), dynamic light scattering (DLS) and SDS-PAGE (Fig. 1). During SEC DR0248 eluted at 80.2 ml, corresponding to a molecular weight of approximately 50 kDa, suggesting that DR0248 forms dimers. The oligomeric state of the protein was evaluated by DLS and the distribution of the hydration radius shows a sharp peak either by mass or intensity analysis at 3.76 ± 0.93 nm, with an estimated protein mass of 74.9 ± 19.5 kDa consistent with that of a dimer.

Since no convincing hit was obtained in the initial crystallization trials, we selected 39 additives, including multivalent ions, salts and nucleotides, for a thermal shift assay to identify those that could stabilize DR0248 and promote crystallization. The most significant results were obtained for transition metals such as cobalt(II), zinc, cadmium, manganese(II) and nickel(II). These metals induce a T_m shift of at least 3 K, where the T_m corresponds to half of the protein in solution being unfolded. These metals were used to set up crystallization trials with DR0248, and better shaped crystals were obtained when the protein was pre-incubated with 1 mM zinc chloride. Measurement of the X-ray fluorescence spectrum confirmed the presence of zinc in the crystal obtained (Fig. 2). After pre-incubation with 1 mM zinc chloride, DR0248 crystals were obtained in two conditions: with DDAO and with C12E8.

Three different data sets were collected at three non-overlapping positions of a crystal obtained with DDAO (Fig. 3a). The peak and

Figure 2
X-ray fluorescence of DR0248 crystals grown in the presence of zinc. Data were measured on beamline ID29 at ESRF.

Figure 3
Single crystals of DR0248 obtained from crystallization conditions consisting of (a) 0.1 M ammonium tartrate, 15% (w/v) PEG 3350, 0.2% (v/v) DDAO, 1 mM zinc chloride and (b) 0.1 M ammonium tartrate, 14% (w/v) PEG 3350, 0.24 mM C12E8, 1 mM zinc chloride.

inflection-point data were combined in a two-wavelength MAD experiment and a third data set was collected at 12.7 keV in order to achieve higher resolution. Peak and inflection-point energies were estimated from an X-ray energy absorption scan near the absorption edge. The crystal diffracted to a maximum resolution of 2.24 Å for the native data set and belonged to space group $C222_1$, with unit-cell parameters $a = 98.4$, $b = 129.9$, $c = 59.2$ Å (Fig. 4*a*). The peak data set showed lower diffraction quality than the inflection-point data set, as indicated by poorer scaling statistics (higher $R_{\text{p.i.m.}}$ and R_{merge} ;

Table 1), which resulted in a lower anomalous signal. Because we could not detect any evident sign of radiation damage (such as an increase in crystal mosaicity, B factor or unit-cell dimensions) or any significant improvement in data statistics by including fewer frames, we concluded that the crystal did not present the same quality at all positions. Analysis of the anomalous signal showed that CC_{anom} fell below 30% at 3.4 Å resolution, and data to 3.5 Å resolution were used to locate the zinc substructure. *SHELXD* located the positions of two

Table 1
Diffraction data and statistics for DR0248–DDAO and DR0248–C12E8 crystals.

	DR0248–DDAO			DR0248–C12E8
	Peak	Inflection point	High energy	
Energy (keV)	9.668	9.665	12.700	12.700
Beamline	ID29, ESRF			
Detector	PILATUS 6M-F			
Space group	$C222_1$			$P2_12_12_1$
Unit-cell parameters (Å)				
a	98.2	98.4	98.4	51.6
b	129.6	129.9	129.9	87.2
c	59.2	59.2	59.2	108.2
Solvent content (%)	65.3	65.4	65.4	46.2
Matthews coefficient (Å ³ Da ^{−1})	3.54	3.56	3.56	2.29
Resolution (Å)	49.2–2.47 (2.58–2.47)	47.2–2.48 (2.59–2.48)	47.2–2.24 (2.32–2.24)	100.0–1.83 (1.88–1.83)
Total No. of observations	176244	177040	111209	197939
No. of unique observations	13828	13694	18588	43613
Completeness (%)	99.6	99.6	99.3	99.5
Multiplicity	12.7	12.9	6.0	4.5
Mean $I/\sigma(I)$	18.1 (2.6)	30.3 (4.6)	14.1 (2.0)	18.8 (1.7)
$R_{\text{merge}}^{\dagger}$	0.095 (0.931)	0.052 (0.480)	0.056 (0.712)	0.038 (0.758)
$R_{\text{p.i.m.}}^{\ddagger}$	0.039 (0.395)	0.021 (0.196)	0.037 (0.466)	0.029 (0.571)
$CC_{\text{anom}}(1/2)^{\S}$ (40–3.7 Å) (%)	64.2	69.2		
$CC_{\text{anom}}(\text{pk/ip})^{\P}$	80.0			

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. [‡] $R_{\text{p.i.m.}} = \sum_{hkl} \{ [1/N(hkl) - 1]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl) \}$. [§] $CC_{\text{anom}}(1/2)$ is the anomalous correlation coefficient within the data set. [¶] $CC_{\text{anom}}(\text{pk/ip})$ is the anomalous correlation coefficient across the peak and inflection-point data sets.

Figure 4
DR0248 crystal diffraction patterns: (a) crystal grown in 0.1 M ammonium tartrate, 15% (w/v) PEG 3350, 0.2% (v/v) DDAO, (b) crystal grown in 0.1 M ammonium tartrate, 14% (w/v) PEG 3350, 0.24 mM C12E8. 1 mM zinc chloride was added prior to setting up the drops. The protein:precipitant ratio is 2:1. Data were measured on ID29 at ESRF.

sites in the asymmetric unit, with CC_{all} and CC_{weak} of 39.10 and 24.70, respectively. Initial phases were calculated with *SHELXE* using the higher resolution data set, and autotracing succeeded in placing a 162-residue polyalanine model from a total of 238 residues.

Eventually, a new crystal form was obtained using 100 mM ammonium tartrate, 14% (w/v) PEG 3350, 0.24 mM C12E8 (Fig. 3*b*). Crystals obtained in this condition diffracted to 1.83 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 51.6$, $b = 87.2$, $c = 108.2$ Å (Fig. 4*b*). Molecular replacement was performed using the DR0248 structure solved by a MAD experiment as a search model. Model refinement and completion of the two structures is under way.

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