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Crystallization of the DdrB–DNA complex from *Deinococcus radiodurans*

The remarkable ability of members of the *Deinococcus* family to recover from extreme DNA damage is in part owing to their robust DNA-repair mechanisms. Of particular interest is their ability to repair hundreds of double-strand DNA breakages through a rapid and efficient mechanism involving novel proteins that are uniquely found in *Deinococcus* spp. One such protein, DdrB, which is thought to play a role early in DSB repair, has been crystallized in complex with ssDNA and data have been collected to 2.3 Å resolution.

1. Introduction

Deinococcus radiodurans is able to withstand and recover from extreme doses of ionizing radiation which shatter its chromosome into hundreds of fragments (Zahradka *et al.*, 2006). DdrB is a protein unique to the *Deinococcus* family that is thought to be implicated in recovery following extensive damage (Tanaka *et al.*, 2004). Early characterization of the protein revealed its single-stranded DNA (ssDNA) binding activity (Norais *et al.*, 2009; Sugiman-Marangos & Junop, 2010) and, as it is highly upregulated following both irradiation and desiccation (Tanaka *et al.*, 2004), it was thought that it played a role as a DNA-damage-induced functional equivalent to canonical single-stranded binding protein (SSB; Norais *et al.*, 2009). Structural characterization subsequently revealed that DdrB in fact possesses a novel fold that is distinct from the structurally conserved OB-fold present in SSB (Sugiman-Marangos & Junop, 2010). In addition to binding ssDNA, DdrB has recently been shown to promote the annealing of complementary strands of DNA *in vitro* (Xu *et al.*, 2010). Here, we report the crystallization and data collection of a DdrB–ssDNA cocrystal complex, the structure solution from which will provide valuable information with regard to the mechanism through which DdrB is able to carry out its biological functions. Cocrystallization of protein–DNA complexes adds an additional dimension to the already arduous task of protein crystallography, as characterization of crystals containing protein but not DNA typically only takes place after diffraction-quality crystals have been optimized and X-ray data have been collected. Optimization of the pH of the protein buffer prior to screening and the use of a fluorescently labelled DNA substrate significantly reduced the number of false positives encountered in the search for a condition that maintains protein–DNA interactions during crystallography.

2. Materials and methods

2.1. Cloning and protein expression

A C-terminal truncation of DdrB from *D. radiodurans* was generated by PCR amplification of the gene DR0070 incorporating a stop codon following residue 144. This PCR product was incorporated into the expression vector pET151-D-TOPO as per the manufacturer's protocol (Invitrogen) and expressed as an N-terminal 6×His fusion in *Escherichia coli* BL21(DE3). Purification was carried out as described previously (Sugiman-Marangos & Junop, 2010).

Briefly, cell pellets were lysed by sonication in lysis buffer (20 mM Tris pH 8.0, 1 M sodium chloride, 5 mM imidazole) and clarified by centrifugation at 48 000g. Soluble lysate was purified by Ni-IMAC FPLC, exchanged into a low-salt buffer (20 mM Tris pH 8.0, 150 mM potassium chloride) and treated with TEV protease to cleave the N-terminal 6×His tag. The cleaved 6×His tag and TEV protease were separated from DdrB_{1–144} by a second pass over the Ni-IMAC column and the protein was exchanged into the final crystallization buffer (20 mM Tris pH 6.0, 100 mM potassium chloride).

2.2. Electrophoretic mobility-shift assay

Electrophoretic mobility-shift assays (EMSAs) were performed in EMSA buffer [20 mM Tris pH 8.0, 150 mM sodium chloride, 10% (v/v) glycerol] by mixing 100 µM unlabelled and Cy5-labelled 50 b ssDNA for 15 min with increasing amounts of DdrB_{1–144} (0, 0.1, 1, 10, 20 and 50 µM) in a 15 µl reaction volume. Both unlabelled and Cy5-labelled ssDNA substrates were obtained from Integrated DNA Technologies (Iowa, USA). The reactions were resolved by electrophoresis on a 10% (w/v) polyacrylamide native Tris/borate/EDTA (TBE) gel running at 80 V for 1 h.

2.3. Crystallization

Initial crystallization screening was carried out over a wide range of protein/DNA concentrations and ratios using ssDNA substrates of various sequences and lengths. Approximately 2000 crystallization experiments were set up by hand in 24-well screw-cap plates (Qiagen) and were primarily performed with kits specifically formulated for the crystallization of protein–nucleic acid complexes (Nucleix from Qiagen and Protein–Nucleic Acid Complex Crystal Screen from KeraFAST), although additional screens were also tested (The Classics Suite and JCSG Core Suites I, II, III and IV from Qiagen). Crystal hits were screened by both X-ray diffraction and visually by monitoring the incorporation of a fluorescent 5'-labelled Cy5 50 b poly-dT ssDNA substrate.

Crystals of DdrB_{1–144} in complex with ssDNA were grown at 293 K using the hanging-drop vapour-diffusion method. Initial crystals were observed approximately 48 h after the drops were set up and continued to grow over the next 2–3 d. DdrB and ssDNA were premixed [1.34 µl DdrB (830 µM) in protein buffer (20 mM Tris pH 6.0, 100 mM potassium chloride) and 0.12 µl 50 b poly-dT (2000 µM)] and were allowed to incubate at 298 K for 10 min before being mixed with 1.5 µl crystallization buffer [50 mM MES pH 5.6, 300 mM potassium

chloride, 10 mM magnesium chloride, 5% (v/v) PEG 8000] and dehydrated over 250 µl 1.35 M ammonium sulfate. No additional cryoprotectant was necessary to prevent ice-crystal formation during data collection.

2.4. Data collection

Crystals of DdrB_{1–144} and ssDNA were flash-cooled to 100 K in a nitrogen stream and diffraction data were collected at a wavelength of 1.1 Å on the X29A beamline of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. A total of 360 images were collected using an ADSC Quantum 315 detector at a distance of 300.0 mm for 1 s exposures with a 0.5° oscillation width.

3. Results and discussion

Crystallization of the apoprotein was performed using full-length DdrB (Sugiman-Marangos & Junop, 2010); however, the main chain of the protein could only be traced for amino-acid residues 1–144. This was consistent with previous reports that the C-terminus of DdrB is likely to be disordered (Norais *et al.*, 2009), in addition to being lacking in functional importance during damage recovery (Bouthier de la Tour *et al.*, 2011). A C-terminal truncation mutant was therefore constructed for cocrystallization with DNA to improve crystal packing through elimination of a large disordered segment.

A number of initial attempts to crystallize DdrB_{1–144} in complex with ssDNA produced diffraction-quality crystals; however, these structures failed to capture the protein–nucleic acid interaction, as determined upon analysis of the resulting electron-density maps. To differentiate between protein crystals with and without DNA bound, a technique described by Georgescu and coworkers was implemented (Georgescu *et al.*, 2008). Crystal hits shown to be protein by X-ray diffraction were screened for incorporation of ssDNA by crystallization with a Cy5-labelled DNA substrate, appearing blue upon visual inspection with a light microscope. Direct interactions between the Cy5 label and DdrB were considered to be unlikely, as EMSAs performed with labelled and unlabelled substrates demonstrated no significant differences (Fig. 1). In order to increase efficiency, broad screening could have been performed using the labelled substrate; additionally, a second DNA substrate using a different fluorophore could have been implemented to rule out direct interactions between the label and the protein, as described previously (Georgescu *et al.*, 2008).

Figure 1

Electrophoretic mobility-shift assays of 50 b ssDNA substrates incubated with increasing concentrations of DdrB_{1–144}. 100 µM unlabelled (a) and Cy5-labelled (b) ssDNA were incubated with 0, 0.1, 1, 10, 20 and 50 µM DdrB_{1–144} and resolved by 10% polyacrylamide native TBE gel electrophoresis. No major differences were observed as a result of the incorporation of a 5' Cy5 label.

Early crystallization trials involved both short (8–10 b) and long (48–52 b) DNA substrates based on estimates reported in the literature of the number of bases required to fully occupy a DdrB pentamer. As DdrB has not been reported to display sequence specificity in its interaction with ssDNA, we decided to perform our trials with a repetitive sequence of DNA consisting of a unique base. This was performed to facilitate the eventual modelling of the DNA in the electron density, as there would be no ambiguity in identifying the identity of the bases. Poly-dT was ultimately selected as it does not have a propensity for self-association (as is the case with dG) and it has been used successfully in the crystallization of other proteins that bind ssDNA (Chen *et al.*, 2008; Chan *et al.*, 2009).

The isoelectric point of DdrB_{1–144} calculated by the ExPASy ProtParam tool (Artimo *et al.*, 2012) is 7.17. Preliminary crystallization trials were carried out in protein buffer at a pH of 8.0. This was not originally a concern as EMSAs had been performed at this pH and the overall negative charge of the protein did not appear to have an effect on DNA binding (Sugiman-Marangos & Junop, 2010). In an attempt to increase the strength of the protein–DNA interaction and potentially decrease charge repulsion and therefore the overall solubility of the complex, a final buffer-exchange step into 10 mM Tris pH 6.0 was appended to the original purification protocol. As pH 6.0 is outside the range in which Tris is most effective as a buffer, a more appropriate buffer such as MES could have been used. Re-screening at a lower pH over 500 μ l 1.5 M ammonium sulfate produced a hit from Nucleix screen condition No. 5 [200 mM potassium chloride, 10 mM magnesium chloride, 50 mM MES pH 5.6, 5% (w/v) PEG 8000], yielding small crystals that were confirmed to be protein by X-ray diffraction (Fig. 2*a*). Before proceeding with optimization, identical drops were set up with Cy5-labelled ssDNA, which produced blue crystals, confirming the incorporation of the DNA into the crystal (Fig. 2*b*).

Diffraction-quality crystals were optimized by systematic screening of the various components of the crystallization solution and the concentration of ammonium sulfate in the well solution (Fig. 2*c*); the use of ssDNA substrates 1–2 bases shorter or longer than 50 b did not appear to have any measurable effect on the quality or extent of diffraction observed. Modulation of the concentration of potassium chloride in the crystallization solution and the concentration of ammonium sulfate in the well solution appeared to have the greatest

Table 1

Data collection.

Values in parentheses are for the highest resolution shell.

Space group	<i>P</i> 3 ₂
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 110.7, <i>c</i> = 58.8
Matthews coefficient (Å ³ Da ^{−1})	2.61
Molecules in asymmetric unit	6
Resolution range (Å)	50.0–2.30 (2.34–2.30)
Observed reflections	203021
Unique reflections	35736 (1844)
Multiplicity	5.7 (5.6)
Completeness (%)	99.9 (100.0)
$\langle I/\sigma(I) \rangle$	17.4 (2.3)
<i>R</i> _{merge} [†] (%)	6.8 (72.6)
Wilson <i>B</i> factor (Å ²)	58.3

[†] *R*_{merge} is defined as $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

effect on the size and quality of the crystals grown. While dehydration over 1.5 M ammonium sulfate consistently produced showers of small crystals, dehydration over 1.30–1.35 M ammonium sulfate significantly reduced the number of nucleation events and allowed the growth of larger crystals. Most drops contained a mixture of two morphologies of triangular prisms differentiated by the relative rate of growth of the crystal faces. The more common morphology was the one in which the length of the prism grew much more rapidly, producing long but relatively two-dimensional crystals; the less common morphology produced a more three-dimensional crystal that was shorter in length but with a larger triangular base. Both crystal morphologies produced visible diffraction patterns; however, the latter crystal morphology consistently diffracted to higher resolution.

Diffraction data were collected to approximately 2.3 Å resolution on the X29A beamline at the NSLS. Data were processed with *HKL-2000* (Otwinowski & Minor, 1997) to 2.4 Å resolution in space group *P*3₂, with unit-cell parameters *a* = 110.7, *b* = 110.7, *c* = 58.8 Å, α = 90, β = 90, γ = 120° (Table 1). Molecular replacement was performed with the *PHENIX* software package (Adams *et al.*, 2010) using the structure of the DdrB pentamer from *D. geothermalis* with waters removed as a search model (PDB entry 4exw; 72% amino-acid identity; Sugiman-Marangos & Junop, 2010), yielding a top solution with a log-likelihood gain (LLG) of 134.55. In total, four other solutions were reported, with LLGs of 113.91, −13.09, −28.92 and −83.46. Analysis of peaks in the resulting *F*_o − *F*_c map revealed the

Figure 2 Crystals of the DdrB_{1–144}–ssDNA complex obtained by the hanging-drop vapour-diffusion method. Initial screening yielded a shower of small crystals from Nucleix condition No. 5 [200 mM potassium chloride, 10 mM magnesium chloride, 50 mM MES pH 5.6, 5% (w/v) PEG 8000] (*a*), which were reproduced using the Cy5-labelled ssDNA substrate (*b*). Optimization of the crystallization condition and dehydration solution yielded larger diffraction-quality crystals (*c*). The length of the scale bar represents approximately 100 μ m.

Figure 3

Stereo-image representation of the $F_o - F_c$ OMIT map (3σ) calculated following molecular replacement with *PHENIX*, indicating the presence of bound ssDNA in the structure solution.

presence of electron density representative of ssDNA (Fig. 3). Model building and structure refinement are currently in progress.

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