Purification, crystallization and preliminary crystallographic investigation of FrnE, a disulfide oxidoreductase from *Deinococcus radiodurans*

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In prokaryotes, Dsb proteins catalyze the formation of native disulfide bonds through an oxidative folding pathway and are part of the cell machinery that protects proteins from oxidative stress. *Deinococcus radiodurans* is an extremophile which shows unparalleled resistance to ionizing radiation and oxidative stress. It has a strong mechanism to protect its proteome from oxidative damage. The genome of *Deinococcus* shows the presence of FrnE, a Dsb protein homologue that potentially provides the bacterium with oxidative stress tolerance. Here, crystallization and preliminary X-ray crystallographic analysis of FrnE from *D. radiodurans* are reported. Diffraction-quality single crystals were obtained using the hanging-drop vapour-diffusion method with reservoir solution consisting of 100 mM sodium acetate pH 5.0, 10% PEG 8000, 15–20% glycerol. Diffraction data were collected on an Agilent SuperNova system using a microfocus sealed-tube X-ray source. The crystal diffracted to 1.8 Å resolution at 100 K. The space group of the crystal was found to be $P2_122_1$, with unit-cell parameters $a = 47.91$, $b = 62.94$, $c = 86.75$ Å, $\alpha = \beta = \gamma = 90^\circ$. Based on Matthews coefficient analysis, one monomer per asymmetric unit is present in the crystal, with a solvent content of approximately 45%.

1. Introduction

Many membrane and secretory proteins incorporate disulfide bonds to help maintain their structure and function (Shouldice *et al.*, 2011). Disulfide-bond formation during folding of some of these proteins is assisted by protein-disulfide isomerases in eukaryotes and Dsb (Disulfide bond) proteins in prokaryotes (Collet & Bardwell, 2002). In eukaryotic cells, disulfide catalysis occurs in the endoplasmic reticulum, whereas in prokaryotes the process takes place in the periplasmic space (Shouldice *et al.*, 2011). The prokaryotic oxidative folding machinery has been best characterized in *Escherichia coli*, and six members named DsbA–DsbE and DsbG have been identified (Collet & Bardwell, 2002). DsbA and DsbB incorporate native disulfides in proteins through an oxidative pathway, whereas DsbC, DsbG and DsbD correct non-native disulfide bonds through an isomerase pathway (Messens & Collet, 2006). Dsb proteins are part of the thioredoxin superfamily, and usually contain a conserved thioredoxin fold and a thiol-rich active-site motif CXXC (Collet & Bardwell, 2002; Shouldice *et al.*, 2011). It has been shown that mutations in *dsb* genes lead to incorrect folding of various proteins, including proteins involved in oxidative stress tolerance (Bardwell *et al.*, 1991; Cumming *et al.*, 2004; Mossialos *et al.*, 2006).

Deinococcus radiodurans is a Gram-positive bacterium from the Deinococcaceae family. It shows extraordinary resistance to DNA-damaging agents, including ionizing radiation, desiccation and genotoxic chemicals (Blasius *et al.*, 2008; Slade & Radman, 2011; Misra *et al.*, 2013). It has the ability to tolerate massive damage to its genome without any measurable loss of cell viability (Battista, 2000). The extraordinary radioresistance of *D. radiodurans* has been attributed to many factors, including the high copy number of the multipartite genome, efficient DNA strand-break repair and the presence of carotenoids, pyrroloquinoline quinone and intracellular manganese complexes (Misra *et al.*, 2004; Rajpurohit *et al.*, 2008; Daly, 2009; Krisko & Radman, 2013). Recently, it has been argued that the protection of proteins from oxidative damage is the key to the radiation resistance shown by *D. radiodurans* (Daly, 2009; Krisko

& Radman, 2013). Dsb proteins have been shown to be the part of the cell machinery that protects proteins from oxidative damage in other systems (Roszczenko *et al.*, 2012; Denoncin *et al.*, 2014). The *D. radiodurans* genome also encodes putative Dsb homologues (Heras *et al.*, 2009); however, very little is known about their roles.

FrnE, a putative Dsb homologue encoded on DR_0659 (hereafter referred to as drFrnE) in the genome of *D. radiodurans*, has recently been characterized (Khairnar *et al.*, 2013). It has been observed that a sublethal concentration (100 μM) of cadmium induces the transcription of DR_0659 by nearly sevenfold (Joe *et al.*, 2011) and mutants lacking this protein showed a ~ 15 -fold decreased tolerance to cadmium (Khairnar *et al.*, 2013). A dr0659 mutant of *D. radiodurans* showed a sixfold lower resistance to γ -radiation and a twofold higher sensitivity to 40 mM H_2O_2 compared with the wild type. drFrnE was characterized as a disulfide isomerase and its role in the protection of malate dehydrogenase from thermal inactivation has been demonstrated (Khairnar *et al.*, 2013). These observations suggest that drFrnE plays an important role in oxidative stress tolerance of *D. radiodurans*, possibly by protecting the damaged cellular proteins from inactivation. The exact mechanism of the disulfide-bond formation and stabilization catalyzed by drFrnE is not known and therefore structure elucidation of drFrnE was undertaken. Here, we report the crystallization and preliminary crystallographic investigation on drFrnE from *D. radiodurans*.

2. Materials and methods

2.1. Overexpression and purification of drFrnE

Recombinant drFrnE was purified from *E. coli* BL21 (DE3) pLysS cells as described in Khairnar *et al.* (2013). In brief, *E. coli* cells expressing drFrnE protein were grown in Luria–Bertani (LB) medium supplemented with 100 μM ampicillin at 310 K. At an OD_{600} of ~ 0.6 , protein expression was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and the cell culture was further incubated for 4 h at 310 K. The cells were harvested by centrifugation, resuspended in cold lysis buffer (50 mM Tris, 300 mM NaCl pH 8.0) and lysed by sonication on ice. Cell debris was removed by centrifuging the cell lysate at 11 000g for 10 min. The clear supernatant containing soluble proteins was mixed with Ni^{2+} -NTA (nitrilotriacetic acid) agarose (Qiagen, India) and kept at 277 K for 2 h with gentle shaking to allow binding of the His-tagged drFrnE

protein. The protein-bound Ni^{2+} -NTA resin was then packed in a column (Econo-Pac, Bio-Rad, India) and washed extensively with lysis buffer containing an increasing concentration of imidazole. The drFrnE protein was finally eluted with elution buffer (50 mM Tris, 300 mM NaCl, 250 mM imidazole pH 8.0) and fractions containing drFrnE protein were analyzed by SDS–PAGE. Pure fractions were pooled and dialyzed against 50 mM HEPES pH 7.0. Pure dialyzed protein was concentrated to 20 mg ml^{-1} using Vivaspin centrifugal concentrators (10 000 Da MWCO, Sartorius AG, Germany) before setting up crystallization trials.

2.2. Crystallization

The preliminary crystallization screening of drFrnE protein was performed by the sitting-drop vapour-diffusion method in 96-well crystallization plates (Crystal Quick RW, three well, Greiner Bio-One, Germany). Commercially available screens including The AmSO₄ Suite, The JCSG Core Suites I–IV and The Cryos Suite (Qiagen, India) were used. The crystallization drops were prepared by mixing 0.9 μl protein solution with 0.9, 0.8 and 0.7 μl reservoir solution in three different wells using a Cy-Bi HTPC robot (Analytik Jena AG, Germany). The reservoir wells contained 75 μl of the individual screen solution. The crystallization plates were incubated at 295 K in an imager/incubator (Formulatrix, Indonesia). Crystals of drFrnE appeared in several conditions: (i) plate-shaped crystals with reservoir consisting of 16% PEG 8000, 20% glycerol, 40 mM potassium dihydrogen phosphate in 2–3 d, (ii) rod-shaped crystals in about 2 weeks from 20% PEG 6000, 100 mM MES pH 5.0 and (iii) rod-shaped crystals from 30% PEG 6000, 100 mM HEPES pH 6.5 in 2–3 weeks. The conditions obtained in the initial hits were optimized manually using the hanging-drop vapour-diffusion method in 24-well plates (Greiner Bio-One, Germany). PEGs with different molecular weights (PEG 400, 1000, 3350, 6000 and 8000) were tried and PEG 8000 was found to be the most effective precipitant for obtaining good-quality single crystals. Different buffers, MES, MOPS, HEPES, Tris, sodium acetate and imidazole (100 mM each), in the pH range 4.5–7.0, were also explored. Further optimization was achieved by using an increasing amount of glycerol, which reduced the number of crystals appearing in each drop. The final crystallization condition used to obtain diffraction-quality single crystals consisted of 10% PEG 8000, 100 mM sodium acetate pH 5.0, 15–20% glycerol.

Figure 1

Rod-shaped crystals of drFrnE obtained in a condition consisting of 10% PEG 8000, 100 mM MES buffer pH 5.0, 20% glycerol. These crystals did not diffract well.

Figure 2

Thick, plate-shaped crystals of drFrnE obtained in a condition consisting of 10% PEG 8000, 100 mM sodium acetate buffer pH 5.0, 20% glycerol. Crystals diffracted to 1.8 Å resolution.

Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.54056
Temperature (K)	100
Detector	Titan CCD
Crystal-to-detector distance (mm)	61
Rotation range per image (°)	1
Total No. of frames	224
Exposure time per image (s)	300
Space group	$P2_12_1$
Unit-cell parameters (Å, °)	$a = 47.91$, $b = 62.94$, $c = 86.75$, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	1.1
Resolution range (Å°)	15.58–1.8 (1.9–1.8)
Total No. of reflections	161924 (13812)
No. of unique reflections	24699 (3482)
Completeness (%)	99.1 (98.2)
Mean $I/\sigma(I)$	13.9 (1.7)
Multiplicity	6.6 (4.0)
No. of molecules in the asymmetric unit	1
$R_{\text{merge}}^{\dagger}$ (%)	7.2 (48.7)
CC^* (%)	99.9 (93.0)
Overall B factor from Wilson plot (Å ²)	20.55

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the measured intensity of each individual reflection and $\langle I(hkl) \rangle$ is the mean value of the intensity.

2.3. Data collection and processing

X-ray diffraction data of drFrnE crystals were collected in-house on an Agilent SuperNova system using a microfocus sealed-tube X-ray source equipped with multilayer optics and a four-circle kappa goniometer operated at 50 kV and 0.8 mA (Cu $K\alpha$, $\lambda = 1.5406$ Å). The diffraction data were collected at 100 K using a temperature-controlled liquid-nitrogen jet. Since the crystallization condition contained 15–20% glycerol, no other cryoprotectant was added. The crystal was flash-cooled directly in liquid N₂. Diffraction data were collected at a crystal-to-detector distance of 61 mm with 1° oscillation per frame and an exposure time of 300 s. A total of 224 frames were processed using the *CrysAlisPro* software suite (v1.171.37.33; Agilent Technologies, Oxford, England). Scaling and merging were

performed using *SCALA* as implemented in the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

The drFrnE protein from *D. radiodurans* was overexpressed in *E. coli* BL21 (DE3) pLysS cells. The recombinant drFrnE was purified using Ni-NTA affinity chromatography. The drFrnE protein was concentrated to 20 mg ml^{−1} in 50 mM HEPES pH 7.0 and used for crystallization. 8–12% PEG 8000 with different buffers was found to be effective in obtaining good-quality single crystals. The rod-shaped crystals obtained in 100 mM MES pH 5.0, 100 mM MOPS pH 5.0 and 100 mM HEPES pH 6.5 buffers did not diffract well (Fig. 1). Large, thick, plate-like crystals appeared in 3–4 d in 10% PEG 8000, 100 mM sodium acetate pH 5.0, 15–20% glycerol which diffracted well (Fig. 2). The glycerol present in the crystallization buffer also served as a cryoprotectant. A drFrnE crystal of approximately 2 × 1 × 0.5 mm in size was used to collect diffraction data. The crystal diffracted to 1.8 Å resolution at 100 K (Fig. 3). The crystal belonged to space group $P2_12_1$, with unit-cell parameters $a = 47.91$, $b = 62.94$, $c = 86.75$ Å. The data-collection and processing statistics are given in Table 1. Based on the molecular mass of the protein (29 561 Da) deduced from the nucleotide sequence and the unit-cell volume (261 588 Å³), the Matthews coefficient (Matthews, 1968) for drFrnE was calculated using the *CCP4* suite. The calculated Matthews coefficient of 2.21 Å³ Da^{−1} and the solvent content of 44.44% suggested the presence of one monomer per asymmetric unit. The diffraction data will be used to solve the structure of drFrnE using the molecular-replacement method.

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Figure 3

Diffraction image of drFrnE crystal from data obtained using an in-house Agilent SuperNova X-ray diffractometer system with a Titan CCD detector.