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Expression, purification, crystallization and preliminary crystal structure analysis of the *Deinococcus radiodurans* organic hydroperoxide-resistance protein

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The organic hydroperoxide-resistance protein (DR1857) from *Deinococcus radiodurans* has been expressed, purified and crystallized. The crystals are suitable for X-ray analysis, diffract to at least 2.3 Å resolution, have unit-cell parameters a = 45.7, b = 59.6, c = 49.7 Å, β = 90.43° and belong to space group $P2_1$. The calculated Matthews coefficient of 2.1 Å^3 Da^{-1} coupled with a calculated solvent content of approximately 42% is consistent with the presence of a homodimer in the asymmetric unit. Here, the methods used in the overexpression and purification of the protein are described and details of crystallization conditions and preliminary X-ray diffraction are provided.

1. Introduction

The reactive oxygen species (ROS) that are generated when organisms are under conditions of oxidative stress include the hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and organic hydroperoxides (OHPs). The latter are chemically reactive byproducts of bacterial oxygen metabolism and can themselves generate free radicals and other ROS. The presence of ROS can cause such serious damage to cellular macromolecules (Atichartpongkul *et al.*, 2001 and references therein) including nucleic acids, proteins and lipids that it can often lead to cell death (Mongkolsuk *et al.*, 1998). Cellular detoxification of ROS is thus of great importance for the viability of all organisms (Tenhaken *et al.*, 1995). Microorganisms have therefore evolved several defence systems to limit the effects of oxidative stress. These include systems for DNA and protein repair, recombination mechanisms and those that prevent damage by neutralizing ROS before their presence becomes deleterious (Atichartpongkul *et al.*, 2001).

The non-pathogenic red-pigmented Gram-positive bacterium *Deinococcus radiodurans* (DEIRA) is well known for its extreme resistance to ionizing radiation and other damaging assaults such as ultraviolet (UV) radiation, desiccation and the presence of ROS (Mattimore & Battista, 1996; Battista, 1997). Attempts to understand the mechanisms DEIRA might use to achieve its extraordinary extremophilic capability have made the bacterium the subject of intense study.

The complete genome sequence of DEIRA strain R1 is available (White *et al.*, 1999) and a thorough analysis of this genome has been carried out (Makarova *et al.*, 2001). At the heart of the capacity of DEIRA to withstand extreme irradiation is undoubtedly a phenomenal ability to repair double-stranded DNA breaks (DSBs) in its genome (Minton, 1994; Makarova *et al.*, 2001). Nevertheless, the genome of DEIRA also contains a plethora of genes that code for proteins that detoxify cells of the ROS that occur under conditions of oxidative stress (Makarova *et al.*, 2001). It may also be that prevention of the DNA damage caused by ROS plays a role in the extremophilic nature of the bacterium.

In bacteria, the best characterized system involved in the detoxification of OHPs is the alkyl hydroperoxide reductase Ahp (Niimura *et al.*, 1995; Poole, 1996; Wood *et al.*, 2002; Wood, Poole *et al.*, 2003). Recently, a new family of proteins that appear to complement those of the Ahp system has been identified (Mongkolsuk *et al.*, 1998). These new proteins are known as organic hydroperoxide-resistance proteins (OHRPs) because deletion of the gene encoding this protein causes *Xanthomonas campestris* pv. *Phaseoli* to be sensitized to OHPs such as *t*-butylhydroperoxide (*t*-BOOH) and cumene hydroperoxide. The OHRP-family members encoded by *Ohr* genes are generally around 140 amino acids in length, are biologically active as homodimers (Lesniak *et al.*, 2002; Rosa Cussiol *et al.*, 2003) and their sequences contain two invariant cysteine residues near positions 60 and 120 in the polypeptide chain. The former is found in a conserved ACF motif, while the environment of the latter is generally the VCP or related motif that is also found in many peroxiredoxins. This, and the fact that OHRP sequences also contain two invariant arginine residues, suggests that OHRPs function in a similar fashion to thiol-dependent 2-Cys peroxidases (Hofmann *et al.*, 2002; Wood,

Schröder *et al.*, 2003). Strong evidence has emerged to support this hypothesis (Lesniak *et al.*, 2002; Rosa Cussiol *et al.*, 2003).

Ohr genes are related to those of the stress-inducible *OsmC* family, with sequence identities of around 30% (Atichartpongkul *et al.*, 2001). Based on recent evidence, the two protein families have very similar structures (Choi *et al.*, 2003; Lesniak *et al.*, 2003) and functions (Conter *et al.*, 2001). The reasons why some bacteria express both OHRP and OSMC proteins are as yet unclear.

As part of a structural genomics project focusing on gene products that may be important in understanding the extremophilic nature of DEIRA strain R1, we have overexpressed, purified and crystallized the product of the gene DR1857. The protein has been predicted to be highly expressed by DEIRA (Karlin & Mrázek, 2001) and has been annotated as being a member of the OHRP family. It is hoped that analysis of its three-dimensional structure and comparison of this with the recently reported structure of an OHRP from *Pseudomonas aeruginosa* (Lesniak *et al.*, 2002) can be used to further characterize its role in the mechanism of cellular detoxification of organic hydroperoxides. The X-ray diffraction data collected from the crystals described here is the initial step towards this goal.

2. Methods and results

2.1. Overexpression and purification of DR1857

The organic hydroperoxide-resistance protein from *D. radiodurans* ($OHRP_{Dr}$) was amplified by *Pfu* DNA polymerase, subcloned into a Gateway pDest17 vector (Invitrogen) containing a 2.6 kDa N-terminal fusion tag that includes a hexahistidine region and transformed into *Escherichia coli* strain BL21 (DE3) (Novagen). The bacteria were grown in 1 l Luria–Bertani (LB) broth containing 50 µg ml^{-1} ampicillin to a cell density of between 0.6 and 0.8 OD at 595 nm. Production of $OHRP_{Dr}$ was induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 310 K. The cells were harvested by centrifugation at 4000*g* for 20 min at 277 K and resuspended in 40 ml of a buffer containing 20 m*M* sodium phosphate pH 7.2, 0.5 *M* NaCl (buffer *A*) supplemented with 20 m*M* $MgCl_2$, 10 µg ml^{-1} DNAseI, 100 µg ml^{-1} lysozyme and a 'complete mini EDTA-free' cocktail of anti-proteases (Roche). They were then lysed on ice for 30 min and the cell debris was removed by centrifugation at 18 000*g* for 30 min at 277 K.

The resulting supernatant was applied onto a metal-chelating column previously loaded with 0.1 *M* nickel chloride (Amersham Pharmacia Biotech Inc) pre-equilibrated in buffer *A*. $OHRP_{Dr}$ was eluted using a 50 ml linear gradient from buffer *A* to a buffer consisting of 20 m*M* sodium phosphate pH 7.2, 0.5 *M* NaCl and 500 m*M* imidazole using a flow rate of 1 ml min^{-1}. $OHRP_{Dr}$ was eluted as a single peak at an imidazole concentration of 400 m*M*. The fractions containing recombinant $OHRP_{Dr}$ were then pooled, dialysed (7.5 kDa molecular-weight cutoff) against a storage buffer consisting of 20 m*M* Tris–NaCl pH 7.2, 200 m*M* NaCl, concentrated to 10 mg ml^{-1} (Centricon YM-10) and stored at 253 K. Subsequent mass-spectrometry experiments (data not shown) confirmed that recombinant $OHRP_{Dr}$ had been obtained with a high degree of purity. Intriguingly, however, SDS–PAGE analysis of the pure protein carried out under non-reducing conditions revealed two bands of similar intensity, both of which migrated close to the 17 kDa molecular weight expected for $OHRP_{Dr}$ plus its fusion tag (Fig. 1). Repeating the analysis under reducing conditions revealed only the upper of the two bands. These results are very similar to observations made during studies on both cTPXI from *Saccharomyces cerevisae* (Chae *et al.*, 1994) and OHRP from *Xylella fastidiosa* (Rosa Cussiol *et al.*, 2003).

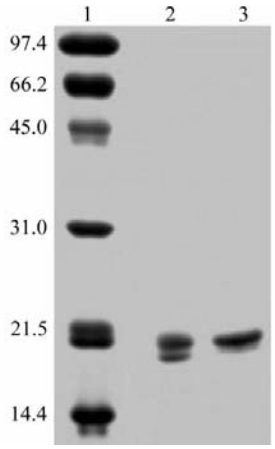

Figure 1
SDS–PAGE analysis of purified recombinant $OHRP_{Dr}$ obtained as outlined in the text. Lane 1 shows bands resulting from molecular-weight markers (kDa; Biorad). Lane 2 shows the two bands obtained when $OHRP_{Dr}$ was analysed under non-reducing conditions. In lane 3 a reducing agent was added to the buffer, resulting in a single band that corresponds to $OHRP_{Dr}$ in its reduced-disulfide form. The lower of the two bands in lane 2 may therefore correspond to $OHRP_{Dr}$ in its oxidized disulfide form and our purified sample may thus contain both redox isomers of the protein.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell (2.42–2.3 Å).

X-ray wavelength (Å)	0.933
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 45.7, b = 59.6, c = 49.7, β = 90.43
Resolution range (Å)	35.6–2.3
Total No. reflections collected	37030
No. unique reflections	10656
Data completeness (%)	93.7 (78.6)
R_{sym} (%)	5.9 (15.0)
Mean $I/\sigma(I)$	17.0 (6.3)

The lower of the two bands seen in our experiments may thus represent the oxidized (*i.e.* closed S—S) state of $OHRP_{Dr}$, while the upper band may represent $OHRP_{Dr}$ with its active-site disulfide reduced.

2.2. Crystallization and preliminary X-ray data collection and analysis

Crystals of native $OHRP_{Dr}$ were obtained at 277 K. They appeared after several months from 2 µl drops containing a 50:50 mixture of the protein stock solution and a reservoir solution made up of 30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$ which were equilibrated against 1 ml of the reservoir solution. The crystals obtained are generally thin rods around 10–20 µm thick that typically form clumps. It was nevertheless possible to extract a single crystal from these. After cryoprotection achieved by soaking the crystals in a buffer consisting of 0.08 *M* Tris–HCl pH 8.5, 0.16 *M* $MgCl_2$, 24%(*w*/*v*) PEG 4000 and 20%(*v*/*v*) glycerol, they were flash-frozen in a stream of gaseous nitrogen at 100 K. X-ray diffraction data were collected on beamline ID14-EH2 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The diffraction images were indexed and integrated with the program *MOSFLM* (Leslie, 1992) and the resulting intensities were scaled and merged using the program *SCALA* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

As can be seen from Table 1, the crystals of $OHRP_{Dr}$ obtained have unit-cell parameters $a = 45.7, b = 59.6, c = 49.7$ Å, $\beta = 90.43°$ and belong to space group $P2_1$. They thus represent a different crystal form to that obtained for OHRP from *P. aeruginosa* (Lesniak *et al.*, 2002). A calculated Matthews coefficient (Matthews, 1968) of 2.1 Å^3 Da^{-1} coupled with a solvent content of approximately 42% is consistent with an active homodimer making up the asymmetric unit. Production of the selenomethionyl derivative of $OHRP_{Dr}$ is currently

under way in order to provide experimental phases for use in structure determination. It is hoped that elucidation of the structure of $OHRP_{Dr}$ in both its oxidized and reduced disulfide states will be possible. This, coupled with the structural information already available on similar systems (Lesniak *et al.*, 2002), should allow a full characterization of the mechanism used by OHRPs in the detoxification of organic hydroperoxides.

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