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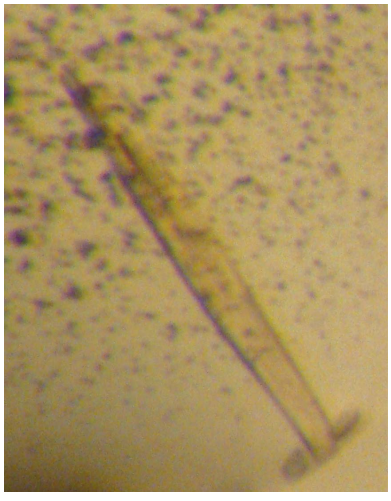

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Expression, purification, crystallization and preliminary X-ray crystallographic studies of alkyl hydroperoxide reductase (AhpC) from the cyanobacterium *Anabaena* sp. PCC 7120

Alkyl hydroperoxide reductase (AhpC) is a key component of a large family of thiol-specific antioxidant (TSA) proteins distributed among prokaryotes and eukaryotes. AhpC is involved in the detoxification of reactive oxygen species (ROS) and reactive sulfur species (RSS). Sequence analysis of AhpC from the cyanobacterium *Anabaena* sp. PCC 7120 shows that this protein belongs to the 1-Cys class of peroxiredoxins (Prxs). It has recently been reported that enhanced expression of this protein in *Escherichia coli* offers tolerance to multiple stresses such as heat, salt, copper, cadmium, pesticides and UV-B. However, the structural features and the mechanism behind this process remain unclear. To provide insights into its biochemical function, AhpC was expressed, purified and crystallized by the hanging-drop vapour-diffusion method. Diffraction data were collected to a maximum *d*-spacing of 2.5 Å using synchrotron radiation. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 80, b = 102, c = 109.6 Å. The structure of AhpC from *Anabaena* sp. PCC 7120 was determined by molecular-replacement methods using the human Prx enzyme hORF6 (PDB entry 1prx) as the template.

1. Introduction

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes. On the basis of the number of conserved cysteine residues in the N-terminal region of the protein, the Prx proteins are divided into three classes: typical 2-Cys Prxs, atypical 2-Cys Prxs and 1-Cys Prxs. The typical 2-Cys Prxs are the largest class and are identified by the conservation of two redox-active cysteines: the peroxidatic cysteine (generally near residue 50) and the resolving cysteine (near residue 170) (Wood *et al.*, 2002). The atypical 2-Cys Prxs use the same mechanism as the typical 2-Cys Prxs but are functionally monomeric (Seo *et al.*, 2000). In the last class, 1-Cys Prxs, only the peroxidatic cysteine (Cys47) is conserved and these Prxs do not contain a resolving cysteine (Choi *et al.*, 1998).

AhpC is a key component of a large family of thiol-specific antioxidant (TSA) proteins distributed among prokaryotic and eukaryotic organisms. AhpC is known to scavenge a variety of peroxides and reactive oxygen, nitrogen and sulfur species (Chae *et al.*, 1994; Nguyên-nhu & Knoops, 2002). A 2.5-fold accumulation of AhpC/TSA was observed by two-dimensional gel electrophoresis in *Anabaena doliolum* following exposure to 321 K, suggesting a role in protection against heat stress (Mishra, Bhargava *et al.*, 2009). Furthermore, it has been demonstrated that enhanced expression of AhpC in *Escherichia coli* offers protection against heat, salt, pesticides, heavy metals and UV-B radiation (Mishra, Chaurasia *et al.*, 2009)

Since 1998 several crystal structures of peroxiredoxins have been published, including those of 2-Cys Prxs and atypical 2-Cys Prxs (Wood *et al.*, 2003). For AhpC, the Protein Data Bank (PDB) contains nine crystal structures originating from human, bacteria and yeast. Cyanobacteria, an ancient and morphologically diverse group of photosynthetic prokaryotes, are the largest and most widely distributed group of photosynthetic prokaryotes on Earth, contributing significantly to the global CO_2 and N_2 budget (Fritsch, 1907). However, to date the PDB does not contain any structure of a cyanobacterial AhpC. Considering the important functions of AhpC in protection against abiotic stresses (Mishra, Chaurasia *et al.*, 2009),

we have expressed, purified and crystallized AhpC from the cyanobacterium *Anabaena* sp. PCC 7120.

2. Materials and methods

2.1. Cloning of the AhpC gene from *Anabaena* sp. PCC 7120

Genomic DNA from *Anabaena* sp. PCC 7120 (also called *Nostoc* sp. PCC 7120; Taxonomy ID 103690) was isolated as described previously (Srivastava *et al.*, 2007). An open reading frame *alr4404* putatively encoding AhpC was cloned as described previously (Mishra, Chaurasia *et al.*, 2009). In brief, the gene was amplified by polymerase chain reaction (PCR) using genomic DNA as template with the pair of primers Pf (5′-CG**GAATTC**CCATGGCTCTCCGTCTTGGT-3′) and Pr (5′-ATTT**GCGGCCGC**TTACTTGTTAGGTTGAGGAGT-3′). The bases shown in bold represent *Eco*RI and *Not*I recognition sites, respectively. PCR was performed in a 25 µl reaction mixture for 30 cycles. Denaturation was performed at 367 K for 90 s, annealing at 335 K for 60 s and primer extension at 345 K for 120 s using standard PCR conditions [100 ng DNA, 2.5 µl 10× PCR buffer with 15 m*M* $MgCl_2$, 200 µ*M* dNTPs, 10 pmol of each primer and 0.2 U *Taq* DNA polymerase in an iCycler (Bio-Rad, USA)]. The amplified PCR product was gel-purified using the freeze–thaw method. Briefly, the band of interest was excised from the 0.8% low-melting agarose gel (Sigma) and frozen at 193 K for about 10 min. The gel containing the band was thawed at 338 K and TE-saturated phenol was added to the melted gel piece. The freeze–thaw cycle was then repeated three times. After the third thawing, the tube was centrifuged at 13 000*g* for 20 min at 277 K and the aqueous layer was removed to a new tube. Residual phenol was removed by the addition of an equal volume of chloroform and 2-propanol in a 24:1 ratio followed by gentle inversion and centrifugation at 12 000*g* at 298 K (room temperature). This process was repeated twice; the supernatant was then transferred to a new tube and a 1/10 volume of 3 *M* sodium acetate was added. To the same tube, an equal volume of 2-propanol was added and the whole mixture was kept at 253 K for 2 h for precipitation of DNA. The precipitated DNA was collected by centrifugation at 12 000*g* for 20 min at 277 K. The DNA pellet was washed by the addition of 70% ethanol followed by centrifugation at 13 000*g* for 10 min at 298 K. Finally, the pellet was dissolved in TE buffer (10 m*M* Tris–HCl pH 8.0 and 1 m*M* EDTA).

2.2. Construction of the expression vector

The purified PCR product was digested with *Eco*RI and *Not*I (NEB, USA) and the resultant DNA fragment was cloned into the expression vector pGEX-5X-2 digested with the same restriction enzymes. The plasmid pGEX-5X-2-*ahpC* was introduced into *E. coli* DH5α. After cultivating the bacteria in LB medium supplemented with 100 µg ml^{-1} ampicillin, the plasmid was isolated and the DNA sequence of *alr4404* was confirmed by sequencing (Mishra, Chaurasia *et al.*, 2009).

2.3. Purification of AhpC

For purification of AhpC (NCBI accession No. NP_488444.1), *E. coli* BL21 cells harbouring the pGEX-5X-2-*ahpC* plasmid were grown in 1 l LB medium supplemented with 100 µg ml^{-1} ampicillin in a 2 l Erlenmeyer flask on an orbital shaker (200 rev min^{-1}) at 310 K. When the absorption of the culture reached a value of A_{600} = 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Germany) was added to a final concentration of 0.5 m*M* and the culture was grown at 293 K overnight. The bacterial pellet from 1 l culture was resuspended in a 1/10 volume of lysis buffer consisting of 20 m*M* NaH_2PO_4 pH 7.4 and 0.5 *M* NaCl and was then subjected to sonication for 60 s on ice using a Branson Sonifier. The cell lysate was centrifuged at 20 000*g* for 30 min at 277 K and the resulting supernatant was used in further purification steps.

The cleared cell lysate was loaded onto a 5 ml GSTrap FF column (GE Healthcare, Sweden) equilibrated with binding buffer (1× PBS pH 7.3) at 277 K at a flow rate of 0.5 ml min^{-1} using an ÄKTAprime chromatography system (GE Healthcare, Sweden). The column was washed with ten column volumes of binding buffer at a flow rate of 1 ml min^{-1} to remove unbound proteins. GST-AhpC was eluted from the column at 277 K with five column volumes of elution buffer (50 m*M* Tris–HCl pH 8.0, 10 m*M* reduced glutathione). The eluted GST-AhpC fraction was concentrated by ultrafiltration (Vivaspin 20, GE Healthcare, Sweden) and the buffer was exchanged to 50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* $CaCl_2$ using a 5 ml HiTrap desalting column (GE Healthcare, Sweden).

For cleavage of the GST tag, the GST-AhpC fusion protein solution was incubated with 10 U mg^{-1} factor Xa protease (GE Healthcare, Sweden) for 16 h at 295 K. After digestion, the protein solution was loaded onto a 5 ml GSTrap FF column pre-equilibrated with 50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* $CaCl_2$. The cleaved AhpC protein was collected in the flowthrough fraction and was further purified by size-exclusion chromatography using a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare, Sweden) equilibrated with 50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* $CaCl_2$. Fractions containing AhpC were collected and pooled and the purified protein was concentrated to 13 mg ml^{-1} by ultrafiltration using a filter with a 10 kDa molecular weight cut-off (Vivaspin 20, GE Healthcare, Sweden) to a final volume of 800 µl. The purity of the target AhpC protein was evaluated by SDS–PAGE (Fig. 1). The protein concentration was determined by the Bradford assay using BSA as the standard (Sigma, USA).

2.4. Crystallization

Initial crystallization screening was performed in 96-well sitting-drop plates (MRC-Wilden, UK) with solutions 1–48 of the Crystal Screen kit and the full Crystal Screen 2 kit (Hampton Research, USA) using a Mosquito pipetting robot (TTP LabTech, UK). The

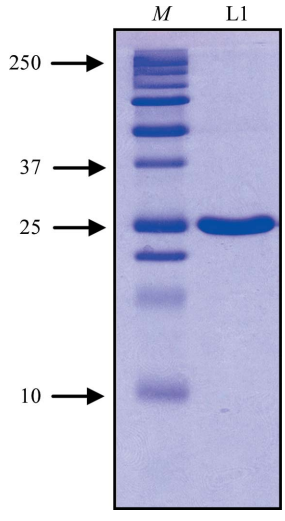

Figure 1
SDS–PAGE (12%) analysis of purified cyanobacterial AhpC. Lane *M*, molecular-weight markers (kDa); lane L1, purified AhpC. The gel was stained with Coomassie Brilliant Blue R-250.

drops contained 100 nl purified AhpC solution at 13 mg ml^{-1}, into which 100 nl reservoir solution was pipetted. Initially, crystals were obtained under three conditions after about two weeks at 291 K. Conditions (i) and (ii) consisted of 0.2 *M* ammonium acetate, 30%(*w*/*v*) polyethylene glycol 4000 and 0.1 *M* sodium citrate tribasic dehydrate pH 4.6 or pH 5.6, respectively, and condition (iii) consisted of 0.2 *M* ammonium acetate trihydrate, 30%(*w*/*v*) polyethylene glycol 8000 and 0.1 *M* sodium cacodylate trihydrate pH 6.5. The initial screening conditions were systematically optimized by modifying the protein concentration, the pH and the type of PEG used. Hanging drops consisting of 2 µl protein solution and 2 µl reservoir solution were equilibrated over 500 µl reservoir solution using 24-well Linbro plates.

2.5. X-ray data collection

Crystals were transferred into mother liquor containing a 5% higher concentration of polyethylene glycol before being mounted free-standing in a cryoloop and vitrified in liquid nitrogen. Thereafter, the crystals were kept under liquid nitrogen in a storage dewar. For transport to the synchrotron the crystals were transferred into a cryogenic 'dry-shipper' dewar (CX100, Taylor Wharton, USA) which maintains a temperature close to 83 K. X-ray diffraction data to a maximum *d*-spacing of 2.5 Å were collected from a single crystal on beamline ID23-1 at the ESRF, Grenoble, France. The crystal was rotated in steps of 0.55° per image over a total of 110°. The data were indexed and integrated with *XDS* (Kabsch, 2010), merged with *POINTLESS* (Evans, 2006) and scaled using *SCALA* (Evans, 2006). *TRUNCATE* (French & Wilson, 1978) was used to transform the intensities to structure factors.

3. Results and discussion

Recombinant AhpC protein from the cyanobacterium *Anabaena* sp. PCC 7120 was expressed in *E. coli* and purified to homogeneity by GST-affinity chromatography. Following cleavage with factor Xa (GE Healthcare, Sweden), which leaves six extra amino acids (GIPGIP) in front of the starting methionine, the calculated molecular weight of the protein was 24 232 Da. The protein preparation was finalized by size-exclusion chromatography. Optimization of the initial crystallization conditions resulted in a final crystallization buffer composed of 0.2 *M* sodium acetate trihydrate, 0.1 *M* sodium cacodylate trihydrate pH 6.5 and 20%(*w*/*v*) polyethylene glycol 8000. Single diffraction-quality crystals grew at 291 K in hanging drops consisting of 2 µl AhpC protein at 13 mg ml^{-1} and 2 µl optimal crystallization buffer equilibrated against 500 µl of the same buffer. The crystals reached dimensions of approximately 0.6 × 0.1 × 0.03 mm in about four weeks (Fig. 2). From a single crystal, X-ray diffraction data were collected to a maximum resolution of 2.5 Å. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 80, b = 102, c = 109.6 Å. Crystal parameters and data-processing statistics are summarized in Table 1.

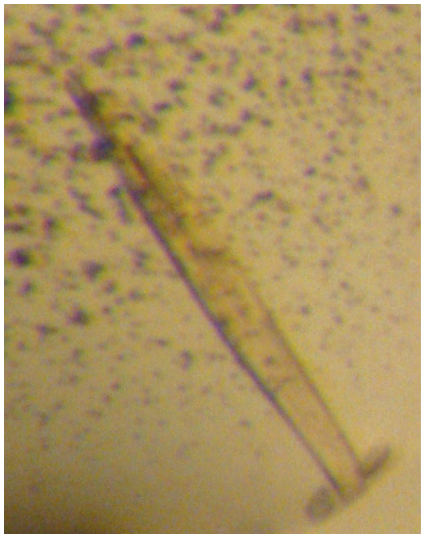

Figure 2
The crystal used for AhpC data collection. The crystal grew at 291 K after four weeks to maximum dimensions of approximately 0.6 × 0.1 × 0.03 mm.

Table 1
Data-collection statistics for AhpC from *Anabaena* sp. PCC 7120.

Values in parentheses are for the highest resolution shell.

No. of crystals	1
Beamline	ID23-1, ESRF Grenoble
Wavelength (Å)	0.97930
Detector	ADSC Q315
Crystal-to-detector distance (mm)	390.4
Rotation range per image (°)	0.55
Total rotation range (°)	110
Resolution range (Å)	54.8–2.50 (2.64–2.50)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 80.0, b = 102.0, c = 109.6
Mosaicity (°)	1.4
Total No. of measured intensities	112447 (14302)
Unique reflections	30539 (4416)
Multiplicity	7.9 (3.2)
Mean $I/\sigma(I)$	15.0 (5.5)
Completeness (%)	97.5 (97.8)
$R_{\rm merge}$† (%)	6.8 (21.3)
$R_{\rm meas}$ or $R_{\rm r.i.m.}$‡ (%)	7.9 (25.3)
Overall B factor from Wilson plot (Å^2)	35.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm meas} = R_{\rm r.i.m.} = \sum_{hkl}[N/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity of all observations i of reflection hkl and N is the multiplicity.

Pairwise sequence comparison of AhpC from *Anabaena* sp. PCC 7120 and hORF6, a human peroxiredoxin with known crystal structure, shows 52% sequence identity over 222 aligned amino acids (PDB entry 1prx; Choi *et al.*, 1998). With hORF6 as the template, we have determined the structure of cyanobacterial AhpC using the *BALBES* molecular-replacement pipeline (Long *et al.*, 2008). The cyanobacterial AhpC structure will deepen our understanding of the relationships between protein structure and function within this important family of proteins. An article describing the structural details will be published elsewhere.

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