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Cloning, overexpression, purification, crystallization and preliminary X-ray diffraction analysis of an atypical two-cysteine peroxiredoxin (SAOUHSC_01822) from *Staphylococcus aureus* NCTC 8325

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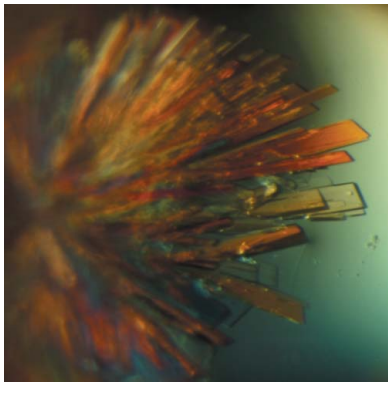
An atypical two-cysteine peroxidase, SAOUHSC_01822, from the virulent *Staphylococcus aureus* strain NCTC 8325 plays a major role in the reponse of the bacterium to oxidative stress. The protein was cloned, expressed, purified to homogeneity and crystallized. The protein was crystallized from 2 *M* ammonium sulfate, 0.1 *M* Na HEPES pH 7, 2%(*v*/*v*) PEG 400. A complete diffraction data set was collected to 2.3 Å resolution using a Rigaku MicroMax HF007 Cu $K\alpha$ X-ray generator and a Rigaku R-AXIS IV^{++} detector. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 43.50, b = 149.35, c = 73.73 Å, β = 104.4°, and contained four molecules in the asymmetric unit.

1. Introduction

Intracellular pathogenic bacteria encounter a vast array of different toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) during the process of pathogenesis and disease progression. The intraphagosomal production of ROS and RNS is highly important for the eradication of intracellular pathogens by the host innate defence system. ROS and RNS exhibit broad-spectrum toxicity and are crucial for the potent microbicidal activity of neutrophils and other phagocytes (Miller & Britigan, 1997). On the other hand, in order to achieve a successful infection event most pathogenic bacteria manifest specific and effective enzymatic pathways that render the host oxidative defence mechanism inactive. Amongst these, peroxiredoxins (Prxs) play a key role during disease progression and pathogenesis. Peroxiredoxins are basically thiol peroxidases and are members of a family that also includes tryparedoxin peroxidase (TryP), alkyl hydroperoxide reductase C (AhpC) and bacterioferritin comigratory protein (Hofmann *et al.*, 2002).

Prxs exert their protective role in cells through their peroxidase activity ($ROOH + 2e^- + 2H^+ \rightarrow ROH + H_2O$). Prxs use redox-active cysteines to reduce peroxides and can be divided into two categories based on the number of catalytically active cysteines: the 1-Cys and 2-Cys Prxs (Chae *et al.*, 1994). Based on structural and mechanistic data, the 2-Cys Prxs can be further subdivided into typical and atypical 2-Cys Prxs (Wood *et al.*, 2003). The peroxidase reaction is composed of two steps centred around a redox-active cysteine called the peroxidatic cysteine. In the first common step the peroxidatic cysteine attacks the peroxide substrate to form cysteine sulfenic acid (Cys-SOH). The second step is basically the resolution of the cysteine sulfenic acid, which distinguishes the three categories of Prxs. In the typical 2-Cys Prxs the peroxidatic cysteine sulfenic acid is resolved by the resolving cysteine residue of the adjacent monomer, forming an intermolecular disulfide linkage which is further resolved by cell-specific biothiols. In contrast, in the atypical 2-Cys Prxs the peroxidatic cysteine sulfenic acid is resolved by the resolving cysteine residue of the same monomer, forming an intramolecular disulfide bond which is subsequently resolved by cellular thioredoxins. Unlike the 2-Cys Prxs, the 1-Cys Prxs contain no resolving cysteine and the cysteine sulfenic acid is resolved by some unknown electron donor.



Staphylococcus aureus is a versatile pathogenic bacterium that is capable of rapidly developing or acquiring multiple antibiotic resistance and is now recognized as a worldwide health problem (Fey *et al.*, 2003). Like all other well known intracellular pathogens, this bacterium also produces a number of antioxidant enzymes when entrapped in the host phagosome to paralyze the oxidative defence mechanism of the host. In a recent global proteomic study, the concentration of a putative atypical 2-Cys peroxiredoxin from *S. aureus* (SaPrx) was found to be augmented after nitric oxide stress, suggesting a protective role of this protein against the host toxic reactive nitrogen intermediates during intraphagosomal growth conditions (Hochgräfe *et al.*, 2008). In this way, this protein may confer prolonged persistence of the bacterium within the host system, leading to fatal systemic infections, and may represent a future drug target against this pathogen. Therefore, we have focused our attention on structural and mechanistic studies of this enzyme. The present work reports the cloning, overexpression, purification, crystallization and preliminary X-ray diffraction studies of SaPrx (SAOUHSC_01822) from *S. aureus* NCTC 8325.

2. Materials and methods

2.1. Cloning

The sequences corresponding to the entire open reading frame of SaPrx were amplified by PCR using *S. aureus* NCTC 8325 genomic DNA as the template with the primer pair 5′-CCCAAGCTTTTAAATATTTTTGTATGCAGC-3′ (forward primer with a *Hin*dIII recognition site) and 5′-CGCGGATCCATGACTGAAATAACATTCAAAGG-3′ (reverse primer with a *Bam*HI recognition site). The purified PCR product was cloned into the *Bam*HI and *Hin*dIII sites of the expression vector pQE30 (Qiagen, USA), which adds six consecutive histidines to the N-terminus of the desired protein. The recombinant DNA was then transformed into *Escherichia coli* SG13009 (pREP4) cells and subsequently selected on ampicillin/kanamycin plates. The positive clones were verified by DNA sequencing. The desired construct has HHHHHHGSM at its N-terminus, where the two additional amino acids G and S were introduced by the *Bam*HI restriction-site nucleotides (GGATCC) between the His_6 tag and the first amino acid M of the native protein.

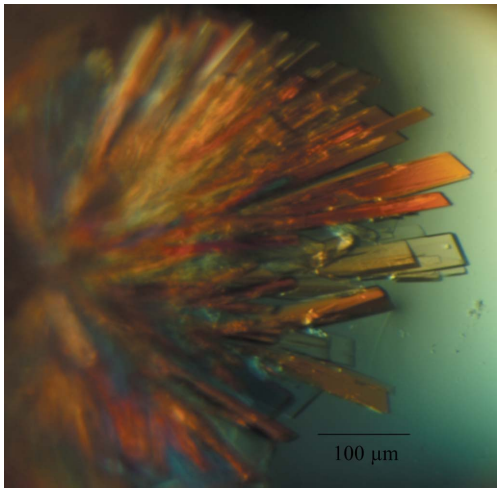


Figure 1
Good diffraction-quality crystals of SaPrx as grown in 2 *M* ammonium sulfate, 0.1 *M* Na HEPES pH 7.0, 2%(*v*/*v*) PEG 400.

2.2. Overexpression and purification

The positive clone harbouring the desired construct of SaPrx was grown in Luria broth supplemented with 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} kanamycin at 310 K for 3 h, during which the A_{600} reached 0.6, induced with 100 µ*M* IPTG and grown for a further 4 h at 310 K in order to obtain the maximum protein yield. The cells from a 1 l culture were harvested and then resuspended in buffer *A* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 10 m*M* imidazole) containing 0.1 m*M* each of leupeptin, pepstatin and aprotinin, and 0.02 m*M* phenylmethylsulfonyl fluoride (PMSF). The suspension was lysed by ultrasonication on ice and the lysate was centrifuged at 14 000 rev min^{-1} for 40 min. The supernatant was loaded onto Ni-Sepharose High Performance affinity matrix (GE Healthcare Biosciences) pre-equilibrated with buffer *A*. The column was then washed extensively with buffer *A* followed by buffer *B* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 50 m*M* imidazole) to remove non-specifically bound contaminants. The protein was finally eluted with buffer *C* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 300 m*M* imidazole). The eluted protein was subjected to size-exclusion chromatography using Superdex 200 prep-grade matrix in a 16/70 C column (GE Healthcare Biosciences) on an ÄKTAprime Plus system (GE Healthcare Biosciences) equilibrated with buffer *D* (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* DTT). 2 ml fractions were collected at a flow rate of 1 ml min^{-1}. The fractions containing the desired protein were pooled together. The protein concentration was estimated by the method of Bradford (1976) and the purity was verified by 15% SDS–PAGE.

2.3. Crystallization

The purified protein was concentrated to 60 mg ml^{-1} in buffer *D* using a 5 kDa cutoff Vivaspin 20 concentrator (GE Healthcare). Preliminary screening for initial crystallization conditions was performed by the hanging-drop vapour-diffusion method using Crystal Screen and Crystal Screen 2 (Hampton Research, USA) at 298 K by mixing 2 µl droplets of concentrated protein solution with an equal volume of reservoir solution in 24-well Linbro plates (Hampton Research, USA). Crystals obtained from the conditions (i) 2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5 and (ii) 2 *M* ammonium sulfate, 0.1 *M* Na HEPES pH 7.5, 2%(*v*/*v*) PEG 400. Conditions were further optimized using various concentrations of ammonium sulfate *versus* a pH range of 7–8.5 in the presence or absence of 2%(*v*/*v*) PEG 400. Crystals of good diffraction quality (Fig. 1) appeared in about 2 d from 2 *M* ammonium sulfate, 0.1 *M* Na HEPES pH 7, 2%(*v*/*v*) PEG 400.

2.4. Data collection and processing

Before data collection, the crystals were quick-soaked in a cryoprotectant solution containing 10%(*v*/*v*) glycerol, 2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.0 and 2%(*v*/*v*) PEG 400 and flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data were collected on an in-house Rigaku R-AXIS IV^{++} image-plate detector using Cu *Kα* X-rays generated by a Rigaku Micromax HF007 rotating-anode generator. The crystal-to-detector distance was kept at 150 mm and the crystal was rotated through a total of 180° with 0.5° rotation per frame. The crystal diffracted to a resolution of 2.3 Å. The 360 frames were processed with *XDS* (Kabsch, 1993) in space group *P*2 and the resulting R_{merge} was 4.7% overall. The space group was determined to be $P2_1$ observing the systematic absences and was confirmed with *POINTLESS* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Scaling was performed

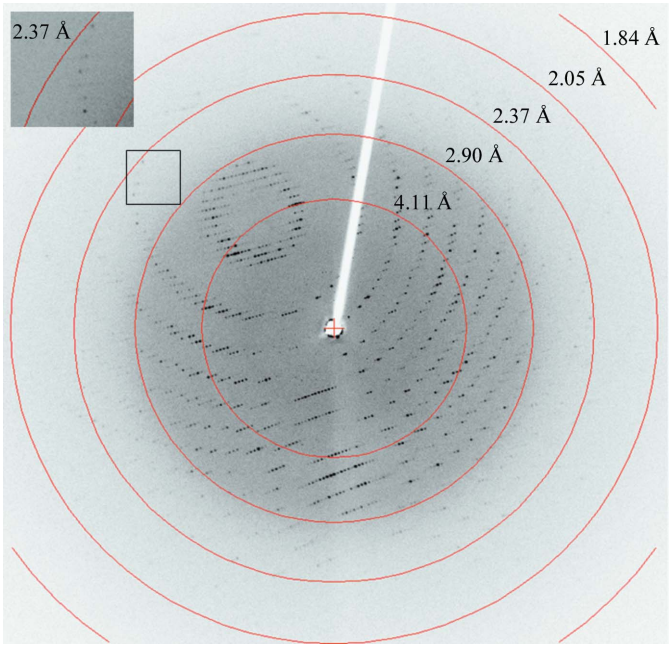


Figure 2
Diffraction image of a typical SaPrx crystal.

using *SCALA* (Evans, 1993) from the *CCP*4 suite. The final statistics of data collection and processing are tabulated in Table 1.

3. Results and discussion

SaPrx, an atypical 2-Cys peroxidase from *S. aureus*, was successfully cloned in *E. coli* and purified to homogeneity with an N-terminal His_6 tag. The molecular weight of monomeric His_6 SaPrx of 18.04 kDa predicted from the sequence was confirmed by 15% SDS–PAGE. The protein was crystallized from 2 *M* ammonium sulfate, 0.1 *M* Na HEPES pH 7, 2%(*v*/*v*) PEG 400. SaPrx crystals diffracted to 2.3 Å resolution (Fig. 2) and belonged to a monoclinic space group, with unit-cell parameters a = 43.50, b = 149.36, c = 73.74 Å, β = 104.4°. Although the highest probability of six molecules per asymmetric unit suggested a Matthews coefficient of 2.15 $Å^3$ Da^{-1} (Matthews, 1968), *MOLREP* (Vagin & Teplyakov, 1997) found the best solution to contain four molecules per asymmetric unit with a V_M of 3.22 $Å^3$ Da^{-1} (solvent content of 61.85%). The tetrameric association was supported by analysis of the packing of the molecules in the unit cell with no unoccupied electron density. The phase was obtained using the crystal structure of a thiol peroxidase from *Streptococcus pneumoniae* (PDB code 1psq; 52% identity; R. Kniewel, J. Buglino, V. Solorzano, J. Wu & C. D. Lima, unpublished work). The resulting R factor decreased to 40.5% and the correlation coefficient was improved to 0.64. The final model building and restrained refinement using *REFMAC*5 (Murshudov *et al.*, 1997) are currently in progress.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 43.50, b = 149.36, c = 73.74, β = 104.4
Resolution (Å)	19.85–2.3 (2.4–2.3)
Total No. of observations	143640 (17570)
No. of unique reflections	40106 (5709)
Completeness (%)	99.4 (97.4)
Multiplicity	3.4 (3.1)
Average $I/\sigma(I)$	20.6 (6.1)
R_{merge}† (%)	0.047 (0.341)
Monomer per ASU (Z)	4
Matthews coefficient (V_M) ($Å^3$ Da^{-1})	3.22
Solvent content (%)	61.85

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of a reflection and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

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