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Crystallization and preliminary X-ray diffraction analysis of an oxidized state of Ohr from *Xylella fastidiosa*

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Xylella fastidiosa organic hydroperoxide-resistance protein (Ohr) is a dithiol-dependent peroxidase that is widely conserved in several pathogenic bacteria with high affinity for organic hydroperoxides. The protein was crystallized using the hanging-drop vapour-diffusion method in the presence of PEG 4000 as precipitant after treatment with organic peroxide (*t*-butyl hydroperoxide). X-ray diffraction data were collected to a maximum resolution of 1.8 Å using a synchrotron-radiation source. The crystal belongs to the hexagonal space group $P6_522$, with unit-cell parameters $a = b = 87.66$, $c = 160.28$ Å. The crystal structure was solved by molecular-replacement methods. The enzyme has a homodimeric quaternary structure similar to that observed for its homologue from *Pseudomonas aeruginosa*, but differs from the previous structure as the active-site residue Cys61 is oxidized. Structure refinement is in progress.

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1. Introduction

An increase in reactive oxygen species (ROS) is an important component of the defence response against microbial infection in plants and animals. ROS can cause damage to macromolecules such as DNA, lipids and proteins (Akaike *et al.*, 1992; Tenhaken *et al.*, 1995). This host response has led bacteria to evolve several complex mechanisms to detoxify ROS such as hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{-\cdot}$) and organic hydroperoxides (OHPs). The cellular enzymatic defences for detoxification of free radicals and H_2O_2 are well characterized and several structures of proteins involved in this mechanism have been solved. However, the structures of bacterial proteins involved in OHP metabolism are comparatively poorly characterized. Alkyl hydroperoxide reductase (AhpR) is frequently considered to be the main protein in OHP metabolism (Wood *et al.*, 2002). This enzyme has the ability to convert OHPs into their respective alcohols at the expense of NADH or NADPH. AhpR consists of two subunits: the reductase subunit F (AhpF) and the catalytic subunit C (AhpC) (Poole & Ellis, 1996; Poole, 1996). AhpC is a thiol-dependent peroxidase that belongs to a large family of peroxidases named peroxiredoxins (Chae *et al.*, 1994). Mongkolsuk *et al.* (1998) characterized a novel gene in the phytopathogen *Xanthomonas campestris* pv. *phaseoli* involved in OHP detoxification, denominated *ohr* (organic hydroperoxide resistance). The deletion of this gene in *X. campestris* rendered the mutants highly sensitive to OHPs but not to other oxidants. Additionally, the expression of *ohr* was highly induced by OHPs, but was not induced by a superoxide generator and was only weakly induced by H_2O_2. Recently, it was observed that organic hydroperoxide-resistance protein (Ohr) but not AhpR seemed to play a significant role in OHP resistance in *Bacillus subtilis* (Fuangthong *et al.*, 2001) and that *ohr* overexpression in an *Escherichia coli ahpC-ahpF* double mutant caused it to revert to the hypersensitive to OHPs phenotype (Mongkolsuk *et al.*, 1998).

At present, several proteins similar to Ohr have been characterized in other organisms. Ohr belongs to a family of proteins that are present only in bacteria, most of which are pathogenic to plants or animals (Ochsner *et al.*, 2001; Fuangthong *et al.*, 2001; Rince *et al.*, 2001; Cussiol *et al.*, 2003).

Analysis of Ohr-protein sequences from several bacteria showed that all homologues possess two conserved cysteine residues located in domains that show different characteristics in their amino-acid composition (Cussiol *et al.*, 2003). The C-terminal cysteine (Cys125 in *X. fastidiosa*) is located among several hydrophilic residues and is part of a Val-Cys-Pro motif which is also present in all proteins of the peroxiredoxin family (Mongkolsuk *et al.*, 1998; Cussiol *et al.*, 2003). On the other side, the cysteine residue located at the N-terminus (Cys61 in *X. fastidiosa*) is surrounded by several hydrophobic residues and is inserted into a new motif: Ala-Cys-Phe.

Both cysteines are essential for the peroxidase activity of Ohr (Lesniak *et al.*, 2002; Cussiol *et al.*, 2003).

Recently, the X-ray structure of homodimeric Ohr from *P. aeruginosa* with a novel α/β fold was reported (Lesniak *et al.*, 2002). The Ohr structure contains two active-site pockets on opposite sides of the dimer. The two conserved cysteines (Cys60 and Cys124) in each active site come from the same monomer and the distance between the S atoms is 3.6 Å. The entrances to the pockets are rich in hydrophobic side chains, which may be related to the high specificity of Ohr towards OHPs. Cys124 is located at the bottom of the cavity, while the side chain of Cys60 is solvent-exposed. The three-dimensional structure of *P. aeruginosa* Ohr was solved using crystals grown in the presence of dithiothreitol (DTT) and the electron-density maps showed a DTT molecule bound to each active site near Cys60. The N^{ε} atom of Arg18 makes a hydrogen bond to a DTT O atom. Arg18 is highly conserved among Ohr proteins and has been shown to play a role in catalysis, since its replacement by glutamine led to a significant decrease in OHP reduction (Lesniak *et al.*, 2002).

X. fastidiosa is the causative agent of a number of economically important crop and citrus diseases and possesses an Ohr protein (Simpson *et al.*, 2000). Two-dimensional gel-electrophoresis/mass-spectrometry analysis revealed that Ohr is highly abundant in whole cell extract and in the extracellular fraction (Smolka *et al.*, 2003), indicating an important role in pathogen resistance to host defences. Ohr from *X. fasdidiosa* is a thiol-dependent peroxidase and catalyzes the following reaction: 2*R*SH + *R*OOH $\rightarrow$ *R*SS*R* + *R*OH + H_2O (Cussiol *et al.*, 2003). The enzyme is able to decompose hydroperoxides and is about 10–20 times more efficient in the removal of organic hydroperoxide than of hydrogen peroxide. Additionally, Ohr only decomposes peroxides in the presence of dithiols such as DTT. No decomposition of peroxides is detected when DTT was replaced by monothiols such as GSH, 2-mercaptoethanol or cysteine (Cussiol *et al.*, 2003).

We report here the crystallization and preliminary X-ray analysis of Ohr from *X. fastidiosa* in an oxidized state. The structure was solved by molecular-replacement methods and structure refinement is in progress. The analysis of the Ohr structure in the oxidized state should provide insights into the enzymatic mechanism of this protein and may provide reasons for the higher efficiency of this protein towards organic hydroperoxides as well as towards dithiol compounds.

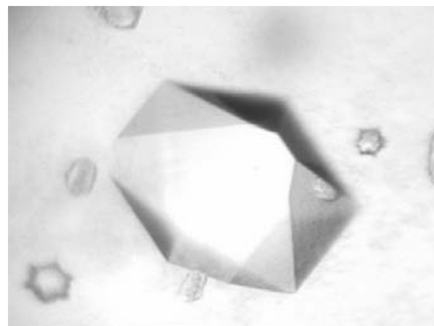

Figure 1
Crystal of Ohr from *X. fastidiosa*.

2. Methods

2.1. Cloning

The 432 base-pair *ohr* gene (NP_299113.1) was amplified by PCR from the cosmid XF-07F02 used in the *X. fastidiosa* genome-sequencing project (Simpson *et al.*, 2000) and cloned in the pET15b vector (Novagen) using *Nde*I–*Bam*HI restriction sites. The resulting pET15b/*ohr* was sequenced in an Applied Biosystems ABI Prism 377 96 to confirm that the construction was correct.

2.2. Expression and purification

Escherichia coli BL21 (DE3) strain harbouring pET15b/*ohr* plasmid was grown (50 ml) overnight in LB medium containing 100 µg ml^{-1} ampicillin at 310 K and transferred to 1 l of fresh LB/amp medium and cultured further at 310 K until the OD_{600} reached 0.6–0.8. Expression was induced with 1 m*M* of IPTG and the cells were harvested after 4 h of incubation at 310 K. The cell pellet was resuspended in starting buffer (20 m*M* sodium phosphate buffer pH 7.4). Seven cycles of 30 s sonication followed by 30 s on ice were applied to the cell suspension. The cell extract was kept on ice during treatment with 1% streptomycin sulfate for 15 min. The suspension was centrifuged at 31 500*g* for 30 min to remove nucleic acid precipitate. Finally, the cell extract was applied to a nickel-affinity column and purified with an imidazole gradient as described by the manufacturer (Hi-trap column; Amersham-Pharmacia Biotech). We obtained ~50 mg of pure protein from 1 l of cell culture. The purity of the protein was confirmed by SDS–PAGE and the peroxidase activity was measured using the FOX method (Wolf, 1994). The purified protein was concentrated to 10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5.

2.3. Crystallization

After treatment with 10 m*M* *t*-butyl hydroperoxide (*t*-BOOH) at 310 K for 1 h, the samples were used in crystallization trials by the hanging-drop vapour-diffusion method. Initial screening was performed at 293 K using Crystal Screen and Crystal Screen II from Hampton Research, mixing equal volumes (2 µl) of protein solution (10 mg ml^{-1} in 5 m*M* Tris–HCl) and reservoir solution. Several conditions were refined and the optimal conditions were obtained with the reservoir solution consisting of 25% PEG 4000 and 0.1 *M* Tris–HCl pH 8.7. The crystals reached dimensions of 0.25 × 0.25 × 0.05 mm after two weeks (Fig. 1).

2.4. Data collection and processing

A crystal cryoprotected with 20% glycerol was cooled to 110 K and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B-CPR at the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. The wavelength was set to 1.453 Å and a MAR CCD detector was used to record the oscillation data with $\Delta\varphi = 1.0°$. The data set was processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Kabsch, 1988; Blessing, 1995) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Ohr crystallization trials using protein without OHP treatment resulted in crystals of poor diffraction quality. In previous work, Cussiol *et al.* (2003) showed the existence of two bands of purified protein on SDS–PAGE and that the amount of each band was dependent on the redox state of Ohr. We found that pre-treatment of Ohr with 10 m*M* *t*-BOOH resulted in the formation of several crystal forms of high quality. *t*-BOOH treatment was chosen because this experimental condition converts Ohr to a highly homogenous oxidized state (Cussiol *et al.*, 2003). These data and those obtained by Lesniak *et al.* (2002) suggest that treatment of Ohr with reductants or oxidants is important in order to maintain the protein in a homogeneous state suitable for crystallization procedures. The crystals studied here were obtained from protein exposed to a large excess of peroxide. Therefore, we expect that after treatment with 10 m*M* *t*-BOOH, Ohr will be in a highly oxidized state, probably as the sulfinic acid (*R*-SO_2) or sulfonic acid (*R*-SO_3).

The best crystal diffracted to 1.8 Å resolution and belonged to space group $P6_522$, with unit-cell parameters $a = b = 87.66$, $c = 160.28$ Å. Table 1 summarizes the data-collection statistics.

The protein structure was solved by molecular-replacement methods with the program *AMoRe* (Navaza, 2001) from the *CCP*4 package using the atomic coordinates of *P. aeruginosa* Ohr (Lesniak *et al.*, 2002) as the search model (PDB code 1n2f; 58% sequence identity). The solution shows one dimer in the asymmetric unit, in agreement with the Matthews coefficient calculation (Matthews, 1968), giving $V_M = 2.8$ Å^3 Da^{-1} and a solvent content of 55.4%. Initial rigid-body refinement was carried out using *AMoRe*, yielding an *R* factor of 0.470. Model completion and refinement are currently in progress.

The *X. fastidosa* Ohr tertiary and quaternary structures are similar to those observed for its counterpart from *P. aeruginosa*, but differ in the redox state of the N-terminal active-site residue. In our case the N-terminal cysteine (Cys61) is oxidized, while in the previous structure of *P. aeruginosa* the corresponding residue (Cys60) is in a reduced state. We expect that the structure of *X. fastidiosa* Ohr will assist in understanding the catalytic mechanism concerning the high specificity for OHPs and dithiol compounds in this important protein family.

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

Space group	$P6_522$
Unit-cell parameters (Å)	$a = b = 87.66$, $c = 160.28$
Resolution limits (Å)	34.3–1.80 (1.90–1.80)
Total No. reflections	372394
No. unique reflections	34390
Completeness (%)	99.9 (99.9)
Multiplicity	10.8 (11.1)
R_{sym} (%)	7.4 (32.8)
$\langle I/\sigma(I)\rangle$	6.2 (2.2)

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