Structural Insights into Enzyme–Substrate Interaction and Characterization of Enzymatic Intermediates of Organic Hydroperoxide Resistance Protein from Xylella fastidiosa

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Organic hydroperoxide resistance proteins (Ohr) belong to a family of proteins that possess thiol-dependent peroxidase activity endowed by reactive cysteine residues able to reduce peroxides. The crystal structure of Ohr from Xylella fastidiosa in complex with polyethylene glycol, providing insights into enzyme–substrate interactions is described herein. In addition, crystallographic studies, molecular modeling and biochemical assays also indicated that peroxides derived from long chain fatty acids could be the biological substrates of Ohr. Because different oxidation states of the reactive cysteine were present in the Ohr structures from X. fastidiosa, Pseudomonas aeruginosa and Deinococcus radiodurans it was possible to envisage a set of snapshots along the coordinate of the enzyme-catalyzed reaction. The redox intermediates of X. fastidiosa Ohr observed in the crystals were further characterized in solution by electrospray ionization mass spectrometry and by biochemical approaches. In this study, the formation of an intramolecular disulfide bond and oxidative inactivation through the formation of a sulfonic acid derivative was unequivocally demonstrated for the first time. Because Ohr proteins are exclusively present in bacteria, they may represent promising targets for therapeutical drugs. In this regard, the structural and functional analyses of Ohr presented here might be very useful.

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Keywords: Ohr crystal structure; fatty acid peroxide; organic peroxide; thiols; sulfonic acid

Introduction

Oxidative burst is an important component of the host response against bacterial infection in plants and animals. Oxidants can cause damage to macromolecules such as DNA, lipids and proteins.¹ ² In response to this oxidative burst, several complex mechanisms have evolved in bacteria to detoxify H₂O₂, superoxide anion radicals and organic hydroperoxides (OHP).³–⁵ Lipids are major targets during oxidative burst as free radicals can directly attack unsaturated and polyunsaturated fatty acids in membranes and initiate lipid peroxidation. In addition to the non-enzymatic process, lipid peroxidation may also be catalyzed by enzymes such as lipoxygenases and dioxygenases in response to pathogen infection.⁶ A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can significantly alter the properties of membrane-bound proteins.⁷ ⁸ Therefore, lipid peroxidation is an extremely deleterious process.

Abbreviations used: AhpC, alkyl hydroperoxide reductase subunit C; AhpE, alkyl hydroperoxide reductase subunit F; AhpR, alkyl hydroperoxide reductase; OHP, organic hydroperoxides; DrOhr, organic hydroperoxide-resistance protein from Deinococcus radiodurans; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Ohr, organic hydroperoxide-resistance protein; Ohrₘ₀, mildly oxidized Ohr; Ohrₒₒ, fully oxidized Ohr; PaOhr, organic hydroperoxide-resistance protein from Pseudomonas aeruginosa; PEG, polyethylene glycol; ESI, electrospray ionization; t-BOOH, tert-butyl hydroperoxide.

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Insights into Ohr–Substrate Interaction

Two crystal structures of Ohr from *X. fastidiosa* are reported here. In the first structure (1.8 Å resolution), the reactive cysteine is oxidized to a sulfonic acid (Cys-SO$_3$H), which has never been described before. The second structure (2.4 Å resolution) corresponds to a mixture of different oxidation states, as confirmed by mass spectrometry experiments. Structural analyses together with biochemical approaches provide a detailed characterization of the redox intermediates and the catalytic cycle of Ohr proteins. We also describe that sulfonic acids in XfOhr are only formed after long periods of incubation with OHP in large excess and that this reaction leads to oxidative inactivation. Finally, these multiple approaches provided insights into enzyme–substrate interactions.

**Results**

The crystal structures of *X. fastidiosa* Ohr

Structures of *X. fastidiosa* Ohr were determined in different oxidative states from two crystals, whose proteins were fully oxidized (XfOhr$_{fo}$) in one case, and mildly oxidized (XfOhr$_{mo}$) in the other. In both cases, the final atomic models include two crystallographically independent monomers (referred to as A and B) forming a homodimer. The XfOhr$_{fo}$ model was refined at 1.8 Å resolution ($R_{factor}=0.196$) and the quality of the electron density allowed modeling of amino acid residues 2 to 143 (out of 143) in monomer A and 3 to 143 in monomer B. The XfOhr$_{mo}$ model was refined at 2.4 Å resolution to a crystallographic R factor of 0.192. Amino acid residues 3 to 143 were modeled in both monomers. As expected, both models present very similar tertiary and quaternary structures. The overall rms deviation for 276 C$^\alpha$ positions between XfOhr$_{fo}$ and XfOhr$_{mo}$ dimers is 0.22 Å. Analysis of the packing shows that residues involved in crystal contacts are different in monomers A and B. Superposition of XfOhr$_{fo}$ and XfOhr$_{mo}$ structures shows that the contacts involving monomer A are almost identical in both structures. In the case of monomer B, crystallographic neighbors are slightly closer in the XfOhr$_{mo}$ structure (a rigid body displacement) but no significant conformational change is observed in the contact regions (data not shown). Global structural analysis and comparisons with homologous structures were performed using the high resolution XfOhr$_{fo}$ structure. A superposition of the structures of *X. fastidiosa* Ohr and its counterparts from *Pseudomonas aeruginosa* (PaOhr, 66% identity) and *Deinococcus radiodurans* (DrOhr, 50% identity) results in overall rms deviations of 1.00 Å (268 C$^\alpha$ aligned) and 1.31 Å (262 C$^\alpha$ aligned), respectively (Figure 1). As expected, no significant differences were found in the global structure. As previously described, Ohr is an elliptically shaped homodimer with the two monomers tightly intertwined. Two six-stranded β-sheets surround a two-helix central core forming a barrel-like

and the detoxification of its products is crucial to bacterial survival and proliferation in the host. OHP is one of the most toxic products of lipid peroxidation that possesses bactericidal properties.

Alkyl hydroperoxide reductase (AhpR) is commonly considered the most important defense protein in OHP detoxification. AhpR is capable of converting OHP into their respective alcohols at the expense of NADH or NADPH. This enzyme system consists of two subunits: alkyl hydroperoxide reductase F (AhpF) and alkyl hydroperoxide reductase C (AhpC). AhpC is a thioldependent peroxidase that belongs to a large family of enzymes named peroxiredoxins.

In addition to AhpR, organic hydroperoxide resistance protein (Ohr) has also been implicated in the defense of bacteria against OHP-induced stress. The first description of Ohr came from studies of the phytopathogen *Xanthomonas campes-tris* pv. phaseoli. The deletion of the Ohr gene from *X. campes-tris* rendered the mutants highly sensitive to OHP but not to other oxidants. Additionally, ohr expression was highly induced by OHP, but not by a superoxide generator and was only weakly induced by H$_2$O$_2$. Furthermore, Ohr but not AhpR seems to play a significant role in OHP resistance in *Bacillus subtilis* and ohr overexpression in *Escherichia coli* double mutant AhpC/AhpF reverted the phenotype of hypersensitive to OHP. Recently, Klomsiri et al. showed that the Ohr and not AhpC is required for the adaptive response of *X. campes-tris* pv. phaseoli to alkyl hydroperoxide derived from linoleic acid. Collectively, these results indicate that Ohr plays a primary role in OHP detoxification.

Ohr proteins are only present in bacteria, most of which are pathogenic to plants and animals. Ohr from *Xylella fastidiosa* (XfOhr), the causative agent of crops and citrus diseases, is a thiol-dependent peroxidase that catalyzes the following reaction:

\[
2RSH + ROOH \rightarrow RSSR + ROH + H_2O.
\]

The enzyme is able to decompose OHP at least one order of magnitude more efficiently than H$_2$O$_2$. Additionally, *in vitro* XfOhr only decomposes peroxides in the presence of dithiols such as DT. No decomposition of peroxides is detected when DT is replaced by monothiols such as GSH, 2-mercaptoethanol or cysteine.

Analysis of Ohr-protein sequences from several bacteria showed that all homologues possess two conserved cysteine residues. The replacement of cysteine for serine residues abolishes the peroxidase activity (1.00 Å (268 C$^\alpha$ aligned) and 1.31 Å (262 C$^\alpha$ aligned), respectively (Figure 1). As expected, no significant differences were found in the global structure. As previously described, Ohr is an elliptically shaped homodimer with the two monomers tightly intertwined. Two six-stranded β-sheets surround a two-helix central core forming a barrel-like...
Each β-sheet is composed of three strands from one monomer and three from the other. Four short helices complete the dimer organization. The two conserved cysteine residues that compose the active site come from the same monomer, the two active site pockets being located on opposite sides of the dimer.

Active site comparisons and basis for the substrate binding

Despite the overall similarity of their structures, PaOhr, DrOhr and the two forms of X. fastidiosa Ohr show interesting differences in their active sites. In the structure of PaOhr, the catalytic cysteine residues are in the reduced state (Cys-SH) and a bound DTT molecule was found in the two active site pockets. The conserved Arg18 (N\text{H}1) from one subunit forms a hydrogen bond with the reactive Cys60 (S\text{g}) from the other subunit and also forms a salt bridge with the invariant Glu50. Arg18 appears to play an important role in the catalytic mechanism by lowering the pK\text{a} value of the Cys60 thiol, since the R18Q mutation severely compromises the enzyme activity.\textsuperscript{14} In the structure of DrOhr the distance between the Cys (S\text{r}) atoms of the two catalytic cysteine residues (Cys57 and Cys121) is 2.8 Å, which is consistent with a mixture of a disulfide bond (2.2 Å) and dithiol configuration (3.7 Å). In this structure, the loop containing the Arg15 (equivalent to Arg18 in PaOhr) adopts a conformation that moves the arginine away from the active site pocket.\textsuperscript{18}

In the XfOhr\textsubscript{es} structure the electron density maps clearly showed the Cys61 (equivalent to Cys60 in PaOhr and Cys57 in DrOhr) fully oxidized to a sulfonic acid group (Cys-SO\textsubscript{3}H) (Figure 2(a)). On the other hand, in the XfOhr\textsubscript{mo} structure the electron density maps suggested that the reactive cysteine was present in more than one oxidative state, similarly to DrOhr. The \( F_o - F_c \) electron density map clearly showed the presence of additional atoms when a Cys-SH was modeled (Figure 2(b)), nevertheless a sulfonic acid group also did not fit well to the electron density after refinement. Setting the occupancies of the oxygen atoms to 0.4 we were able to model the SO\textsubscript{3} group. An additional peak in the \( F_o - F_c \) electron density map suggesting a disulfide form was also observed (Figure 2(c)). These results indicated that a mixture of different oxidation states was present in the crystal. In fact, mass spectrometry experiments performed under conditions where XfOhr\textsubscript{mo} crystals grew showed that Cys61 is in a mixture of sulfhydryl, disulfide and overoxidized forms (see Supplementary Data). Since different enzyme redox intermediates were present in the XfOhr\textsubscript{mo} crystal, the resolution of this structure provided important information on the catalytic intermediates, as will be further discussed below.

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**Figure 1.** Overall structural comparison between Ohr from different species. Stereo ribbon diagram illustrating the superposition of Ohr from *X. fastidiosa* (red), *P. aeruginosa* (green) and *D. radiodurans* (blue). *X. fastidiosa* Ohr catalytic cysteine residues are represented in CPK.
Interestingly, in XfOhr<sub>fo</sub> and XfOhr<sub>mo</sub> structures the loop containing Arg19 (equivalent to Arg18 in PaOhr and Arg15 in DrOhr) follows the conformation found in reduced PaOhr structure, bringing the arginine side-chain close to the active site (Figure 3). In XfOhr<sub>fo</sub>, Arg19 conformation is further stabilized by a number of interactions with the SO<sub>3</sub>H group of the overoxidized Cys61, in addition to the salt bridge with the conserved Glu51 (see Supplementary Data, Figure S1A). The catalytic cysteine residues are engulfed and surrounded by several hydrophobic amino acids (see Figure S1B of Supplementary Data).

During the last refinement cycles, the Fourier difference maps clearly showed an elongated electron density in the entrance of both active site pockets. The electron density was interpreted as corresponding to a polyethylene glycol (PEG) molecule derived from the crystallization solution (see Materials and Methods). An ethylene glycol polymer with 17 non-hydrogen atoms could be modeled, which corresponds to a PEG molecule of approximately 400 Da (Figure 4(a)). It is well known that commercially available PEG reagents contain a mixture of molecules of different molecular masses, which could explain the electron density for a lower molecular mass PEG than that used for crystallization. Another possibility is that only a portion of the molecule is ordered in the crystal and thus visible in the electron density maps.

The XfOhr active site pocket perfectly accommodates a PEG molecule that lies above the Arg19 side-chain and is involved in several hydrophobic contacts (Figure 4(b)). Notably, side-chains of residues Phe68, Phe96*, Val36* and Pro126 undergo significant displacement when compared to equivalent residues in PaOhr and DrOhr structures (Figure 4(c)). Furthermore, the entire loop Gly90–Phe96 undergoes a displacement upon PEG binding (not shown).

Very similar electron density was found in both active sites of both structures (XfOhr<sub>fo</sub> and XfOhr<sub>mo</sub>), the position and conformation of the modeled PEG molecules being essentially the same (not shown). Monomers A and B are involved in different crystal contacts and the entrance of both active sites is highly exposed to the solvent, their structures not being stabilized by the crystal packing. These results suggest that the PEG binding is not a mere artifact induced by crystallization, but it may mimic the binding of the endogenous substrates. In fact, recent works have shown that PEG molecules can occupy sites and channels that naturally belong to long chain fatty acids.<sup>19–21</sup> Interestingly, a peroxide molecule derived from oleic acid can be easily modeled into the PEG electron density with the peroxide function falling very close to Cys61 (Figure 5 and Figure S1C of Supplementary Data). Therefore, we propose here that physiological substrates of Ohr proteins may

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**Figure 2.** Oxidation states of the reactive cysteine in the XfOhr<sub>fo</sub> and XfOhr<sub>mo</sub> structures. 2F<sub>o</sub>−F<sub>c</sub> maps are shown in blue and contoured at 1.5σ while F<sub>o</sub>−F<sub>c</sub> maps are shown in red and contoured at 2.8σ. (a) XfOhr<sub>fo</sub> shows Cys61 fully oxidized to sulfonic acid. (b) XfOhr<sub>mo</sub> refinement with the cysteine in the reduced form clearly shows the presence of additional density. (c) A sulfonic acid group could be modeled when the occupancies of the oxygen atoms were set to 0.4. Final refinement of XfOhr<sub>mo</sub> also shows an additional peak in the F<sub>o</sub>−F<sub>c</sub> electron density map suggesting a disulfide form.

**Figure 3.** Active site of X. fastidiosa Ohr. In X. fastidiosa Ohr the loop containing the Arg19 (equivalent to Arg18 in PaOhr and Arg15 in DrOhr) adopts a conformation as in the reduced PaOhr structure (Ohr<sub>fo</sub> is shown in red, Ohr<sub>mo</sub> in yellow, PaOhr in green and DrOhr in blue). The DrOhr atomic coordinates (PDB code 1USP) does not include the side-chain atoms of Arg15. We modeled a rotamer to illustrate the loop movement.
possess an elongated shape, such as peroxides derived from long chain fatty acids and lipoamide. Accordingly, XfOhr was able to reduce hydroperoxides derived from long chain fatty acids such as oleic acid, as observed by preliminary experiments (data not shown). Unfortunately, we could not establish enzymatic parameters for the decomposition of peroxide derived from oleic acid among other reasons because the solvents used to solubilize this oxidant, inhibited XfOhr activity. We are working on the establishment of a suitable protocol to determine the catalytic constants.

Characterization of Ohr catalytic intermediates

As described in the previous section, Cys61 of XfOhrfo is in the sulfonic acid form, which is not reducible by standard reducing agents such as DTT and thioredoxin. It is well known that when thiol-dependent peroxidases have their reactive cysteine

Figure 4. PEG binding in the X. fastidiosa Ohrfo active site pocket. (a) Final $2F_o - F_c$ map contoured at 1.5σ showing the PEG electron density. (b) Protein–PEG hydrophobic interactions according to LIGPLOT analysis. Hydrophobic interactions are shown in green. (c) Stereo image of active site residues superposition of XfOhrfo (red), PaOhr (green) and DrOhr (blue). The asterisk (*) refers to residues from the other monomer.

Figure 5. Model of binding of a fatty acid hydroperoxide to X. fastidiosa Ohr. A hydroperoxide derived from oleic acid (OPZ cis-hydroperoxide octadec 10 enoic acid) was manually modeled based on the PEG electron density. Note that the oxygen atoms of the peroxide function (PF) fall nearby the reactive cysteine side-chain (Cys61).
residues over oxidized they are rendered inactive unless sulfiredoxin is present in the reaction mixture. Thus, we decided to verify whether XfOhr is susceptible to oxidative inactivation by treatment with a large excess of peroxides. Since XfOhr can accept electrons from lipoic acid, XfOhr activity can be followed by decay in $A_{340\ nm}$ due to NADH absorption according to the pathway described below. A similar approach was described for a thiol-dependent peroxidase from *Mycobacterium tuberculosis*.

NADH → lipoamide dehydrogenase (bovine)
→ lipoamide → Ohr → peroxide

Initially, XfOhr activity was determined in the presence of various concentrations of peroxides and no inactivation was observed even when large excess of peroxides were employed (Figure 6(a)). Next, we decided to analyze the kinetics of inactivation after pre-incubation of XfOhr with amounts of peroxides equivalent to those employed in the crystallization trials (17$t$-BOOH:1XfOhr and 1$t$-BOOH:1XfOhr). After various intervals of incubation (indicated in the X-axis of Figure 6(b)), the excess of peroxide was removed by ultra-filtration and XfOhr activity was determined. Considerable inactivation was achieved only after 3 h of pre-incubation and only when XfOhr was incubated with large excess of $t$-BOOH (Figure 6(b)). Reversely, XfOhr was highly resistant to inactivation by H$_2$O$_2$, even under a condition where this peroxidase was extensively inactivated by $t$-BOOH (Figure 6(c)).

These findings are consistent with our previous observation that only OHP, but not H$_2$O$_2$, can over oxidize XfOhr. This observation was based on the fact that in non-reducing SDS-PAGE the disulfide intermediate of XfOhr migrates faster than the other states. In fact, it is well known that proteins with an intra-molecular disulfide bond migrate faster in non-reducing SDS-PAGE than the other oxidation states. Therefore, we decided to mix XfOhr and peroxides in the same proportion (17 peroxides:1 XfOhr) used to obtain the XfOhr$_{cr}$ crystals (Figure 7(a)). Two bands were detected for the non-treated XfOhr, which probably corresponds to the dithiol (band b) and the intra-molecular disulfide bond (band a), as described. XfOhr treated with DTT migrated preferentially at position b (lane 2), whereas H$_2$O$_2$ induced formation of band a (lane 7), as described. XfOhr treated with diamide also provoked the formation of band a (lane 9). XfOhr pretreated with H$_2$O$_2$ or diamide and then treated with DTT migrated preferentially at position b (lanes 8 and 10, respectively), as would be expected for an intra-molecular disulfide reduction. These results represent further experimental evidence that an intramolecular disulfide is a redox intermediate of XfOhr because diamide induces only a disulfide bridge as an oxidation product.

Interestingly, treatment of XfOhr with a large excess of OHPs did not induce the formation of band a (lanes 3 and 5), suggesting again that in this case XfOhr is over oxidized. In fact, no alteration in XfOhr migration was detected for the samples previously oxidized with OHPs and then treated.
with DTT (lanes 4 and 6), which is consistent with the fact that the sulfonic acids are not reducible by DTT. It is important to emphasize here that OHPs induce formation of band a, but only when added at stoichiometric amounts.

Since reduced and over oxidized forms of Ohr are expected to migrate to the same position (band b) in SDS-PAGE, we decided to investigate the formation of sulfhydryl groups in this anti-oxidant protein after treatment of Ohr with various reductant compounds. Formation of sulfhydryl groups in XfOhr was monitored by reaction with DTNB, the Ellman’s reagent. About 60% of XfOhr sulfhydryl groups reacted with DTNB after reduction with DTT (Figure 7(b)). These results probably reflect the fact that XfOhr cysteine residues are embedded in the polypeptide structure (see Figure S1B of Supplementary Data) and therefore their accessibility to DTNB would be difficult. In fact, all the XfOhr sulfhydryl groups reacted with DTNB when reduction by DTT was carried out with protein denatured by 1% (w/v) SDS (data not shown). Again, the crystallization conditions used to obtain XfOhrmo were simulated and oxidants were added in large excess over XfOhr. As expected, all of the oxidants decreased the amount of XfOhr sulfhydryl groups (Figure 7(b)). After elimination of the excess of these oxidants, XfOhr was treated with DTT in an attempt to regenerate their sulfhydryl groups. Regeneration of sulfhydryl groups only occurred when XfOhr was treated with oxidants under conditions expected to generate the intramolecular disulfide intermediate: that is H2O2 and diamide. Regeneration of sulfhydryl groups did not occur for XfOhr treated with OHP, which is consistent with the observation that only these compounds can over oxidize and inactivate XfOhr when added in large excess. The fact that H2O2 and diamide-treated XfOhr were reducible by DTT is further experimental evidence that an intra-molecular disulfide bond is in fact an intermediate of the catalytic cycle (Figure 7(b)). Accordingly, isoeletrofocusing gels indicated that more acidic forms of XfOhr were formed only when this protein was exposed to OHP for long intervals (data not shown).

In the structure of DrOhr, reactive cysteine is present as a mixture of oxidative states, among them an intramolecular disulfide form, which is consistent with our proposal that this intermediate is only generated under mild oxidative conditions. So far, we have presented results that further indicate that this intermediate is in fact formed in solution. To unequivocally demonstrate the identity of redox intermediates of XfOhr in solution, mass spectrometry experiments with native and trypsin-digested enzyme were carried out (see Supplementary Data, Figures S2 and S3 and explanatory text for experimental details). In agreement with our biochemical assays, we observed intramolecular disulfide bond formation by mass spectrometry experiments when XfOhr was treated with H2O2 and diamide (Table 1). t-BOOH also induced intramolecular disulfide bond formation but only when added at much lower doses (1 t-BOOH: 1 XfOhr). Contrary to the differential reactivity towards cysteine residues, both H2O2 and t-BOOH oxidized methionine residues (Table 1).

**Discussion**

A detailed characterization of the molecular mechanism of a protein belonging to the Ohr family was performed using multiple approaches. XfOhrmo structure and biochemical assays have unequivocally shown that an intramolecular disulfide is a redox intermediate of XfOhr in solution (Table 1). Furthermore, the elucidation of XfOhr structure in complex with PEG and comparisons with Ohr from other species, which are in different oxidative states, provides a set of snapshots along the coordinate of the enzyme-catalyzed reaction
(Figure 8). In the reduced form (Figure 8(i)), the Arg19 containing loop is closed and the respective guanidine group is stabilized by a salt bridge with conserved Glu51 and an H-bond with Cys61 (Sg), as seen in PaOhr structure.14 Arg19 is likely to make a major contribution to the stabilization of the Cys61 thiolate anion. In the next step (Figure 8(ii)), the substrate binds to the active site. In vitro, different types of peroxides can be reduced by Ohr,13,14 but according to the structure of XfOhr–PEG complex, the active site can better accommodate elongated molecules which interact with the hydrophobic

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ND, not determined.

a Control Ohr activity (without any pre-treatment) was considered to be 100%, therefore, 0% of inactivation. The values described in this column were obtained by the difference between control activities minus the activity after Ohr treatment by high doses (17 oxidant:1 XfOhr) of oxidants (see the legend to Figure 6(c)).

b Recovery of sulfhydryl groups after exposure of XiOhr to high doses of oxidants (17 oxidants:1 XiOhr). Experimental details are described in the legend to Figure 7(b). Recovery was calculated considering the amount of sulfhydryl group in the protein reduced by DTT as 100%.

c Redox states described in this column were proposed based on the fast migration on intra-molecular disulfide bond relative to the other oxidative states.13 Again, XiOhr was treated with high doses of oxidants (17:1).

d Oxidative states determined by native and trypsin digested XiOhr under various conditions. See Supplementary Data for experimental details.

e Disulfide was only observed when XiOhr was mildly oxidized (1 XiOhr:1 t-BOOH).

Structure described by Lesniak et al.14

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Figure 8. Proposed molecular mechanism of lipid hydroperoxides reduction by Ohr. (i) Reduced form of the enzyme (Cys61-S\(^{\cdot}\)). The guanidine group of Arg19* is stabilized by a salt bridge with conserved Glu and an H-bond with Sg of Cys61, as seen in PaOhr structure.14 Arg19 is likely to make a major contribution to the stabilization of the Cys61 thiolate anion. In the next step (Figure 8(ii)), the substrate binds to the active site. In vitro, different types of peroxides can be reduced by Ohr,13,14 but according to the structure of XfOhr–PEG complex, the active site can better accommodate elongated molecules which interact with the hydrophobic...
moiety of the Arg19 side-chain, stabilized by a number of additional hydrophilic interactions (Figure 4). Remarkably, the oxygen atoms of the peroxide function of modeled fatty acid peroxide appear nearby Cys61 (Figure 5). After peroxide decomposition (Figure 8(iii)), Cys61 is oxidized to sulfenic acid (Cys-\(\text{SOH}\)) which is quickly attacked by the sulphydryl group of the resolving cysteine (Cys125) forming an intra-molecular disulfide. The loop containing Arg19 detaches, releasing the product (an alcohol derivative) and consequently, Ohr adopts the open conformation, as seen in DrOhr structure.14,15 Finally, the last step (Figure 8(iv)) involves the reduction of the disulfide by a redox partner and a rearrangement of the loop, bringing the Arg19 side-chain back to the active site. The detailed molecular mechanism involved in this step remains to be elucidated, since the biological partner of Ohr protein is not known, although lipoamide has been considered as a candidate.13,18

According to this model, Ohr can be over-oxidized to sulfonic acid (Cys-\(\text{SO}_2\text{H}\)) if the sulfenic acid (Cys-\(\text{SOH}\)) intermediate reacts with two peroxide molecules, probably in two steps. Since the sulfenic acid of Cys61 is probably very reactive towards Cys125, its over oxidation to sulfonic acid should occur only when very high concentrations of peroxide are employed, which is consistent with our data obtained through several biochemical assays (Table 1).

At this point, it is elucidative to compare Ohr and OsmC structures. Four structures from three different proteins belonging to different species (E. coli, Mycoplasma pneumoniae and Thermus thermophilus) were reported.16,17,27,28 The overall quarten-ary structures are very similar, but variations can be observed specially in the N-terminal region corresponding to the loop where arginine 19 is located in XfOhr. The N-terminal loops in OsmC proteins are longer and contain a more extended \(\beta\)-strand. Whereas Glu51 is conserved in all OsmC structures, the function of Arg19 of XfOhr is replaced by a non-conserved arginine residue that is located farther away (Arg39 in E. coli).16,17,27 Furthermore, other residues in the active site of Ohr are replaced in OsmC. As an example, OsmC presents a higher content of aliphatic amino acids (e.g. Phe) and other substitutions, such as tyrosine 58 and 127 of XfOhr are replaced in OsmC for His and Val residues, respectively. These differences, among others, are reflected in the different shapes of OsmC and Ohr active sites.16,17,27

Interestingly, in the case of OsmC protein from T. thermophilus, the reactive cysteine is also over oxidized but in a sulfinate (Cys-\(\text{SO}_2\text{H}\)) instead of a sulfonate state (Cys-\(\text{SO}_3\text{H}\)).17 In this case, one of the oxygen atoms of the sulfinate is stabilized by two hydrogen bonds with N\(^\circ\) of arginine 37. In the case of XfOhr, no evidence of sulfinic acid formation was obtained by crystal structure and by mass spectrometry experiments (see Supplementary Data, Figures S1A, S2 and S3 and explanatory text). Instead, our results indicate that the formation and stabilization of a sulfonate is strongly favored. Two oxygen atoms (OD1 and OD2) of the sulfonylated cysteine in XfOhr\(_{19}\) structure form H-bonds with the N\(^\circ\) and N\(^\circ\)\(^1\) of arginine 19, respectively. The third oxygen atom (OD3) interacts with the sulfur of Cys125 (Figure S1A of Supplementary Data).

Further experimental support for the strong stabilization of sulfonylated cysteine came from mass spectrometry experiments. In fact, peaks corresponding to the XfOhr dimer during mass spectrometry experiments were detected only when this enzyme was pre-treated under conditions in which the sulfonlated cysteine is expected to form (data not shown).

The hydrophobicity of the active site (see Figure S1B of Supplementary Data) might prevent cysteine residues from being exposed to high concentrations of hydrophilic peroxides, such as \(\text{H}_2\text{O}_2\), and consequently avoid over oxidation of this residue to sulfonate state. In fact, all of the reduced cysteine residues in XfOhr were only detected when this protein was previously denatured by SDS (data not shown). The hydrophobicity of the XfOhr active site may be related to the fact that this peroxidase is only reduced by dithiols like DTT and DHLA but not by monothiols,13 because these reductants are hydrated. Since the reduction of a disulfide bond by a monothiol requires two molecules of reductants whereas reduction by a dithiol requires only one molecule, less water might need to be removed, when compounds with two sulfhydryl groups are utilized as substrates by XfOhr. In any case, apparently there is no steric restraint for the accommodation of two monothiol molecules into the XfOhr active site as shown by molecular modeling (data not shown). It is also possible that this specificity relies on the higher redox potentials of dithiols in comparison with monothiols.

In addition to hydrophobicity, our studies also suggested that the biological Ohr substrate/partner can be molecules of elongated shape. In this regard, the heterologous system containing NADH, bovine lipoamide dehydrogenase and lipoamide, was shown to efficiently support XfOhr peroxidase activity. In a similar approach described for a thiol-dependent peroxidase from Mycobacterium tuberculosis, Bryk et al.24 utilized a system containing lipoamide bound through an amide linkage to dihydrolipoamide succinyltransferase, which was more efficient than the heterologous one. This kind of structural organization situates the lipoamide molecule in a manner to construct an elongated shaped arm-like structure. The genome analysis shows that the lipoamide containing enzymes are present in X. fastidiosa and thus it may be a possible XfOhr reductant system.13

The results presented here also support the hypothesis that peroxides derived from long chain fatty acids can be substrates of Ohr (Figures 4
and 5). These insights into enzyme–substrate interaction came in part from the analysis of XfOhr structures in complex with PEG (Figure 5). Interestingly, electron density from a PEG molecule was only observed in XfOhr amongst all OsmC and Ohr structures described. DrOhr crystallization condition contains PEG 4000,16 the same PEG used to obtain XfOhr crystals. We believe that because in the DrOhr structure the N-terminal Arg loop adopts an open conformation that corresponds to the situation of substrate release (Figure 8(iv)), this should avoid PEG/substrate binding. In the case of PaOhr, the catalytic cysteine residues are in the reduced state (closed conformation), suitable for PEG/substrate binding (Figure 8(ii)). However, the presence of a DTT molecule in the active site might have avoided the accommodation of a PEG molecule, even when this precipitant was present in the mother liquor. Finally, the absence of PEG molecules in the OsmC structures might be related with the differences in the shapes of their active sites. It is noteworthy the fact that in E. coli OsmC, a phenylalanine residue is bound to the entrance of the active site, suggesting that the substrate of these enzymes might be aromatic hydroperoxides,27 instead of elongated and hydrophobic molecules. Finally, the exclusive presence of PEG in the active site of XfOhr among all OsmC/Ohr described structures perhaps is somehow related with the fact that only this enzyme possesses their reactive cysteine residues in the sulfonate state which provoked a stronger stabilization of the closed conformation of the Arg loop (Figure S1A of Supplementary Data).

In cells, OHPs should be generated by oxidation of double bonds in both mono and polyunsaturated fatty acids. Despite that the majority of the bacterial fatty acids are saturated, a considerable fraction of these biomolecules are monounsaturated in certain pathogenic bacteria.29-31 Recently, nuclear magnetic resonance spectroscopy studies revealed that X. fastidiosa also possesses a large amount of oleic (18:1), palmitoleic (16:1) and vaccenic (18:1) fatty acids. Despite that the majority of the bacterial fatty acids are saturated, a considerable fraction of these biomolecules are monounsaturated in certain pathogenic bacteria.29-31 Recently, nuclear magnetic resonance spectroscopy studies revealed that X. fastidiosa also possesses a large amount of oleic (18:1), palmitoleic (16:1) and vaccenic (18:1) fatty acids.32 We are currently investigating the reduction of peroxides derived from unsaturated fatty acids by Ohr. Nevertheless, preliminary results indicate that peroxide derived from linoleic acid is reduced by XfOhr with efficiency comparable with t-BOOH, therefore, at least one order of magnitude higher than H2O2.

Table 2. Crystal parameters and data collection statistics

<table>
<thead>
<tr>
<th>Ohr crystals</th>
<th>Ohrmo</th>
<th>Ohrmo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P6122</td>
<td>P6122</td>
</tr>
<tr>
<td>Unit-cell parameters (Å)</td>
<td>a = b = 87.6 and c = 160.2</td>
<td>a = b = 91.5 and c = 157.6</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>34.30–1.80 (1.90–1.80)</td>
<td>79.31–2.40 (2.53–2.40)</td>
</tr>
<tr>
<td>Total no. reflections</td>
<td>370,764</td>
<td>117,837</td>
</tr>
<tr>
<td>No. unique reflections</td>
<td>34,330</td>
<td>15,924</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.9)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>10.8 (11.1)</td>
<td>7.4 (7.1)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>7.4 (32.8)</td>
<td>6.1 (26.7)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>6.2 (2.2)</td>
<td>10.3 (2.8)</td>
</tr>
</tbody>
</table>

* Oliveira et al.33

Materials and Methods

Crystalization and data collection

X. fastidiosa Ohr was expressed, purified and crystallized as described.33 In an attempt to obtain the crystal structure of XfOhr in different oxidative states, after purification, XfOhr was incubated with several redox agents such as H2O2, tert-butyl hydroperoxide (t-BOOH), diamide and DTT at different concentrations. Afterwards, crystallization trials were performed using the hanging-drop vapor diffusion method and crystals were obtained from protein samples incubated with 10 mM or 0.58 mM of t-BOOH at 310 K for 1 h. These treatments correspond to molecular proportions of 17 t-BOOH to 1 XfOhr and 1 t-BOOH to 1 XfOhr, respectively. The optimal crystallization condition was obtained with the reservoir solution consisting of 25% (w/v) PEG 4000 and 0.1 M Tris–HCl buffer (pH 8.7). The crystals obtained from samples treated with 10 mM t-BOOH (XfOhrmo) reached 0.25 mm × 0.25 mm × 0.05 mm after two weeks. The crystals obtained from samples treated with 0.58 mM t-BOOH (XfOhrmo) reached 0.20 mm × 0.20 mm × 0.05 mm after eight weeks. The crystals, cryo-protected by 25% glycerol, were cooled to 110 K and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B at the Brazilian Synchrotron Light Laboratory. Data sets were indexed using the program MOSFLM34 and the resulting intensities were scaled and merged using the program SCALA35,36 from the CCP4 package.37 Unit cell parameters and data collection statistics are shown in Table 2. The data for XfOhrMo have been reported by Oliveira et al.33 and are repeated here for comparison.
Determination of XfOhr activity by lipoamide system

An assay to measure XfOhr activity was developed based on the ability of this enzyme to utilize lipoic acid as substrate. XfOhr aliquots (300 µM) were pretreated for one week at 4 °C with the following oxidants: diamide, H₂O₂ or l-BOOH at 1:1 stoichiometry (oxidant/protein). The excess of oxidants was removed by PD-10 desalting columns (Amersham Biosciences). XfOhr was also submitted to similar pre-treatment with H₂O₂. XfOhr activity was then measured by NADH consumption under the conditions described above using the lipoamide assay system.

Structure determination and refinement

The structures were solved by molecular replacement using the program AMoRe. The XfOhrfo structure was solved using the atomic coordinates of P. aeruginosa Ohr (PDB code 1N2F) as the search model. Crystallographic refinement was carried out using the CNS package. Initial model for XfOhrmo was obtained from the XfOhrfo coordinates. Structure refinement was performed using the program REFMAC 5.0. In both cases, refinement cycles were alternated with visual inspection of the electron density maps and model rebuilding with the program O. During the final cycles water molecules were introduced using the program Water Pick from the CNS package for the XfOhrfo structure and ARP/wARP in the case of XfOhrmo. At this stage of the XfOhrmo refinement, the Fo − Fc electron density map clearly showed the presence of additional atoms when the reactive cysteine was modeled in the reduced form (Cys-SH), nevertheless a sulfonic acid (Cys-SO₃H) also showed the presence of additional atoms when the reactive cysteine was modeled in the reduced form (Cys-SH), nevertheless a sulfonic acid (Cys-SO₃H).

Table 3. Refinement statistics of X. fastidiosa Ohr crystals

<table>
<thead>
<tr>
<th>Ohr crystals</th>
<th>Ohrfo</th>
<th>Ohrmo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflections</td>
<td>32,616</td>
<td>15,130</td>
</tr>
<tr>
<td>Working</td>
<td>1714</td>
<td>794</td>
</tr>
<tr>
<td>Main-chain</td>
<td>2342</td>
<td>2294</td>
</tr>
<tr>
<td>Non-hydrogen atoms (except waters)</td>
<td>256</td>
<td>182</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>19.6:21.4</td>
<td>19.2:24.3</td>
</tr>
<tr>
<td>r.m.s.d. values</td>
<td>0.005</td>
<td>0.012</td>
</tr>
<tr>
<td>Bonds</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Angles</td>
<td>94.1</td>
<td>95.6</td>
</tr>
<tr>
<td>Additional, allowed regions</td>
<td>5.9</td>
<td>4.4</td>
</tr>
<tr>
<td>PDB code</td>
<td>1ZB9</td>
<td>1ZB8</td>
</tr>
</tbody>
</table>

Determination of sulfhydryl groups content in XfOhr

XfOhr samples (5 mM Tris–HCl buffer (pH 7.5)) were treated with redox agents as described above at the same concentrations for 90 min or one week. After treatment the samples were diluted to 0.58 nM and mass spectrometric experiments were performed in a ESI quadrupole time of flight mass spectrometer (Q-Tof Ultima API, Micromass) coupled with a capillary HPLC system (CapLC; Micromass, UK). For protein mass measurement experiments, the samples (6 µl, corresponding to 3.5 nmol) were desalted on-line using a C18 trap column (Optipak C18; Waters). The protein was eluted from the trap column using a 1:1 water/acetonitrile+0.1% (v/v) formic acid solution. The protein spectra were deconvoluted using the MaxEnt 1 program as supplied by the manufacturer.

For the LC-MS/MS analysis, the samples were digested with trypsin for 4 h at 37 °C in 50 mM NH₄HCO₃. The peptides (6 µl) were desalted on-line and eluted using a gradient of 0–60% (v/v) acetonitrile for 1 h. The spectra were acquired using the Data Directed Analysis, selecting the doubly and triply charged peptides for MS/MS experiments. All the MS/MS spectra were processed using the Proteinlynx Global Server software and the MASCOT search engine (Matrix Science, Boston).

Electrospray ionization mass spectrometry

Protein sample solutions in 5 mM Tris–HCl buffer (pH 7.5) were treated with reductant agents as described above at the same concentrations for 90 min or one week. After treatment the samples were diluted to 0.58 nM and mass spectrometric experiments were performed in a ESI quadrupole time of flight mass spectrometer (Q-Tof Ultima API, Micromass) coupled with a capillary HPLC system (CapLC; Micromass, UK). For protein mass measurement experiments, the samples (6 µl, corresponding to 3.5 nmol) were desalted on-line using a C18 trap column (Optipak C18; Waters). The protein was eluted from the trap column using a 1:1 water/acetonitrile+0.1% (v/v) formic acid solution. The protein spectra were deconvoluted using the MaxEnt 1 program as supplied by the manufacturer.

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† http://www.pymol.org


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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.03.054

**References**


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