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Structural and Functional Characterization of Ohr, an Organic Hydroperoxide Resistance Protein from *Pseudomonas aeruginosa*

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Pathogenic bacteria have developed complex strategies to detoxify and repair damage caused by reactive oxygen species. These compounds, produced during bacterial aerobic respiration, as well as by the host immune system cells as a defense mechanism against the infectious microorganisms, have the ability to damage nucleic acids, proteins, and phospholipid membranes. We have determined the crystal structure of Pseudomonas aeruginosa Ohr, a member of a recently discovered family of organic hydroperoxide resistance proteins. Using in vitro assays, we demonstrate that Ohr is a novel hydroperoxide reductase, converting both inorganic and organic hydroperoxides to less toxic metabolites. Structure-based mutagenesis reveal that the Ohr catalytic mechanism is similar to that of the structurally unrelated peroxiredoxins, directly utilizing highly reactive cysteine thiol groups to elicit hydroperoxide reduction.

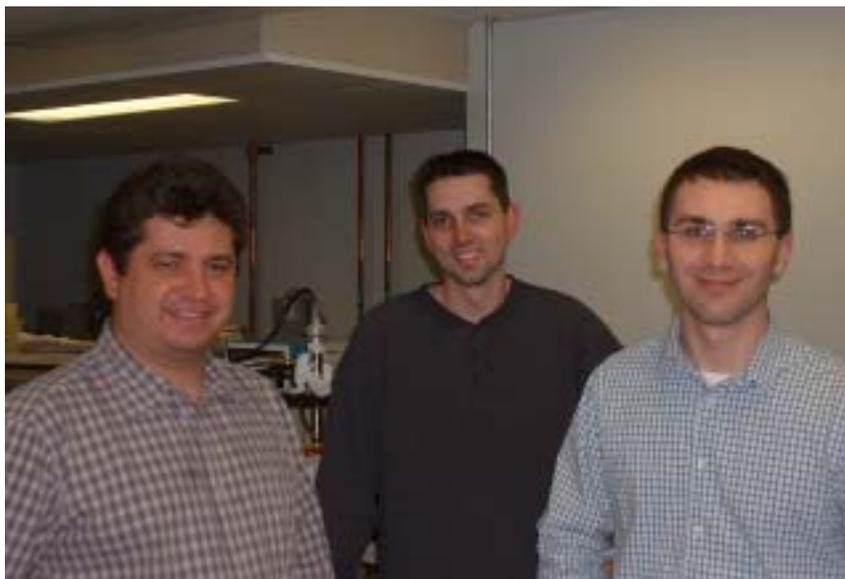
Pseudomonas aeruginosa, a versatile and ubiquitous Gram-negative bacterium, is one of the major causes of opportunistic human infections, such as bacteremia in burn victims, urinary tract infections in catheterized patients, pneumonia, as well as a significant cause of morbidity and mortality in cystic fibrosis patients. During the course of host infection, *P. aeruginosa* is exposed to a variety of reactive oxygen species, including organic hydroperoxides. Their detoxification is essential for bacterial survival and proliferation, and genes involved in protection against organic peroxide toxicity play important roles in host-pathogen interactions.

A recently described family predicted to have such properties encompasses the organic hydroperoxide resistance (Ohr) and the osmotically inducible (OsmC) proteins. Prior to our experiments, the molecular mechanism that Ohr and OsmC utilize to protect bacteria from organic hydroperoxides had not been identified. We, therefore, determined the crystal structure of *P. aeruginosa* Ohr revealing an oval-shaped molecule that lacks significant structural similarity to other proteins. Ohr is a tightly folded a/b homodimer with two

putative active sites located at the monomer interface on opposite sides of the molecule. Each of the active sites contains two invariant cysteines, which are ideally located to form an intramolecular disulfide bond. The structural details of the Ohr active site suggest that the molecule functions as an organic hydroperoxide reductase, which was confirmed using *in vitro* enzyme assays.

Structure based mutagenesis was

used to clarify the enzymatic mechanism and the role of the invariant cysteine residues, documenting that Cys-60 is indispensable for Ohr enzymatic activity, and that Cys-124, while not absolutely required for activity, is necessary for the maintenance of a high peroxidase reaction rate. We postulate that the interaction between Cys-60 and Arg-18 in Ohr has a major role in lowering the pK_a of the thiol side chain of Cys-60, polarizing and stabilizing it in the ionized state,



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which is more nucleophilic and therefore more reactive than the unionized form. The increased reactivity of the Cys-60 thiol group allows it to function as the initial attacking residue as follows: First, Cys-60 reacts with a peroxide molecule (ROOH), in the process becoming transiently oxidized to a

cysteine sulfenic acid (Cys-60-SOH) intermediate. A molecule of peroxide is simultaneously reduced to its corresponding alcohol (ROH). Second, the Cys-60-SOH group quickly reacts with the reduced thiol group of Cys-124, forming an intramolecular disulfide bond and releasing a molecule of water: Cys-

60-SOH + HS-Cys-124 \rightarrow Cys-60-S-S-Cys-124 + H₂O. Finally, oxidized Ohr is regenerated back to its enzymatically active, reduced state using a yet unidentified endogenous protein or a small-molecule reductant.

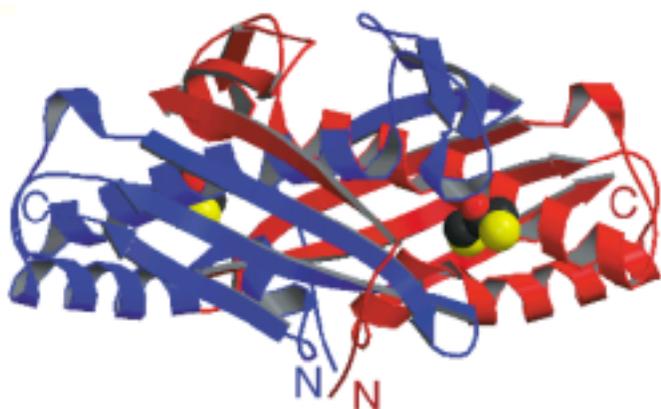


Figure 1. A Structure of the Ohr dimer bound to DTT. One monomer is in red, the other in blue. DTT is shown in CPK format, with oxygen atoms in red, carbon atoms in black and sulfur atoms in yellow.

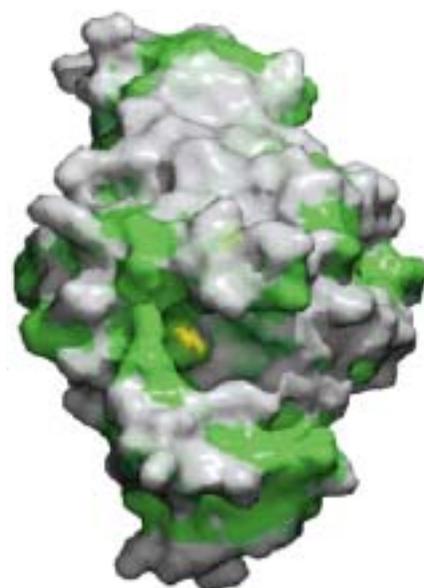


Figure 2. Molecular surface rendering of the Ohr dimer. The catalytically active Cys-60 (yellow) lies at the bottom of the active-site pocket, which is outlined with hydrophobic residues (green).