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Backbone and side chain ^1H , ^{13}C , and ^{15}N NMR assignments for the organic hydroperoxide resistance protein (Ohr) from *Burkholderia pseudomallei*

Garry W. Buchko

Biological Sciences Division, Pacific Northwest National Laboratory, Mail-Stop K8-98, P.O. Box 999, Richland, WA 99352, USA

Stephen N. Hewitt

Department of Medicine, University of Washington, Seattle, WA 98185-7185, USA

Alberto J. Napuli

Department of Biochemistry, University of Washington, Seattle, WA 98185-7185, USA

Wesley C. van Voorhis

Department of Medicine, University of Washington, Seattle, WA 98185-7185, USA

Department of Biochemistry, University of Washington, Seattle, WA 98185-7185, USA

Peter J. Myler

Seattle Biomedical Research Institute, 307 Westlake Avenue N, Seattle, WA 98109-5219, USA

Abstract

Burkholderia pseudomallei is a NIAID Category B microorganism responsible for melioidosis. Here we report backbone and side chain NMR assignments for the 139-residue, homodimeric, organic hydroperoxide resistance protein (Ohr) from this organism.

Keywords

Melioidosis; Infectious diseases; Biological warfare agent; Perdeuterated proteins; Host-pathogen interactions; Seattle Structural Genomics Center for Infectious Disease (SSGCID)

Biological context

The mission of the Seattle Structural Genomics Center for Infectious Disease (SSGCID) is to determine structures for target proteins from NIAID Category A-C agents and emerging and re-emerging infectious disease organisms (www.ssgcid.org). One of the agents included in this list is *Burkholderia pseudomallei*, an aerobic, Gram-negative, soil-dwelling bacterium responsible for the infectious disease melioidosis (Wiersinga et al. 2006). Melioidosis (aka Whitmore's disease) is a serious health problem in Southeast Asia and Northern Australia,

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Biological Sciences Division, Pacific Northwest National Laboratory, Mail-Stop K8-98, P.O. Box 999, Richland, WA 99352, USA
garry.buchko@pnl.gov.

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accounting for 40% of all sepsis-related mortality in northeast Thailand. *Burkholderia pseudomallei* is also of concern to the US Centers for Disease Control and Prevention because of its potential use in biological warfare and biological terrorism. Present in the sequenced genome of *B. pseudomallei* is an open reading frame for a 139-residues protein with high sequence similarity to organic hydroperoxide resistance proteins (*Bp*-Ohr). Ohr proteins convert toxic exogenous and endogenous organic hydroperoxides (OHPs) into less toxic metabolites. Because OHP detoxification appears to be an important component in host-pathogen interactions (Mongkolsuk et al. 1998) and Ohr proteins are exclusively observed in bacteria (Atichartpongkul et al. 2001), these proteins are potential drug targets as their inactivation may assist the host in defeating a pathogenic attack. A three-dimensional structure of *Bp*-Ohr will serve as a useful blueprint for structure-based drug design of new therapeutics. Towards determining the solution structure of *Bp*-Ohr, we have assigned the majority of the backbone and side chain resonances for *Bp*-Ohr in the reduced state, the first reported solution assignments for an Ohr protein.

Materials and methods

Cloning, expression, and purification

The *Bp*-Ohr gene (YP_336021.1) was amplified using the genomic DNA of *B. pseudomallei* strain 1710b (Q3JK82) and the oligonucleotide primers 5'-GGGTCCTGGTTTCGATGAACATTCTGTACAAGACGGCG-3' (forward) and 5'-CTTGTTTCGTGCTGTTTATTACGCGACGACGAGGCGCACC-3' (reverse; Invitrogen, Carlsbad, CA). The amplified *Bp*-Ohr gene was then inserted into the *NraI/PmeI* digested expression vector AVA0421 such that a 21-residue tag containing six consecutive histidine residues was present at the N-terminal of the expressed protein. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) cells (Novagen, Madison, WI) by a heat shock method. Expression of double-labeled (^{13}C , ^{15}N) and triple-labeled ($\sim 50\%$ ^2H , ^{13}C , ^{15}N) protein was induced with isopropyl β -D-1-thiogalactopyranoside at 298 K following previously described protocols (Buchko et al. 2005) using cells grown in minimal media (750 mL) with 100% H_2O and $\sim 50\%$ D_2O , respectively. A sample containing only ^{13}C - and ^{15}N -labeled cysteine was also prepared to assist the assignment (Buchko et al. 2005). Labeled *Bp*-Ohr was purified with a conventional, two-step protocol involving Ni-NTA affinity purification (Qiagen, Valencia, CA) followed by gel-filtration chromatography on a Superdex75 HiLoad 26/60 column (Amersham Pharmacia Biotech, Piscataway, NJ) that simultaneously exchanged *Bp*-Ohr into the buffer used for the NMR studies (100 mM NaCl, 20 mM Tris, 1.0 mM dithiothreitol, pH 7.1).

Nuclear magnetic resonance spectroscopy

The NMR data on the double- and triple-labeled samples (~ 1.5 mM) were collected at 298 K using Varian Inova -600, -750, and -800 spectrometers equipped with triple resonance probes and pulse field gradients. The double-labeled (^{13}C , ^{15}N) samples were used to collect NOESY data and the triple-labeled ($\sim 50\%$ ^2H , ^{13}C , ^{15}N) samples used to collect backbone assignment data. Pulse sequences for the two- and three-dimensional experiments were from Biopack (Varian) using the following experiments: HNCOC, HNCACB CBCA(CO)NH, C(CO)NH-TOCSY, HBHA (CO)NH, ^{15}N HSQC-NOESY and ^{13}C -HSQC-NOESY. To assist the acquisition of chemical shifts for the $^{13}\text{C}\beta$ atoms the HNCACB experiment was optimized to detect the β -carbons ($\tau_{\text{CC}} = 7$ ms, $\text{D1} = 2$ s). The NOESY experiments were used extensively to assist proton side chain assignments due to poor proton sensitivity in the backbone experiments using the triple-labeled ($\sim 50\%$ ^2H , ^{13}C , ^{15}N) sample. An overall rotational correlation time (τ_{c}) for *Bp*-Ohr was estimated from backbone amide ^{15}N $T_{1\rho}/T_1$ ratios (Szyperski et al. 2002). To determine if oxidation/reduction of a predicted disulfide bond in the protein resulted in significant structural changes, ^1H - ^{15}N HSQC spectra were collected

on ^{15}N -labeled *Bp*-Ohr (~1 mM) at 0, 1, 10, 50, and 100 mM DTT. All NMR data was processed using Felix2007 (MSI, San Diego, CA) software and analyzed with the program Sparky (v3.115). Uncorrected ^1H , ^{13}C , and ^{15}N chemical shifts were referenced to DSS (DSS = 0 ppm) using indirect methods and deposited in the BioMagResBank database (www.bmrb.wisc.edu) under the accession number BMRB-16144. Note that *Bp*-Ohr contains a N-terminal, 21-residue tag and these residues have been labeled with an asterisk in Fig. 1.

Results and discussion

While monomeric *Bp*-Ohr has a molecular weight of 16.7 kDa, (14.4 kDa plus a 2.3 kDa, 21-residue N-terminal tag), both size exclusion chromatography and NMR spectroscopy were consistent with a protein twice the size. Using a flow rate of 2.5 mL/min, *Bp*-Ohr eluted off a 26/60 Superdex75 size exclusion column with a retention times of 76 min, a value characteristic of an ~35 kDa protein. NMR spectroscopy provided additional evidence that *Bp*-Ohr formed a dimer in solution. First, the estimated isotropic overall rotational correlation time (τ_c) for the protein, as inferred from ^{15}N spin relaxation times (Szyperski et al. 2002), was 13.1 ns at 298 K, a value consistent with a protein in the 30 kDa range. Second, poor magnetization transfer was observed for most of the 3D backbone assignment experiments using the double-labeled sample (^{13}C , ^{15}N) sample. Such experimental difficulties are typical of proteins with molecular weights > 30 kDa (Kay and Gardner 1997; Buchko et al. 2005). On the other hand, magnetization transfer was improved significantly using the triple-labeled sample (~50% ^2H , ^{13}C , ^{15}N) in combination with TROSY, enabling unambiguous assignment of most of the amide cross peaks in the ^1H - ^{15}N HSQC spectrum of *Bp*-Ohr as shown in Fig. 1. The observation that *Bp*-Ohr forms a dimer in solution corroborates with the structures of other organic hydroperoxide resistance proteins in the RSCB Protein Data Bank showing the protein crystallizing as a homodimer (Lesniak et al. 2002; Meunier-Jamin et al. 2004).

As illustrated in Fig. 1, most of the cross peaks in the ^1H - ^{15}N HSQC spectrum of *Bp*-Ohr could be unambiguously assigned. Excluding the first seven residues in the N-terminal tag, amide cross peaks were unidentifiable for L49 and two small regions of the protein, L59-S60 and Y124-S125. These latter two regions are adjacent to the two conserved cysteine residues in the protein, C57 and C123, essential for enzyme activity (Lesniak et al. 2002). Missing amide resonances in the ^1H - ^{15}N HSQC spectrum may reflect local disorder in these regions, or, motion on the intermediate timescale (ms to μs ; Buchko et al. 2005). Because the data was collected in the presence of reducing agent, 1 mM DTT, it is unlikely that the disorder about these two regions reflect partial reduction of the C57-C123 disulfide bond. This was confirmed by the analysis of the $^{13}\text{C}^\beta$ chemical shifts for the cysteine residues. The cysteine $^{13}\text{C}^\beta$ chemical shifts normally differ by approximately 10 ppm in the oxidized (~40 ppm) and reduced (~30 ppm) states and the $^{13}\text{C}^\beta$ chemical shift for C57 was 29.1 ppm (C123 $^{13}\text{C}^\beta$ was ambiguous). Furthermore, titration of ^{15}N -labeled *Bp*-Ohr (~1 mM) with 0-100 mM DTT showed no perturbations in the ^1H - ^{15}N HSQC spectra indicating that either the protein was originally isolated in the reduced form, or, there are no major structural changes in the protein's backbone in the reduced and oxidized form as suggested by crystal structures of both states (Meunier-Jamin et al. 2004). Therefore, the absence of ^1H - ^{15}N HSQC cross peaks adjacent to C57 and C123 may reflect intermediate time scale motion that is often associated with binding and catalysis in many proteins (Buchko et al. 2005).

To identify regions of helical and β -strand secondary structure in *Bp*-Ohr, the deviation of the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ chemical shifts from random coil values were calculated. These values are plotted in Fig. 2 and illustrate that the protein contains six β -strands and three α -helices. The sequential alignment of these elements of secondary structure is the same as observed for other organic pyrophosphate resistant proteins (Lesniak et al. 2002; Meunier-Jamin et al. 2004): three β -strands—two α -helices— α -helix— β -strand. Preliminary analyzes of the NOESY data

indicate that the β -strands form one large β -sheet, $\beta 6:\beta 5:\beta 4:\beta 1:\beta 2:\beta 3$, with $\beta 6:\beta 5$ parallel and the remaining strands anti-parallel. By comparison with the published homodimer crystal structures, $\beta 6:\beta 5:\beta 4$ is derived from one monomer and $\beta 1:\beta 2:\beta 3$ from the other.

Assignment of most of the backbone and side chain resonances for *Bp*-Ohr will make it possible to eventually determine its solution structure using conventional NMR-based methods. Such efforts will require slightly more exotic NMR experiments to separate the intermolecular NOEs from the intramolecular NOEs and these efforts are currently in progress. More importantly, these NMR assignments for *Bp*-Ohr are the first for an Ohr protein and make possible detailed studies of protein-substrate interactions for an organic hydroperoxide resistance protein in solution. Consequently, efforts to better characterize the molecular mechanism of OHP detoxification and the search for drugs to inactivate Ohr proteins will be facilitated using chemical shift perturbation (Buchko et al. 2005) and other powerful types of NMR experiments (Zuiderweg 2002).

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Fig. 1.

The ^1H - ^{15}N HSQC spectrum for double-labeled *Bp*-Ohr (~1.5 mM) collected at a proton resonance frequency of 750 MHz, 298 K, in 100 mM NaCl, 20 mM Tris, 1 mM DTT, pH 7.1. Size exclusion chromatography and the estimated correlation time indicate that the 160-residue protein (21-residue tag) is a dimer in solution. The residues for the tag are labeled with an asterisk and assigned side chain resonances identified with a horizontal line. The inset is an expansion of the congested region spectrum

Fig. 2.
Analysis of the observed $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ chemical shift deviations from random coil values for Bp-Ohr where $\Delta\delta^{13}\text{C} = \delta_{\text{Random coil}} - \delta_{\text{Observed}}$. Solid = $^{13}\text{C}^\alpha$, open = $^{13}\text{C}^\beta$. (Random coil values obtained from cns_solve_1.1)