Crystal Structures of the NO- and CO-bound Heme Oxygenase from Neisseria meningitidis

IMPLICATIONS FOR O₂ ACTIVATION*  

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Jonathan Friedman‡, Latesh Lad‡, Rahul Deshmukh§, Huiying Li‡, Angela Wilks§, and Thomas L. Poulos‡‡  
From the ‡Department of Molecular Biology and Biochemistry, Department of Physiology and Biophysics, and Program in Macromolecular Structure, University of California, Irvine, California 92697 and §Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201  

Heme oxygenases catalyze the oxidation of heme to biliverdin, carbon monoxide, and free iron while playing a critical role in mammalian heme homeostasis. Pathogenic bacteria such as Neisseria meningitidis also produce heme oxygenase as part of a mechanism to mine host iron. The key step in heme oxidation is the regioselective oxidation of the heme α-meso-carbon by an actuated Fe(II)-OOH complex. The structures of various diatomic ligands bound to the heme iron can mimic the dioxygen complex and provide important insights on the mechanism of O₂ activation. Here we report the crystal structures of N. meningitidis heme oxygenase (nm-HO) in the Fe(II), Fe(II)-CO, and Fe(II)-NO states and compare these to the NO complex of human heme oxygenase-1 (Lad, L., Wang, J., Li, H., Friedman, J., Bhaskar, B., Ortiz de Montellano, P. R., and Poulos, T. L. (2003) J. Mol. Biol. 330, 527–538). Coordination of NO or CO results in a reorientation of Arg-77 that enables Arg-77 to participate in an active site H-bonded network involving a series of water molecules. One of these water molecules directly H-bonds to the Fe(II)-linked ligand and very likely serves as the proton source required for oxygen activation. Although the active site residues differ between nm-HO and human HO-1, the close similarity in the H-bonded water network suggests a common mechanism shared by all heme oxygenases.

Because of part of the toxicity of free heme, it is necessary for a number of organisms to degrade heme to harmless products that can be readily excreted. In mammals, heme is converted in a series of enzymatic steps to the glucuronidated form of bilirubin, which is readily excreted. Heme oxygenase (HO) initiates this process by catalyzing the oxidation of free heme to biliverdin. Biliverdin reductase next reduces biliverdin to bilirubin (1) followed by glucuronidation of bilirubin by UDP-glucuronosyltransferase (2). Two human isoforms of HO have been identified to date. HO-1 is a 32-kDa protein, which is anchored to the microsomal membrane through a C-terminal hydrophobic tail (3). HO-1 is found at highest levels in the spleen where recycling of erythrocyes takes place but may also function as a cytoprotective molecule against oxidative stress, because biliverdin and its reduced form, bilirubin, may work as potent physiological antioxidants (1). In contrast, HO-2 is a constitutively expressed 36-kDa protein existing primarily in the testes and in some regions of the brain (4). This finding has led to the proposal that one role of HO-2 is the production of carbon monoxide, which is controversially thought to act as a neuronal messenger by stimulating guanylate cyclase activity (5–10).

HO also is present in some pathogenic bacteria such as Corynebacterium diphtheriae, Neisseria meningitidis, and Pseudomonas aeruginosa (11–15). In bacteria, heme oxygenase is a soluble cytoplasmic protein, which takes part in the mining of iron from host heme. Iron is a necessary nutrient required for the survival of most pathogenic bacteria, and the ability of these bacteria to acquire sufficient amounts of iron during infection is essential for such pathogens to propagate disease. Currently, the most studied heme assimilation systems are those of Gram-negative bacteria (16). These bacteria express highly specific outer membrane receptors that bind different heme-containing compounds, extract heme from these compounds, and then transport heme into the bacterial cytoplasm (17, 18). In the Gram-negative bacterium N. meningitidis, a heme oxygenase (nm-HO) also is necessary for use of iron from imported heme and appears to be transcriptionally linked with an outer membrane heme receptor (11). Besides being essential in iron assimilation, nm-HO protects N. meningitidis from heme toxicity by degradation of heme (12).

The overall HO reaction involves a complex sequence of transformations that consumes 3 molecules of O₂ and 7 electrons (Fig. 1). The first electron reduces the ferric heme iron to the ferrous state, which is then followed by binding of a O₂ molecule to form a Fe(II)-O₂ complex. The addition of a second electron and one proton to the oxygen complex generates a reactive hydroperoxy intermediate, Fe(III)-OOH. The specific proton donor and mechanism of O₂ activation in nm-HO is currently unknown. Furthermore, unlike P450s and peroxidases, heme oxygenases do not utilize a Fe(IV)-O oxyferryl intermediate as the active hydroxylating species but instead utilize the electronophilic Fe(III)-OOH hydroperoxy intermediate (19–21), which directly attacks the α-meso-carbon of heme. Of particular interest in the HO reaction cycle is the control of regioselectivity such that only the α-meso-heme position is oxygenated. This differs from the nonenzymatic oxygenation of heme in which all
The heme oxygenase active site prevents the oxidation of all of the meso-heme positions with the exception of the /H9251-meso-position because this heme edge is the most accessible. Furthermore, hydrogen-bonding interactions within the active site may help to orient molecular oxygen toward the /H9251-meso-carbon. Important insights have been gained in HO function by solution of the human HO-1 (22), rat HO-1 (23), and nm-HO (24) crystal structures. The structure of the oxy-HO complex is important for further probing the catalytic mechanism. Unfortunately, stability of the oxy-complex precludes the ready determination of the crystal structure. Of the other two common Fe(II) ligands, CO and NO, NO is the best molecular mimic for O₂ binding. Both O₂ and NO prefer a bent geometry, whereas CO prefers a linear geometry when bound to Fe(II) porphyrins (25–28). Moreover, high resolution structures of globins complexed with O₂ and NO show very similar binding geometries and local interactions in the heme pocket (29). As a result, we chose the NO complex as the best molecular mimic for the oxy-complex and the present paper describes the structure of the NO and CO complexes of Fe(II)-nm-HO.

**MATERIALS AND METHODS**

**Expression of nm-HO**—The Escherichia coli BL21(DE3) strain carrying pWMZ1651 (hemO) was grown overnight at 37 °C in 10 ml of LB medium containing 100 mg/liter ampicillin. The culture was used to inoculate 1 liter of fresh LB-ampicillin media and grown at 37 °C to a final A₆₀₀ nm 0.6–0.8. Expression was induced by the addition of isopropl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. The cells were grown further for 4 h at 28–30 °C and harvested by centrifugation (5500 rpm for 15 min). The resulting cell pellet was green due to the conversion of endogenous E. coli heme to biliverdin by the expressed nm-HO protein.

**Purification of nm-HO**—The cell pellet was resuspended in 20 mm...
Tris-HCl (pH 7.8) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and was allowed to stir at 4 °C for 1 h. Post-stirring cells were lysed by sonication for 1 min for 4–5 times and centrifuged at 18,000 rpm for 1 h. The soluble fraction was applied to a Sepharose-Q Fast Flow column previously equilibrated with 20 mM Tris-HCl (pH 7.6). The column was washed with three volumes of 20 mM Tris-HCl (pH 7.6), and the protein was eluted in the same buffer using a linear gradient of NaCl from 0 to 500 mM. The protein elutes at a concentration of 150–200 mM NaCl, and the peak fractions were pooled together based on SDS-PAGE analysis.

Reconstitution of nm-HO with Heme—The purified protein was dialyzed against 20 mM Tris-HCl (pH 7.6) (2 × 1003 liters) at 4 °C followed by reconstitution with a 2.0–2.5 molar excess of hemin, which produces the nm-HO-heme complex. The sample was then applied to a Sepharose-Q Fast Flow column previously equilibrated with 20 mM Tris-HCl (pH 7.6), and the protein was eluted in the same buffer using a linear gradient of NaCl from 0 to 500 mM. Peak fractions were pooled together based on the highest absorbance ratios at 406/280 nm. The pooled fractions were dialyzed against 20 mM Tris-HCl (pH 7.6) (2 × 1003 liters) and then further purified by S-100 gel filtration chromatography. The nm-HO protein was concentrated by Amicon filtration unit and stored at −80 °C.

Crystal Growth—Optimized conditions for crystallization consisted of vapor diffusion with the reservoir containing 0.1 M Tris-HCl (pH 8.5), 0.2 M sodium acetate, and 32.5% poly(ethylene glycol) 3350, and sitting drops consisted of 5/9262 reservoir buffer and 5/9262 protein stock at 23 mg/ml. Crystals grew within 7 days at room temperature. Single crystals were immediately frozen in a stream of nitrogen, and data were collected using a R-AXIS IV image plate detector. The crystals belong to tetragonal space group P4_2_2_1 with cell dimensions of a = b = 63.17 Å, c = 100.38 Å with 1 molecule/asymmetric unit.

Generation of NO-Bound nm-HO Complex—Crystals of nm-HO were subjected to anaerobic treatment by cycling between extensive degas-
sing and purging with pure N₂ gas for 30–60 min. Fresh dithionite and sodium nitrite solutions were made with degassed water in sealed serum vials. NO-bound crystals were prepared by soaking with 10 mM dithionite and 5 mM sodium nitrite for 30 min inside a glove box before being flash-frozen for data collections.

**Generation of CO-bound nm-HO Complex**—Crystals of nm-HO were soaked with freshly prepared 10 mM dithionite and then placed in a CO high pressure cell (Stanford Synchrotron Radiation Laboratory, beamline 1–5) and incubated at 65 p.s.i. for 10–15 min. The cell was depressurized for 10 s, and the CO-bound crystals were flash-frozen for data collections.

**Data Collection**—All of the data were collected using an in-house R-AXIS IV imaging plate detector equipped with a rotating copper anode x-ray generator with Osmic optics. Crystals were maintained at −160 °C in a stream of nitrogen (Crystal Logic, Los Angeles, CA). Data sets consisted of 130 frames with a φ-rotation of 1°/frame. High resolution data of the NO-bound nm-HO complex were collected using a single crystal at the Advanced Light Source (Berkeley, CA) beamline 5.0.2. Data were integrated and scaled with DENZO and SCALEPACK (30) with rejections performed using ENDHKL (Louis Sanchez, California Institute of Technology) in conjunction with SCALEPACK.

**Model Building and Refinement**—All of the structures were determined by the molecular replacement method using CNS (31) in which the protein moiety of nm-HO (PDB code, 1J77) was the search model. Protein atoms were initially refined by simulated annealing followed by a few cycles of conjugate gradient minimization, water picking, and temperature factor refinement. The program O (32) was used for further adjustment and modeling of protein atoms, ligands, and water molecules. All of the refinements were done with CNS. Backbone geometry was checked in PROCHECK (33), and none of the residues was in the disallowed region. The Fe-ligand distances and geometry were not restrained during refinement. Data collection and refinement statistics for each structure are summarized in Table I. Protein Data Bank coordinates have been deposited in the Protein Data Bank (PDB codes 1P3T, 1P3U, 1P3V).

**RESULTS**

**Reduced Fe(II) Structure**—Unlike human HO-1 where the distal helix moves closer to the heme upon reduction (34), there is little change in structure in nm-HO upon reduction from Fe(III) to Fe(II). The only significant change is that the distal water ligated in the Fe(III) structure is absent in the Fe(II) structure.

**CO and NO Fe(II) Complexes**—Fig. 2 illustrates the H-bonded network in the CO and NO complexes in addition to the human HO-1 NO complex. The nm-HO NO and CO complexes are very similar with the exception that the natural tendency of NO to bend enables the NO to form a better H-bond with Wat1. Given the similarity between O₂ and NO, it is very likely that the oxy and hydroperoxy complexes form a similar H-bonded network. The water network is similar in the HO-1-NO complex with the exception...
that the key side chains participating in the H-bonded network are different. Arg-77 and Asn-118 in nm-HO correspond to Arg-85 and Asp-140 in HO-1. As shown in Fig. 3, Asp-140 participates directly in the H-bonded network in HO-1 while in nm-HO Arg-77 serves this function. In addition, a conformational change is required to position Arg-77 for H-bonding to Wat3. Arg-77 and Asn-118 occupy two alternate conformations. In the Fe(III) and Fe(II) states, Arg-77 is in the “up” position pointing away from the heme. In the Fe(II)-CO and Fe(II)-NO complexes, Arg-77 moves to the “down” position where it participates in the active site H-bonded network (Fig. 4). In order for Arg-77 to move to the down position, Asn-118 must also move to avoid steric crowding. In the nm-HO Fe(II) structure, the electron density for Arg-77 is weak precluding precise positioning of the side chain. However, the electron density for Asn-118 is well defined, and because Arg-77 cannot point down with Asn-118 positioned as shown in the Fe(II) structure (Figs. 2A and 3A), we assume that Arg-77 must point up in the Fe(II) structure. Therefore, it appears that ligand binding triggers a set of conformational changes that forms a rigid H-bonded network involving Ser-117, Arg-77, and three ordered solvent molecules. The solvent molecules also must rearrange upon ligation. For example, Wat3 moves approximately 3 Å in the NO and CO complexes while Wat1 and Wat2 move less than 1 Å. As in HO-1, a combination of steric effects and H-bonding to ordered solvent helps bend the ligand toward the α-meso-heme carbon, which accounts for why both HO-1 and nm-HO hydroxylate only the α-meso-carbon.

**DISCUSSION**

The purpose of this study was to use NO and CO as probes that mimic the oxy and hydroperoxy complexes of nm-HO to better understand how nm-HO activates molecular oxygen and controls regioselective heme hydroxylation. The picture that emerges is very similar to that of human and rat HO-1 (36). It now appears that a common feature of HOs is to establish a rigid network of distal pocket H-bonded water molecules that ensures proper proton delivery to the distal O atom of the iron bound O2 ligand. Furthermore, these H-bonds coupled with steric factors bend the ligand toward the α-meso-heme carbon for regioselective hydroxylation. Further steric control is provided by the distal pocket masking all but the α-meso-carbon from electrophilic attack by the Fe(III)-OOH intermediate.

Of the most studied HOs, human and rat HO-1 and cd-HO from *C. diphtheriae*, nm-HO is the outlier. nm-HO has the least conserved distal pocket environment and has Asn-118 in place of the catalytically essential Asp-140 in HO-1. As a result, the side chains participating in the distal pocket H-bonded network are different in nm-HO than in human HO-1 (Fig. 3). This clearly illustrates that the precise identity of the side chains is not a critical factor in controlling HO activity. Furthermore, the precise charge of side chains also appears not to be important. In HO-1, Asp-140 H-bonds with Wat1 while Ser-117 serves this function in nm-HO. Moreover, in nm-HO, movement in the side chain of Arg-77 is triggered upon ligand binding and may be required for the activation of O2. Interestingly, HO-1 also has a homologous Arg (Arg-85) but Arg-85 in
HO-1 remains fixed in place in the Fe(III), Fe(II), and Fe(II)-NO structures (34). A common feature between HO-1 and 

nm-HO is Wat1, which directly H-bonds to the distal ligand. We suggest that the key to HO activity is the proper H-bonding donor/acceptor relationship and polarization of Wat1 such that Wat1 serves as a proton donor to dioxygen (Fig. 5). Stabilization of the hydroperoxy intermediate also is critical to allow electrophilic attack on the a-meso-heme carbon as opposed to cleavage of the peroxide O=O bond. It now is known that

altering the H-bonded network by mutating Asp-140 to Ala converts HO-1 into a peroxidase (37), very likely because the 

regioselectivity of heme oxygenation by nm-HO is primarily controlled by steric strain, because Ser-117 and Gly-120 both block access to all of the 

meso-positions with the exception of the a-meso-carbon.

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