Roles of Distal Asp in Heme Oxygenase from Corynebacterium diphtheriae, HmuO

**A WATER-DRIVEN OXYGEN ACTIVATION MECHANISM**

Toshitaka Matsui, Momoko Furukawa, Masaki Unno, Takeshi Tomita, and Masao Ikeda-Saito

From the Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira, Aoba, Sendai 980-8577, Japan

Heme oxygenases found in mammals, plants, and bacteria catalyze degradation of heme using the same mechanism. Roles of distal Asp (Asp-136) residue in HmuO, a heme oxygenase of Corynebacterium diphtheriae, have been investigated by site-directed mutagenesis, enzyme kinetics, resonance Raman spectroscopy, and x-ray crystallography. Replacements of the Asp-136 by Ala and Phe resulted in reduced heme degradation activity due to the formation of ferryl heme, showing that the distal Asp is critical in HmuO heme oxygenase activity. D136N HmuO catalyzed heme degradation at a similar efficiency to wild type and D136E HmuO, implying that the carboxylate moiety is not required for the heme catalysis by HmuO. Resonance Raman results suggest that the inactive ferryl heme formation in the HmuO mutants is induced by disruption of the interaction between a reactive Fe—OOH species and an adjacent distal pocket water molecule. Crystal structural analysis of the HmuO mutants confirms partial disappearance of this nearby water in D136A HmuO. Our results provide the first experimental evidence for the catalytic importance of the nearby water molecule that can be universally critical in heme oxygenase catalysis and propose that the distal Asp helps in positioning the key water molecule at a position suitable for efficient activation of the Fe—OOH species.

Heme oxygenase (HO) catalyzes regiospecific conversion of heme (iron protoporphyrin IX) to o-biliverdin, CO, and free iron (1–3). Mammalian HO, which has been extensively studied, exists as two isomers, a 33-kDa inducible HO-1 and a 36-kDa constitutive HO-2. Primary functions of HO-1 are excess heme catabolism and antioxidant defense, whereas that of HO-2 is proposed to generate CO that has been recognized as a physiological messenger molecule (2, 4–6). Higher plants, algae, and cyanobacteria utilize o-biliverdin from their HO reactions for the synthesis of light-harvesting pigments (7–9). In some pathogenic bacteria, HO degrades host heme so as to acquire iron required for their survival and proliferation (10–13). Despite the varied physiological functions, the heme degradation by HO proceeds through the same mechanism (Fig. 1). The three successive steps of O2 activation involving the uptake of a total of seven electrons is performed by the substrate heme itself as evident from the absence of any other cofactor in HO (3). The first step of the HO reaction is the regiospecific hydroxylative cleavage of the porphyrin a-meso carbon atom. The resulting a-meso-hydroxyheme immediately reacts with another O2 to yield verdoheme and CO. Further O2 activation cleaves the heme macro cycle to afford biliverdin and free ferrous iron. Among the three distinct oxygenation steps, the first meso-hydroxylation is of particular importance due to its sharp contrast to O2 activation by cytochrome P450 monoxygenase.

The O2 activation both in HO and P450 is initiated by reduction of the ferric heme iron to the ferrous state, which allows rapid binding of O2 (Fig. 2). Further one-electron reduction and protonation of the ferrous-O2 complex yields a ferric hydroperoxy species (Fe—OOH). In P450 as well as peroxidase and catalase, the O—O bond of Fe—OOH is heterolytically cleaved to afford a reactive oxo ferryl heme. On the other hand, the Fe—OOH species in HO is now widely accepted as the hydroxylating reagent of the heme, and the ferryl heme is inactive in the porphyrin a-meso hydroxylation (14, 15). The terminal oxygen of Fe—OOH is supposed to attack the a-meso-carbon in HO rather than liberate as hydroxide (or more possibly as water) in P450. In comparison to the better characterized mechanism of the ferryl heme formation in P450 and peroxidase enzymes, little is known about how the HO enzyme enables the meso-hydroxylation by Fe—OOH.

The crystal structure of the heme-HO complex reveals salient aspects of the architecture of the HO protein that appear to promote the meso-hydroxylation (16). The heme group in HO is sandwiched between two helices termed as “proximal” and “distal” helices. The proximal helix contains an axial His ligand of heme, which is essentially neutral, as determined by spectroscopic measurements (17). The formation of ferryl heme should be less enhanced by the neutral His ligand in HO than a deprotonated His, histidinate, ligand in peroxidase (18). The distal helix of HO, which is kinked above the heme plane around two conserved Gly residues (Gly-139 and Gly-143 in human HO-1), is in close contact with the heme group to sterically restrict access to all the meso positions except for the meso.
Distal Asp in a Bacterial Heme Oxygenase, HmuO

α-meso-carbon, leading to the α-regiospecificity of HO catalysis. The steric constraints imposed by the distal α-helix on the bound dioxygen direct the terminal oxygen atom of the ferric-O₂ complex toward the heme α-meso-carbon and makes its Fe—O—O angle acute (∼110°) to allow van der Waals contact of the terminal oxygen with the α-meso-carbon (19, 20). The unique structure of the distal helix should be critical in promoting meso-hydroxylation by Fe—OOH because replacement of either of the two Gly diminishes the HO activity with partial formation of the ferryl species (21).

Further mutational studies on polar residues in the distal helix of HO-1 have uncovered another critical residue, Asp-140, whose replacement leads to dominant formation of the inactive ferryl heme (22, 23). The distal Asp is located ~8 Å from the heme iron but forms an H-bond network to an iron-bound water ligand through an intervening water molecule(s). Because this distal Asp mutation is not expected to disrupt the distal helix structure, it has been proposed that Asp-140, its carboxylate moiety in particular, is crucial in activation of Fe—OOH through the intervening water molecules. The distal Asp is highly conserved in most mammalian, plant, and bacterial HOs. However, two bacterial HOs, HemO from Neisseria meningitidis and PigA from Pseudomonas aeruginosa, 2 lack this distal Asp. The absence of a carboxyl residue in their distal pockets has been confirmed in the crystal structures of HemO and PigA (24, 25). Despite the absence of the distal Asp residue, both HemO and PigA utilize Fe—OOH in hydroxylating the heme meso-carbon to form meso-hydroxyheme as mammalian HO-1 (25, 26). These findings have raised questions about the general importance of the distal Asp carboxylate for mammalian HO-1, and thus, a reassessment is needed to accurately delineate how Fe—OOH hydroxylates the heme meso-carbon.

To this end, a series of distal Asp (Asp-136) mutants of HO from Corynebacterium diphtheriae (HmuO) have been characterized by enzymatic reaction, resonance Raman scattering, and x-ray crystallography. HmuO, a 24-kDa soluble HO, is one of the most structurally and enzymatically well characterized bacterial HOs (10, 27–29). Despite its low sequence identity to human HO-1 (33%), the crystal structure of HmuO reveals a distal Asp conserved at a position similar to that in HO-1 (29), thereby offering an opportunity for us to examine whether the carboxylate of the distal Asp residue is significant in HO catalysis. We have found that the distal Asp of HmuO is critical in positioning a distal pocket water molecule at the vicinity of Fe—OOH and that this nearby water can promote heme meso-hydroxylation by Fe—OOH without help by the distal Asp carboxylate. Our results suggest a universal, water-driven activation mechanism of Fe—OOH for all the HO enzymes including HemO and PigA.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids of Asp-136 Mutants of HmuO—Construction of pMW-HmuO, the synthetic expression plasmid for HmuO wild type, was described elsewhere (28). The full-length synthetic hmuO gene was designed with unique restriction sites convenient for cassette mutagenesis. To synthesize the expression plasmid of Asp-136 mutants, the wild type pMW-HmuO was excised with SpeI and MscI, and the resulting larger fragment of the digests was gel-purified. A synthetic 47-residue nucleotide, 5′-CTAGTTGCTCACACAGCCCTCTGAGTCGCTACCTCGGTGCCCTCTCGGGTGG-3′, and its complementary 43-residue nucleotide, 5′-CCACCCGAGAGGGCACCGAGGTAGCGTGCTAGTTGCTCACACAGCCCTCTGAGTCGCTACCTCGGTGCCCTCTCGGGTGG-3′, both containing the sense and antisense codon for Ala136, respectively, as indicated by the underlined bases, were annealed to make a double-stranded DNA. This double-stranded DNA was ligated to the larger SpeI/MscI fragment to form pMW-HmuO containing the distal Asp mutants in the same way except for codons corresponding to the residues 136, AAT (Asn), GAA (Glu), and TTC (Phe). All final coding sequences of these mutant plasmids were confirmed by DNA sequencing (Sawady).

Protein Expression, Purification and Reconstitution with Heme—Escherichia coli expression and purification of the HmuO mutant proteins, except D136F, were carried out as described previously (28). D136F was expressed in inclusion bodies, different from the other mutants that were expressed in the soluble form. Using BL21(DE3)pLysS and mild induction with 30 μM isopropyl 1-thio-β-D-galactopyranoside at 25 °C increased the soluble form more than half of the total D136F HmuO protein expressed. All the purified proteins were homogeneous as judged by SDS-polyacrylamide gel electrophoresis. Heme reconstitution of each purified HmuO mutant was performed as described previously (28). Note that the Asp-136 mutants, especially D136F, required longer incubation with ferric heme for proper reconstitution than the wild type. The heme complexes of the purified proteins were stored at ~80 °C until use.

Spectroscopic Measurements—UV-visible absorption spectra were recorded on an Agilent 8453 spectrophotometer at 20 °C. Resonance Raman spectra were obtained with the 413.1-nm line of a krypton ion laser (Spectra Physics) or 441.6-nm line of a helium-cadmium laser (Kinmon Electronics). Raman spectra were detected with a CCD camera attached to a single polychromator (Ritsu Yoyogakagu, DG-1000). The Fe—N—O vibrations, (ν(Fe—NO), (ν(Fe—N—O), and (ν(N—O)), were determined by isotope-sensitive Raman lines of the NO-bound ferric heme-myoglobin complexes with (15N)O, (14N)NO, and (15N)O gases. To avoid photoreactions, the laser power was tuned at 2–10 milliwatts, and the accumulation time was 5 min. Raman spectra were calibrated with indene and CCl₄, and the spectral accuracy was within ±1 cm⁻¹.

H₂O₂-supported Reactions—All the reactions with H₂O₂ were performed at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0. To 1 ml of solution of ~5 μM ferric heme-HmuO complex was added varied amount of H₂O₂. Product ratios of verdoheme and ferryl heme were estimated from the absorbance increase at 680 nm in the absence and presence of o-cresol. At this wavelength, absorption of the ferric and ferryl heme is negligible, so that the amount of verdoheme formed can be determined. o-Cresol does not react with verdoheme but immediately reduces the ferryl heme to the ferric state. Multiple turnovers of the ferric heme with H₂O₂ resulted in almost complete formation of verdoheme even in D136F HmuO.

Reaction rates with H₂O₂ were determined by using a UNISOKU RSP-601 stopped-flow apparatus equipped with a built-in rapid scan spectrophotometer at 20 °C. Typically, 5 μM ferric enzymes were mixed with equal volumes of H₂O₂ solution (0.5–10 mM). Absorbance decay at 404 nm was well fitted to a single exponential function. Linear relationships of the observed reaction rates with concentrations of H₂O₂ indicate that the H₂O₂ binding to the heme iron is involved in a rate-determining step, and their slopes give apparent reaction rate constants (k_app). The k_app values are summations of formation rates of the verdoheme (kᵥ) and ferryl heme (kᵥ), and ratios of kᵥ to kᵥ are equal to the product ratios of verdoheme to ferryl heme (Equations 1–4). The kᵥ and kᵥ values, thus, can be calculated from k_app and the product ratios.
and NADPH was added CPR (final concentration, 0.1 mM) and D136A HmuO formed crystals suitable for high resolution x-ray structural analysis under conditions similar to those used for the heme complex of wild type HmuO. Diffraction data for D136E were collected at 95 K on an ADSC Quantum 4R CCD detector using 0.98 Å synchrotron radiation at BL6A of the EMBL Hamburg Source. The major product looks like an oxo ferryl heme (Fe^{IV}=O), which is a common intermediate for the formation of verdoheme and ferryl heme (Fig. 2).

**RESULTS**

**Coordination Structures of Hemes in HmuO**—The distal Asp of HmuO (Asp-136) has been replaced by Glu, Asn, Ala, and Phe. The Asp-136 mutants of HmuO have been expressed in *E. coli*, purified, and reconstituted with ferric heme by a slight modification of a method described for the wild type HmuO (29). The mutant proteins were concentrated to ~20 mg/ml (D136E and D136N) or 10 mg/ml (D136A) in 20 mM HEPES, pH 7.0. D136E crystals were grown at 20 °C by a hanging drop vapor diffusion method from a solution mixed with an equal volume of the protein stock solution and a reservoir solution containing 50 mM MES (pH 6.1), 2.2 M ammonium sulfate, 0.23 M sodium iodide, and 0.1% 80 °C, its high spin ferric heme even on ice and without precipitation solution was converted to a low spin species in a few days. The low spin form of D136F HmuO easily aggregated upon the addition of ~1 M ammonium sulfate. Diffraction data for D136E were collected at 95 K on an ADSC Quantum 4R CCD detector using 0.98 Å synchrotron radiation at BL6A of Photon Factory, and those for D136N and D136A mutants were collected at 95 K on an ADSC Quantum 210 CCD detector using 1.00 Å synchrotron radiation at NW12 of Photon Factory-Advanced Ring. Data were integrated, merged, and processed with HKL2000 (30). Structures were determined by the method of molecular replacement and rigid body refinement by CNS (31) using a starting model that was constructed from the structure of the ferric heme complex of wild type HmuO (Protein Data Bank code 1IW0) by mutating Asp-136 to Ala. The model was further refined using the maximum-likelihood target and the translation-libration-screw refinement (32) with the program REFMAC5 (33). Throughout the model building and refinement process, 19% of the reflections were excluded to monitor the R_{free} value. Drawings were made by PyMOL.  

\[ k_1 = \frac{[\text{verdoheme}]}{[\text{ferryl heme}]} \] (Eq. 4)  

The low spin form of D136F HmuO easily aggregated upon the addition due to its denaturation during crystallization. Although D136F HmuO is also observed in the H2O2 reaction of distal Asp mutants of HmuO, it has been reported that H2O2 supports the first oxygenation of ferric heme to yield α-meso-hydroxylme, which is immediately converted to verdoheme IXα in the presence of O2 (14, 28). As shown in Fig. 4a, aerobic incubation of ferric wild type HmuO and H2O2 caused a decrease in the Soret band and concomitant appearance of a broad absorption band centered at 680 nm, typical for verdoheme formation. On the contrary, the Soret peak of ferric D136F HmuO rapidly shifted to 412 nm with the appearance of prominent visible bands at 526 and 549 nm (Fig. 4b). Verdoheme is a minor product in the D136F mutant as judged by the appearance of only a small band around 680 nm. The major product looks like an o xo ferryl heme (Fe^{IV}=O), which was confirmed by regeneration of the starting ferric heme by the addition of electron donors such as α-cresol. The ferryl heme is not capable for the heme meso-hydroxylation and is also observed in the H2O2 reaction of distal Asp mutants of mammalian HO-1 (14, 22, 23). These results indicate that the distal Asp in HmuO is crucial for the proper heme degradation as in mammalian HO-1. The distal Asp is likely to enhance meso-hydroxylation by a ferric hydroperoxy-heme complex (Fe=OOH), which is a common intermediate for the formation of verdoheme and ferryl heme (Fig. 2).

Reactions of other ferric Asp-136 mutants with H2O2 also gave mixtures of verdoheme and ferryl heme with varied product ratios (Table I). The verdoheme ratio (% verdoheme) was highest in wild type HmuO, indicating Asp as the best residue for the meso-hydroxylation. Because the D136N substitution (Fig. 4c) as well as D136E does not significantly suppress the verdoheme formation, the carboxylate moiety of distal Asp does not seem to be required for the HmuO catalysis. On the other hand, D136A HmuO (Fig. 4d) afforded an approximately equimolar mixture of the two products, and Phe-136 most effectively hampered the proper heme degradation. Thus, high polarity and/or H-bonding ability of a residue at position 136 appear to facilitate the meso-hydroxylation. These results are
in contrast to the previous proposal for mammalian HO-1 that the distal Asp (Asp-140) carboxylate is indispensable for the heme meso-hydroxylation (22, 23). The distal Asp mutations in rat HO-1 compared with HmuO have much more drastic effects on the H$_2$O$_2$-supported reactions: Verdoxhemes ratios are roughly estimated to be 100% for wild type and D140E, 20% for D140N, and 0% for D140A and D140F HO-1. The major difference compared with the HmuO mutants is dominant formation of the ferryl heme species in D140N and D140F HO-1.

The Asp-136 substitutions affect not only the product distribution of the H$_2$O$_2$ reactions but also reactivity of the heme-HmuO complex with H$_2$O$_2$ (Table II). Observed reaction rates linearly depend on the H$_2$O$_2$ concentration, suggesting the H$_2$O$_2$ binding to the heme iron as a major rate-determining step. Table II summarizes apparent rate constants ($k_{app}$) and individual formation rates of the verdoheme ($k_1$) and ferryl heme ($k_2$) derived from Equations 1–3. Inspection of Table II reveals that as the verdoheme ratio (% verdoheme) decreases in the Asp-136 mutants, the $k_{app}$ value significantly increases (up to 330-fold increase for D136F HmuO). The higher reactivity of the Asp-136 mutants can be attributed to the facile H$_2$O$_2$ binding to the heme iron, which is consistent with simultaneous increase in $k_1$ and $k_2$ (Table II). These results imply that the Asp-136 substitutions make the heme iron more accessible for H$_2$O$_2$. The more pronounced increase in $k_2$ than $k_1$ would be due to preferred conversion of Fe—OOH to ferryl heme rather than verdoheme.

**Heme Degradation by Reductive Activation of Molecular Oxygen**—Although a physiological electron donor of HmuO is yet to be identified, mammalian NADPH-cytochrome P-450 reductase (CPR) is known to support the heme degradation by the bacterial enzyme (27, 28). The CPR-supported heme degradation by wild type and D136F HmuO were examined to assess roles of distal Asp in the reductive activation of O$_2$ (Fig. 5). The CPR reactions were carried out with 3.5 mol eq of NADPH; 7 electrons were required for stoichiometric conversion of ferric heme to biliverdin (Fig. 1). The ferric heme in wild type HmuO was immediately reduced to form ferrous-O$_2$ heme and finally resulted in almost stoichiometric formation of biliverdin having broad absorbance around 685 nm (Fig. 5a). The ferric D136F mutant was similarly converted to ferrous-O$_2$ heme by NADPH/CPR (Fig. 5b). After the consumption of NADPH, however, absorbance increase at 685 nm was approximately half that observed for the wild type. There remained starting ferric heme, as evident from the sharp absorption band at 404 nm. Although further addition of NADPH diminished the remaining ferric heme, the yield of biliverdin was not remarkably increased.

The CPR-dependent reaction by D136F HmuO was drastically affected by co-incubation of superoxide dismutase and catalase (Fig. 5c), which did not have any appreciable effects on the heme degradation catalyzed by the wild type enzyme. Oxy-ferrous heme formed was no longer degraded in D136F HmuO, and 85% of the starting ferric heme was recovered after consumption of 7 mol eq of NADPH. The autooxidation of ferrous-O$_2$ heme was much faster for D136F than wild type HmuO (20 and 0.31 h$^{-1}$, respectively), and NADPH appeared to be mostly used up to reduce the auto-oxidized oxy heme in D136F HmuO. Although catalase alone successfully inhibited the biliverdin formation, Soret peak of D136F was gradually shifted from 412 nm (oxyferrous heme) to 421 nm during the incubation without superoxide dismutase (data not shown), strongly supporting the significant production of superoxide anion via the autooxidization. Thus, D136F HmuO in the absence of superoxide dismutase and catalase (Fig. 5b) partially oxidizes the heme macrocycle by utilizing exogenous H$_2$O$_2$ generated through the dismutation of superoxide.

CPR reduces oxy HmuO ~50-fold slower than rat oxy HO-1, and the deleterious effect of the D136F mutation seems to arise from the inefficient reduction of oxy HmuO by CPR. The unidentified authentic electron donor of HmuO in C. diphtheriae might overcome the instability of oxy heme by its rapid reduction. Thus, although the distal Asp is found to be critical in stabilizing the ferrous oxy form, elucidation for its role in the activation of endogenously produced Fe—OOH is not feasible at present.

**Resonance Raman Analysis of the NO-bound Heme Complexes**—Effects of the Asp-136 mutations on the immediate vicinity of ligands bound to the heme iron have been studied by resonance Raman analyses of the iron-ligand vibrations using the NO-bound ferrous heme HmuO complex (Table III). The stable NO complex has been used as a model for the metastable O$_2$ complex and highly reactive Fe—OOH species, because the FeNO unit is bent like FeO$_2$ and FeO(OH) units (36, 37), whereas the FeCO unit is upright. Wild type HmuO exhibits $\nu$(Fe—NO) and $\delta$(Fe—N—O) at 563 and 1600 cm$^{-1}$, respectively. These frequencies are typical for hexacoordinate NO complexes, indicating the proximal His remains coordinated (38, 39). The Asp-136 substitutions cause high frequency shift of $\nu$(N—O) without any changes in $\nu$(Fe—NO) and $\delta$(Fe—N—O). The extent of the $\nu$(N—O) shifts is roughly correlated to the decrease in the verdoheme production; i.e., only a few wavenumbers' shift in D136N and D136A, and the largest 27 cm$^{-1}$ shift occurs in D136F HmuO (Tables II and III).

Considering the unaltered $\nu$(Fe—NO) and $\delta$(Fe—N—O), there should be no significant changes in the geometry of the Fe—NO units caused by the mutations (Table III). The same $\nu$(Fe—His) frequency of the ferrous heme complex is indicative of similar electron donation from the axial His ligand in the Asp-136 mutants (Table I). Therefore, the $\nu$(N—O) shift in the Asp-136 mutants can be attributed to varied electronic polarization of the FeNO unit. The high frequency shift of $\nu$(N—O) is indicative of less positive environment of the FeNO unit (40).
The crystal structure of the NO-bound ferrous-heme complex of wild type HmuO reveals that the terminal oxygen atom of the FeNO unit is H-bonded with a water molecule, which consists a H-bond network including Asp-136. A similar ligand-water interaction was also found in the crystal structure of ferrous-O2 wild type HmuO. Such an H-bond donation by the nearby water would lower \( \nu(N=O) \) in the wild type by decreasing the bond order of NO. The significant shift to a higher frequency of \( \nu(N=O) \) in the D136F mutant, thus, should be caused by disruption of the NO-water interaction. The small shifts of \( \nu(N=O) \) are indicative of lesser interference of the NO-water interaction in the D136A and D136N mutants. This can be also the case for FeOOH, and Asp-136 is suggested to help the reactive intermediate to accommodate the H-bond donation by the nearby water molecule.

Crystal Structures of Asp-136 HmuO Mutants—To further elucidate the mutation-induced changes in the water network, crystal structures of the Asp-136 HmuO mutants have been examined. We have successfully crystallized the ferric heme complexes of D136E, D136N, and D136A HmuO (Fig. 6). Unfortunately, D136F HmuO was not stable enough for the crystallization (see “Experimental Procedure”). The data collection and refinement statistics are listed in Table IV. The D136N and D136A mutants as well as the wild type crystallized in a space group \( P_{21} \), and their asymmetric units consisted of three protein molecules (molecules A, B, and C). The D136E mutant gave C2 crystals containing two protein molecules in the asymmetric unit (molecules A and B). In a given mutant crystal, the three or two protein molecules in each asymmetric unit had essentially the same conformation in all the enzymes, so that the structural descrip-
Distal Asp in a Bacterial Heme Oxygenase, HmuO

### Table III
**Comparison of Fe—N—O vibration modes for HmuO adducts** (in cm⁻¹ units)

| HmuO  | v(Fe—NO) | δ(Fe—N—O) | v(N—O) |
|-------|----------|------------|--------|
| WT    | 563      | 453        | 1600   |
| D136N | 563      | 453        | 1603   |
| D136A | 563      | 453        | 1604   |
| D136F | 563      | 453        | 1627   |

**Discussion**

**Roles of Distal Asp in HmuO—** The Asp-136 substitutions do not alter coordination and spin states of heme incorporated in HmuO as revealed by absorption and resonance Raman spectroscopy (Table I, Fig. 3). Product analysis of the H₂O₂-dependent heme degradation (Fig. 4 and Table II) shows that although the major product in the wild type enzyme is verdoheme, a significant amount of the inactive ferryl heme is produced by D136A and D136F HmuO. These observations demonstrate that the distal Asp is also critical in the bacterial HmuO catalysis, as has been proposed for the corresponding Asp-140 in mammalian HO-1. The carboxylate moiety of the distal Asp, however, is not required for promoting the proper meso-hydroxylation by HmuO because D136N as well as D136E HmuO affords verdoheme with H₂O₂ at a similar efficiency to the wild type enzyme. Even in D136A HmuO, half of the products is the verdoheme, whereas the other half is the ferryl heme. The non-requirement of the carboxylate is contrast to the previous conclusions on mammalian HO-1 (22, 23) but consistent with the fact that the other two bacterial HO, HemO from *N. meningitidis* and PigA from *P. aeruginosa*, do not have carboxyl residues in the distal heme pocket (24).

The distal Asp in HmuO and HO-1 is connected to the heme iron ligand water (W0) through H-bonds with one or two intervening water molecules (16, 29, 41). Because the water molecule in the vicinity of W0 is expected to directly interact with Fe—OOH, the nearby water (W1) appears to be critical in the activation of the reactive Fe—OOH intermediate. The current results provide the first experimental support for the catalytic importance of this nearby water. Although the W1 water is found in the crystal structures of wild type and D136E HmuO, the water molecule is only partially present in D136A HmuO (Fig. 6). Resonance Raman spectroscopy suggests almost complete loss of W1 in D136F HmuO (Table III). The disappearance of the nearby water is correlated with the increase in the ferryl heme formation (Table II), suggesting a major functional role of the distal Asp in HmuO in stabilizing the nearby water to control the reaction of Fe—OOH. The ordered water molecules close to the O₂ binding site are found in all the HO enzymes, including HemO and PigA (16, 24, 25, 29, 41), so that its importance in the HO catalysis is likely universal.

Considering the rapid autooxidation of oxy D136F HmuO, the nearby water molecule appears to stabilize the ferrous-O₂ complex through a H-bond to a distal oxygen atom of the FeO₂ unit (20). This water molecule most likely functions as a proton donor in the formation of the Fe—OOH species, as one-electron reduction of ferrous-O₂ wild type rat HO-1 directly affords Fe—OOH at 77 K (15). Similar cryo-reduction of oxy D140A HO-1 yielded a peroxy (Fe—OO⁻) complex, whose conversion to Fe—OOH requires annealing at 200 K (42). Thus, in wild type HO-1, there should be an efficient proton donor for the peroxy complex.

The nearby water likely functions as a proton donor in the activation of the Fe—OOH species as well. The Fe—OOH species of wild type HO-1 is further protonated, as shown by ENDOR spectroscopy, and then converted to meso-hydroxyheme at 215 K (42). Formation of meso-hydroxyheme from Fe—OOH also exhibits a significant solvent isotope effect (2.3 at 215 K (43). In D140A HO-1, the ENDOR signal of this second proton is also observable but noticeably broader than in the wild type, suggesting distal pocket disorder. Further annealing produces an EPR-silent species in the D140A mutant, suggesting ferryl heme formation. Disappearance (or high mobility) of the nearby water is consistent with the disorder to...
yield ferryl heme rather than *meso*-hydroxyheme.

Comparison with Mammalian HO-1—In comparison to HmuO, the distal Asp mutations in mammalian HO-1 have more drastic effects on the heme degradation (22, 23). Dominant formation of the ferryl heme even in D140N and D140A HO-1 had implied a critical function of the Asp carboxylate in the activation of Fe—OOH. One may think that HO-1 needs the distal Asp carboxylate for the *meso*-hydroxylation, whereas HmuO does not. In fact, the solvent isotope effect of rat HO-1 in the conversion of the protonated Fe—OOH to hydroxyheme (2.3 at 215 K) is consistent with presence of a carboxyl donor acting as a general acid catalyst (43). This elemental step is, however, slower in HmuO than rat HO-1, suggesting higher activation energy for HmuO. Thus, HO-1 should demand less help of the general acid catalyst.

5 R. M. Davydov, T. Matsui, M. Ikeda-Saito, and B. M. Hoffman, unpublished results.

---

**Table IV**

Statistics of data collection and structure refinement for the ferric heme complexes of D136E, D136N, and D136A HmuO mutants

| Mutant   | D136E | D136N | D136A |
|----------|-------|-------|-------|
| Protein Data Bank code | 1WNX  | 1WNW  | 1WNV  |
| Beam line | PF BL6A | PF-AR | PF-AR |
| Crystal data |       |       |       |
| Space group | C2    | P2₁   | P2₁   |
| Cell parameters |       |       |       |
| a (Å) | 106.151 | 54.059 | 54.129 |
| b (Å) | 63.722  | 63.101 | 62.781 |
| c (Å) | 72.296  | 107.164 | 107.993 |
| β (degree) | 130.282 | 101.03 | 101.00 |
| Molecules in asymmetric unit | 2 | 3 | 3 |
| Resolution range (Å) | 50.0–1.85 | 50.0–1.70 | 50.0–1.85 |
| Total observations | 150,232 | 304,911 | 250,744 |
| Unique reflections | 34,677 | 75,503 | 59,737 |
| Mosaicity (degree) | 0.150 | 0.196 | 0.164 |
| I/σ | 0.064 (0.326) | 0.073 (0.316) | 0.088 (0.332) |
| Completeness (%) | 99.9 (100.0) | 97.3 (95.7) | 98.3 (97.6) |
| R-factor | 0.150 | 0.196 | 0.164 |
| R-free | 0.201 | 0.233 | 0.204 |
| Bond length (Å) | 0.022 | 0.022 | 0.021 |
| Bond angle | 1.888 | 1.926 | 1.777 |
Alternatively, varied stability of the water network nicely explains the different mutational effects between HmuO and HO-1. The nearby water molecules in HO-1 are stabilized not only by the distal Asp but also by multiple H-bonds with surrounding water molecules as shown in Fig. 6a. NMR studies have shown that the H-bond network in HmuO is more stable than in human HO-1 (44, 45). Without the additional stabilization by the distal Asp, however, the less stable nearby water in HO-1 could be highly labile. On the other hand, the complete removal of the nearby water molecules in HmuO requires large and hydrophobic Phe residue because of the highly stabilized distal pocket H-bond network.

A crystallographic study by Lad et al., however, found that D140A human HO-1 retained the nearby water (36). They proposed that the side chain of Asp-140 works only as a H-bond acceptor to prevent protonation of Fe—OOH by the nearby water and that its substitution other than Glu allows water to act as a proton donor to exclusively afford ferryl heme in the H2O2 reaction. This proposal contradicts the aforementioned experimental results; (i) an efficient proton donor is essential both in the formation and activation of Fe—OOH in wild type HO-1, (ii) D140A HO-1 lacks the proton donor, and (iii) 50% formation of verdoheme by D136A HmuO (Table II).

The human HO-1 crystal consists of two distinct forms, open and closed (16, 36). The distal pocket water network of the D140A mutant was retained only in its closed form, and that in the open form was substantially disturbed to lose the nearby water. Although the closed form of human wild type HO-1 is likely representative of its solution structure, the open conformation might be predominant in the D140A HO-1 mutant. To test this hypothesis, we have measured resonance Raman spectra of the NO-bound ferrous heme complexes of rat HO-1. Unfortunately, the ν(N—O) mode of the D140A HO-1 NO complex was not detected, possibly because of strong overlapping vibrations. The ν(N—O) signal was successfully observed for D140F HO-1 at 1628 cm⁻¹, which is considerably higher than 1590 cm⁻¹ in wild type HO-1. The high frequency shift as observed for D136F HmuO (Table III) indicates loss of the nearby water, and thus, the destabilization of the key water molecule can be a major cause of the ferryl heme formation, at least in rat D140F HO-1 and likely in rat D140A HO-1 as well.

**Activation of Fe—OOH by a Proton Donor**—The remaining significant question is how the protonation by the nearby water activates Fe—OOH to promote meso-hydroxylation. The proximal oxygen atom of Fe—OOH might become protonated in HO, because the protonation of the distal oxygen is normally thought to enhance ferryl heme formation as shown in Fig. 8a (18). The proximal protonation would either make the terminal oxygen more positive during the transition state of its electrostatic attack to the meso-carbon (atom 3) or promote homolytic cleavage of the hydroperoxo O—O bond and generate a hydroxide radical to attack the meso-carbon (46). However, in the crystal structure of the oxy form of HmuO (20), a precursor of Fe—OOH, the nearby water is H-bonded to the distal oxygen atom. The proximal oxygen of FeO2 is tightly covered by Gly-135 and Gly-139 with a H-bond with an amide NH of Gly-139. Thus, the proximal protonation is less likely, although high flexibility of the distal helix might allow the drastic rearrangement of the H-bond network.

The water molecule nearby the distal oxygen of Fe—OOH might facilitate a nucleophilic attack by forming a H-bond acceptor. However, the deprotonation of the distal oxygen is not feasible, because the nearby water should not abstract the distal proton to form the highly basic peroxy (Fe—OO⁻) complex (15). Alternatively, the nearby water could control the Fe—OOH conformation (Fig. 8b). In the ferrous-O2 structure (20), the terminal oxygen of FeO2 points exactly to the a-meso carbon with an acute Fe—O—O bond of 110°. As a result, the terminal oxygen of FeO2 is only 3.4 Å from the a-meso carbon atom. Because one electron reduction of the ferrous O2 complex is expected to slightly stretch the O—O bond, the terminal oxygen of Fe—OOH could be in contact with the a-meso-carbon. Only in the presence of the contact, the protonation of the terminal oxygen atom may help hydroxylate the meso-carbon. In addition to the steric pressure imposed by the distal helix that determines the unique FeO2 geometry, the nearby water could fine-tune the Fe—OOH conformation as well. To address the precise mechanism of the Fe—OOH activation, a crystal structural analysis of the reactive Fe—OOH species is in progress.

In summary, we have examined catalytic importance and roles of Asp-136 in a bacterial HO from *C. diphtheriae*, HmuO. The distal Asp of HmuO is important to stabilize a critical water molecule located at the vicinity of the Fe—OOH species, whereas its carboxyl moiety is not strictly required. Activation of Fe—OOH by the nearby water can be universal among all the HO enzymes including HOs lacking carboxylate in their distal heme pockets. The stringent requirement of the distal Asp carboxylate proposed for mammalian HO-1 may actually come from less stability of the nearby water.

**Acknowledgments**—We thank Dr. T. Yoshida for the wild type HmuO gene and NADPH cytochrome P450 reductase protein, members of BL6A and NW12 of Photon Factory for assistance during the diffraction data collection, Dr. H. Fujii for comments, and an anonymous reviewer for suggestions to clarify the effects of the D136F mutation on the CPR reaction. Synchrotron radiation experiments were conducted under the approval of 2003G118 and 2002G139 at Photon Factory. Some x-ray diffraction data on the mutants were also collected at BL44B2 of SPring-8. Part of the work was conducted at the Hybrid Nano-Material Research Center of the Institute of Multidisciplinary Research for Advanced Materials, Tohoku University.

**REFERENCES**

1. Tenhunen, R., Marver, H. S., and Schmid, R. (1969) *J. Biol. Chem.*, **244**, 6388–6394
2. Yoshida, T., and Kikuchi, G. (1978) *J. Biol. Chem.*, **253**, 4224–4229
3. Ortiz de Montellano, P. R. (1998) *Annu. Rev. Pharmacol. Toxicol.*, **31**, 543–549
4. Yoshida, T., Bio, P., Cohen, T., Muller, R. M., and Shibahara, S. (1988) *Eur. J. Biochem.*, **171**, 457–461
5. Mains, M. D. (1997) *Annu. Rev. Pharmacol. Toxicol.*, **37**, 517–554
6. Poss, K. D., and Tonegawa, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.*, **94**, 10925–10930
7. Beale, S. I., and Cornejo, J. (1984) *Arch. Biochem. Biophys.*, **235**, 371–384
8. Tomita, H., Fujii, T., Yoshida, and M. Ikeda-Saito, unpublished result.
Roles of Distal Asp in Heme Oxygenase from Corynebacterium diphtheriae, HmuO: A WATER-DRIVEN OXYGEN ACTIVATION MECHANISM
Toshitaka Matsui, Momoko Furukawa, Masaki Unno, Takeshi Tomita and Masao Ikeda-Saito

J. Biol. Chem. 2005, 280:2981-2989.
doi: 10.1074/jbc.M410263200 originally published online November 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410263200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 18 of which can be accessed free at
http://www.jbc.org/content/280/4/2981.full.html#ref-list-1