Heme oxygenase (HO) catalyzes the first step in the heme degradation pathway. The crystal structures of apo- and heme-bound truncated human HO-2 reveal a primarily α-helical architecture similar to that of human HO-1 and other known HOs. Proper orientation of heme in HO-2 is required for the regioselective oxidation of the α-mesocarboxyl group. This is accomplished by interactions within the heme binding pocket, which is made up of two helices. The iron coordinating residue, His45, resides on the proximal helix. The distal helix contains highly conserved glycine residues that allow the helix to flex and interact with the bound heme. Tyr154, Lys199, and Arg203 orient the heme through direct interactions with the heme propionates. The rearrangements of side chains in heme-bound HO-2 compared with apoHO-2 further elucidate HO-2 heme interactions.

Heme oxygenase (HO) catalyzes the degradation of heme to free iron, carbon monoxide, and biliverdin in the presence of NADPH-dependent cytochrome P450 reductase. Seven electrons are transferred, and three molecules of oxygen are consumed in the degradation of one molecule of heme to biliverdin. Biliverdin reductase reduces biliverdin to bilirubin. This is accomplished by interactions within the heme binding pocket, which is made up of two helices. The iron coordinating residue, His45, resides on the proximal helix. The distal helix contains highly conserved glycine residues that allow the helix to flex and interact with the bound heme. Tyr154, Lys199, and Arg203 orient the heme through direct interactions with the heme propionates. The rearrangements of side chains in heme-bound HO-2 compared with apoHO-2 further elucidate HO-2 heme interactions.

The abbreviations used are: HO, heme oxygenase; HEPPS, 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid; HRM, heme regulatory motif; GST, glutathione S-transferase; r.m.s.d., root mean square deviation.
The first three-dimensional structure of human HO-1 was determined for the truncated protein in 1999 (30). Subsequently, many HO-1 crystal structures have been reported, including rat HO-1, biliverdin-associated HO-1, ferrous/ferrous-NO-bound HO-1, and some HO-1 variants (31–33). These structures led to significant advances in the study of the HO-1 catalytic mechanism (34). The HO-2 used in this study is the soluble portion of HO-2 with a mutation of Cys127 to an alanine and is missing residues 264–316 and will be referred to as HO-2 in this report. Here we report the first apo- and heme-bound HO-2 crystal structures at 2.4- and 2.6-Å resolution, respectively.

**EXPERIMENTAL PROCEDURES**

**Cloning, Overexpression, and Purification of Human HO-2**

The full-length human HO-2 cDNA in a pGEX-4T-2 (Amersham Biosciences GE Healthcare) GST gene fusion vector was kindly provided by Dr. Mahin D. Maines (University of Rochester, School of Medicine). The truncated HO-2 lacking the three HRMs and the membrane binding region was generated by replacing Cys-127 with Ala-127 and putting a stop codon “TAA” right before Cys-265, which will be referred to as HO-2 in this report. Here we report the first apo- and heme-bound HO-2 crystal structures at 2.4- and 2.6-Å resolution, respectively.

**Crystal Structure of Apo- and Heme-bound HO-2**

Crystal Preparation—Crystals of the apoHO-2 were grown using the hanging drop method with 1.5 μl of a 5 mg ml⁻¹ protein solution (50 mM KCl, 50 mM Tris-HCl, pH 7.5) mixed with 1.0 μl of well solution containing 40% polyethylene glycol 1.5K (Fluka, St. Louis, MO), 200 mM potassium glutamate, and 100 mM triethanolamine, pH 8.5, at 277 K. The heme-bound HO-2 crystals were grown using the hanging drop method with 1.5 μl of 5 mg ml⁻¹ protein solution in 50 mM KCl, 50 mM Tris-HCl, pH 7.5, mixed with 1.0 μl of well solution containing 33% polyethylene glycol dimethlyether 500 (Sigma-Aldrich), 20 mM MgCl₂, and 100 mM HEPPS, pH 8.5, at 277 K.

X-ray Data Collection and Structure Solution—X-ray diffraction data for the apoHO-2 structure was collected at a wavelength of 0.97933 Å at the General Medicine and Cancer Institutes Collaborative Access Team (GM/CA-CAT) beamline 23-ID-D located at the Advanced Photon Source at Argonne National Laboratory. The diffraction data for the heme HO-2 structure was also collected at the GM/CA-CAT beamline 23-ID-B utilizing a wavelength of 0.97946 Å. Both data sets were indexed and scaled using HKL2000 (35). Molecular replacement was carried out with MOLREP using human heme oxygenase-1 (Protein Data Bank code 1N45) as the initial model (36). An initial apoHO-2 model was partially built with ARP/WARP resulting in a structure with 54.7% of the possible residues positioned (37). The apoHO-2 structure was completed with several rounds of manual building with Coot and restrained refinement in REFMAC (38, 39). The refined apoHO-2 structure was used as the molecular replacement model for the heme-bound HO-2 structure. The heme-bound HO-2 model was then completed as described for apoHO-2.

The quality of the final structures were assessed using MOLPROBITY and PROCHECK (40, 41). The final model of apoHO-2 was refined to a resolution of 2.40 Å with an R-factor
Crystal Structure of Apo- and Heme-bound HO-2

TABLE 1

Crystal parameters, x-ray data collection, and refinement statistics

|                        | Human apoHO-2 | Human heme HO-2 |
|------------------------|---------------|-----------------|
| Space group            | P2_12121      | P2_12121        |
| Unit cell parameters (Å)| A = 75.769, B = 86.017, C = 97.753 | A = 74.977, B = 85.094, C = 97.846 |
| Data collection statistics |                |                 |
| Wavelength (Å)         | 0.97933       | 0.97946         |
| Resolution range (Å)   | 50.0–2.3 (2.38–2.30) | 40.96–2.32 (2.4–2.32) |
| Number of reflections (measured/unique) | 203,168/28,128 | 278,069/24,030 |
| Completeness (%)       | 95.9 (78.2)   | 85.6 (27.4)     |
| Rmerge                  | 0.08200       | 0.08390         |
| Redundancy              | 7.3 (3.9)     | 11.6 (1.8)      |
| Mean I/σ(I)             | 21.89 (1.93)  | 19.2 (0.776)    |
| Refinement and model statistics |                |                 |
| Resolution range        | 49.15–2.40 (2.46–2.40) | 40.96–2.61 (2.68–2.61) |
| No. of reflections (work/test) | 25,217 | 19,515 |
| Rmerge                  | 0.201         | 0.205           |
| Rfree                   | 0.253         | 0.256           |
| r.m.s.d. bonds (Å)      | 0.011         | 0.006           |
| r.m.s.d. angles (°)     | 1.255         | 0.951           |
| ESU from Rmerge (Å)     | 0.249         | 0.305           |
| B factor, Wilson plot (Å) | 59.62        | 65.96           |
| No. of protein molecules/atoms | 2/3,621     | 2/3,563         |
| No. of waters           | 154           | 86              |
| No. of ions             | 0             | 0               |
| No. of auxiliary molecules | 2 Triton X-100 | 2 Hemin         |
| Ramachandran plot (%)   |                |                 |
| Most favorable region   | 93.2          | 94.7            |
| Additional allowed region | 6.8         | 5.3             |
| Generously allowed region | 0.0         | 0.0             |
| Disallowed region       | 0.0           | 0.0             |
| PDB code                | 2Q32          | 2QPP            |

a ESU, estimated standard uncertainty.

RESULTS

HO-2 Overall Structure—The HO-2 that was used in this study lacked the two C-terminal HRM domains (265–316), and had Cys-127 mutated to Ala. These alterations to HO-2 were done to increase the likelihood of crystallization. Two molecules of HO-2 were observed in the asymmetric unit of both apoHO-2 and heme-bound HO-2. Out of the 264 residues in the truncated apoprotein, 212 residues were observed in molecule A, and 218 residues were observed in molecule B. Molecule A of apoHO-2 was lacking electron density for Met1–Met28 and Thr243–Lys264. In all previous HO-1 structures, the C-terminal residues also have not been observed.

In the B molecule of heme-bound HO-2, the heme was modeled at 75% occupancy. Elongated positive electron density was observed near the heme in both molecules of the heme-bound HO-2 structure. Compared with the A molecule the heme pocket of the B molecule was more disordered. The four pyrrole rings of the heme in the B molecule were restrained during refinement to form a plane. There was no need to restrain the heme in molecule A beyond the default REFMAC parameters. In addition to REFMAC restrained refinement the heme-bound HO-2 was also modeled using ensemble refinement using CNS (Protein Data Bank code 2RGZ) (43, 44). The disorder of the heme in the heme pocket of the B molecule can be seen in the 16-conformer ensemble refinement (supplemental Fig. 1).

The secondary structural elements of HO-2 are almost exclusively α-helical, with short loop segments that connect adjoining α-helixes (Fig. 2). The overall structure of HO-2 is nearly identical to those of human (30) and rat HO-1 (45), the bacterial HOs (Neisseria meningitidis HmuO (46), Corynebacterium diphtheriae HemO (47), and Pseudomonas aeruginosa PigA (48)), as well as the Synechocystis sp. HOs (49, 50). These similarities include the kinked distal helix and heme binding site. Like HO-1 (45, 51) and HmuO (34), the overall fold is maintained even in the absence of substrate heme.

Distal Helix—A flexible, highly conserved sequence (Gly159–Asp-Leu-Ser-Gly-Lys164), which is an integral part of the heme binding pocket, allows the distal helix to bend and come into close proximity to the heme (30, 32, 45, 46, 51–54). This glycine-rich flexible region in heme-bound HO-2 allows the backbone atoms of Gly159 to interact with the heme iron through a...
water molecule. Gly^{159} in apoHO-2 is in the same position as observed in heme-bound HO-2. The catalytically important Asp^{160} that resides on the distal helix is relatively unchanged in both apo- and heme-bound HO-2 (55, 56). The most striking differences between the distal helix in apoHO-2 and heme-bound HO-2 are found in the amino acids near the bound heme. In heme-bound HO-2, Tyr^{154} repositions to form a hydrogen bond with the propionate located in the heme pocket. The side chains of Thr^{155} and Arg^{156} in heme-bound HO-2 move away from the heme allowing more room in the heme pocket (Fig. 3). The expansion of the heme pocket caused by the movement of Thr^{155} and Arg^{156} is not observed in apoHO-2.

In the previously solved heme-bound human HO-1 structure, the two HO-1 monomers in the asymmetric unit were observed in slightly different conformations (30). Molecule A of the heme-bound HO-1 was considered to be in a closed conformation while molecule B was in an open conformation, which was proposed to be important in binding heme and/or releasing products (30). The difference between the open and closed conformation is the position of the distal helix with respect to the heme pocket. In the open conformation, the distal helix has moved away from the heme pocket causing a slightly larger heme pocket compared with the closed conformation. In the HO-2 structures the movement of the distal helix occurs at Gly^{163}. Gly^{163} moves away from the heme pocket, causing the heme pocket to adopt the open conformation. By comparing the overall r.m.s.d. of the heme-bound HO-1 and HO-2 α-carbons it is apparent that both apoHO-2 and heme-bound HO-2 are in the open conformation (Table 2). The movement of the distal helix at Gly^{163} is seen when the apo- and heme-bound HO-2 structure is compared with the closed heme-bound HO-1 structure (supplemental Fig. 2). All other significant backbone atom deviations between HO-2 and HO-1 are located in the loops between helices.

Proximal Helix (His Ligand Side)—Unlike the distal helix, the proximal helix lacks any flexible region that would allow the helix to translocate during heme binding. The heme iron-coordinating histidine, His^{45}, resides on the proximal helix (Fig. 4). His^{45} of heme-bound HO-2 coordinates the heme iron through an imidazole nitrogen at a distance of 2.1 Å in the A molecule. The distance between the imidazole nitrogen of His^{45} and the heme iron was restrained during refinement to a distance of 2.1 Å. A hydrogen bonding interaction with Glu^{49} serves to stabilize the iron-coordinating histidine. A carboxyl oxygen from Glu^{49} forms a hydrogen bond with the imidazole nitrogen of
His$^{45}$ that is not coordinating the heme iron at a distance of 3.5 Å in molecule A and 3.8 Å in molecule B. The interaction between His$^{45}$ and Glu$^{49}$ is not present in the apoHO-2 structure. Without a bound heme, the imidazole ring of His$^{45}$ of apoHO-2 is no longer restrained and adopts a different conformation than the one that is observed in heme-bound HO-2 (Fig. 4). There is little change in the proximal helix with the exception of the movements of His$^{45}$ and Glu$^{49}$.

**Hydrophobic Region**—The residues that make up the hydrophobic pocket, Val$^{54}$, Phe$^{53}$, Phe$^{57}$, and Phe$^{234}$ remain unchanged in both apo- and heme-bound HO-2. In the heme-bound HO-2, the heme $\alpha$-meso edge is positioned so that it is directed toward the residues that make up the hydrophobic region of the heme pocket. Unexplained positive electron density was observed between the hydrophobic region and the heme $\alpha$-meso edge of the heme in the heme-bound HO-2 structure. We were unable to identify the compound giving rise to the density, and the density was left un-modeled (Fig. 5A). In the same region that the unexplained density was found in heme-bound HO-2, a molecule of Triton X-100 was observed in apoHO-2. The Triton X-100 molecule occupied a position near the hydrophobic region of the heme pocket where the heme $\alpha$-meso edge would occupy (Fig. 5B). The tetramethylbutyl group of the Triton X-100 was in the same position as the unexplained density in heme-bound HO-2.

**Heme Protein Interactions**—In HO-2 and other known HOs, the $\alpha$-, $\beta$-, and $\gamma$-meso edges of the heme group are positioned in the interior of the heme pocket with the $\delta$-meso edge exposed to the solvent. In order for the hydroperoxo form of HO-2 to specifically catalyze electrophilic attack on the heme $\alpha$-mesocarbon, the heme group needs to be properly oriented in relation to the heme pocket. The way that HO-2 seems to orientate the bound heme is through interaction with the propionate groups. One of the heme propionate groups is buried in the heme pocket, whereas the other is solvent-exposed. In the heme-bound HO-2 structure, there are three residues that play crucial roles in heme coordination (Tyr$^{154}$, Lys$^{199}$, and Arg$^{203}$). Tyr$^{154}$ is located on the distal helix with its OH group 2.7 Å in molecule A and 2.9 Å in molecule B from one of the carboxyl oxygens of the buried propionate. Located on the A6 helix, Arg$^{203}$ coordinates the solvent-exposed propionate at a distance of 3.0 Å in both the A and B molecules. While Lys$^{38}$ is too distant to form a hydrogen bond with the solvent-exposed propionate in the crystal structure, a shift in the position of the propionate could cause the formation of a hydrogen bond. These hydrogen bonds correctly orient the heme and place the $\alpha$-meso edge carbon in position for hydroxylation (Fig. 4).

**DISCUSSION**

The two structures we present are of the core domain of human HO-2. These truncated human HO-2 structures contain the region responsible for heme recognition, binding, and degradation, elucidating the important structural features of HO-2 and improving our understanding of the catalytic mechanism of HO-2. The structures indicate that the catalytic cores of HO-1 and HO-2 are structurally conserved, and the major
differences underlying the diverse physiological functions of HO-1 and HO-2 may lie in the C terminus of the two proteins.

Hydrophobic Cavity—The conformation of the HO-2 heme pocket can be directly affected by the hydrophobic cavity. One possible reason that both HO-2 structures were observed in an open conformation could be due to the Triton X-100 in apoHO-2 and the un-modeled electron density in heme-bound HO-2. The Triton X-100, which occupies a position near the hydrophobic cavity, might hinder the heme pocket from assuming a closed conformation. Although there is no definitive evidence, it seems likely that the core domains of HO-1 and HO-2, which are responsible for the heme binding and degradation, share a similar fold. Both HO-1 and HO-2 structures consist mostly of α helices. Overall, the secondary structure of HO-2 closely resembles apoHO-1 and heme-bound HO-1.

It has been shown that mutating Asp140 to an Ala converts HO-1 into a peroxidase (56). It is proposed that the Asp140 to Ala mutant cannot stabilize the peroxide bond and will lead to an Fe$^{3+}$-O oxyferryl center instead of the Fe$^{3+}$-OOH reactive intermediate (57). Asp140 stabilizes the Fe$^{3+}$-OOH intermediate through a hydrogen bonding network with water molecules located near the heme and the Fe$^{3+}$-OOH intermediate. In the high resolution heme-bound HO-1 structure, the water molecules thought to be involved in this hydrogen bond network were observed in the closed conformation (51). The open conformation of heme-bound HO-1 has a much less ordered solvent structure with some water thought to be at a partial occupancy. This disorder in the open confirmation may explain why the solvent structure is not seen in the heme-bound HO-2 structure. Even though the entire hydrogen network is not resolved in the heme-bound HO-2 structure, Asp160 still adopts the same conformation as Asp140 in the heme-bound HO-1 model and is thought to act in a similar fashion.

Although the C-terminal HRM region shows the most significant sequence divergence between HO-1 and HO-2, most of the key amino acids involved in heme binding, Lys$^{18}$, His$^{45}$, Glu$^{49}$, Tyr$^{154}$, Gly$^{159}$, Asp$^{160}$, Gly$^{163}$, Lys$^{199}$, and Arg$^{203}$ are observed in similar positions in the heme-bound HO-2 structure when compared with heme-bound HO-1. The major differences in the crystal structure of apoHO-2 and heme-bound HO-1 are seen in the helices that directly interact with the heme. The proximal helix of apoHO-2 moves slightly down-
ward, and the distal helix moves upward relative to hemebound HO-1. In heme-bound HO-2, the same displacement of the distal helix is observed when compared with heme-bound HO-1.

Regioselectivity — When free heme is oxidized by ascorbate in an aerobic environment, all four of the heme meso carbons are oxidized with equal frequency (58). The oxidation of heme by HO-1 can also be driven with ascorbate without cytochrome P450 reductase, but the reaction only results in the oxidation of the heme α-mesocarbon, just as in the reaction performed in the presence of the reductase (59). Spectroscopic, crystallographic, and enzymatic studies have provided important information on the steric and electronic factors that control regioselectivity in the bacterial and mammalian HOs (34, 59, 60). The crystal structure described here provides additional insight into how the oxidation of the heme in HO-2 is limited to the α-mesocarbon. The distal helix is positioned so that the helix is in close proximity across the entire width of the heme. This sterically hinders access to the β-, γ-, and δ-mesocarbons. In order for the distal helix to restrict access to the β-, γ-, and δ-mesocarbons the heme has to be properly positioned in the heme pocket. As seen in other α-hydroxylating HOs, the interactions between Tyr154, Lys199, Arg203, and to a lesser extent Lys38, and the heme propionates are responsible for the position of the heme in the heme pocket. Disrupting these side chain-heme interactions can result in the loss of regioselectivity (59–62).

Heme oxygenase from P. aeruginosa (pa-HO or PigA), unlike mammalian HOs, oxidizes the heme at the δ-mesocarbon (61–63). Asn19, Phe117, Lys132, and Trp158 of pa-HO interact with the heme instead of Tyr154, Lys199, and Arg203 in heme-bound HO-2. The position of the heme in pa-HO is rotated by ~100° counterclockwise placing the δ-mesocarbon in the same position as the α-meso in HO-2 (48, 61). Lys132 coordinates the heme propionate in pa-HO, and the distal helix restricts access to all but the δ-mesocarbon. It is apparent that the position of the heme in relation to the heme pocket is the cause of the regioselectivity of HO-2.

An Implication for Catalysis — HO-2, unlike globins, does not have a distal histidine or any other polar residues that stabilize the iron-bound ligand. The only moiety that is in position to stabilize the distal water ligand is the carbonyl oxygen of Gly159. Asp160, which interacts indirectly with the iron hydroperoxy intermediate through hydrogen-bonded water molecule, is hydrogen-bonded to a water molecule in a similar position to a water molecule seen in heme-bound HO-1. Both molecules of heme-bound HO-2 were in the open conformation when compared with heme-bound HO-1. The open confirmation of heme-bound HO-1 did not have a fully resolved solvent structure (51). It is likely that the open conformation of heme-bound HO-2 would act in a similar manner to the open confirmation of heme-bound HO-1, and the entire solvent structure would not be observed. It is likely that when HO-2 binds heme, the heme pocket closes, trapping the necessary water molecules that then form a hydrogen-bonding network with Asp160. The water molecule network could act as a proton-transport system required to form the Fe3+–OOH intermediate, which subsequently attacks the α-mesocarbon. This H-bonding network also appears to be involved in promoting conformational dynamics associated with catalysis (42).

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