Solution $^1$H NMR Investigation of the Active Site Molecular and Electronic Structures of Substrate-bound, Cyanide-inhibited HmuO, a Bacterial Heme Oxygenase from Corynebacterium diphtheriae*$^{[S]}$

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The molecular structure and dynamic properties of the active site environment of HmuO, a heme oxygenase (HO) from the pathogenic bacterium Corynebacterium diphtheriae, have been investigated by $^1$H NMR spectroscopy using the human HO (hHO) complex as a homology model. It is demonstrated that not only the spatial contacts among residues and between residues and heme, but the magnetic axes that can be related to the direction and magnitude of the steric tilt of the FeCN unit are strongly conserved in the two HO complexes. The results indicate that very similar contributions of steric blockage of several meso positions and steric tilt of the attacking ligand are operative. A distal H-bond network that involves numerous very strong H-bonds and immobilized water molecules is identified in HmuO that is analogous to that previously identified in hHO (Li, Y., Syvitski, R. T., Auclair, K., Wilks, A., Ortiz de Montellano, P. R., and La Mar, G. N. (2002) J. Biol. Chem. 277, 33018–33031). The NMR results are completely consistent with the very recent crystal structure of the HmuO-substrate complex. The H-bond network/ordered water molecules are proposed to orient the distal water molecule near the catalytically key Asp136 (Asp140 in hHO) that stabilizes the hydroperoxy intermediate. The dynamic stability of this H-bond network in HmuO is significantly greater than in hHO and may account for the slower catalytic rate in bacterial HO compared with mammalian HO.

Heme oxygenase (HO) is an α-helical enzyme that carries out the highly stereoselective conversion of hemin to a-biliverdin, iron, and CO, excising CO from the a-meso position (1). In contrast to the better understood heme peroxidases and cytochromes P450, which pass through the common ferryl intermediate, the reactive form of HO is a ferric hydroperoxy intermediate (2–4). In mammals, the ~300-residue membrane-bound enzyme occurs as an inducible HO-1, whose primary roles are iron homeostasis and heme catabolism (5, 6), whereas the constitutive HO-2 has been proposed (7) to generate CO as a neural messenger. In higher plants, algae, and cyanobacteria, HO generates the open tetrapyrrrole as light-harvesting pigments (8). HO has also been identified in several pathogenic bacteria, where its role appears to be the essential “mining” of iron from hemes in the host (9, 10). Plant and bacterial HO-1s are soluble and somewhat shorter (~200 residues) (9, 10) than mammalian HO (11). Among the characterized bacterial HO-1s, sequence homology to the more extensively studied mammalian HO varies from relative high (33% sequence identity/70% similarity) for HmuO from Corynebacterium diphtheriae (10) to low (~25%) for HemO from Neisseria meningitides (9).

The remarkable recent progress in understanding the functional properties of HO based on mutagenesis and spectroscopic studies (3, 4, 12–14), of a slightly truncated, soluble, and completely active recombinant mammalian HO, has been considerably enhanced by the successful x-ray crystallographic characterization of the substrate complexes of first human HO (hHO), followed by rat HO (15, 16). These structures shed light on a key determinant of the α-stereoselectivity, in that the distal histidine hemes the same as to sterically completely block access to the β- and δ-meso positions and partially block access to the γ-meso positions (15–17). Although no distal residue that would stabilize the hydroperoxy unit could be identified, the occurrence in the crystal of a localized water molecule H-bonded to the distal histidine Asp140 carboxylate, together with the observation that mutating Asp140 to a non-anionic side chain abolishes HO activity (12, 14), has led to the proposal that the water molecule may be sufficiently stabilized in its crystallographically defined position to serve as the weak H-bond donor to stabilize the hydroperoxy unit.

Solution $^1$H NMR characterization of hHO and its substrate complex has contributed to the understanding of the structure/function relationship of HO (18–22). An annoying, but functionally irrelevant property of the mammalian HO-1s is that binding of the native substrate, protoporphrin (PH; R = vinyl in Fig. 1), leads to ~1:1 orientational isomerism about the α/γ-axis (18–20), which leads to spectral congestion and limits both the range and reliability of structural characterization. Nevertheless, the pattern of dipolar shifts for the protons on the proximal helix allowed determination of the orientation of the major magnetic axis, which could be correlated with a ~20° tilt of the FeCN in the direction of the α-meso position (19, 20).
To the reference x and y, whose acceptor could be identified in the allowed sufficiently definitive and extensive assignments to demonstrated that water molecules were in the immediate vicinity (19,21,24) the axial ligand toward the α-meso position contributes to the stereoelectrostatics of the reaction. Two-dimensional 1H NMR of a hHO complex with the 2-fold symmetric substrate (21), 2H2O at 20, 25, and 30 °C from the initial magnetization recovery of a standard inversion-recovery pulse sequence. The distance of proton H from the iron, RH, was estimated from the relation RH = Rpp/(T1p/T1pp), using the heme for the α-meso–H for H+ (Rpp = 4.8 Å and T1p = 50 ms) as reference (20, 21, 32). Steady-state NOES from HmuO-DMDH-CN in 2H2O were recorded with and without saturation of the solvent resonance for 300 ns using 3:9:19 detection (33). NOESY spectra (mixing time of 40 ms, 10–40 °C) (34) and Clean-TOCSY spectra (25, 35; spin lock of 15 and 30 ms) (35) using MLEV-17 (36) were recorded over a bandwidth of 14 kHz (or 28 kHz) (NOESY) and 14 kHz (TOCSY) with recycle times of 1 s (or 0.33 s) using 512 r1 blocks of 128 and 250 scans, each consisting of 2048/4096 points. Two-dimensional data sets were processed using Bruker XWIN software on a Silicon Graphics Indigo work station and consisted of 30’sine-squared bell apodization in both dimensions and zero filling to 2048 × 2048 data points prior to Fourier transformation.

Magnetic Axes—The magnetic axes (Fig. 1) were determined by a least-squares search for the minimum in the error function (21, 32, 37) (Equation 1).

\[
F/n = \sum_{i=1}^{a} \delta_{\text{tripolar}} - \delta_{\text{dipolar}}^2 
\]  

The calculated dipolar shift in the reference coordinate system, \( x', y', z' \) (or \( R', \theta', \phi' \)), is given by Equation 2, with \( \Delta x_0 = 2.48 \times 10^{-3} \text{ mmol} \) and \( \Delta x_0 = 0.58 \times 10^{-3} \text{ mmol} \) of the axial and rhombic anisotropies of the diagonal paramagnetic susceptibility tensor taken from the isoelectronic met-cyano-myoglobin complex (37).

\[
\delta_{\text{tripolar}} = (24\pi N)^{-1}(2\Delta_x\sin\theta\cos\phi)R^3
\]

\[
+ 3\Delta_x\sin\theta\cos\phi(R^{-1})^3
\]

\[
3\Delta_x\sin\theta\cos\phi(R^{-1})^3
\]

\[
\delta_{\text{dipolar}}^2 = \delta_{\text{tripolar}}^2 + \delta_{\text{dipolar}}^2 + \delta_{\text{zonal}}^2
\]

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\delta_{\text{dipolar}}^2 = \delta_{\text{tripolar}}^2 + \delta_{\text{zonal}}^2
\]

\[
\delta_{\text{dipolar}}^2 = \delta_{\text{tripolar}}^2 + \delta_{\text{zonal}}^2 + \delta_{\text{dipolar}}^2
\]

The symbols \( \delta_{\text{dipolar}} \), \( \delta_{\text{zonal}} \), and \( \delta_{\text{dipolar}} \) are the chemical shifts of an unfolded tetrapeptide (38) relative to DSS and the effect of secondary structure (39) and ring currents (40) on the shift, respectively.

RESULTS

The initially assembled HmuO-PH-CN complex exhibits two sets of hyperfine shifted resonances (data not shown; see Sup-
HmuO complexes. The crowded region between 10 and 15 ppm is a hyperfine aromatic cluster. The homologous assignments are patterned with the assignments of the observed sequential backbone NOESY contacts in hHO and HmuO compared in Fig. 3 (18–20). The essentially identical shift pattern for major isomer of the PH substrate is evidence for the same heme orientation in HmuO and hHO (see below).

**Heme Assignments**—Dipolar contacts were observed in a set of pyrrole substituents on both HmuO-PH-CN and HmuO-DMDH-CN (data not shown) that are identical to those reported in detail for the analogous hHO complexes (21). The remarkably similar hyperfine shifts for a given substituent in the HmuO and hHO complexes are in evidence in the data provided in Table I. The essentially identical shift pattern for the major isomer of the PH substrate is evidence for the same heme orientation in HmuO and hHO (see below).

**Proximal and Distal Helices**—Standard backbone NOE connectivities (data not shown; summarized in Fig. 2) among TOCSY-detected side chains locate two helical fragments (I and II) for which numerous side chains exhibit moderate-to-large hyperfine shifts. Fragment I is Ala–Z–Ala–2–AMX–3–Z–Ala––4–Z–Ala––5–Z–Ala––6–Z and/or Hα-N(3) NOE connectivities characteristic of helices (41) as described for hHO (20, 21).

The resolved portions of the 600-MHz 1H NMR spectra of equilibrated hHO-PH-CN and HmuO-PH-CN (19, 20) are compared in Fig. 3 (A and B, respectively). Similarly, the traces of HmuO-DMDH-CN and hHO-DMDH-CN (21) are compared in Fig. 3 (C and D, respectively). The very close similarity of the pattern of resolved resonances in the hHO and HmuO complexes is quite apparent. The homologous assignments are connected by dashed lines between the two hHO and the two HmuO complexes. The crowded region between 10 and 15 ppm for HmuO-DMDH-CN in 1H2O is expanded in Fig. 4A. The relevant homologous portions of the amino acid sequence for the two HOs are illustrated in Fig. 2. The nonselective T1 values for well resolved peaks of interest in hHO-DMDH-CN and HmuO-DMDH-CN (as well as in the PH complexes) are the same. In particular, the low-field labile proton peaks Gly139 NH and upfield Ser138 CαH and CβH exhibit T1 values indistinguishable from those of Gly143 NH and Ser142 CαH and CβH (~50, 85, and 50 ms, respectively) reported previously (20, 21).

Comparison in Fig. 3 of the NMR spectra of the complexes of the two HOs shows that the patterns of shifts are so similar in the two proteins that it is highly advantageous to pursue assignments on the basis of the comprehensive and definitive assignments previously reported for hHO complexes (20–22). Hence, two-dimensional NMR data are presented only to define an important distal H-bond network/aromatic cluster as just recently characterized in hHO. We initially (and trivially) assign the heme, followed by locating hyperfine shifted protons that arise from TOCSY-detected side chains placed on sequentially assigned backbone via the standard N(–N(3), β-N, αβ-N, and/or β-Hα-N NOE connectivities characteristic of helices (41) as described for hHO (20, 21).

**Fig. 2.** Sequence portions of interest for HmuO (with the homologous residues in hHO in italics) and schematic representation of the observed sequential backbone NOESY contacts in hHO-PH-CN for portions of the proximal helix (I), the distal helix (II), two additional helical fragments (IV and V), and one non-helical fragment (VI), which participate in the distal H-bond network and aromatic cluster. Helix III, identified in hHO, was not located in HmuO-PH-CN.

| I  | II |
|----|----|
| hHO | HmuO |
| NNi±1 | αNi±1 |
| βNi±1 | αNi±3 |
| αi±H3 |

| III |
|-----|
| hHO | HmuO |
| NNi±1 | αNi±1 |
| βNi±1 | αNi±3 |
| αi±H3 |

| IV |
|----|
| hHO | HmuO |
| NNi±1 | αNi±1 |
| βNi±1 | αNi±3 |
| αi±H3 |

| V |
|----|
| hHO | HmuO |
| NNi±1 | αNi±1 |
| βNi±1 | αNi±3 |
| αi±H3 |

| VI |
|----|
| hHO | HmuO |
| NNi±1 | αNi±1 |
| βNi±1 | αNi±3 |
| αi±H3 |

Solution Active Site Structure of HmuO

[Image: Figure 2]
hHO (20, 21), indicates that Phe$^{201}$ is slightly shifted away from Ala$^{23}$. Finally, note that 3-CH$_3$ in HmuO-PhCN exhibits weak NOESY cross-peaks to Phe$^{208}$ (which also exhibits strong NOESY cross-peaks to the 2-vinyl group) (data not shown), whereas the small Phe$^{214}$ cross-peak to 3-CH$_3$ is not seen in hHO-Ph-CN (20, 21) (but the strong 2-vinyl cross-peak to Phe$^{214}$ is observed). This indicates that the conserved Phe 208/ Phe$^{214}$ at the pyrrole A/B junction is slightly closer to position 2 in HmuO than in hHO. Moreover, NOESY cross-peaks of the labile protons for NH$_2$ of Gln$^{38}$ in hHO (Fig. 5B) (20, 21) are absent in the HmuO complex (Fig. 5A), but strong contacts with some aliphatic protons are present as expected because of the Gln$^{38}$ → Leu$^{33}$ replacement in HmuO. The chemical shifts of the two HmuO complexes, as well as of the two hHO complexes (20, 21), for these assigned residues are compared in Table II, where we also include the predicted dipolar shifts for the residues in the hHO complex. The observed inter-residue and heme-residue dipolar contacts are summarized schematically in Fig. 6.

The NOESY and TOCSY data (data not shown; summarized in Fig. 2) indicate that helical fragment II is represented by Val-$\text{AMX}_{1-2}$-Leu$_{3}$-$\text{N}_{4}$-$\text{AMX}_{5-6}$-$\text{AMX}_{7}$-$\text{Gly}_{1-8}$ (Fig. 2), where AMX$_{i-2}$ is in contact with a two-spin aromatic ring; Val$_{1}$, Leu$_{3}$, and AMX$_{7}$ exhibit moderate-to-large high-field dipolar shifts; and Gly$_{1-8}$ exhibits strong low-field dipolar shifts. Both the sequence and the dipolar shift pattern identify (20, 21) this as a key portion of the distal helix Val$^{131}$-Gly$^{139}$ (analogous to Thr$^{135}$-Gly$^{143}$ in hHO). As shown in the slices through 8-CH$_3$ in HmuO and hHO (Fig. 7, A and B), the strong contact with C$_{6}$H of Val$^{131}$ is conserved (relative to C$_{6}$H of Thr$^{135}$). However, the weak contacts between 8-CH$_3$ and NH of the adjacent conserved Leu$_{134}$ and Gly$_{135}$ in HmuO (residues 138 and 139 in hHO) observed in the hHO complex (Fig. 7B) (20, 21) are not detectable in HmuO (Fig. 7A) and indicate a small movement of the distal helix near its kink away from 8-CH$_3$ in HmuO relative to hHO. Finally, slices through similarly relaxed (T$_2$ ∼ 85 ms) Ser$^{138}$ C$_{1-2}$ in HmuO (Fig. 7C) and Ser$^{142}$ in hHO (Fig. 7D) indicate that the Ser is slightly farther from heme 6-H$_{2}$ in HmuO relative to the hHO complex. The chemical shifts for helix II residues, together with data from hHO complexes (20, 21), are listed in Table II. The observed inter-residue and heme-residue contacts are illustrated schematically in Fig. 6.

**H-bond Network/Aromatic Cluster**—The HmuO complexes, like the hHO complexes (21, 22), exhibit a set of strongly low-field shifted labile proton peaks (Figs. 3 and 4A), which (with the unique exception of HmuO Gly$^{139}$ NH/hHO Gly$^{143}$ NH) exhibit negligible paramagnetic relaxation, so their strong low-field bias must be attributed to strong hydrogen bonds (42). Three sequential fragments are easily recognized (summarized in Fig. 2) by their remarkably similar arrangements compared with the three characterized fragments labeled IV–VI in hHO-DMDH-CN (21). The analogous fragment III could not be recognized as easily in the HmuO complex (but see “Discussion”). The helical fragment IV, Z-$\text{Gly}_{1-2}$-$\text{AMX}_{3-4}$-$\text{AMX}_{5-6}$, exhibits strong low-field NH shifts for Gly$_{1}$ and AMX$_{5-6}$ (Fig. 8, B and D; summarized in Fig. 2), as found for Ala$^{160}$-Phe$^{166}$ in hHO (21). In agreement with the assignment of Leu$^{152}$-Tyr$^{161}$ to fragment IV, a three-spin TOCSY ring makes contact with AMX$_{3-4}$ (Fig. 9D), and two spins of a three-spin aromatic ring make contact with AMX$_{5-6}$ (Fig. 9C), which must arise from Tyr$^{141}$. The three TOCSY/NOESY peaks of the AMX$_{1-3}$ side chain exhibit the unusual pattern (Fig. 9, B–D) that one cross-peak becomes narrower, whereas the other broadens as the
temperature of Trp 50). Moreover, comparison of the 3:9:19 trace (33) without the carboxylate of Asp 136 (homologous to Asp 140 in hHO) is the acceptor for NH groups of Gly159 and His128 N in hHO) cannot be identified by comparison with hHO. The relative positions of the donors and acceptors in the strong H-bonds in HmuO and hHO (21) are compared in Table III.

Acceptors for Strong H-bond Donors—Having demonstrated for HmuO a remarkably conserved arrangement on the distal side of the heme for three of the four fragments involved in the H-bond network/aromatic cluster in hHO (21), one can speculate that the acceptors for these strong H-bond donors in hHO are also homologous in HmuO. Thus, sequence comparison indicates that the carboxylate of Glu196 (homologous to Glu423 in hHO) is the acceptor for NH groups of Arg130 and Ala96 (22) and that the carbamoyl group of Asp136 (homologous to Asp140 in hHO) is the acceptor for Tyr161 O6. Similarly, His142 N in hHO as donor to Glu202 in hHO (22) indicates that Glu196 is the acceptor for His139 N in HmuO. Even though fragment III, identified in hHO (21), could not be assigned in HmuO, the sequence homology suggests that the Asp166 carboxylate (homologous to Asp192 in hHO) cannot be identified by comparison with hHO. The relative positions of the donors and acceptors in the strong H-bonds in HmuO and hHO (21) are compared in Table III.

Labile Proton Exchange—Comparison of the resolved low-field 1H NMR trace of HmuO-DMDH-CN in 1H2O in Fig. 4A with that of the complex 20 min (Fig. 4B) and 4 h (Fig. 4C) after transfer into 2H2O shows that, whereas Tyr55 OH has a half-life <5 min, other strong H-bonding proton exchange time-half-lives not only for peptide NH groups (30 min for Arg127, -30 days for Phe160, and 2 h for peak a), but also for side chain labile protons (4 h for N H of His128 and -2 h for N H of Trp50). Moreover, comparison of the 3:9:19 trace (33) without (Fig. 4A) and with (Fig. 4B) saturation of the bulk water resonance shows significant magnetization transfer to the low-field peaks (22, 43), as shown in the difference trace in Fig. 4C. The magnetization transfer to the four labile protons shown to exchange slowly with water must arise from NOEs between these labile protons and "immobilized" water molecules (43), as we previously observed for hHO-DMDH-CN (22). The magni-
tude of the NOEs is ~10% for His128 NH and ~25% for Phe160 NH, Trp59 NH, and peak a. For the other peaks that exhibit magnetization transfer from water, it is not possible at this time to differentiate between chemical exchange and NOEs as the origin of the magnetization transfer (43).

Magnetic Axes and Cyanide Tilt—The completely conserved pattern of large dipolar shifts for proximal helix residues (Table II) and conserved contacts with the heme (Fig. 6) in HmuO relative to the hHO complexes can arise only if HmuO·PH·CN and hHO·PH·CN possess very similar orientation for the major magnetic axis (32). A direct determination of the magnetic axes for HmuO·PH·CN using the present 1H NMR data and the recently available HmuO crystal coordinates2 leads to α = 234 ± 16, β = 18 ± 2, and κ = 47 ± 12, which can be compared with reported values of α = 234 ± 12, β = 20 ± 3, and κ = 25 ± 13 for hHO-DMDH-CN (21). The

\[
\begin{array}{cccc}
\text{Proton} & \text{HmuO·substrate·CN} & \text{hHO·substrate·CN} \\
\hline
1-\text{CH}_3 & 4.91 & 8.98 & 4.95 & 8.95 \\
2-\text{CH}_3/vinyl & 15.63 (H_{\alpha}), -4.35, -4.80 (H_{\beta}) & 20.87 & 15.68 (H_{\alpha}), -4.22, -4.23 (H_{\beta}) & 21.37 \\
3-\text{CH}_3 & 19.42 & 18.65 & 19.63 & 18.25 \\
4-\text{CH}_3/vinyl & 9.87 (H_{\alpha}), 0.43, 0.80 (H_{\beta}) & 9.58 & 10.09 (H_{\alpha}), 1.77, 2.52 (H_{\beta}) & 8.51 \\
5-\text{CH}_3 & 8.09 & 10.18 & 9.04 & 9.78 \\
6-H_s & 10.12, 9.59 & 10.20, 9.76 & 11.75, 10.62 & 11.02, 10.24 \\
7-H_s & -1.00, -0.74 & -1.04, -0.78 & 0.40, 0.40 & -0.05, 0.12 \\
7-H_s & -0.66, 0.52 & -0.82, 0.42 & -0.45, -0.45 & -0.91, -0.01 \\
8-\text{CH}_3 & 10.37 & 8.19 & 10.48 & 8.29 \\
\alpha-meso-H & -3.63 & -2.10 & -5.10 & -2.83 \\
\beta-meso-H & * & * & 7.60 & 6.80 \\
\gamma-meso-H & * & * & 3.84 & 1.89 \\
\delta-meso-H & 7.24 & * & 7.13 & 7.65 \\
\hline
\end{array}
\]

* R = vinyl in Fig. 1.
* R = methyl in Fig. 1.
* Data are from Ref. 20.
* Data are from Ref. 21.
* Not assigned.
| Residue⁹ | Proton | HmuO · substrate · CN | hHO · substrate · CN | δexp[ppm]|  |
|---|---|---|---|---|---|
| **Helix I** | | | | | |
| Ala¹⁹/Lys²² | NH | 7.96 | 8.03 | 7.93 | 7.88 | 1.58 |
| C_H | 6.39 | 6.50 | 6.65 | 6.18 | 3.66 |
| C_H₂ | 2.60 | 2.66 |  |
| Gln²⁹/Glu²³ | NH | 9.25 | 9.24 | 9.19 | 9.00 | 1.34 |
| C_H | 4.87 | 4.97 | 4.86 |  | 1.13 |
| Ala³⁹/Val⁴ⁱ | NH | 8.54 | 8.62 | 8.65 | 8.65 | 1.05 |
| C_H | 4.87 | 4.96 | 4.1 |  | 0.45 |
| His⁶⁰/His⁶⁵ | NH | 10.46 | 10.73 | 10.03 | 10.15 | 2.66 |
| C_H | 4.86 | 5.82 | 4.01 | 4.68 | 2.21 |
| C_H₂ | 9.77 | 10.02 | 9.24 | 8.95 | 5.53 |
| C_H₃ | 10.31 | 10.73 | 9.99 | 10.74 | 8.80 |
| Glu³¹/Thr³⁶ | NH | 9.99 | 10.14 | 9.71 | 9.85 | 2.67 |
| C_H | 5.68 | | 4.95 | 5.20 | 1.84 |
| C_H₂ | 3.36 | 2.92 | 3.43 | 5.05 | 5.11 | 1.28 |
| Lys²³/Gln²⁷ | NH | 7.95 | 7.94 | 7.70 | 8.05 | 0.77 |
| C_H | 4.85 | 4.85 | 3.75 |  | 0.03 |
| Ala³²/Ala²⁸ | NH | 6.77 | 6.80 | 7.12 | 7.30 | −0.39 |
| C_H | 2.38 | 2.45 | 2.25 | 2.42 | −1.63 |
| C_H₂ | −2.27 | −2.24 | −2.25 | −2.14 | −3.79 |
| Glu³³/Glu³⁸ | NH | 6.38 | 6.42 | 5.85 | 6.18 | −1.01 |
| C_H | −0.25 |  | −0.60 | −0.42 | −0.59 |
| Ala²⁰⁰/Ala²⁰⁶ | NH | 8.79 | 8.71 | 8.70 |  | −0.39 |
| C_H | 3.64 | 3.62 | 3.41 | 5.57 | 0.71 |
| C_H₂ | 0.62 | 0.61 | 0.97 |  |  |
| Phe³⁰⁷/Phe³⁰⁸ | NH | 6.36 | 6.43 | 6.50 | 6.43 | −0.41 |
| C_H | 6.04 | 6.10 | 5.85 | 5.82 | −0.95 |
| Phe³⁰⁸/Phe³¹⁴ | NH | 7.52 | 7.47 | 8.08 |  | −0.72 |
| C_H | 2.83 | 2.80 | 2.94 |  | −1.76 |
| C_H₂ | 2.14 | 2.18 | 2.11 | −0.83 | −0.47 |
| C_H₃ | 6.35 | 6.13 | 6.62 | 6.83 | −1.19 |
| C_H₄ | 6.06 | 5.92 | 6.95 | 6.35 | −1.55 |
| C_H₅ | 6.37 | 6.28 | 7.78 | 6.27 | −1.38 |
| **Helix II** | | | | | |
| Tyr³⁵/Tyr³⁴ | C_H | 6.64 | 6.64 | 6.71 | 6.25 | −0.52 |
| C_H₂ | 6.30 | 6.27 | 6.45 | 6.56 | −0.34 |
| Val³¹/Thr³⁵ | NH | 7.13 | 7.26 | 6.74N | 6.74 | −0.71 |
| C_H | 2.07 | 1.97 | 2.65 | 2.39 | −1.83 |
| C_H₂ | 1.23 | 1.18 | 3.60 | 3.35 | −0.98 |
| C_H₃ | −0.85, 0.85 | −0.90, 0.80 | 0.01 | −0.10 | −2.02 |
| Arg³³²/Arg³³⁶ | NH | 8.27 | 8.27 | 5.09 |  | −0.31 |
| C_H | 5.06 | 2.63 | 2.51 | 2.79 | −0.16 | 0.0 |
| C_H₂ | 9.84 | 9.73 | 9.56 | 9.51 | 0.36 |
| N_H | 8.46 | 8.46 | 7.83 | 7.46 | 0.07 |
| C_H | 6.82 | 6.80 | 7.04 | 7.10 | 0.06 |
| OH | 11.50 | 11.51 | 10.84 | 10.38 | 0.08 |
| Leu³³⁸/Leu³³⁹ | NH | 8.30 | 8.34 | 7.12 | 7.05 | −0.48 |
| C_H | 3.49 | 3.51 | 3.08 | 3.12 | −0.53 |
| C_H₂ | −1.18 | −1.10 | −0.37 | −0.40 | −0.16 |
| C_H₃ | 1.05 | 1.08 | 0.65 | 0.55 | −0.72 |
| C_H₄ | 0.23 | 0.25 | 0.70 | 0.55 | −0.84 |
| C_H₅ | −0.66 | −0.63 | 0.00 | −0.07 | −1.68 |
| Gly³³⁵/Gly³³⁹ | NH | 5.73 | 5.79 | 5.95 | 5.90 | −1.07 |
| C_H | 0.20, −0.25 |  | −0.70 | −0.07 | −1.71 |
| Asp³³⁶/Asp³⁴⁰ | NH | 10.16 | 10.07 | 9.65 | 9.79 | 1.61 |
| C_H | 8.25 | 8.24 | 7.43 | 7.72 | 5.49 |
| C_H₂ | 4.00 | 4.02 | 4.05 | 3.82 | −0.02 |
| Ser³³⁹/Ser³⁴² | NH | 8.09 | 8.07 | 8.05 | 8.22 | −0.02 |
| C_H | 2.80 | 2.88 | 2.60 | 2.81 | −1.60 |
| C_H₂ | −1.43 | −1.21 | −3.35 | −2.59 | −6.90 |
| C_H₃ | −2.77 | −2.60 | −5.25 | −4.07 | −6.20 |
| Gly³³⁸/Gly³⁴³ | NH | 16.40 | 16.52 | 15.92 | 16.10 | 3.38 |
| C_H | 8.71 | 9.03 | 7.86 | 8.05 | 8.25 |
| C_H₂ | 8.46 | 8.61 | 7.61 | 7.65 | 0.47 |
| **Helix V** | | | | | |
| Tyr³⁷⁵/Tyr³⁶⁶ | NH | 8.72 | 8.74 | 9.11 | 9.06 | 0.05 |
| C_H | 3.71 | 3.71 | 3.81 | 3.81 | 0.02 |
| C_H₂ | 7.30 | 7.32 | 7.83 | 7.76 | 0.17 |
| C_H₃ | 6.95 | 6.96 | 7.04 | 6.99 | 0.33 |
| OH | 17.32 | 17.29 | 16.84 | 16.70 | 0.59 |
| Thr³⁵⁶/Val³⁵⁹ | NH | 8.46 | 8.32 | 8.39 | 8.30 | 0.10 |
| C_H | 6.63 | 6.63 | 6.89 | 6.89 | −0.03 |
| C_H₂ | 2.91 | 2.91 | 3.90 | 3.90 | −0.04 |
| C_H₃ | 0.58 | 0.58 | 1.01 | 1.01 | −0.07 |
| Residue$^a$ | Proton | HmuO · substrate · CN $^{b}$ | hHO · substrate · CN $^{b}$ | $\delta_{\text{dipolar}}/$ |
|------------|--------|-------------------------------|-----------------------------|-------------------|
|            |        | PH$^b$ | DMDH$^b$ | PH$^{b,d}$ | DMDH$^{b,e}$ |
| Fragment VI |        |        |        |        |        |
| Asn$^{79}$/His$^{84}$ | NH | 11.50 | 11.51 | 7.64 | 0.10 |
| Arg$^{79}$/Arg$^{85}$ | NH | 12.85 | 12.86 | 13.00 | 13.03 | 0.16 |
| Ala$^{90}$/Lys$^{96}$ | C,H | 4.60 | 4.55 | 4.49 | 0.26 |
| Gly$^{83}$/Ala$^{87}$ | C,H | 3.93 | 3.94 | 3.70 | 0.14 |
| Val$^{82}$/Ala$^{88}$ | C,H | 7.99 | 7.99 | 3.88 | 0.11 |
|             |        |        |        |        |        |
| Helix IV |        |        |        |        |        |
| Leu$^{159}$/Leu$^{164}$ | NH | 7.03 | 7.06 | 7.41 | 7.45 | 0.26 |
| Gly$^{159}$/Ala$^{165}$ | C,H | 10.17 | 10.22 | 12.05 | 12.09 | 0.30 |
| Phe$^{160}$/Phe$^{166}$ | C,H | 3.86 | 3.97 | 2.03 | 0.36 |
| Aromatic rings |        |        |        |        |        |
| Phe$^{92}$/Phe$^{97}$ | C,H | 3.86 | 3.97 | 7.21 | 6.23 | 0.28 |
| C,H | 7.21 | 7.59 | 7.45 | 0.47 |
| C,H | 7.91 | 7.68 | 7.50 | 0.53 |
| Trp$^{96}$/Trp$^{101}$ | N,H | 11.30 | 11.33 | 10.05 | 10.04 | 0.09 |
| Trp$^{96}$/Tyr$^{55}$ | N,H | 11.43 | 11.36 | 7.58 | 7.58 | 0.28 |
| C,H | 6.51 | 6.51 | 6.51 | 0.28 |
| C,H | 6.63 | 6.63 | 6.63 | 0.28 |
| C,H | 6.30 | 6.30 | 6.30 | 0.28 |

$^a$ Residues are given as HmuO/hHO. Chemical shifts (in ppm) are referenced to DSS in $^1$H$_2$O and 100 mM phosphate, pH 7.3, at 30 °C.

$^b$ R = vinyl in Fig. 1.

$^c$ R = methyl in Fig. 1.

$^d$ Data are from Ref. 20.

$^e$ Data are from Ref. 21.

$^f$ Dipolar shift (in ppm) was calculated on the basis of the magnetic axes determined using $\delta_{\text{dipolar}}/$ for hHO · DMDH · CN (21) and the hHO · PH · H$_2$O crystal structure (15).

$^g$ Not uniquely assignable due to spectral congestion.

magnetic axes for both complexes yield similarly excellent correlation between the $\delta_{\text{dipolar}}$/ and $\delta_{\text{dipolar}}$/ for the input proximal side residues (data not shown; see Supplemental Material). In each case, $z$ is tilted $-20^\circ$ toward the $\gamma$-meso position. The $z$ direction is oriented toward the proximal side (20, 32); hence, the FeCN vector ($-z$ direction) is tilted toward the $\alpha$-meso position. Therefore, a direct contribution to stereoselectivity from the tilt due to direct steric interactions with the ligand in the direction of the $\alpha$-meso position is operative in both hHO and HmuO.

**DISCUSSION**

**PH Orientation**—The orientation of PH in both hHO (19, 20) and HmuO complexes is similarly rotationally disordered about the $\alpha'$-$\gamma$-meso axis in the initially formed complex, with very similar $-3:1$ ratios at equilibrium and with the same heme orientation dominating in each complex in solution. Notably, the heme orientation found in the HmuO·PH·H$_2$O crystal structure$^2$ is the same as the dominant isomer in solution, whereas that in the hHO·PH·H$_2$O crystal structure (21) is the minor form in solution (19, 20). The resolution upon using DMDH rather than PH for HmuO is less dramatic than for hHO because, in contrast to hHO (20, 21), there are no detectable changes in the intrinsic line width of the signals in the DMDH relative to the PH complexes of HmuO, only the loss of the second set of minor compound signals. The narrower lines for the HmuO complex compared with the HO complex are attributed in part to a reduction in size (216 versus 265 residues) relative to hHO. An example of the narrower line widths in the HmuO complex compared with the hHO complex is the detection of the complete Ser138 TOCSY connections (data not shown; see Supplemental Material), including the NH-C$_{6}$H correlation missing in hHO-DMDH-CN (21), despite unchanged paramagnetic relaxation.

**Utility of hHO as a Homology Model**—The similarity in the
The close similarity of the environments of the individual heme methyls not only reflects the numerous completely conserved contacts, but allows the ready identification of the HmuO residues whose nature has been dramatically altered compared with hHO residues, i.e. hHO Ile211 -> HmuO His205 in Fig. 5 (C and D) and hHO Gln38 -> HmuO Leu33 in Fig. 5 (A and B). Fragment III (Fig. 2) could not be located in analogy with hHO because fragment III possesses three aromatic residues (Phe95, Trp96, and Tyr97) in hHO that could be easily identified (21) by their contacts with fragment IV, and these residues on fragment III are substituted by aliphatic residues (Lys89, Leu90, and Asn91) in HmuO. The cluster of aromatic side chains, i.e. those of HmuO/HO Tyr53/Tyr58, Phe160/Phe166, Tyr161/Phe167, Tyr130/Tyr134, Tyr133/Tyr137, and Phe42/Phe47, are again largely conserved in the two HO proteins (Fig. 6). Finally, the acceptor for all but one (Tyr161 O-H) of the assigned strong H-bond donors could be identified in HmuO complexes solely on the basis of sequence homology. We therefore conclude that a structurally characterized HO complex will serve as a valuable homology model to facilitate the assignment of residues involved in many details of the active site structure in a related HO.

The sequence homology between HmuO and hHO is relatively high (33% identity and 70% similarity if conservative substitutions are included) (10). Other bacterial HO s, such as HemO from N. meningitides (44), exhibit less sequence homology to mammalian HO s, but still exhibit a structure (45) that is related to that characterized in the two mammalian HO s (15, 16) and one bacterial HO (45) and exhibit different details of the active site. To date, 1H NMR data on HemO (44, 45) have not been reported to allow comparisons.

Comparison with the HmuO-PH-H2O Crystal Structure—The 1H NMR data are consistent with the crystal structure2 for HmuO to the same degree previously found for the same hHO complexes (20, 21). The proximal helix is strongly conserved.
but the distal helix exhibits dipolar shifts that deviate from those predicted by the relatively robust magnetic axes in the same fashion as found for hHO (see Supplemental Material) (20, 21). The loss in solution of dipolar contacts between the NH groups of Leu134 and Gly135 and 3-CH₃ and the weakening of contacts between Ser138 CH/H and 6-H (Fig. 7B) relative to
predictions based on the crystal structure\(^2\) suggest possibly only a small (0.5–1.0 Å) movement of the distal helix near its kink. However, similar differences in the distal helix position have been observed in the two non-equivalent molecules in the hHO-DMDH-CN crystal (15) and may simply represent the intrinsic mobility of the distal helix.

The distal H-bond network in the HmuO complex,\(^2\) as in the case of HHO-DMDH-CN, is not readily discerned in the crystal structure (14, 15). However, once the donor NH and OH groups have been identified by \(^1\)H NMR, the crystal structure readily identifies the probable acceptors. The proposed acceptors for the strong H-bonds in HmuO are the Glu\(^{57}\), Asp\(^{86}\), Asp\(^{136}\), and Glu\(^{196}\) carboxylates, based solely on the homology to HHO-DMDH-CN NMR data (21) and the hHO-DMDH-CN crystal structure (15) and completely confirmed in the HmuO crystal structure (Fig. 6).\(^2\) The crystallographic geometry (distance and angle)\(^2\) for these H-bonds in HmuO is summarized in Table III, where they can be compared with similar data on HHO-DMDH-CN and HHO-DMDH-CN. Their dispositions are far from ideal (42) to allow the strong H-bond so obvious in the 1H NMR data, as shown in Table III. This non-ideal orientation of the donors and acceptors may be simply the result of the intrinsic uncertainties in the crystallographic positions of the two interacting units. The acceptor for the new strong H-bond from Tyr\(^{161}\) O\(_\text{H}\) is identified in the crystal\(^2\) as the side chain of Gln\(^{46}\) (Fig. 6). Similar strong H-bonds, as yet unassigned, appear in the \(^1\)H NMR spectra of apo-HO in both mammals and bacteria,\(^3\) indicating that the H-bond network plays a key role in the structures of both the apo-HO and substrate complexes.

A structural difference of possible significance between the HmuO cyanide-ligated complex and the crystal structure of the aquo-ligated complex\(^2\) is the relative position of fragment IV relative to the distal helix II. This same difference was previously observed in the hHO complex (20, 21). Thus, although moderate intensity NOEs are observed between the Tyr\(^{161}\) ring and Gly\(^{139}\) NH (i.e. \(r_{ij} > 4\) Å), the crystal structure indicates \(r_{ij}/H1012< 6\) Å. This same difference was previously observed for the analogous hHO complexes involving the homologous Phe\(^{167}\) ring and Gly\(^{143}\) NH (20, 21). Thus, the aromatic cluster appears to move ~2–3 Å closer to the distal helix in the cyanide complex in solution compared with the aquo complex in the crystal. This difference may be due to the different ligands used in the alternate studies in solution (CN\(^-\), a H-bond acceptor) and in the crystal (H\(_2\)O, a H-bond donor).

Ordered Water Molecules—NOEs indicative of nearby immobilized water molecules (Fig. 4, A, B, and C') (43) are observed for the NH groups of Arg\(^{79}\) and Phe\(^{160}\) and the N\(_\text{H}\) groups of His\(^{128}\) and Trp\(^{150}\). Water molecules are indeed observed close to the NH groups in the crystal structure.\(^2\) Similar water NOEs are observed for the NH groups of the homologous Arg\(^{85}\) and Phe\(^{166}\) in hHO and His\(^{132}\) N\(_\text{H}\) (the Tyr homolog of Trp\(^{150}\) was not assigned in hHO) (22). Hence, both HO\(_2\)s are characterized

\(^2\) Y. Li, R. T. Syvitski, G. C. Chu, M. Ikeda-Saito, and G. N. La Mar, unpublished data.
Comparison of Dynamic Properties of HmuO and hHO—The very close structural homology between HmuO and hHO apparent in both the solution $^1$H NMR data and crystal structures is, however, in contrast to the highly differential dynamic properties of the two enzymes. On the one hand, the rate of exchange with $^3$H$_2$O of homologous labile protons involved in the strong H-bonds differs significantly for the two enzymes, with HmuO exhibiting significantly reduced rates. Comparison of the homologous NH groups shows that the half-lives for a residue are ~4 h for HmuO His$_{128}$ N$_H$ and 45 min for hHO His$_{132}$ N$_H$ (22), 30 min for HmuO Arg$_{48}$ NH and 25 min for hHO Arg$_{48}$ NH (22), and ~700 h for HmuO Phe$_{167}$ NH. Moreover, the Tyr$_{161}$/Tyr$_{157}$ OH groups, which exhibit saturation transfer due to exchange, exhibit a much smaller saturation factor (~10%) in HmuO than in hHO (~40%), dictating a much slower exchange rate in HmuO relative to the Phe$_{167}$ ring in hHO. The ~700 factor decrease in the Phe$_{167}$/Phe$_{157}$ NH exchange rate indicates that the dynamic stability near fragmentation IV is ~4 kcal greater in HmuO than in hHO (46). Although the extremely low-field shifts for the labile protons of the H-bond networks are very similar in the two HO complexes (Table III), indicating that the individual H-bonds are remarkably strong, other factors that contribute to the stability of the folding in the environment of the network are clearly much weaker in hHO than in HmuO.

The $^1$H NMR data provide other indicators for a dynamically more stable (and hence, less flexible) HmuO than hHO. On the one hand, two new and strong H-bonds are observed, one of which could be uniquely attributed to Tyr$_{161}$ O$_H$ (which substitutes for Phe$_{167}$ in hHO). The likely acceptor, Gln$_{46}$, is suggested by the HmuO crystal structure, although the strong low-field bias due to H-bonding suggests a stronger or more flexible single acceptor. In fact, the crystal structure of HmuO$_{PH}$H$_2$O$_2$ places a water molecule within 3 Å of this OH. The sequence origin of the other strong H-bond (peak c at 12.4 ppm in Fig. 4A) is not identified, but has no homolog in hHO. Nevertheless, these two strong H-bonds in HmuO are incrementally more mobile than those conserved relative to hHO (21, 22). The second observation is that the Tyr$_{161}$ ring, unlike the Phe$_{167}$ ring, exhibits slow ring reorientation about the C$_p$–C$_p$ bond, as evidenced by the resolution of two C$_p$ H peaks at low temperature, whereas an averaged C$_p$ peak is observed for Phe$_{167}$ at all temperatures for hHO complexes (20, 21). The decreased mobility of Tyr$_{161}$ in HmuO relative to the Phe$_{167}$ ring in hHO (21) in 2-fold reorientation supports a tighter and more constrained distal environment in HmuO than in hHO.

Role of the H-bond Network—The conservation of the H-bond network/aromatic cluster/ordered water molecules in HmuO relative to hHO argues for important functional roles. The existence of a network of water molecules that includes water molecules near the catalytically critical distal helix Asp$_{136}$/Asp$_{140}$ (12, 14) supports the notion that water provides the stabilizing H-bond to the novel hydperoxy intermediate. Interaction of Asp$_{136}$ with the distal ligand via two water molecules has been recently characterized in the crystal structure of the rat HO-PH$_2$N$_2$O$_2$ complex (23). The presence of a water/H-bond network that extends from the distal pocket through the enzyme to its surface on the opposite side from the substrate-binding pocket (22) suggests that the channel may funnel the required nine protons to the active site in a controlled manner.

The greater dynamic stability of the pocket near the catalytically important Asp$_{136}$/Asp$_{140}$ is also apparent in two other

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**TABLE III**

| Donor | Acceptor | N(O)–H–O $^d$ | N(O)–H–O $^e$ |
|-------|----------|---------------|---------------|
| Arg$^7$/Arg$^9$/N$_H$ | Glu$^7$/Glu$^8$ | 3.3 | 125 $^+$ |
| Ala$^9$/Lys$^{26}$/N$_H$ | Glu$^5$/Glu$^6$ | 2.7 | 177 $^+$ |
| Tyr$^{157}$/Tyr$_{153}$ OH | Glu$^5$/Glu$^6$ | 3.8 | 158 $^+$ |
| Gly$^{159}$/Ala$_{185}$/N$_H$ | Asp$_{186}$/Asp$_{187}$ | 3.2 | 133 $^+$ |
| Phe$_{160}$/Phe$_{166}$/N$_H$ | Asp$_{166}$/Asp$_{187}$ | 2.9 | 133 $^+$ |
| Glu$_{196}$/Glu$_{202}$ | 2.5 | 155 $^+$ |
| Asp$_{136}$/Asp$_{140}$ | 3.0 | 129 $^+$ |
| Tyr$_{161}$/Tyr$_{157}$/OH | Asp$_{186}$/Asp$_{187}$ | 2.7 | 155 $^+$ |
| Tyr$_{161}$/Tyr$_{157}$/OH | Asp$_{136}$/Asp$_{140}$ | 4.0 | 115 $^+$ |
| His$^{132}$/His$_{132}$/N$_H$ | Glu$^{166}$/Glu$_{165}$ | 2.8 | 161 $^+$ |
| Tyr$_{161}$/OH | Glu$_{196}$/Glu$_{202}$ | 4.1 | 160 $^+$

$^a$ Chemical shift (in ppm) referenced to DSS in $^3$H$_2$O and 100 mM phosphate, pH 7.3, at 35 °C as HmuO–OH–CN/H–H–O–OH–CN, with the data for hHO taken from Ref. (21).

$^b$ Donor (HmuO/hHO) identified by the present $^1$H NMR data for HmuO–OH–CN, and as previously reported for hHO–OH–CN (21).

$^c$ Acceptor (HmuO/hHO) identified for the hHO complex by the crystal structure of hHO–PH$_2$N$_2$O$_2$ (15), proposed for the HmuO complex based on the sequence and structure homology of HmuO to hHO and as confirmed by the HmuO–PH–H$_2$O crystal structure (Footnote 2), except for the Tyr$_{161}$/OH and Glu$_{196}$/C–O–H bond, which could be identified solely in the HmuO–PH–H$_2$O crystal structure.

$^d$ N(O)–O distance as indicated in the HmuO–PH–H$_2$O crystal structure (Footnote 2).

$^e$ N(O)–H–O bond angle as indicated in the HmuO–PH–H$_2$O crystal structure (Footnote 2).

$^f$ Not present in hHO.
observed. The greater dynamic stability of the distal pocket in HmuO relative to hHO, witnessed in both slower labile proton exchange and aromatic ring reorientation, may be responsible for the ~4 factor slower turnover rate in HmuO (30) than in mammalian HOS (2, 11). More extensive NMR studies of both the dynamic properties of the distal side and the distribution of oriented water molecules in HmuO and other HO complexes are in progress.

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