The presence of variable static hemin orientational disorder about the α-γ-meso axis in the substrate complexes of mammalian heme oxygenase, together with the incomplete averaging of a second, dynamic disorder, for each hemin orientation, has led to NMR spectra with severe spectral overlap and loss of key two-dimensional correlations that seriously interfere with structural characterization in solution. We demonstrate that the symmetric substrate, 2,4-dimethyldeuterohemin, yields a single solution species for which the dynamic disorder is sufficiently rapid to allow effective and informative 1H NMR structural characterization. A much more extensive, effective, and definitive NMR characterization of the cyanide-inhibited, symmetric heme complex of human heme oxygenase shows that the active site structure, with some minor differences, is essentially the same as that for the native protohemin in solution and crystal. A unique distal network that involves particularly strong hydrogen bonds, as well as inter-aromatic contacts, is described that is proposed to stabilize the position of the catalytically critical distal helix Asp-140 carboxylate (Liu, Y., Koenigs, J. L., Poulos, T. L., Loehr, T. M., and Ortiz de Montellano, P. R. (2000) J. Biol. Chem. 275, 34501–34507). The potential role of this network in placing a water molecule to stabilize the hydroperoxy species and as a template for the condensation of the distal helix upon substrate binding are discussed.

Mammalian heme oxygenase (HO) is a ~300-residue, membrane-bound, non-heme enzyme that, using heme as cofactor and substrate, catalyzes the regiospecific conversion of heme to α-biliverdin, iron, and CO (1). The physiological roles of HO are heme catabolism (HO-1) (2–4) and the generation of CO as a putative neural messenger (HO-2) (5, 6). Detailed mechanistic (7–13) and spectroscopic (13–17) studies of the fully active recombinant, soluble 265-residue portion of HO-1 have shown that, in contrast to heme peroxidase and cytochrome P450, HO does not act through a ferryl intermediate. Recent crystal structures (18, 19) of the substrate-bound, water-ligated complexes of a more truncated 233-residue human HO, hHO (20), and the complete rat HO (18), rHO, have revealed a largely helical enzyme that confirms the binding of heme by His-25 and locates a highly bent distal helix that is sufficiently close to the heme to sterically block all but the α-meso position (see Fig. 1) for attack. Detailed studies have established that the reactive intermediate in HO catalysis is the hydroperoxy-heme (11) rather than the ferryl heme common to peroxidases and cytochromes P450 (21). Although the present structural information provides a ready interpretation of the stereoselectivity of the heme cleavage in terms of steric blocking of all but the α-meso position by the distal helix, the environmental influences that stabilize the Fe-O-OH rather than Fe=O species are incompletely understood.

Recent studies with both hHO (22) and rHO (23) mutants have shown that elimination of the carboxylate of Asp-140 on the distal helix completely abolishes HO activity and leads to formation of a catalytically incompetent ferryl species. Asp-140 is not oriented toward the heme iron and cannot directly interact with a heme ligand. However, the crystal structure (20) of hHO-PH-H2O (PH = protohemin, with R = vinyls in Fig. 1) identified a localized distal water molecule that bridges the ligated water and Asp-140 carboxylate and led to the proposals (22, 23) that such a localized water molecule may play the crucial role in stabilizing the Fe-O-OH species. It is reasonable that such an important role for a distal water is based on an extensive network of interactions that maintain the optimal Asp-140 side chain orientation. The characterization of such a distal network of hydrogen bonds would identify additional sites for more subtly modulating HO activity by mutagenesis.

Solution 1H NMR investigations of the hHO and rHO have contributed to our understanding of the enzyme properties (24–26). By far the most informative NMR spectra were observed (24–26) for the low spin ferric, substrate-bound, cyanide-inhibited complex, HO-PH-CN, which serves as a model for HO-heme-O2, except that CN−, in contrast to the bent Fe-O-O unit, prefers to bind normal to the heme (27). The paramagnetism of the iron leads to significant hyperfine shifts (27, 28) for the iron ligands and nearby residues that both improve resolution and provide a wealth of molecular and
where the position of a nucleus is given by \( R, \theta', \Omega \), where \( \alpha, \beta, \gamma \) are the axial and rhombic anisotropies of the paramagnetic susceptibility tensor. \( \chi \) and \( \Gamma(\alpha,\beta,\delta) \) is the Euler rotation that converts the reference coordinate system, \( x', y', z' \), into the magnetic coordinate system, \( x, y, z \), of interest.

A number of active site residues in hHO-PH-CN have been assigned so that the orientation of the magnetic axes (\( \Gamma(\alpha,\beta,\gamma) \) in Equation 1) can be determined (26). The major magnetic axis, which correlates with the Fe-CN tilt from the heme normal (27), was shown to rotate a large \( \sim 20^\circ \) tilt of the ligand in the general direction of the \( \alpha \)-meso position (26). Thus, two distinct steric influences contribute to the regioselectivity of the heme cleavage: the placement of the distal helix so close to the heme as to block access to all but the \( \alpha \)-meso positions (20), and the steric tilt of the diatomic ligand toward the \( \alpha \)-meso positions (25, 26). A steric tilt of the Fe-Oz unit had been proposed earlier on the basis of resonance Raman spectroscopy (31), but the direction of the tilt could not be determined. The NMR studies confirmed an active site structure for hHO-PH-CN in solution that is very similar to that of hHO-PH-H2O in the crystal (26), with two exceptions. The major (\( \sim 75\% \)) species in solution has the heme rotated by \( 180^\circ \) about the \( \alpha-\gamma \)-meso axis relative to the crystal (25, 26) to that in the crystal (20), and a distal aromatic cluster appeared closer to the heme in solution (26) than in the crystal. Finally, we observed (26) a series of strongly low field shifted (\( 9-17 \) ppm) labile protons that are too weakly relaxed to exhibit any significant hyperfine shifts, and hence their low field bias likely represents unusually strong, and by implication, important, hydrogen bonds (32). These H-bond signals could not be assigned, although the furthest low field shifted labile proton was shown to reside near the distal aromatic cluster (26).

These initial NMR studies (24–26), moreover, revealed some serious obstacles to extracting the maximum information from the \( ^1H \) NMR spectral parameters of the native PH complex. On the one hand, it was shown that the protohemin orientation is equilibrium (static) disordered about the \( \alpha-\gamma \)-meso axis in a 55:45 ratio in hHO (24), and in a \( 3:1 \) ratio in hHO (25), with the minor form in solution for each case possessing the heme orientation found in the crystal structures (18, 20). This disorder has only marginal relevance for HO function, because the location of the \( \alpha \)-meso position in the protein matrix is conserved. However, the alternate heme orientations induce sufficiently large differences in hyperfine shifts for a large number of the active site residue protons so as to severely complicate the resolution of one- and two-dimensional NMR spectra (24–26). Moreover, even the individual species with a given heme orientation exhibited a second, dynamic equilibrium heterogeneity resulting from interconversion of two (or more) species (26). This latter interconversion is fast on the NMR chemical shift time scale but results in severe broadening or complete loss of the signals for numerous residues.

Our interests here are to structurally characterize by NMR a unique HO complex free of heme disorder, which also exhibits significantly reduced line broadening from the dynamic equilibrium heterogeneity. Such a complex can serve as the reference for future solution NMR structural studies of hHO mutants, apo-hHO, and the ligand-free hHO-substrate complex, as well as for other natural genetic variants such as the bacterial HOs (33–35). The symmetric hemin, 2,4-dimethyldeuteroporphyrin was purchased from Mid-Century Chemicals and the iron incorporated to yield DMDH by standard procedures (36). DMDH was titrated into apo-hHO to a 1:1 stoichiometry in the presence of a 10-fold molar excess of KCN in a 90% \( ^1H \)O, 10% \( ^2H \)O solution buffered at pH 8.0 with 100 mM phosphate. The final hHO-DMDH-CN complex was \( \sim 1.5 \) mM.

**EXPERIMENTAL PROCEDURES**

**Protein Sample**—The 265-residue soluble portion of hHO was expressed and purified as described previously (7). 2,4-Dimethyldeuteroporphyrin was purchased from Mid-Century Chemicals and the iron incorporated to yield DMDH by standard procedures (36). DMDH was titrated into apo-hHO to a 1:1 stoichiometry in the presence of a 10-fold molar excess of KCN in a 90% \( ^1H \)O, 10% \( ^2H \)O solution buffered at pH 8.0 with 100 mM phosphate. The final hHO-DMDH-CN complex was \( \sim 1.5 \) mM.

**NMR Spectroscopy**—\(^1H \)NMR data were collected on a Bruker AVANCE 600 spectrometer operating at 600 MHz. Reference spectra were collected in both \( ^1H \)O and \( ^2H \)O over the temperature range 10–40 °C at a repetition rate of 1 s–1 using a standard one-pulse sequence with saturation of the water solvent signal. Chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the water resonance calibrated at each temperature. Non-selective \( T_1 \) values were determined in both \( ^1H \)O and \( ^2H \)O at 20, 25, and 30 °C for the initial magnetization recovery of a standard inversion-recovery pulse sequence. The distance of proton \( H \), from the iron, \( R_{H} \), was estimated from the relation \( R_{H} = R_{H}^0 [T_1(T_2)]^{1/2} \), using the heme for the \( \alpha-\gamma \)-H for \( H^* \) (\( R_{H}^0 = 4.6A \) and \( T_2 = 50ms \) as reference). NOESY (37) spectra (mixing time, 40 ms; \( 10-40 \) °C) and Clean-TOCSY (38) spectra (25, 35, 40 °C, spin lock of 15 and 30 ms) using MLEV-17 (39) were recorded over a bandwidth of 14 (or 28) KHz (NOESY) and 14 KHz (TOCSY) with recycle times of \( 1s \) or \( 0.33s \), using 512/21 blocks of 128 and 250 scans each consisting of 2048 2D points. Two-dimensional data sets were processed using Bruker XWIN software on a Silicon Graphics Indigo work station and consisted of 30° sine-squared-bellapodization in both dimensions, and zero-filling to 2048 × 2048 data points prior to Fourier transforms.

**Magnetic Axes**—The magnetic axes (see Fig. 1) were determined by a least-squares search for the minimum in the error function (27, 30, 40) (Equation 2).

\[ F_{mn} = \sum_{i=1}^{n} \left[ \delta_{obs}(i) - \delta_{calc}(i) \right]^2 \]  

(Eq. 2)

The calculated dipolar shift in the reference coordinate system, \( x', y', z' \), or \( R, \theta', \Omega \), is given by Equation 1, with \( \Delta x_{\alpha} = 2.48 \times 10^{-9} \) mmol and \( \Delta x_{\beta} = -0.58 \times 10^{-9} \) mmol as the axial and rhombic anisotropies of the diagonal paramagnetic susceptibility tensor taken from the isoelectronic metMbCN complex (39). The observed dipolar shift, \( \delta_{obs}(i) \) is given by Equation 3.

\[ \delta_{obs}(i) = \delta_{calc}(i) - \delta_{DSS}(dia) \]  

(Eq. 3)

\( \delta_{DSS}(obs) \) is the chemical shifts, in ppm, referenced to DSS, for the paramagnetic hHO-DMDH-CN complex, and \( \delta_{DSS}(dia) \) is determined from the available molecular structure for the presumed isostructural hHO-PH-H2O complex (26).
in hHO-DMDH-CN follows standard procedures in low spin ferric hemins/hemoproteins containing native protohemin (27). Thus, NOESY spectra allow the detection of all expected dipolar contacts among TOCSY-connected pyrrole substituents and the meso-Hs in a circular fashion (data not shown) as reported for the metMbCN complex with the same symmetric heme (45). The substrate shifts at 25 °C are listed in Table I, where they can be compared with similar data for the same complex of native PH. It is important to note that the symmetric DMDH, unlike PH, does not have a unique numbering system. Hence, we will label the heme methyls in DMDH on the basis of the position of the similarly situated methyl in the major isomer hHO-PH-CN complex in solution. Because the PH orientation of the major isomer of hHO-PH-CN (26) in solution is rotated 180° about the α-γ-meso axis of PH in the crystal (20) of hHO-PH-H$_2$O, our present numbering requires the comparison of the solution 1→8 substituent environments with the crystallographic 8→1 positions, respectively.

**Sequential Assignments**—Six fragments, labeled I–VL, could be uniquely identified by sequential backbone NOESY connectivities (see Figs. 3 and 4) among TOCSY-detected (data not shown; see Supplemental Data) spin systems, five of which (I–V) exhibited the N$_{3}$-N$_{4}$-α, α-N$_{4}$-β, β-N$_{5}$, γ-N$_{5}$, and/or α-β-γ connectivities indicative of portions of helices (44).

The Proximal Helix—The eight-residue helical fragment I, Z-AMX-$\gamma$-Val-$\gamma$-AMX-$\gamma$-Thr-$\gamma$-Ile-Ala-$\gamma$-Z (Z long chain), contains the uniquely low field contact shifted axial His-25 backbone as i+3 (data not shown; see Supplemental Data). This proximal helix contains the strongly up-field dipolar-shifted side residues Ala-$\gamma$-S (i+6) and Gly-$\gamma$-S (i+7) that make the expected NOESY contacts to 2-CH$_3$ and 3-CH$_3$. The improved resolution allowed the detection of the complete Val$_{24}$, and at least the C$_{3}$-H of Glu-$\gamma$ in the DMDH, but not in the PH complex (26). The observed NOESY cross-peak pattern is summarized in Fig. 5. The dipolar shifted complete proximal Phe-$\gamma$-207 (only ring seen in native complex (Ref. 26)), the Phe-$\gamma$-214 ring, and a complete Ile-$\gamma$-211 (not resolved in native complex (Ref. 26)) spin systems were detected and assigned on the basis of their expected NOESY contacts to the heme and proximal helix. The chemical shifts for the newly assigned residues are listed in Table II, and the data for the remainder of the proximal helices are provided (and compared with the data for hHO-PH-CN, in Supplemental Data).

**The Distal Helix**—The 10-member helical fragment II, AMX-$\gamma$-Thr-$\gamma$-Z-$\gamma$-AMX-$\gamma$-Thr-$\gamma$-Leu-$\gamma$-N$_{2}$-$\gamma$-AMX-$\gamma$-Z-$\gamma$-AMX-$\gamma$-Z-$\gamma$-N$_{2}$-$\gamma$ (N is only peptide NH of residue N$_{i}$, Z = long chain) is unique to the fragment Tyr-$\gamma$-34–Gly-$\gamma$-143, and contains the strongly relaxed and dipolar-shifted signals of Leu-$\gamma$-138–Gly-$\gamma$-143, of which the Leu-$\gamma$ and Gly-$\gamma$ NHs exhibit the expected dipolar contacts to the 8-CH$_3$ (data not shown; see Supplemental Data). Two TOCSY-detected two-spin aromatic rings show NOESY cross-peaks to the backbone of residues i and i+3, confirming the assignment of Tyr-$\gamma$-34 (with ring contacts to 8-CH$_3$) and Tyr-$\gamma$-37. The complete Asp-$\gamma$-140 with the expected dipolar shifts is identified (see Fig. 4 (A and C)). It is noted that the TOCSY connectivities of neither the backbone of Tyr-$\gamma$-134, Tyr-$\gamma$-137, nor the side chain of Asp-$\gamma$-140 could be observed in hHO-PH-CN (26). It was not possible to detect TOCSY cross-peaks to the strongly low field shifted and severely relaxed (T$_{1}$−50 ms) Gly-$\gamma$-134 NH. However, strong NOESY cross-peaks of N$_{i}$-$\gamma$ to a TOCSY-detected (not seen in hHO-PH-CN) pair of strongly low field shifted non-labile protons locate the Gly-$\gamma$-143 C$_{\alpha}$Hs. It was not possible to observe either TOCSY or NOESY cross-peaks from the Gly-$\gamma$-139 NH to the C$_{\alpha}$Hs. Moreover, although it was possible to locate a candidate for the Gly-$\gamma$-144 NH by the its expected NOESY cross-peak to the
Gly-143 NH, neither Gly-144 C_H could be located by TOCSY.

A complete TOCSY-detected Val exhibited the NOESY cross-peak to the heme 5-CH_3 (predicted 8-CH_3 in crystal (Ref. 20)) as expected for Val-146. Both N_\text{-}N_{\text{i}} and \beta_\text{-}N_{\text{i}} cross-peak locate the NH of Leu-147, for which TOCSY encompasses the majority of the predicted, very strongly down-field shifted, side

| TABLE I | Chemical shifts of heme protons in hHO-DMDH-CN and hHO-PH-CN |
|---------|---------------------------------------------------------|

| Proton | hHO-DMDH-CN | hHO-PHC-CN^a |
|--------|--------------|--------------|
| 1-CH_3 | 8.95         | 4.95         |
| 2-CH_3/vinyl | 21.37      | 15.68(H_\text{a}), 4.22, 4.23 (H_\text{m}) |
| 3-CH_3 | 18.25        | 19.63        |
| 4-CH_3/vinyl | 8.51       | 10.09(H_\text{a}), 1.77, 2.52 (H_\text{m}) |
| 5-CH_3 | 9.78         | 9.04         |
| 6-H_\text{a} | 11.02, 10.24 | 11.75, 10.62 |
| 6-H_\text{m} | -0.05, 0.12  | 0.40, 0.40   |
| 7-H_\text{a} | 13.13, 7.02  | 15.87, 5.68  |
| 7-H_\text{m} | -0.91, -0.01 | -0.45, -0.45 |
| 8-CH_3 | 10.48        | 10.48        |
| α-Meso-H | -2.83        | -5.10        |
| β-Meso-H | 6.80         | 7.60         |
| γ-Meso-H | 1.89         | 3.84         |
| δ-Meso-H | 7.65         | 7.13         |

^a Data are from Ref. 25.

FIG. 2. Resolved portions of the 600-MHz $^1$H NMR spectra in $^1$H_2O, pH 8.0, at 25 °C of the freshly prepared hHO-PH-CN complex with ~1:1 heme orientational disorder (A) and the equilibrated hHO-PH-CN complex with the ~3:1 equilibrium heme disorder (26) (B). The spectrum of hHO-DMDH-CN under the same conditions is given in C, where the insets to this trace show the improved resolution afforded at other temperatures. The peaks are labeled M_\text{i}, H_i, and m_i, h_i for methyls (M_\text{i}, m_\text{i}) and single protons (H_i, h_i) for the major (or only for DMDH) and minor heme orientation where i compound to pyrrole positions 1–8 at meso positions a–e, or the sequence position of an amino acid.
When briefly changing the 1H2O solution to 2H2O (data not shown), these strongly low field shifted labile protons are retained in the newly assigned distal helix residues are included in Table II. Less than half of the residues could be sequence-specifically assigned in hHO-PhCN (26). The shifts for the previously assigned residues are listed (and compared with those for hHO-PhCN) in Supplemental Data.

H-bonding Fragments—The signals of ~15 labile protons with insignificant paramagnetic relaxation resonate to the low field of 9 ppm. TOCSY spectra reveal that 2 are Trp ring N and 13 are peptide NHs; 3 fail to exhibit any TOCSY connectivities (data not shown; see Supplemental Data). Several of these strongly low field shifted labile protons are retained briefly upon changing the 1H2O solution to 2H2O (data not shown) confirming their role in H-bonds. The majority of these labile protons will be shown to arise from four fragments, designated III–VI (of which three, III–V, correspond to portions of helices), pertinent to the distal cavity (residues 92–98, 163–167, 57–62, and 84–87). The chemical shifts of the residues are listed in Table II, and the pattern of dipolar contacts among residues in the four sequential fragments and to the heme and/or distal helix is illustrated schematically in Fig. 5.

The labile protons at 11.71 (i+4), 9.51 (i+5), and 9.13 ppm (i+2) are part of the helical fragment III connectivities, (Na)2-Leu1-Ala2-AMX3-AMX4-AMX5-AMX6-NH7, as shown in Fig. 3 (A–D), where the backbone of residues i+3 to i+5 each make contact to a TOCSY-detected aromatic ring. This unique pattern identifies the segment Asp-92–Gly-98 that includes the aromatic rings of Phe-95, Trp-96, and Tyr-97. The inconsequentially hyperfine-shifted segment III members do not, and are not expected to, exhibit dipolar contacts to either heme or the distal helix. A five-member helical segment IV involves labile protons at 12.05 (i+1) and 11.58 (i+2) with sequential connectivities described by: Leu–Ala1–AMX2–AMX3–N4, and illustrated in Fig. 3 (D–G), with an obvious missing α (under solvent) for j+2. The residues exhibit small-to-moderate low field hyperfine shifts but minimal paramagnetic relaxation (see Table II). This fragment is uniquely identified as Leu–Phe–Leu and the Gly-163 NH, and the backbone of j+2 and j+3 make the contact to two three-spin aromatic rings of Phe-166 (Fig. 3F) and Phe-167 (Fig. 3C). It is important to note that Phe-166 was initially proposed to be a Tyr because only one ring TOCSY peak could be detected in hHO-PhCN (26); two are clearly observed in hHO-DMDHCN (see Supplemental Data and “Discussion”). The Leu-164 C, and the Gly-163 NH make weak dipolar contact with the Trp-96 NeH of fragment III (see Fig. 3F), and the rings of Phe-166 and Phe-167 make dipolar contact with the distal helix (fragment II) via the Asp-140 side chain (as expected; see Fig. 4A) and the Gly-143 NH (not expected), respectively. The dipolar contacts are summarized schematically in Fig. 5.

Two shorter fragments encompass the low field peptide labile protons 9.06 (k+1) of fragment V and 13.05 (m+1) and 9.46 (m+2) of fragment VI. The helical fragment V shows the TOCSY/NOESY connectivity AMX2-Val6-1-Ala12-2-Ala13-3-Ala14-4-Ala15-5-Ala16-6-NH7, where AMX6 makes contact to a two-spin aromatic ring (see Fig. 4B). This portion is unique to Tyr-58–Glu-62. The C, Hs of Tyr-58 exhibit strong NOESY cross-peaks to the lowest field (16.7 ppm), but paramagnetically inconsequentially influenced, labile proton (Fig. 4F) with no TOCSY peaks that identifies the Tyr-58 ring OH. The Tyr-58 ring exhibits NOESY cross-peaks to both the ring of Phe-166 on fragment IV (Fig. 4B) and the NH of Arg-85 (on fragment VI (Fig. 3H); see below)) (as expected from the crystal structure) and the Tyr-58 OH displays the expected NOESY cross-peak to Tyr-137 and Asp-140 on the distal helix (fragment II; see Supplemental Data). NOESY cross-peaks from both the Ile-57 C, and Tyr-58 NH to a two-spin aromatic ring locate the Tyr-114 ring. TOCSY/NOESY characterize a non-helical fragment VI as AMX13-Z14-Z15-Ala16-17 (Z = long chain), with N-N connectivities shown in Fig. 3(D, F, and H), where AMX13 makes contact to an aromatic ring proton with no TOCSY connectivity; the connectivities are summarized in Fig. 5. Although the fragment is not unique in the sequence of hHO, NOESY cross-peaks between the NH of residues m+1 and the distal helix (fragment II)
Tyr-137 ring uniquely identify VI as arising from His-84–Ala-87. The His-84 and Arg-85 NH make the expected dipolar contact to the ring and Tyr-137 on the distal helix (fragment II (Fig. 3, G and H)).

A labile proton at 14.7 ppm exhibits strong NOESY cross-peak to two non-coupled protons in the aromatic spectral window, which identify the C6H and C4H of the likely His-132 ring (25). Left unassigned in the 9.5–15 ppm spectral window are two labile protons at 9.6 and 9.9 ppm, which exhibit TOCSY peaks to identify peptides (one an Ala), but do not provide contacts to any currently assigned residues and hence remain unassigned at this time.

Aromatic Residues/Clusters—The rings of six residues on the H-bonding fragments participate in a cluster with inter-ring dipolar contacts that include two additional interacting Phe rings, the proximity of which to Phe-167, Trp-96, and Leu-164 for one (Fig. 4B), and to 3-CH₃ for the other (data not shown), identifies Phe-47 and Phe-37, respectively. The inter-ring contacts are summarized in the chain Tyr-58-Phe-166-Phe-167-Phr-47-Trp-96-Phe-95, where Phe-47 additionally interacts...
**Table II**

*H NMR chemical shifts for newly assigned residues on the proximal and distal helices*

| Residue   | Proton | \( \delta_{obs}(ppm) \) | \( \delta_{calc}(ppm) \) | \( \delta_{calc}/\delta_{obs} \) | \( \delta_{calc}/\delta_{obs} \) |
|-----------|--------|-----------------|-----------------|-----------------|-----------------|
| **Proximal helices** | | | | | |
| Phe-207   | NH     | 8.08            | -0.25           | -0.72           | |
|           | C\(_2\)H | 2.94            | -1.44           | -1.76           | |
|           | C\(_3\)H | 2.11            | -1.1(-0.84)     | -0.83(-0.47)    | |
|           | C\(_4\)Hs | 6.43            | -0.96           | -1.31           | |
|           | C\(_5\)Hs | 5.82            | -0.96           | -0.95           | |
| Ile-211   | NH     | 7.80            | -0.25           | -1.04           | |
|           | C\(_2\)H | 2.61            | -1.21           | -0.97           | |
|           | C\(_3\)H | 1.33            | -0.60           | -0.64           | |
|           | C\(_4\)H | 0.85            | -0.62(-0.52)    | -1.25(-1.08)    | |
|           | C\(_5\)H | 0.41            | -0.58           | -0.51           | |
|           | C\(_6\)H | -0.22           | -1.08           | -0.57           | |
| **Distal helix (II)** | | | | | |
| Tyr-134   | NH     | 8.36            | 0.12            | -0.36           | |
|           | C\(_2\)H | 3.68            | -0.74           | -0.51           | |
|           | C\(_3\)H | 3.07            | -0.31           | -0.43           | |
|           | C\(_4\)Hs | 6.25            | -1.26           | -0.52           | |
|           | C\(_5\)Hs | 6.56            | -0.82           | -0.34           | |
|           | OH     | 10.97           |                | 11.2            | |
| Thr-135   | NH     | 6.74            | -1.79           | -0.71           | |
|           | C\(_2\)H | 2.39            | -2.14           | -1.83           | |
|           | C\(_3\)H | 3.35            | -1.17           | -0.98           | |
|           | C\(_4\)H | -0.10           | -1.78           | -2.02           | |
| Arg-136\(a\) | NH     | 6.59            | -1.57           | -0.71           | |
|           | C\(_2\)H | 3.94            | 0.04            | 0.48            | |
|           | C\(_3\)H | 1.06            | -0.39           | 0.0             | |
|           | C\(_4\)Hs | 1.27            | -0.40           | -0.27           | |
|           | C\(_5\)Hs | 0.37            | -0.49(-0.53)    | -0.33(-0.61)    | |
|           | C\(_6\)H | 2.47            | 0.14            | 2.36            | |
|           | C\(_7\)H | 2.72            | -0.27           | -0.22           | |
|           | N\(_H\) | 9.51            |                | 9.56            | |
| Tyr-137   | NH     | 8.27            | 0.17            | -0.20           | |
|           | C\(_2\)H | 5.09            | 0.99            | 5.11            | |
|           | C\(_3\)H | 2.51            | -0.34           | -0.16           | |
|           | C\(_4\)Hs | 2.79            | -0.25           | 0.00            | |
|           | C\(_5\)Hs | 7.46            | 0.2             | 7.83            | |
|           | C\(_6\)H | 7.10            | 0.19            | 7.04            | |
|           | OH     | 10.38           |                | 10.84           | |
| Asp-140   | NH     | 9.79            | 2.42            | 9.65            | |
|           | C\(_2\)H | 7.72            | 4.49            | 7.43            | |
|           | C\(_3\)H | 4.05            | 1.61            | 1.98            | |
|           | C\(_4\)H | 3.82            | 1.36            | 1.65            | |
| Leu-141   | NH     | 7.19            | -0.19           | 1.21            | |
|           | C\(_2\)H | 4.47            | 1.06            | 1.01            | |
| Val-146   | NH     | 7.88            | 0.06            | 7.78            | |
|           | C\(_2\)H | 3.76            | 0.14            | 3.75            | |
|           | C\(_3\)H | 2.32            | 0.09            | 2.38            | |
|           | C\(_4\)Hs | 0.56            | -0.33           | 0.53            | |
|           | C\(_5\)Hs | 0.71            | -0.60           | 0.77            | |
| Leu-147\(b\) | NH     | 9.02            | 1.15            | 9.08            | |
|           | C\(_2\)H | 6.19            | 1.90            | 7.83            | |
|           | C\(_3\)H | 4.09            | 2.32            | 4.07            | |
|           | C\(_4\)H | 3.18            | 1.97            | 2.90            | |
|           | C\(_5\)H | 7.92            | 0.68            | 5.70            | |
|           | C\(_6\)H | 2.31            | 2.15            | 3.59            | |
| **Fragment V** | | | | | |
| Tyr-58    | NH     | 9.06            | 1.21            | 0.05            | |
|           | C\(_2\)H | 3.81            | -0.19           | 0.02            | |
|           | C\(_3\)H | 2.81            | 0.10            | 0.14            | |
|           | C\(_4\)Hs | 7.76            | 0.73            | 0.17            | |
|           | C\(_5\)Hs | 6.99            | 0.65            | 0.33            | |
|           | OH     | 16.70           |                | 16.84           | |
| Val-59    | NH     | 5.30            | 0.71            | 8.39            | |
|           | C\(_2\)H | 3.40            | -0.06           | 3.37            | |
|           | C\(_3\)H | 1.71            | 0.02            | 1.71            | |
|           | C\(_4\)Hs | 0.26            | -10             | 0.25            | |
|           | C\(_5\)Hs | 0.68            | 0.77            | 0.66            | |
| Ala-60    | NH     | 6.89            | -1.01           | -0.03           | |
|           | C\(_2\)H | 3.90            | -0.07           | -0.04           | |
|           | C\(_3\)H | 1.04            | 0.11            | -0.07           | |
| Leu-61    | NH     | 8.62            | 0.71            | -0.06           | |
|           | C\(_2\)H | 3.53            | -0.37           | -0.10           | |
| Glu-62    | NH     | 7.33            | 0.92            | -0.01           | |
|           | C\(_2\)H | 3.96\(b\)       | -0.21           | -0.01           | |
|           | C\(_5\)H | 2.26\(a\)       | -0.01           | -0.02           | |
| **Fragment VI** | | | | | |
| His-84    | NH     | 7.64            | -0.13           | 0.10            | |
|           | C\(_2\)H | 5.10            | 0.87            | 0.11            | |
|           | C\(_3\)H | 3.41            | 0.60            | 0.08            | |
|           | C\(_4\)H | 3.26            | 0.71            | 0.09            | |
|           | C\(_5\)H | 6.64            | -0.45           | 0.11            | |

*a,b* See previous note.
### Fragment III

| Residue | Proton | HHO-DMDH-CN δ<sub>δ<sub>0</sub>obs</sub> | HHO-DMDH-CN δ<sub>δ<sub>0</sub>calc</sub> | hHO-PH-CN δ<sub>δ<sub>0</sub>obs</sub> | hHO-PH-CN δ<sub>δ<sub>0</sub>calc</sub> |
|---------|--------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Arg-85  | NH     | 13.03                           | 0.16                             | 13.00                            | 0.16                             |
|         | C_H    | 4.49                            | 0.26                             | 4.49                             | 0.26                             |
| Lys-86  | NH     | 9.46                            | 0.14                             | 9.42                             | 0.14                             |
|         | C_H    | 3.70                            | -0.26                            | 1.44                             | 0.14                             |
| Ala-87  | NH     | 8.15                            | 0.13                             | 8.15                             | 0.13                             |
|         | C_H    | 3.88                            | -0.23                            | 1.11                             | 0.11                             |
|         | C_H8   | 1.34                            | -0.21                            | 0.12                             | 0.12                             |

#### Table II—continued

### Fragment IV

| Residue   | Proton | HHO-DMDH-CN δ<sub>δ<sub>0</sub>obs</sub> | HHO-DMDH-CN δ<sub>δ<sub>0</sub>calc</sub> | hHO-PH-CN δ<sub>δ<sub>0</sub>obs</sub> | hHO-PH-CN δ<sub>δ<sub>0</sub>calc</sub> |
|-----------|--------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Leu-164   | NH     | 7.45                            | -0.56                            | 0.26                             | 7.41                             |
|           | C_H    | 5.38                            | 0.81                             | 0.40                             | 5.37                             |
|           | C_H8   | 3.00                            | 0.84                             | 0.45                             | 3.00                             |
|           | C_H8   | 2.15                            | 0.31                             | 0.30                             | 2.13                             |
|           | C_H8   | 1.30                            | 0.07                             | 0.33                             | 1.28                             |
|           | C_H8   | 0.93                            | -0.68                            | 0.42                             | 0.90                             |
| Ala-165   | NH     | 12.09                           | 4.07                             | 0.30                             | 12.09                           |
|           | C_H    | 4.00                            | 0.03                             | 0.31                             | 4.00                             |
|           | C_H    | 1.52                            | 0.35                             | 0.25                             | 1.52                             |
| Phe-166   | NH     | 11.58                           | 3.61                             | 0.42                             | 11.56                           |

**Notes:**
- Observed chemical shift, in ppm, referenced to DSS via the residual solvent signal in H<sub>2</sub>O, 95% H<sub>2</sub>O; 5% D<sub>2</sub>O at pH 8.0 and 30 °C.
- Observed dipolar shift as determined by Equations 3 and 4.
- Calculated dipolar shifts, as determined by Equation 1 using the magnetic axes and the crystal coordinates of molecule A of hHO-PH<sub>2</sub>O (20).
- Either as reported earlier (26), or as deduced here by comparison to hHO-DMDH-CN.
- Protons not stereospecifically assigned, so δ<sub>0</sub>obs and δ<sub>0</sub>calc are determined for both possibilities.
- NH<sub>C</sub>H<sub>3</sub> fragment detected by TOCSY. The C<sub>H</sub>C<sub>3</sub>H<sub>2</sub> fragment was assigned by its NOESY cross-peak to the Arg-136 N<sub>H</sub> assigned on the basis of the crystal structure (see text).
- TOCSY cross-peak connectivity, NH to C<sub>H</sub>H<sub>3</sub>, but not to C<sub>H</sub> or the expected relaxed C<sub>H</sub>H<sub>3</sub> were observed. The C<sub>H</sub> and the C<sub>H</sub>H<sub>3</sub> are, however, could be assigned by their unique predicted low-field dipolar shifts and NOESY cross-peaks to the NH and C<sub>H</sub>H<sub>3</sub>.
- Severe spectral overlap in both TOCSY and NOESY spectra in this spectral window dictates that these assignments are only tentative.
- The C<sub>α</sub>H signal is under the solvent signal.
with Phe-37 (see Fig. 4B). Both Leu-93 and Ala-94 exhibit
NOESY cross-peaks to a complete Trp (N_3H at 10.8 ppm)
which is the only other in hHO, Trp-101, as expected (20).
A very narrow obvious methyl group with no TOCSY cross-peaks
exhibits the expected cross-peaks to C_6H of Leu-164 on fragment
III, and the above assigned Phe-47 ring identifies the
C_6H_3 of Met-51 (data not shown; see Table II). A weakly up-
field shifted complete Val exhibits NOESY cross-peaks to both
the Phe-47 and Phe-37 ring (data not shown), and is assigned to
Val-42. Both the Tyr-58 ring and OH make strong dipolar
contacts with a labile proton (Fig. 4E) with no TOCSY connectivity,
which the crystal structure (20) suggests arises from the
guanidyl group N_3H of Arg-136 (Table II).

The methyls of distal helix Leu-138 make contact with the
adjacent Tyr-134 ring and an additional two-spin ring that
the crystal structure identifies as Tyr-182 (data not shown).
Tyr-182, in turn, exhibits the expected cross-peaks to the
rings of two Phe that the crystal structure indicates must be
the interacting rings Phe-79 and Phe-178. The rings of Phe-
79, Phe-178, and Tyr-182 exhibit strong NOESY cross-peaks
to a methyl that must arise from Leu-83. Another very nar-
row methyl peak with no TOCSY connectivity exhibits the
expected cross-peak to the rings of Tyr-134 on fragment II
and Tyr-182, and must arise from C_6H_3 of Met-186. The
dipolar contacts are shown schematically in Fig. 6, and the
chemical shifts for these residues are provided in the Supple-
mental Data.

Magnetic Axes Determination — The orientation of the mag-
netic axes in hHO-DMDH-CN were determined using the con-
served magnetic anisotropies of isoelectronic low spin met-
MbCN (30), the coordinates of molecule A in the crystal struc-
ture of the 233 fragment hHO-PH_2O complex (20), and the
\( \delta_{\text{dip}} \) for hHO-DMDH-CN. Comparison of the chemical shifts
for the two complexes for residues that could be assigned in
both reveals the same pattern of shifts for the two complexes
(Table II and Supplemental Data). Not surprisingly, the opti-
mized orientation for hHO-DMDH-CN exhibits a tilt of -20° (\( \beta \))
and tilt direction \( \alpha (-z \text{ or } \text{FeCN vector toward } \alpha \text{-meso-H}) \), sim-
ilar to that deduced earlier for hHO-PH-CN (26). The shifts
predicted (see Table II and Supplemental Data Fig. 6S), and
observed \( \delta_{\text{dip}} \) for distal residues agree reasonably for residues
134–142, but deviate significantly for all protons for Gly-143
(as observed before (Ref. 26). For the majority of the residues
on sequential fragments III–VI, the dipolar shifts are small (<0.5 ppm) to negligible (see Table II). Thus, the extreme low
field positions of labile protons in particular is not related to
the paramagnetism of the heme iron, but must arise from strong H-bonds (32).

There are insufficient data available at this time to ade-
quate model the distal helix position by its dipolar shifts. For
this, distance estimates from rise curves must be obtained
which are impractical at this time. However, some insight into
the potential movement of the distal helix is obtained by con-
sidering the Gly-143 N_3H, for which \( \delta_{\text{dip}} \) is considerably less than the predicted 5 ppm (Table II), although
its relaxation (\( T_1 = 50 \) ms) is well predicted (26). A \( \sim 0.5-\AA 
move by Gly-143 N_3H toward the \( \alpha \)-meso position leads to
a negligible change in \( R_{\text{Fe}} \) that is consistent with the observed
\( T_1 \), but to an increase in \( \delta_{\text{dip}} \) to \( \sim 10 \) ppm, which is consistent with \( \delta_{\text{dip}} \).

Extension of Assignments to hHO-PH-CN — The definitive as-
signments achievable for hHO-DMDH-CN, could, in part, be
extended to hHO-PH-CN, primarily on the basis of conserved
NOESY patterns among similarly shifted protons, as listed in
Table II and Supplemental Data. This resulted in the reassign-
ment (26) of the aromatic rings of Tyr-58, Tyr-137, and Phe-166
and the identification of a residue as Leu (Leu-164) rather than
the original Ala (see "Discussion"). As is apparent in Table II,
the chemical shifts for a given residue in the two complexes
are very similar for the hyperfine shifted residues, and essen-
tially the same for residues only weakly influenced by
paramagnetism.

**DISCUSSION**

Advantages of the Symmetric DMDH Substrate — The obser-
vation in hHO-DMDH-CN of the same pattern of NOESY cross-
peaks between the hemin substituents and both proximal and
distal helix residues as in hHO-PH-CN (26) reflects very similar

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**FIG. 5.** Schematic representation of the sequential backbone NOESY contacts for the proximal helix (I), the distal helix (II), and
four fragments (III–V helical) that participate in the distal H-bonding/aromatic cluster network. Solid lines represent observed
connection. Dashed lines show expected connections that are obscured by excessive spectral overlap.
modes of substrate binding for native PH and DMDH. Hence, we conclude that information on the molecular and electronic structures, as well as magnetic properties of the heme deduced for hHO/DMDH, is also valid for hHO-PH. However, the 1H NMR spectra for the former complex are much more informative than for the latter complex for several reasons.

Vastly improved spectral resolution, and hence confidence in and extent of assignments, result upon substituting DMDH for PH. On the one hand, the ~25% “minor” heme orientation is abolished by the symmetric heme. However, the more dramatic resolution afforded by DMDH arises from the fact that the intrinsic dynamic heterogeneity of the hHO-substrate complex is more effectively averaged (i.e. more rapid motion), lending to significant line narrowing, and in turn, the detection of numerous TOCSY cross-peaks crucial to the unambiguous, sequence-specific residue assignment (see Supplemental Data). Thus, the backbone TOCSY connection of distal helix residues Tyr-134, Thr-135, Tyr-137, Asp-140, and Gly-143 is observed in hHO-DMDH-CN, and, although strong candidates for those signals are now available based on observed NOESY cross-peaks in hHO-PH-CN, the crucial TOCSY cross-peaks in this complex still cannot be observed (26).

Notable limitations of the two-dimensional NMR spectra of hHO-PH-CN involve the presently unambiguous assignment of Leu-164 and Phe-166. The former exhibited a single TOCSY cross-peak indicative of an Ala, and the ring exhibited a single TOCSY cross-peak indicative of a Tyr (26). Both residues exhibit additional TOCSY cross-peaks for hHO-DMDH-CN that trace out a complete Leu and Phe.

On the proximal side, essentially the complete, moderately hyperfine shifted, but only inconsequentially paramagnetically relaxed, Ile-211 (Table II) side chain is observed by TOCSY for the DMDH complex (data not shown, see Supplemental Data), with NOESY peaks to 3-CH3 and Ala-28 (data not shown) as predicted by the crystal structure (20). For the PH complex, these TOCSY peaks are all undetectable, although the conserved dipolar shifted and NOESY cross-peaks to Ala-28 C\(^{\alpha}\)-H3 allow the assignment of a few Ile-211 signals (Table II). Because only selective residues/protons lose their TOCSY connectivity in the PH relative to DMDH complex, we must ascribe the loss of these TOCSY peaks to increased linewidths caused by slower interconversion of the dynamic heterogeneity in the PH than DMDH complex. Fortunately, the resolution for the labile protons (and in large part, backbone and side chain protons) involved in the unique H-bonding network, as well as the ring protons from the aromatic cluster, on the distal side can be identified (although less thoroughly, see Table II) for hHO-PH-CN on the basis of the present definitive assignments for hHO-DMDH-CN.

Thus, despite the extensive and unambiguous assignments of the target residues in hHO-DMDH-CN, as well as the dem-

**FIG. 6.** Schematic representation of the relative dispositions among the assigned six sequential fragments I–VI of hHO-DMDH-CN and the heme and the observed dipolar contacts among these fragments and to the heme. The position of eight side chains, shown in rectangles (Phe-37, Val-42, Phe-47, Phe-79, Tyr-114, Phe-178, Tyr-182), as well as the methyl (as triangles) C\(^{\alpha}\)-H3 of Met-51, Met-186, C\(^{\alpha}\)-H3 of Ile-57, and C\(^{\alpha}\)-H3 of Leu-83, which could be uniquely assigned on the basis of the crystal structure. The dashed lines represent the pairs of residues that exhibit the expected NOESY contact; the asterisk on the dotted line between Phe-167 and Gly-143 indicates an observed weak NOESY cross-peak for which the crystal separation is 8 Å.
onstrated conservation of both hyperfine shifts and inter-residue and heme-residue dipolar contacts between the two substrate complexes, it is still not possible to characterize by \(^1\)H NMR the active site environment as thoroughly for the native substrate as for the symmetric 2,4-dimethyldeuterohemin. We conclude, therefore, that future \(^1\)H NMR characterization of the native protohemin, as substrate. The symmetric hemin, more informative using the symmetric hemin DMDH, rather than bacterial HO and mutants of mammalian HO) will be much more effective and due and heme-residue dipolar contacts between the two sub-

TABLE III

| Label proton shift | Donor | Acceptor\(^a\) | N(O)H-O | N(O)-H-O angle |
|-------------------|-------|---------------|---------|----------------|
| ppm               |       |               |         |                |
| 13.02             | Arg-85 N\(_p\)H | Glu-62 CO\(_2\) | 3.5     | 146            |
| 9.45              | Lys-86 N\(_p\)H | Glu-62 CO\(_2\) | 3.1     | 148            |
| 10.38             | Tyr-137 OH | Glu-62 CO\(_2\) | 2.7     | 156            |
| 12.08             | Ala-165 N\(_p\)H | Asp-92 CO\(_2\) | 2.9     | 142            |
| 11.56             | Phe-166 N\(_p\)H | Asp-92 CO\(_2\) | 3.3     | 98             |
| 9.11              | Leu-93 N\(_p\)H | Leu-89 C-O | 2.7     | 156            |
| 11.69             | Tyr-96 N\(_p\)H | Gly-163 C-O | 2.8     | 152            |
| 16.66             | Tyr-58 OH | Asp-140 CO\(_2\) | 4.0(3.5) | 75 (114)\(^b\) |
| 9.47              | Arg-136 N\(_p\)H | Asp-140 CO\(_2\) | 3.9(3.4) | 74 (162)\(^b\) |
|                   | Arg-136 N\(_p\)H | Tyr-58 O\(_s\) | 3.3     | 139            |

\(^a\) Probable acceptor residue as suggested by the crystal structure in hHO-PH\(_2\)O (20).

\(^b\) Data in parentheses represent distance and angles where Asp-140 \(\chi_2\) is rotated by \(-45^\circ\) from that in the crystal structure to optimize the Tyr-58 OH to Asp-140 H-bond.

Active Site Molecular Structure—The pattern of the intra-residue NOESY connections among the five distal residue frag-
Fig. 8. Schematic representation of the inter-fragment strong H-bonds (solid lines) and inter-ring dipolar contacts (dashed lines) among the members of the distal H-bonding/ aromatic cluster network. The H-bonds are represented by directional arrows pointing toward the acceptor functional group. Unless identified otherwise, the H-bond is between the peptide NH and a peptide carbonyl group. The asterisk indicates very strong H-bonds as reflected in strong low field bias to the chemical shifts.

Fig. 9. Stereo views of the distal pocket of hHO-PH$_2$H$_2$O as found in the crystal structure (35), showing the distal helix II (green) and backbone and residues of the four sequential fragments III (orange), IV (dark red), V (dark blue), and VI (purple) (and other aromatic side chains in light blue) in the distal H-bonding/ aromatic cluster network.

ments II–VI deduced completely independently of the crystal structure, as depicted schematically in Fig. 6, as well as the few residues with connections to two or more of the fragments or to the heme (Phe-47, Val-42, Phe-37, Trp-101) the assignment of which was affected on the basis of the crystal structure, confirm an active site environment that is largely consistent with the same structure of the cyanide complex in solution as for the aquo complex in the crystal (20), with three exceptions. First, the orientation of PH differs in solution and crystal, as discussed previously (26), but this point is moot in the DMDH complex. Second, the distal helix Gly-143 NH and, by inference, at least portions of the distal helix are closer to the iron in solution than crystal for hHO (20), with the solution position more closely resembling that in the crystal of rHO (18). However, the finding of the distal helix closer to the iron in solution than crystal is likely not functionally relevant for hHO, because a similar difference in positions of the distal helix is found in the crystals of hHO (20) and rHO (18), for which activities are essentially the same (7, 47). Instead, different positions of the distal helix more likely reflect the intrinsic flexibility of at least portions of the helix for which the exact position is easily perturbed by small environmental influences. The crystal structure of hHO-PH$_2$H$_2$O yielded two molecules in the unit cell with slightly different positions of the distal helix and with part of the distal helix backbone exhibiting disorder (20).

The third difference involves the proximity of the Phe-167 ring, and possibly the Tyr-58 ring (see below), to the distal helix, as reflected in weak NOESY cross-peaks (~5–6 Å) between the ring C$_6$H and C$_7$H to the Gly-143 NH observed in both hHO-PH-CN (26) and hHO-DMDH-CN (data not shown), where the crystal coordinates predict distance >8 Å, and the very strong H-bond between the Tyr OH and carboxylate of Asp-140 (see below). The other contact between fragment IV and the distal helix (Phe-166 ring and Asp-140) is expected (~4 Å). The likely movement of the distal helix closer to the iron by ~0.5 Å would not shorten the Phe-166 ring to Gly-143 distance significantly. A possible role of the distal ligand as an H-bond acceptor in modulating the interaction between the distal helix and the aromatic cluster/H-bonding network will be considered below.

Distal H-bonding Network and Aromatic Interactions—The critical role of Asp-140 in HO activity (13, 22, 23), although connected to the heme ligand in the hHO-PH$_2$H$_2$O crystal structure solely by a H-bonded water (18, 20), emphasizes the potential important role of H-bonding interactions in facilitating the formation of the Fe-O-O-H rather than Fe=O activated species. Numerous peptide NHs and side chain-labile protons exhibit unusually low field shifts (Tables II and III). Because the magnetic axes predict small (~0.5 ppm) to negligible dipolar shifts for the assigned labile protons, their strong low field bias must be interpreted as reflecting strong hydrogen bonds (32).

The presently characterized strong H-bonding network among residues in fragments II–VI, and between fragments IV, V, and VI and the distal helix (II), and the fact that the spatial arrangements of these fragments qualitatively conform to the prediction of the crystal structure (20) (see Fig. 6) allows the use of the crystal structure to identify the candidates for H-bond acceptors to the low field assigned labile protons.

The structural constraints for strong H-bonds are inter-het-
aroatomic distances N–H–O of ~2.5 Å and a near linear N–H–O configuration (32, 48). The crystal structure (20) does not reveal such sites, but for each labile proton, it identifies a unique
candidate, as listed in Table III. The H-bonding distances and angles in Table III are far from ideal for a strong H-bond (32, 48). However, the available resolution of the structure allows for significant uncertainties in the positions of the relevant atoms, and it is very likely that there is some structural rearrangement in the geometries of at least the side chains of residues on the distal fragments II–VI (see above) upon replacing a ligated water H-bond donor in the crystal with diatomic ligated H-bond acceptor such as cyanide (or molecular oxygen). The fact is that the strong H-bonds exist, as reflected in the strong low field bias to the labile proton chemical shift (32) such that more “ideal” H-bond geometries must exist in both hHO-DMDH-CN and hHO-PH-CN. Moreover, as observed in the weak Phe-167–Gly-143 dipolar contact, it is likely that the distal fragments III–VI may all move slightly with respect to the distal helix in the cyanide complex in solution relative to the aquo complex in the crystal.

The locations of the resulting H-bonds among the distal fragments II–VI are displayed schematically in Fig. 8. First and foremost, the Tyr-58 side chain OH serves as a donor to the carboxylate of the catalytically critical Asp-140. Although the geometry of the H-bond is not very favorable in the crystallographic orientation of the carboxylate, a −45° rotation of the carboxylate (or a small movement of the Tyr ring) leads to a much more favorable H-bond interaction (Table III) without interfering with the simultaneous H-bond between the Arg-136 NH and Asp-140 carboxylate (Table III). Second, the other identified strong H-bonds occur exclusively between donors and acceptor of different fragments II–VI (see Fig. 8), indicating that one of the prime roles of the H-bond network is to stabilize the relative positions of these fragments. The “center” of this network appears to be fragment V containing Tyr-58. The helix backbone exhibits the greatest dynamic stability (49) in that the peptide NHs of both Tyr-58 and Val-59 exhibit their respective NOESY cross-peak with undiminished intensity 6 months after dissolution of the complex in 2H2O (data not shown).

The different protein segments for which relative positions are stabilized by strong H-bonds may also be stabilized by interaction of aromatic rings. Thus, fragments IV and V interact via Tyr-58 and Phe-166 ring contacts, whereas the Phe-47 ring interacts with rings on fragment III (Trp-96) and fragment IV (Phe-167), as shown in Fig. 8. The mutation of Asp-140 has already been shown to adversely affect the stability of the Fe-O-OH species (13, 22, 23). The present NMR analysis makes the proposal that the mutation of the side chains of Glu-62, Glu-92, and/or Tyr-58, candidates not obvious from the crystal structure alone, would similarly, but less severely, affect HO activity by disrupting the distal H-bonding network.

It is noteworthy that our present assignments include not only the donor residues, but also the key acceptor residues (i.e., Glu-62, Asp-92, Asp-140) in this network, setting the stage for future NMR studies of the effect of mutation on each of these H-bonds. The acceptors to the four strongest peptide NH H-bonds, Glu-62 and Asp-92, are conserved among mammalian HO and are retained in some (33), but not other (34), bacterial HO. If the water molecule H-bonded to Asp-140 indeed serves as a stabilizing influence for the Fe-O-OH in the activated form of the enzyme, then the network of strong H-bonds can be viewed as a scaffold for facilitating the correct orientation of the Asp-140 side chain for optimal catalysis.

The presently characterized H-bonding network, quite aside from being potentially relevant to HO activity, exhibits some unique properties. Thus, the strong low field bias for the chemical shift of the NH and OH indicates strong H-bonding (32) that is consistent with its presence in both the crystal and solution. Moreover, several of the NHs, including the side chain of Trp-96 and a His, as well as several peptide NHs, exchange sufficiently slowly to be detected in 2H2O (data not shown). The strength of the H-bonding, as reflected both in the chemical shift (32) and their distinctly reduced lability, is all the more remarkable because the crystal structure indicates that these residues are located close to the protein surface. Finally, although the 1H NMR spectra and the two-dimensional maps differ strongly between the apo-HO and the substrate-bound complexes (25), the H-bonding network and aromatic cluster, as reflected in the strong low field shifts, and the aromatic cluster as described herein, appear largely conserved.2 The portion of the hHO-PH2H2O crystal structure (20) that encompasses the presently described H-bond network/aromatic clusters in human heme oxygenase is shown in the stereo view of Fig. 9. The strongly interacting and highly stabilized H-bonding/aromatic cluster network serves to form a wall that interacts with the distal helix, and this wall may be retained in the apo-HO to serve as a template for the distal helix to condense upon the binding of the substrate. Preliminary studies of a complex3 of a bacterial HO-PH-CN complex (33, 50) suggest that the distal H-bond and part of the aromatic cluster network are, in large part, present and may have general role in HO activity.

Mechanistic Implications—As argued previously, partitioning of the ferric hydroperoxide intermediate between hydroxylation of the α-meso carbon of the porphyrin ring and formation of a ferryl (FeIV=O) species represents a critical branchpoint in the function of the family of catalytic hemoproteins (11–13). In the case of peroxidases and cytochrome P450 enzymes (21), for example, the distal active site environment provides residues that hydrogen bond, directly or through intervening water molecules, to the oxygen of the peroxy ligand that is distal to the iron. This hydrogen-bonding pattern facilitates scission of the oxygen-oxygen bond to produce the critical ferryl intermediate. In contrast, the role of the ferric hydroperoxide as the actual oxidizing agent in the case of heme oxygenase appears to require an alternative hydrogen-bonding scheme in which the hydrogen bond to the distal oxygen, if any, is relatively weak. Indeed, it is likely that proton delivery to the oxygen coordinated to the iron facilitates electrophilic porphyrin hydroxylation by making the iron-bound oxygen a better leaving group. The stringent hydrogen-bonding pattern observed in the present NMR experiments is likely to be critical in the fine control of proton delivery that helps to channel the reaction toward the heme oxygenase outcome rather than the formation of a ferryl species. This inference is consistent with the finding that perturbation of the distal hydrogen-bonding pattern by mutation of Asp-140 (22, 23), or of the flexibility of positioning of the distal helix by mutation of Gly-139 or Gly-143 (51), leads to formation of a ferryl species and the acquisition of peroxidase activity at the expense of heme oxygenase activity.

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