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Solution ^1H NMR characterization of substrate-free C. *diphtheriae* heme oxygenase; pertinence for determining magnetic axes in paramagnetic substrate complexes

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Abstract

Proton 2D NMR was used to confirm in solution a highly conserved portion of the molecular structure upon substrate loss for the heme oxygenase from the pathogenic bacterium *Corynebacterium diphtheriae*, HmuO. The chemical shifts for the conserved portion of the structure are assessed as references for the dipolar shifts needed to determine the orientation of the paramagnetic susceptibility tensor, χ , in paramagnetic substrate complexes of HmuO. It is shown that the chemical shifts for the structurally conserved portion of substrate-free HmuO serve as excellent references for residues with only small to moderate sized dipolar shifts in the cyanide-inhibited substrate complex of HmuO, yielding an orientation of χ that is essentially the same as conventionally obtained from large dipolar shifts based on empirical estimates of the diamagnetic reference. The implications of these diamagnetic chemical shifts for characterizing the hydrogen bonding in the physiologically relevant, resting-state, high-spin aquo complex are discussed. The pattern of labile proton exchange in the distal H-bond network of substrate-free HmuO allowed comparison of changes in dynamic stability of tertiary contacts in the substrate-free and substrate-bound HmuO and with the same complexes of human heme oxygenase.

Keywords

Heme oxygenase; *Corynebacterium diphtheriae*; molecular structure; aromatic cluster; magnetic axes

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APPENDIX A: Supplementary Data One table (chemical shifts for all assigned residues), and 13 Figures (aromatic and aliphatic side chain TOCSY, sequential $\text{N}_i\text{-N}_{i+1}$ NOESY contacts for Phe52-Thr54, Arg79-His84 and Glu156-His162, aromatic-aliphatic and inter-aliphatic NOESY contacts, inter-aromatic and aromatic-labile proton contacts for HmuO-DMDH-CN, magnetic axes for HmuO-DMDH-CN, schematic of conserved tertiary contacts in HmuO), total 20 pages.

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1. Introduction

The physiological catabolism of heme is carried out by the non-metal enzyme, heme oxygenase, HO, which utilizes heme as cofactor and substrate to stereospecifically cleave heme at a *meso*-position to generate iron, biliverdin, and CO. [1] The enzyme is widely distributed and has been most extensively studied primarily for the mammalian HOs, where all three products are vital to the organism. [2-5,6] Also studied in some detail are HOs from pathogenic bacteria such as *Corynebacterium diphtheriae*, HmuO, [7-9] where the main function is to secure the required free iron. [5] HOs from plants and photosynthetic bacteria generate the open tetrapyrrole as precursors to light harvesting pigments. [10] The structurally characterized HOs exhibit remarkably conserved alpha-helical folds [8,9,11-15] in spite of considerable sequence variations and share a common mechanism that proceeds via the intermediates, *meso*-hydroxy-protoheme, verdoheme and iron-biliverdin. [1-6]

The unique involvement of the ferric-hydroperoxy, [16,17] rather than the more common oxo-ferryl, as the reactive species has been attributed to the destabilization of heterolytic O-O bond cleavage by weak H-bonding provided by one of a cluster of three conserved ordered water molecules imbedded within an extended H-bond network [8,9,11,12] with several stronger-than-usual H-bonds. [18-20] In mammalian HOs [11,12] and HmuO, [8,9] a key homologous Asp (140 in mammalian HO, 136 in HmuO) is at the center of the coupling between the exogenous ligand and the distal H-bond network via the ordered water molecules. In spite of significant sequence [5] and structural [8,9,11] homology, particularly in the H-bond network, [18,19] the Asp mutation completely abolished activity in mammalian HO, [21,22] but only reduced it to one-half in HmuO. [23] Clearly, in spite of structural similarities, there is a significant difference in the coupling of the distal H-bond network with the exogenous ligand in mammalian HO and HmuO.

Solution ^1H NMR is particularly effective in monitoring the strength of H-bonds, with the labile protons experiencing low-field bias with increasing H-bond strength. [24] A simple system for examining the coupling of the exogenous ligand to the H-bond network through the ordered water molecules is the equilibrium between the resting-state, high-spin, aquo, and the low-spin, hydroxo complexes, where simple deprotonation of the ligated water within a conserved molecular structure converts the H-bond donor ligated water molecule into an H-bond acceptor hydroxide ligand. A recent ^1H 2D NMR study [20] has shown that, upon deprotonation of the axial water in human HO, hHO-1, the low-field bias of the Tyr58 OH, which is a strong H-bond donor to the catalytically critical Asp140, moves ~ 2.7 ppm to lower field, suggesting a very substantial increase in the Tyr58 H-bond donor strength in the hydroxide complex. However, the chemical shift changes cannot be directly related to changes in H-bonding, since both complexes are paramagnetic and protons can experience significant dipolar shifts, [25,26] δ_{dip} , within $\sim 12\text{\AA}$ of the iron. The H-bond strength is reflected only in the *diamagnetic* contribution, $\delta_{\text{DSS}}(\text{dia}^*)$, to the observed chemical shift for a *labile proton* in a complex, $\delta_{\text{DSS}}(\text{obs})$, given by: [18-20]

$$\delta_{\text{DSS}}(\text{dia}^*) = \delta_{\text{DSS}}(\text{obs})\delta_{\text{dip}} \quad (1)$$

Comparisons of H-bond strengths ($\delta_{\text{DSS}}(\text{dia}^*)$) in two complexes require the determination of δ_{dip} in each, [20,27,28] which, in turn, requires the determination of the anisotropy (axial, $\Delta\chi_{\text{ax}}$; rhombic, $\Delta\chi_{\text{rh}}$), and orientation (Euler rotational angles, α , β , γ) of the paramagnetic susceptibility tensor, χ , for each complex. For the aquo complex of particular interest, δ_{dip} values for a pair of complexes (*i.e.*, hHO-1 and HmuO) to be compared, via $\Delta\chi_{\text{ax}}$, provide a unique and direct probe of the relative axial ligand field strength, [20,29,30] which, for the His-Fe-H₂O chromophore, is modulated by the H-bond donor strength of the ligated water

to the ordered water molecule. Hence, the characterization of the H-bonds in the active site of the ferric, resting state HO, both for the ligated water and the distal H-bond network, demands the determination of the anisotropy and orientation of χ for each complex.

There are two NMR routes to determining the anisotropy/orientation [31] of χ . One approach relies on measuring residual dipolar contributions to the observed ^{15}N - ^1H coupling constants, which necessitates a uniformly ^{15}N -labeled HO complex. The alternate approach is to use ^1H 2D NMR to determine $\Delta\chi_s$ and the Euler angles, $(\Gamma(\alpha, \beta, \gamma))$ from a least-squared search for the minimum between the calculated δ_{dip} for *non-labile protons*, given by: [26,32-34]

$$\delta_{\text{dip}}(\text{calc}) = (24\pi\mu_0 N_A)^{-1} [2\Delta\chi_{\text{ax}}(3\cos^2\theta' - 1)R^3 + 3\Delta\chi_{\text{rh}}(\sin^2\theta' \cos 2\Omega')R^3] \Gamma(\alpha, \beta, \gamma), \quad (2)$$

(R, Θ', Ω' are the polar coordinates in an iron-centered coordinate system, x', y', z' , $\Gamma(\alpha, \beta, \gamma)$ is the Euler rotation that transforms the reference into the magnetic coordinate system, x, y, z , in which χ is diagonal), and the observed dipolar shift, $\delta_{\text{dip}}(\text{obs})$, given by:

$$\delta_{\text{dip}}(\text{obs}) = \delta_{\text{DSS}}(\text{obs}) - \delta_{\text{DSS}}(\text{dia}), \quad (3)$$

where $\delta_{\text{DSS}}(\text{obs})$ and $\delta_{\text{DSS}}(\text{dia})$ are the chemical shifts of *non-labile protons* in the paramagnetic complex of interest, and in a suitable diamagnetic structural analog. To date, however, the necessary chemical shifts ($\delta_{\text{DSS}}(\text{dia})$) for a diamagnetic substrate analog have not been reported for either hHO-1 or HmuO.

When $\delta_{\text{dip}}(\text{obs})$ is large (> 1 ppm), $\delta_{\text{DSS}}(\text{dia})$ for non-labile protons can be estimated by the computer program ShiftX, [35] δ_{DSS} ShiftX), if a crystal structure of an isostructural complex is available; the uncertainty in $\delta_{\text{DSS}}(\text{dia})$ can be large (up to ± 0.3 ppm). For the low-spin cyano complexes, sufficient resonances with $|\delta_{\text{dip}}| > 1$ ppm have been assigned in hHO-1 [18] and HmuO [19] complexes to quantitate $\Delta\chi_s$ and $\Gamma(\alpha, \beta, \gamma)$ in spite of the uncertainties in $\delta_{\text{DSS}}(\text{shift X})$. The problem is more serious for the high-spin, aquo complex of interest for which strong paramagnetic relaxation [26,33] obliterates [20,30,36] the residues with sizeable δ_{dip} . However, because relaxation depends more strongly ($\propto R_{\text{Fe}}^{-6}$) on distance from the iron, R_{Fe} , than δ_{dip} ($\propto R_{\text{Fe}}^{-3}$), there will be numerous protons with $R_{\text{Fe}} > 9 \text{ \AA}$ that will exhibit $|\delta_{\text{dip}}| \sim 0.2$ to 0.8 ppm with sufficiently weak relaxation to allow unambiguous assignment. [20,30,36]

Substrate-free HO is diamagnetic. In addition to the intrinsic interest in the molecular structure, H-bonding, and dynamic stability of substrate-free HO relative to the substrate-bound complex, substrate-free HO can provide the critical non-labile proton $\delta_{\text{DSS}}(\text{dia})$ for at least that portion of the HO structure that is strictly conserved upon loss of the substrate. Crystal structures of the substrate-free mammalian HOs have shown [11,12,37,38] that the bulk of the molecular structure, in particular an extended cluster of aromatic residues within a distal H-bond network, are strongly conserved upon substrate loss. A crystal structure of substrate-free HmuO is in progress (M. Unno, and M. Ikeda-Saito, to be published) and preliminary results confirm that the same portion of the structure of HmuO is conserved upon substrate loss as in mammalian HOs. Solution ^1H NMR on substrate-free hHO-1 has shown [39] that, not only are the tertiary contacts among the conserved helices indistinguishable from those in the substrate complexes, but that the relatively strong inter-helical H-bonds for the majority of the substrate-free HOs are unaffected by the substrate binding.

We report here on a ^1H NMR study of substrate-free HmuO with three main goals in mind. The primary goal is to assign a sufficient number of non-labile proton resonances in the conserved molecular structure so as to provide the basis for determining δ_{dip} , via Eq. (3) for the high-spin aquo complex. The second goal is to assess the relative merits of $\delta_{\text{DSS}}(\text{dia})$ obtained by ShiftX [35] and substrate-free HmuO as references for δ_{dip} for determining the orientation of the magnetic axes for the published data [19] for the cyanide-inhibited substrate complex of HmuO. To address the latter goal, however, we must determine initially by NMR the extent to which the solution molecular structure is conserved relative to the substrate complexes in solution. [19] Since the native protohemin, PH, (R = vinyl in Figure 1) leads to loss of both resolution and sensitivity because of orientational isomerism about the α,γ -meso axis, we have used the two-fold symmetric 2,4-dimethyldeuteroheme, (DMDH, R = methyl in Figure 1), that obviates orientational disorder. NMR studies of both hHO-1 and HmuO have shown that structural differences between the PH and DMDH complexes are inconsequential. [18,19] Lastly, the $\delta_{\text{DSS}}(\text{dia}^*)$ in substrate-free HmuO and hHO-1, as well as the substrate complexes for both HOs, will be compared to assess relative H-bond strength. [19] Consideration of the rates of exchange of labile protons provides a limited insight into changes in dynamic stability. [40]

2. Materials and methods

2.1 Sample preparation

Substrate-free HmuO was expressed and purified as previously reported [41] and a ~ 3 mM solution was prepared in $^1\text{H}_2\text{O}$, 100 mM in phosphate, pH ~ 7.0. The cyanide complex of the 2-fold symmetric substrate, 2, 4-dimethyldeuterohemin, DMDH (R = methyl in Figure 1) was prepared as described previously, [19] yielding a ~3 mM HmuO-DMDH-CN solution 100 mM in phosphate at pH 7.0. Samples in $^1\text{H}_2\text{O}$ were converted to $^2\text{H}_2\text{O}$ chromatographically on a Sephadex column. [42]

2.2 NMR Spectroscopy

^1H NMR data were collected on Bruker AVANCE 500 and AVANCE 600 spectrometers operating at 500 MHz and 600 MHz, respectively. Reference spectra were collected in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ over a temperature range of 20-30°C at a repetition rate of 1 s^{-1} using a standard one-pulse sequence with presaturation 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. NOESY spectra (mixing time of 40 ms, 20-30°C) and Clean-TOCSY spectra [43] (25°, 30°C; spin lock time of 20, 35, and 50ms, respectively) using MLEV-17 [44] were recorded over a bandwidth of 12 kHz (NOESY) and 12 kHz (TOCSY) with recycle times of 1 s using 512 t1 blocks of 128 and 256 scans, each consisting of 2048 t2 points. Two-dimensional data sets were processed using Bruker XWIN software on a Silicon Graphics Indigo work station using 30° sine-squared bell apodization in both dimensions and zero filled to 2048×2048 data points prior to Fourier transformation.

2.3 Magnetic axes determination

The anisotropy and orientation of χ in a paramagnetic HO derivative are determined by a least-square minimization of the error function, [18-20,26,32,34,36] F/n ,

$$F/n = \sum_{i=1}^n |\delta_{\text{dip}}(\text{obs}) - \delta_{\text{dip}}(\text{calc})|^2, \quad (4)$$

where $\delta_{\text{dip}}(\text{calc})$ and $\delta_{\text{dip}}(\text{obs})$ are given by Eq. (2) and (3), respectively. We use the crystal coordinates of HmuO-PH- H_2O (PDB access code 1IW0) for R, Θ' and Ω' . [8]; similar

results are obtained using the HmuO PH-O₂ complex. [9] (PDB access code 1VX8) The magnetic anisotropies for a wide range of His/CN⁻ ligated hemoproteins, including globins, peroxidases, and HOs, have been shown [18, 26, 34, 45, 46] $\Delta\chi_s$ to be highly conserved, with $\Delta\chi_{ax} = 2.48 \times 10^{-8} \text{ m}^3/\text{mol}$ and $\Delta\chi_{rh} = -0.58 \times 10^{-8} \text{ m}^3/\text{mol}$ at 25°C. Hence, only 3 parameter searches for the minimum in F/n are required in Eq. (4). The magnetic axes have been determined for the low-spin, cyanide complexes of hHO-1 [18] and the determined $\Delta\chi_s$ were found indistinguishable from those of the iso-electronic myoglobin complex.

Two sources of $\delta_{DSS}(\text{dia})$ for non-labile protons in Eq. (3) are considered. On the one hand, the chemical shift is computed using the ShiftX program [35] using the crystal coordinates of HmuO-PH-H₂O (PDB access code 1IW0), [8] to which must be added the ring current shifts of the porphyrin ring, δ_{prc} , *i.e.*,

$$\delta_{DSS}(\text{dia}) = \delta_{DSS}(\text{ShiftX}) + \delta_{\text{prc}} \quad (5)$$

The values of predicted δ_{prc} have been found valid for interpreting the ring current shifted residues in diamagnetic carbonyl globins. [47] The ShiftX program is reliable for non-labile protons. [35] However, for labile protons large discrepancies can occur between $\delta_{DSS}(\text{obs})$ and $\delta_{DSS}(\text{ShiftX})$, as witnessed for the peptide NHs of Arg85, Ala165, Phe166 of hHO (analogs of ARg79, Gly159, Phe160 in HmuO) which exhibit $\delta_{DSS}(\text{obs})$ 12.4, 11.8, 11.3 ppm, [39] respectively, for substrate-free hHO, while $\delta_{DSS}(\text{ShiftX})$, using the substrate-free hHO crystal structure [37] yields 9.5, 8.6, 8.7 ppm, respectively. For this reason, as well as the possibility that small changes in H-bond strength might occur even for very similar molecular structure, as reflected in chemical shifts for non-labile protons, we exclude chemical shift data for labile protons in the determination of the magnetic axes.

On the other hand, we used the observed chemical shift for substrate-free HmuO, $\delta_{DSS}(\text{substrate-free HmuO})$ for the structurally conserved portion upon loss of substrate, to which is added the porphyrin ring current; [47]

$$\delta_{DSS}(\text{dia}) = \delta_{DSS}(\text{obs:substratefree HmuO}) + \delta_{\text{prc}} \quad (6)$$

Error analysis was carried out as described in detail previously. [48]

3. Results

3.1 ¹H NMR spectral comparisons

The low-field, resolved portion of the 600 MHz ¹H NMR trace of substrate-free HmuO in ¹H₂O is shown in Figure 2A, where it can be compared to the same trace of HmuO-DMDH-CN [19] in Figure 2A'. Peaks are labeled by residue number for peptide NHs and by residue number and position for all other protons. Figures 2B, 2C display the labile proton peak intensity loss with time 25 min (Figure 2B), and 1 hr (Figure 2C) after exchanging ¹H₂O for ²H₂O.

3.2 Target residues and assignment protocols

Crystal structures [37,38] of substrate-free hHO-1 and rat HO-1, as well as solution ¹H NMR of the former, [39] have shown that the molecular structure on the distal side of the HOs is strongly conserved upon loss of substrate; a similar conclusion appears to be valid for HmuO (M. Unno and M. Ikeda-Saito, to be published). We target two classes of residues for assignment. One class of residues consists of those whose labile protons exhibit robust H-bonds (chemical shifts to the low-field of ~9 ppm); these H-bonds are invariably between

two different helices [8,9,11,18,19] so that they reflect, with exquisite sensitivity, the conservation of tertiary structure involving these helices upon loss of substrate. Three helical fragments are assigned sequence-specifically via standard [49] N_i-N_{i+1} , α_i-N_{i+1} , β_i-N_{i+1} , α_i-N_{i+3} and/or $\alpha_i-\beta_{i+1}$ contacts among TOCSY-detected spin systems in a manner identical to that reported for both the HmuO DMDH-CN [19] and hHO-1-DMDH-CN [18] complexes. Hence, 2D NMR data are provided only in Supplementary Data (Supplementary Data Figures S1-S9). This procedure identifies the fragments Phe52-Thr54, Arg79-Leu87 and Leu158-Tyr161 (Supplementary Data Figures S3-S5), and assigns the strong peptide NH H-bonds for Tyr53, Arg79, Ala80, Gly159 and Phe160. It was not possible to detect the $O_\eta H$ for Tyr53. The chemical shifts for labile protons involved in robust H-bonds (strong low-field bias) are provided in Table 1, and the remaining data are listed in Supplementary Data (Supplementary Data Table S1). In each table, we provide the same chemical shift data [19] for the HmuO-DMDH-CN complexes.

The other class of target residues consist of those in the extended cluster of 14 aromatic rings that are present in that portion of the distal pocket whose structure is completely conserved upon substrate loss in mammalian HOs. [11,12,18,19,37,38] The NOESY contacts among TOCSY-detected aromatic rings (Supplementary Data Figures S1, S2) are optimally resolved in ^2H_2O solution. The chemical shifts for this cluster of residues in hHO-1 has been shown [20] to provide sufficient $\delta_{DSS}(\text{dia})$ for hHO-1-DMDH- H_2O to determine robust anisotropy/orientation of χ in spite of only small to moderate $\delta_{dip}(\text{obs})$. The pattern of inter-ring contacts for the aromatic cluster predicted by the crystal structures [8,9] of the substrate complexes, and observed [19] by solution NMR in HmuO-DMDH-CN, is schematically depicted in Figure 3. TOCSY spectra (Supplementary Materials, Figure S1) locate one Phe ring in addition to the Phe52 and Phe160 rings assigned sequence-specifically, five Tyr rings in addition to the sequence-specifically assigned Tyr ring, two four-spin rings indicative of Trps and the weak coupling of two His rings. The located Phe, Tyr and His rings interact among themselves as shown in the NOESY spectrum (not shown, see Supplementary Data Fig. S6) in the precise manner predicted in Figure 3; the chemical shifts are included in Supplementary Data Table S1.

The strong upfield ring currents of the aromatic rings in the cluster result in the partial resolution of several aliphatic side chains on the upfield side of the intense envelope, with the expected contacts to the aromatic cluster as depicted in Figure 3. TOCSY spectra (not shown) locate the isopropyl termini of four Leu (in addition to the sequence-specifically assigned Leu83 and Leu87), the ethyl terminus of one Ile and a complete seven-spin system indicative of an Arg. These aliphatic side chains exhibit the aliphatic-aromatic (Supplementary Materials, Figure S7), and inter-aliphatic (Supplementary Materials, Figure S8) predicted in Figure 3, for Leu72, 77, 137 and 169 and Ile166. The strong Arg side chain interaction with the Trp96 ring can arise only from Arg97. The above-assigned aromatic rings exhibit NOESY contacts to labile protons in the low-field spectral window (not shown, see Supplementary Data Fig. S9) and identify the $O_\eta H$ s of Tyr133, 151, 172, and 176, the $N_\epsilon H$ of His128 and 129, the $N_\epsilon H$ s of Trp50 and 96 and the $N_\epsilon H$ of Arg132. The chemical shifts for the labile protons are listed in Table 1 where they can be compared to the same data for HmuO-DMDH-CN. [19] Extended assignments of the aromatic cluster and the strongly low-field shifted protons in HmuO-DMDH-CN [19] are provided in Supplementary Materials, Figures S10 and S11.

3.3 Magnetic axes determination for HmuO-DMDH-CN

The initial three-parameter determination [19] of α , β , and $\kappa = \alpha + \gamma$, based on the conserved [26,34,46] $\Delta\chi_{ax} = 2.48 \times 10^{-8} \text{ m}^3/\text{mol}$ and $\Delta\chi_{rh} = -0.58 \times 10^{-8} \text{ m}^3/\text{mol}$, using as input only non-labile protons from the proximal helix residues and the crystal structure of HmuO-PH- H_2O (PDB access file 1IW0) [8] and relatively crude empirical estimates [50] of $\delta_{DSS}(\text{dia})$

in Eq. (3) yielded $\alpha = 234 \pm 16^\circ$, $\beta = 18 \pm 3^\circ$, $\kappa = 47 \pm 12^\circ$. With minor extensions of proximal helix assignments and the more reliable $\delta_{\text{DSS}}(\text{dia})$ values based on the ShiftX program, [35] (Eq. (5)), we redetermine $\alpha = 253 \pm 9^\circ$, $\beta = 16 \pm 2^\circ$, and $\kappa = 36 \pm 11^\circ$, with an improved correlation between $\delta_{\text{dip}}(\text{obs})$ and $\delta_{\text{dip}}(\text{calc})$ over that reported previously (Supplementary Materials, Figure S12).

We now address the determination of the orientation of χ for HmuO-DMDH-CN *using only the residues of the aromatic cluster and its aliphatic contacts* with the relatively small $\delta_{\text{dip}}(\text{obs})$ as input to a three-parameter search for α , β and κ . This test is not so much to determine quantitatively the magnetic axes on the basis of such a limited data set, but rather to test the relative merits of $\delta_{\text{DSS}}(\text{dia})$ determined by either Eq. (5) or (6). Hence we carry out only 3-parameter searches for α , β and κ , using the established invariant [18, 26, 34] $\Delta\chi_{\text{ax}} = 2.48 \times 10^{-8} \text{ m}^3/\text{mol}$, and $\Delta\chi_{\text{rh}} = -0.58 \times 10^{-8} \text{ m}^3/\text{mol}$. The same conclusions are reached on the basis of 5-parameter searches (not shown), but with larger uncertainties than for the 3-parameter search. In the first case, we use $\delta_{\text{DSS}}(\text{dia})$ based on the ShiftX program, [35] (Eq. (5)), for which the optimized parameters are: $\alpha = 257 \pm 13^\circ$, $\beta = 15 \pm 2^\circ$, and $\kappa = 52 \pm 16^\circ$. A low residual error function results, $(F/n) = 0.043 \text{ ppm}^2$, and a plot of $\delta_{\text{dip}}(\text{obs})$ vs $\delta_{\text{dip}}(\text{calc})$ exhibits a good correlation as shown in Figure 4A.

In the second case, we use the input $\delta_{\text{dip}}(\text{obs})$ for the same residue set, but with $\delta_{\text{DSS}}(\text{dia})$ based on $\delta_{\text{DSS}}(\text{obs}; \text{substrate-free HmuO})$ (Eq. (6)) to yield: $\alpha = 255 \pm 6^\circ$, $\beta = 14 \pm 1^\circ$ and $\kappa = 33 \pm 12^\circ$, and an excellent correlation between $\delta_{\text{dip}}(\text{obs})$ and $\delta_{\text{dip}}(\text{calc})$, as illustrated in Figure 4B. The orientation of χ presently determined using only the residues in the aromatic cluster as input is within the uncertainties the same as that determined from the large $\delta_{\text{dip}}(\text{obs})$ obtained from the proximal helix. Moreover, using only the aromatic cluster residues as sources of $\delta_{\text{dip}}(\text{obs})$ yields an orientation of χ indistinguishable from that obtained using either $\delta_{\text{DSS}}(\text{dia})$ based on ShiftX [35] (Eq. (5)), or substrate-free as source of $\delta_{\text{DSS}}(\text{dia})$ (Eq. (6)). However, the precision of the determination (residual error function, F/n) is much greater when using Eq. (3) (smaller $F/n = 0.007 \text{ ppm}^2$) than Eq. (5) (larger $F/n = 0.043 \text{ ppm}^2$) to determined δ_{dip} in Eq. (6). Clearly, $\delta_{\text{DSS}}(\text{dia})$ based on chemical shifts from substrate-free HmuO (Eq. (6)) are superior to the ShiftX [35] program (Eq. (5)) estimates. An improved correlation between $\delta_{\text{dip}}(\text{obs})$ and $\delta_{\text{dip}}(\text{calc})$ for residues with small $\delta_{\text{dip}}(\text{obs})$ has also been observed for hHO-1-DMDH-CN [18,39] when using $\delta_{\text{DSS}}(\text{dia})$ based on substrate-free-hHO-1 shift data.

3.4 Comparison of H-bond networks

The values of $\delta_{\text{DSS}}(\text{dia}^*)$ for labile protons for HmuO-DMDH-CN, [19] obtained via Eq. (1) and the δ_{dip} from the optimized magnetic axes, are listed in Table 1 where they can be compared to the $\delta_{\text{DSS}}(\text{dia}^*)$ for substrate-free HmuO via:

$$\delta_{\text{DSS}}(\text{dia}^*) = \delta_{\text{DSS}}(\text{obs}; \text{substrate-free HmuO}) + \delta_{\text{prc}} \quad (7)$$

The values for the δ_{prc} for the labile protons of interest are $< |0.1|$ (Supplementary data, Table S1) so that uncertainties in δ_{prc} do not influence the comparisons of $\delta_{\text{DSS}}(\text{dia}^*)$ between substrate-free HmuO and HmuO-DMDH-CN. Within the uncertainties of $\pm 0.1 \text{ ppm}$ for each total chemical shift, the data in Table 1 reveal that, while $\delta_{\text{DSS}}(\text{obs})$ may differ significantly between substrate-free HmuO and HmuO-DMDH-CN, these differences are due primarily to δ_{dip} for the latter complex. Hence, the detected members of the H-bond network in substrate-free HmuO exhibit essentially the same H-bond strengths as in the HmuO-DMDH-CN complex. [19] Notable, however, is our failure to detect the key Tyr53 OH signal in substrate-free HmuO. For hHO-1, a similar conservation of H-bond strengths in the H-bond network was noted upon loss of the substrate, [18, 39] with the prominent

exception of the Tyr58 side chain, the analog of Tyr53 in HmuO, whose H-bond strength was significantly decreased upon loss of substrate. The $\delta_{\text{DSS}}(\text{dia}^*)$ values for the labile protons in the H-bond network of substrate-free-hHO-1 are included in Table 1 for comparison.

3.5 Labile proton exchange

The rate of intensity loss of low-field resolved labile protons after replacement of $^1\text{H}_2\text{O}$ with $^2\text{H}_2\text{O}$ is followed in the spectra in Figures 2B-2C. The half-lives for exchange for protons substrate-free HmuO are listed in Table 2, where we include for comparison the half-lives for the same protons in HmuO-DMDH-CN [19] and for homologous protons in substrate-free hHO. [39]

4. Discussion

4.1 Scope of assignments

Spectral resolution restricted assignments to portions of only 40 of the 200 residues by solely homonuclear ^1H NMR at 600 MHz. Fortunately, included in these assignments are the majority of the members of the H-bond network with stronger than usual H-bonds and all members of the extended aromatic cluster and some of its key aliphatic side chain contacts whose resolution was expanded by the ring currents. Hence, the majority of our target residues are assigned. More extensive assignments will require heteronuclear labeling and 3D NMR. However, our present interests are limited to the H-bond network and the aromatic cluster, whose successful ^1H -only NMR assignment sets the stage for future magnetic axes determination on other paramagnetic and/or mutant HmuO-substrate complexes.

4.2 Structural conservation upon substrate loss

The crystal structures of HmuO substrate complexes [8,9] identify twelve helices with numbers (consists of residues): helix 1(proximal helix, 7-25), 2(26-34), 3(38-67), 4(74-78), 5(79-92), 6(95-100), 7(103-120), 8(distal helix, 121-152), 9(154-157), 10(158-163), 11(168-183), and 12(186-213). Two types of presently determined NMR parameters argue for conserved molecular structure for the aromatic cluster and the bulk of the H-bond network upon loss of substrate for HmuO in solution. On the one hand are the highly conserved robust H-bonds between pairs of helices as listed in Table 1 (helix origin for both the donors and acceptors are given by italics in parentheses for each residue), implying conserved interactions for helix 3 with helices 5 and 8, helix 5 with helices 6 and 7, helix 7 with helix 12 and helix 8 with helices 11 and 12. On the other hand are the highly conserved pattern and magnitude of inter-residue NOESY cross peaks upon loss of substrate for the extended aromatic cluster depicted in Figure 3 (helical origin of residues are given in italics next to the residue), arguing for conserved interactions for helix 3 with helices 5, 6, 7, 8 and 10, helix 4 with helices 8, 11, helix 5 with helices 6 and 10, and helix 8 with helix 11. The conserved interhelical contacts are summarized schematically in Figure S13 of Supplementary Materials. The conserved NMR observables upon loss of substrate for HmuO in solution are essentially the same as those observed for hHO-1 upon loss of substrate in solution. [18,39] The observed conservation of the aromatic cluster and bulk of the H-bond network is consistent with preliminary results of the crystallographic characterization of substrate-free HmuO (M. Unno and M. Ikeda-Saito, to be published).

We therefore conclude that the structure of the aromatic cluster in substrate-free HmuO is conserved relative to that in substrate complexes and that the chemical shifts, when corrected for the effect of the diamagnetic porphyrin ring current, serve as reliable values for

$\delta_{\text{DSS}}(\text{dia})$ necessary to quantitate $\delta_{\text{dip}}(\text{obs})$ and hence, the orientation of the magnetic axes in the paramagnetic HmuO complex.

Preliminary 1D ^1H NMR spectra as a function of pH for HmuO-DMDH- H_2O reveal (Z. Du, G. N. La Mar and M. Ikeda-Saito, unpublished observations) a much weaker coupling of the distal H-bond network with the ligated water molecule than documented for the same hHO-1 complex. [20] This difference may account for the much weaker dependence of HO activity upon mutation [21,23] of the critical distal Asp (Asp136 in HmuO, Asp140 in hHO-1). The 2D NMR characterization necessary to quantify the H-bond strength in the high-spin complex of HmuO are in progress.

4.3 Orientation of χ in HmuO-DMDH-CN

The chemical shifts of the established conserved structure upon substrate loss for the aromatic cluster, after correcting for the diamagnetic porphyrin ring current, provide reliable values of $\delta_{\text{DSS}}(\text{dia})$ for references to quantitate $\delta_{\text{dip}}(\text{obs})$ via Eq. (6), and in turn, the orientation of χ . The fact that the orientation of χ in HmuO-DMDH-CN determined using only $\delta_{\text{DSS}}(\text{dia})$ via Eq. (6) and the relatively small $\delta_{\text{dip}}(\text{obs})$ for the aromatic cluster, is essentially indistinguishable from that determined [19] from the relatively large $\delta_{\text{dip}}(\text{obs})$ for the proximal helix referenced to $\delta_{\text{DSS}}(\text{dia})$ calculated by the ShiftX program [35] (*i.e.*, Eq. (5)) provide confirmation for both the relatively robust nature of the determined orientation of χ and the validity of using ShiftX program [35] to estimate $\delta_{\text{DSS}}(\text{dia})$ when δ_{dip} is large.

The various magnetic axes determinations indicate that the major magnetic axis, z , is tilted (β) $\sim 15^\circ$ in a direction (α) approximately towards the γ -meso position. Since β is controlled by the Fe-CN bond in the $-z$ direction, [26] the Fe-CN unit is tilted $\sim 15^\circ$ towards the α -meso position. A direct correlation between Fe-CN tilt and the $-z$ axis has been established for the cyanide-inhibited substrate hemin complex of mammalian HO. [12,18] Hence the Fe-CN tilt can be directly related to a steric contribution to the stereoselectivity that cleaves exclusively at the α -meso position in HmuO. The rhombic magnetic axes, defined by $\kappa = \alpha + \gamma = 45 \pm 15^\circ$, are expected to be controlled by the orientation of the axial His imidazole ring, whose prediction [51] of $\kappa = 45^\circ$ is qualitatively consistent with our results.

4.4 Dynamic stabilities

For the more general EX2 mechanism [40], the relative exchange lifetimes for homologous protons in two complexes to be compared relate to the relative dynamic stabilities (shorter lifetime $\rightarrow$ lower dynamic stability) of the interaction whose breaking exposes these protons to exchange. Exchange half-lives were obtained for the labile protons in only three H-bonds in both substrate-free HmuO and HmuO-DMDH-CN, [19] as well as for the homologous residues in substrate-free [39] hHO-1, Arg79 (Arg85)NH $\rightarrow$ Glu57 (Glu62)CO $_2^-$, Phe160 (Phe166)NH $\rightarrow$ Asp86 (Asp92)CO $_2^-$, and His128 (His132)N $_e$ H $\rightarrow$ Glu196 (Glu202)CO $_2^-$, in HmuO (hHO-1); half-lives are listed in Table 2. The three H-bonds monitor the relative dynamic stabilities of the helix-5/helix-3, helix-10/helix-5, and helix-8/helix-12 interactions, respectively.

Qualitative conclusions that can be drawn from the data in Table 2 are that the substrate-free HO is generally less stable dynamically than the substrate complex for both HmuO and hHO-1, [18,39] but the pattern of exchange lifetimes changes are not the same in the two HOs. Comparison of the data for substrate-free HmuO and hHO indicate that the dynamic stability is somewhat greater in hHO. [39] The greater dynamic stability of the helix-5/helix-3 and helix-10/helix-5 interactions in substrate-free HmuO than substrate-free hHO-1, also observed [18,39], for the respective cyanide complexes with DMDH, likely arises because of a key additional strong H-bond in HmuO involving the Tyr161 OH (Phe167 in

hHO-1). The turnover rate of HOs is generally controlled by the very slow product release, [2,6] and this product release likely involves disruption or “opening” of the distal pocket. The generally greater dynamic stability of HmuO than hHO-1 in both substrate [18] and substrate-free [39] complexes is consistent with the factor 4 slower turnover rate in HmuO [52] than hHO-1.[2]

5. Conclusions

The observed strong conservation in solution of inter-helical tertiary contacts of aromatic rings and robust H-bonds for substrate-free HmuO relative to the substrate HmuO complexes confirms a highly conserved molecular structure in solution of HmuO upon substrate loss for all helices not making direct contact to substrate. These conclusions based on our limited ^1H NMR data are completely consistent with preliminary results of a crystal structure of substrate-free HmuO (M. Unno and M. Ikeda-Saito, to be published). The relative exchange rates for labile protons in substrate-free and substrate complex of HmuO indicate a pattern of destabilization of tertiary structure upon substrate loss that is different from that observed in hHO-1 [19] but agrees in that, in general, loss of substrate leads to a decrease in the dynamic stability of the enzyme.

The chemical shifts of residues for the structurally conserved portion of the molecular structure upon loss of substrate serve as reliable references for the dipolar shifts of the paramagnetic, cyanide-inhibited, substrate complex, and allow the use of much smaller dipolar shifts to quantitate the orientation of the paramagnetic susceptibility tensor. The ability to quantitate small dipolar shifts for the aromatic cluster provides the crucial information to quantitate dipolar shifts in the strongly paramagnetic aquo complex of HmuO where the axial anisotropy provides direct information on the strength of the H-bond of the ligated water to the ordered water in the distal H-bond network. [20,30,36] Such a ^1H 2D NMR investigation of the HmuO-DMDH- H_2O is in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HO	heme oxygenase
hHO-1	human heme oxygenase-1
HmuO	<i>Corynebacterium diphtheriae</i> heme oxygenase
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
PH	protohemin
DMDH	2-,4-dimethyldeuterothemin
NOESY	two-dimensional nuclear Overhauser spectroscopy
TOCSY	two-dimensional total correlation spectroscopy
R_{Fe}	distance from the iron

Figure 1.

Orientation of the magnetic axes, x , y , z , relative to the iron-centered reference coordinate system, x' , y' , z' , with z' normal to the heme plane pointing towards the proximal side, and x' , y' , in the heme plane. The two coordinate systems are related by the Euler rotation, $\Gamma(\alpha, \beta, \gamma)$ via $\Gamma(\alpha, \beta, \gamma)[x', y', z'] = [x, y, z]$, where β is the angle of tilt of the major magnetic axes, z , from the heme normal, z' ; α is the angle between the projection of z on the x' , y' plane and the x' axis; $\kappa = \alpha + \gamma$ corresponds to angle between the projection of x' on the x' , y' plane and the x' axis.

Figure 2. The low-field resolved portions of the 600 MHz NMR spectrum of: (A) substrate-free HmuO, and (A') HmuO-DMDH-CN, both in $^1\text{H}_2\text{O}$, 100 mM in phosphate, pH 7.0 at 25°C, and (B) 25 min., and (C) 1 hr., after converting from $^1\text{H}_2\text{O}$ to $^2\text{H}_2\text{O}$. Resonances are labeled by sequence number for peptide NHs and by residue number and position for other protons.

Figure 3.

Schematic depiction of the interacting rings (depicted by ovals) of the extended distal aromatic cluster, whose structure can be expected to be conserved upon loss of substrate. Also labeled are key aliphatic side chains (depicted by rectangles) in contact with these aromatic rings whose upfield ring currents yield partially resolved signals on the upfield side of the diamagnetic envelope. An asterisk indicates entry points into the cluster via sequence-specific assigned residues. Solid lines represent contacts predicted (and observed in substrate-free HmuO) by the crystal structures [8,9] of HmuO substrate complexes. The two Trp are not part of the aromatic cluster but they make contact to Leu87 which is on the sequence-specifically assigned helix 5 that also contains Leu83 (indicated by a dashed line). The helical origin for each residue is given in italics within parentheses.

Figure 4.

Plot of $\delta_{\text{dip}}(\text{obs})$ versus $\delta_{\text{dip}}(\text{calc})$ for the optimized magnetic axes of HmuO-DMDH-CN based on residues in the aromatic cluster and its aliphatic contacts, using: **(A)** the ShiftX program to determine $\delta_{\text{dip}}(\text{obs})$ via Eq. (5), and **(B)** using $\delta_{\text{DSS}}(\text{obs})$ from substrate-free HmuO and Eq. (6). The values $\Delta\chi_{\text{ax}} = 2.48 \times 10^{-8} \text{ m}^3/\text{mol}$ and $\Delta\chi_{\text{rh}} = -0.58 \times 10^{-8} \text{ m}^3/\text{mol}$, shown to be invariant in a wide range of His/CN⁻-ligated ferri-hemoproteins, [26,34] were used for both determinations. The optimized orientation of the magnetic axes are $\alpha = 257^\circ$, $\beta = 15^\circ$ and $\kappa = 52^\circ$ for **(A)**, and $\alpha = 255^\circ$, $\beta = 14^\circ$ and $\kappa = 33^\circ$ for **(B)**.

Table 1
Labile proton chemical shifts reflecting H-bond strength in HmuO and hHO-1 complexes^a

Donor ^b	Acceptor ^c	substrate-free HmuO		HmuO-DMDH- CN		substrate-free hHO-1	
		δ _{DSS} (obs)	δ _{DSS} (dia*) ^d	δ _{DSS} (obs) ^e	δ _{DSS} (dia*) ^f	δ _{DSS} (dia*) ^g	δ _{DSS} (dia*) ^g
Peptide NHs							
Tyr53/58(3) ^h	Ala49/Leu54 C=O(3) ^h	9.86	9.85	9.95	9.81		8.88
Arg79/85(5)	Glu57/62 CO ₂ ⁻ (3)	12.62	12.55	12.94	12.68		12.48
Ala80/Lys86(5)	Glu57/62 CO ₂ ⁻ (3)	10.16	10.10	10.05	9.84		10.40
Ala105/110(7)	Asp210/Glu216 CO ₂ ⁻ (12)	9.53	9.57	9.26	9.40		10.40
Gly159/Ala165(10)	Asp86/Glu92 CO ₂ ⁻ (5)	10.08	9.99	10.32	9.98		11.92
Phe160/166(12)	Asp86/Glu92 CO ₂ ⁻ (5)	11.15	11.02	11.56	11.07		11.41
Side Chains							
Trp50 N _H H	H ₂ O 107 <i>j</i>	9.89	9.86	9.93	9.83		
Tyr53/58 O _H H(3)	Asp136/140 CO ₂ ⁻ (8)	<i>i</i>		17.44	16.22		11.59
Trp96 N _H H(6)	Asp88 CO ₂ ⁻ <i>j</i> (5)	11.32	11.30	11.39	11.30		
Arg112/117 N _H H(7)	Asp196/202 CO ₂ ⁻ (12)	8.63	8.67	8.75	8.84		8.93
His128/132 N _H H(8)	Glu196/202 CO ₂ ⁻ (12)	13.88	13.95	13.68	13.84		14.61
His129 N _H H(8)	H ₂ O 1013	12.86	12.85	12.72	12.73		<i>j</i>
Arg132/136 N _H H(8)	Tyr53/58 O _H (8)	<i>i</i>		9.85	9.12		9.25
Tyr133/137 O _H H(8)	Glu57/62 CO ₂ ⁻ (3)	11.38	11.33	11.62	11.42		10.87
Tyr151 O _H H/h(9)	Leu37 C = O <i>j</i>	<i>i</i>		9.96	10.07		<i>j</i>
Tyr161 O _H H/h(10)	Gln46 C = O <i>j</i> (3)	<i>i</i>		11.08	10.03		<i>j</i>
Tyr176/182 O _H H(11)	His129 N _H /none(8)	11.56	11.62	11.46	11.80		8.60

^a Chemical shifts in ppm, referenced to DSS, in ¹H₂O, 100 mM in phosphate, at pH 7 and 25°C.

^b Donor proton for HmuO/hHO-1, as assigned in ¹H NMR spectra.

^c Acceptor heteroatom for HmuO/hHO-1 as identified in crystals. [8,11]

^d Given by Eq. (7).

e Taken from [18]

f Given by Eq. (7).

g Taken from [39]

h The helical origin for each residue is given in italics within parentheses.

i Not located.

j Not present.

Table 2

Half-lives for labile proton exchange and relative dynamic stabilities of inter-helical contacts in HmuO and hHO-1 complexes.^a

Residue ^b	Substrate-free HmuO	HmuO-DMDH-CN ^c	Substrate-free hHO-1 ^d	hHO-1-DMDH-CN ^e
Arg79/Arg85	$9.0 \pm 1.8 \times 10^2$	$6.0 \pm 1.2 \times 10^2$	$6.0 \pm 2.0 \times 10^1$	$1.5 \pm 0.3 \times 10^3$
Phe160/Phe166	$1.6 \pm 0.4 \times 10^3$	$> 5.4 \pm 1.1 \times 10^5$	$9.0 \pm 2.2 \times 10^2$	$9.0 \pm 2.2 \times 10^2$
His128/His132	$6.0 \pm 2.0 \times 10^1$	$9.0 \pm 1.8 \times 10^3$	$1.2 \pm 0.3 \times 10^2$	$5.4 \pm 1.1 \times 10^3$

^a Half-lives in seconds at 25 °C in ²H₂O, 100mM phosphate, pH 7.0.

^b HmuO/hHO-1

^c Taken from [19]

^d Taken from [53]

^e Taken from [18]