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^{1}H NMR Detection of Immobilized Water Molecules within a Strong Distal Hydrogen-Bonding Network of Substrate-Bound Human Heme Oxygenase-1

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Discrete, nonligated water molecules are often found in the crystal structure active sites of metalloproteins in general, and hemoproteins in particular.[1] Their roles can range from simple impediments to rapid ligation, as in the globins,[2] to participation in important hydrogen-bonding networks in the catalytic cycles of enzymes such as the cytochromes P450.[3] ^{1}H NMR spectroscopy can identify water molecules within an enzyme by the detection of an NOE[4] between the water molecule and specific enzyme protons.[5] Even buried water molecules, however, exchange too fast with the bulk water ($\sim 10^3$ s^{-1}) to yield discrete ^{1}H NMR signals.[5] Nevertheless, water molecules localized within a protein can still be identified by ^{1}H NMR through NOEs between the bulk water signal and individual protein resonances.[5] For nonlabile protons such NOEs are difficult to assign because of severe resolution problems. Enzyme labile protons are easier to assign, but it must be shown that chemical exchange does not contribute to magnetization transfer.[5] Direct (water−enzyme contact), versus indirect NOEs (NOE to a nearby, rapidly exchanging enzyme labile proton), can be differentiated solely on the basis of a molecular structure.[5]

Heme oxygenase-1,[6] HO-1, is a ∼32-kDa membrane-bound enzyme that employes heme as substrate and cofactor in the conversion[7] of heme to iron, CO, and biliverdin IXα. The net reaction consumes three O_2, seven electrons, and nine protons. Recombinant, truncated, soluble, and fully active HO-1 has been shown to act via a hydroperoxy intermediate[8,9] rather than the more common ferryl form. The initial crystal structures of human HO-1,[10] hHO-1, and rat HO-1[11] did not provide information as to how such a novel hydroperoxy intermediate is stabilized. However, the loss of activity upon mutating the conserved Asp140[12,13] and the X-ray detection of a water molecule localized near Asp140[12] lead to the proposal that this water molecule provides the weak H-bond required to stabilize the ligated hydroperoxy species. We have demonstrated[14−16] by ^{1}H NMR that the cyanide-inhibited, substrate-bound hHO-1 complex exhibits[14] an active-site molecular structure that is, for the most part, indistinguishable from that revealed in the earlier crystal structure.[10] However, an extensive H-bond network with very strong H-bonds (labile protons with chemical shift 9−17 ppm) was identified[16] which was not recognizable in the crystal structure. This network involves the catalytically critical Asp140 and extends from the distal pocket through the opposite end of the enzyme, with the NHs of Arg85, Lys86, Ala165, and Phe166 and the side-chain labile protons of Tyr58, Trp96, His132, and Arg136, serving as donors to four carboxylates (Glu62, Asp92, Asp140, Glu202) and one carbonyl (Gly163).[16] We demonstrate here by ^{1}H NMR that numerous other water molecules[5] are localized within this H-bond network.

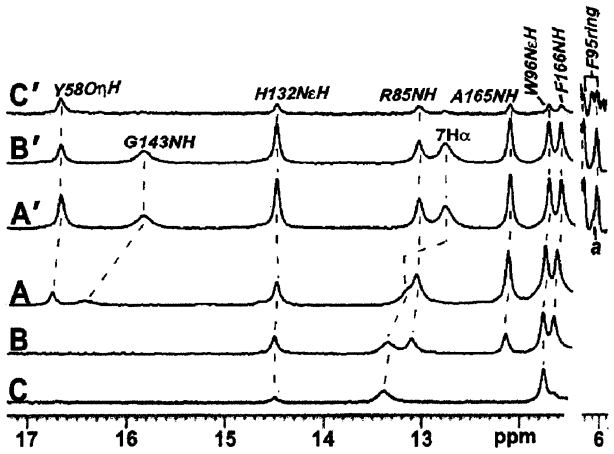

Figure 1. 11.5−17.0 portion of the 600 MHz ^{1}H NMR "soft-pulse" "1:1" spectra of hHO-1−DMDH−CN[4] in 100 mM phosphate, pH 7.0 in: (A) ^1H_2O at 25 °C, (B) 20 min, and (C) 4 h after transferring from ^1H_2O to ^2H_2O at 25 °C. The 11.5−17.0 (apodized with 5 Hz line broadening) and 5.90−6.25 ppm (apodizied by 60° shifted-sine-bell-square) of a "3:9:19" spectra at 35° in ^1H_2O with off-resonance (A′) off-resonance (B′) on-resonance saturation (∼80%) of the water signal, with the difference trace shown in (C′). The difference trace for the 5.90−6.25 ppm portion is enhanced by 4 relative to the trace in A′ and B′. Peaks are labeled as previously assigned. Peak a is an unassigned labile proton.[14−16]

The low-field portion of the sequence-specifically assigned[14−16] ^{1}H NMR spectrum[17,18] in ^1H_2O of ∼1.5 mM hHO-1−DMDH−CN (DMDH = 2,4-dimethyldeuterohemin; a two-fold symmetric heme that obviates heme orientational disorder)[14−16] is shown in Figure 1A. The 25 °C spectra[17,18] of the same sample 20 min (Figure 1B) and 4 h (Figure 1C) after transfer to ^2H_2O show that the majority of the labile protons exhibit exchange lifetimes of minutes to hours.[19] Two additional relevant, slowly exchanging labile protons[16] near 9.0 ppm (Arg136 $N_\epsilon H$ and Lys86 NH) are not shown. This eliminates exchange as the source of any magnetization transfer.[5] Comparison of the 35 °C ^{1}H NMR spectra[20,21] in ^1H_2O with off-resonance (Figure 1A′) and on-resonance (Figure 1B′) irradiation of the ^1H_2O results in the difference trace in Figure 1C′, that reveals ∼10−15% temperature- and pH-independent reduction in intensities for labile protons which are indicative of a saturation factor, SF (due to exchange), or NOE, η (due to dipolar interactions), given by $SF = \eta = (I - I_o)I_o^{-1}$, ($I$, I_o signal intensity with and without saturating ^1H_2O), of −0.1 to −0.2, (including the NH of Lys86 and $N_\epsilon H$ of Arg132), except for the Tyr58 OηH. The reduction in SF or η is linear in the $^2H/^1H$ solvent composition. The larger SF for the Tyr58 OηH (∼−0.4) at 35 °C contains exchange contributions which decrease with temperature until the

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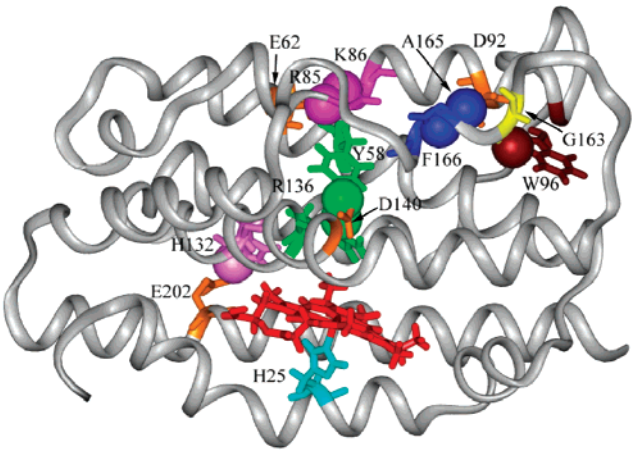

Figure 2. Ribbon representation[10,24] of hHO-1−heme−H_2O. The heme is red and His25 is light blue. The residues providing the eight strong H-bond donors are colored magenta for the peptide NHs of Arg85 and Lys86, blue for the peptide NHs of Ala165 and Phe166, green for the side-chain OH of Tyr58 and $N_\epsilon H$ of Arg136, brown for the side-chain $N_\epsilon H$ of Trp96 and pink for the side-chain $N_\epsilon H$ of His132. The four carboxylate acceptors of Glu62, -202 and Asp92, -140, are shown in gold, while the carbonyl acceptor of Gly163 is shown in yellow.[14] The "ordered" water molecules detected by NOEs are indicated by spheres of the same color as the adjacent donor residue. Six of these eight spheres colored green, magenta, and dark blue overlap significantly in three pairs.

saturation factor approaches that of the other labile protons at 5 °C. Except for Y58 OηH at elevated temperature, exchange with water is completely eliminated, dictating that we are observing slowly exchanging enzyme protons in dipolar contact with either close-by, rapidly exchanging enzyme-labile protons or directly with immobilized water molecules.[5]

Estimates of the interproton distance r_{ij} responsible for the NOE from the cross relaxation rate, σ_{ij}, were determined from the steady-state NOE,[22] $\eta_{j\rightarrow i} = \sigma_{ij}/\rho_i$. Selective relaxation rates,[23] ρ_i, for the labile protons of interest yield values $\rho_i = 15$ to 30 s^{-1} (nonselective ρ_i range from 0.5 to 6.2 s^{-1}), and result in ranges in $\sigma_{ij} = -2$ to -4 s^{-1}. An estimated molecular correlation time, $\tau_r \sim 15-20$ ns, together with $\sigma_{ij} = -0.1\ \gamma^4\hbar^2\tau^{-6}_{ij}\tau_r$, indicate[22] $r_{ij} \sim 3$ Å. Inspection of the crystal structure[10,24] reveals no rapidly exchanging labile enzyme protons within 5 Å of the slowly exchanging labile protons of interest, dictating that we are observing *a direct NOE between the protein and water molecule(s)*.[5] Such immobilized water molecules have been identified by ^{1}H NMR in only a limited range of small biopolymers, and primarily for buried water molecules also detected in the crystal.[5] This is the largest enzyme for which immobilized water molecules have been observed by ^{1}H NMR.

The ribbon structure[10,24] of hHO-1 is illustrated in Figure 2, where H-bond donor protons, Arg85, Lys86, Ala165, and Phe166 NHs, $N_\epsilon H$ of Trp96, $N_\epsilon H$ of His132, $O_\eta H$ of Tyr58, and $N_\epsilon H$ of Arg136, are colored as described below, and the acceptor residues are shown in gold (carboxylates) or yellow (carbonyl). Spheres depict the positions for the water molecules that are consistent with the observed NOE for each of the eight labile protons; they are colored to match donor residues. It is noted that in three cases, for the proton-donor pairs; NHs of Ala165/Phe166 (deep blue), NHs of Arg85/Lys86 (magenta), and OηH of Tyr58/$N_\epsilon H$ of Arg136 (green), the water "spheres" overlap, suggesting that a single water molecule may account for the observed NOEs to each pair of protein labile protons. The H_2O near the Trp96 $N_\epsilon H$ should[10] also be close to the ring of Phe95, one of only two aromatic rings with resolved ring resonances (see 5.9−6.25 ppm window of Figure 1, A′ and B′). In accord with this expectation, saturation of the water resonance leads to detectable NOEs to the Phe95 ring protons (right side of Figure 1C′) Hence, the present ^{1}H NMR data dictate the presence of at least five, and as many as eight, "ordered" water molecules at the positions indicated in Figure 2 with residence times[5] $> \tau_r \sim 20$ ns. Similar water NOEs to other, unassigned slowly exchanging low-field NHs (not shown) suggest that additional water molecules may be immobilized in this H-bond network.

The crystal structure of the substrate-bound hHO[10,12,25] reveals numerous buried water molecules, but none as close to the strong H-bond donor-protons as estimated from NMR, except for that near Asp140 (blue spheres in Figure 2). However, the different ligands used in the crystal structure[10,12,25] (water, H-bond donor) and solution ^{1}H NMR studies[14−16] (H-bond acceptor, CN^-) may account for these differences. The likely role of the strong H-bonding network is to provide a "scaffold" to support the "ordered" water molecules, and these "ordered" water molecules, in turn, both stabilize the hydroperoxy intermediate[8,9] in the reaction pathway and serve as a "water channel" to funnel protons to the catalytic site in a controlled manner.

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