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Solution NMR characterization of magnetic/electronic properties of azide and cyanide-inhibited substrate complexes of human heme oxygenase: Implications for steric ligand tilt

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ABSTRACT

Solution 2D ^{1}H NMR was carried out on the azide-ligated substrate complex of human heme oxygenase, hHO, to provide information on the active site molecular structure, chromophore electronic/magnetic properties, and the distal H-bond network linked to the exogenous ligand by catalytically relevant oriented water molecules. While 2D NMR exhibited very similar patterns of two-dimensional nuclear Overhauser spectroscopy cross peaks of residues with substrate and among residues as the previously characterized cyanide complex, significant, broadly distributed chemical shift differences were observed for both labile and non-labile protons. The anisotropy and orientation of the paramagnetic susceptibility tensor, χ, were determined for both the azide and cyanide complexes. The most significant difference observed is the tilt of the major magnetic axes from the heme normal, which is only half as large for the azide than cyanide ligand, with each ligand tilted toward the catalytically cleaved α-meso position. The difference in chemical shifts is quantitatively correlated with differences in dipolar shifts in the respective complexes for all but the distal helix. The necessity of considering dipolar shifts, and hence determination of the orientation/anisotropy of χ, in comparing chemical shifts involving paramagnetic complexes, is emphasized. The analysis shows that the H-bond network cannot detect significant differences in H-bond acceptor properties of cyanide versus azide ligands. Lastly, significant retardation of distal helix labile proton exchange upon replacing cyanide with azide indicates that the dynamic stability of the distal helix is increased upon decreasing the steric interaction of the ligand with the distal helix.

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1. Introduction

The physiological catabolism of hemin is carried out by the non-metal enzyme heme oxygenase, HO, which cleaves hemin into iron, biliverdin and CO [1]. The enzyme, which is widespread among mammals, plants, cyanobacteria and some pathogenic bacteria, exhibits a conserved α-helical fold characterized by a compact structure except for several large cavities and a distal pocket where the distal helix can exhibit variable position with different ligands [2–8]. Two HO properties are exceptional, selectivity leading solely to α-meso cleavage in mammalian HOs, and the participation of the ferric hydroperoxy as the reactive species; the conventional ferryl species that participates in cytochrome P450 and peroxidases catalysis is inactive in HOs [9–11]. The stereoselectivity is recognized as arising from the manner of seating of the substrate in the active site, where the distal helix is placed sufficiently close to the substrate to sterically shield three meso positions from reaction and sterically tilt/orient the bound oxygen towards the fourth meso position which is attacked [2–8]. The unique participation of the ferric hydroperoxy species that cleaves homolitically is rationalized by distal interactions of the ligand with primarily ordered water molecules [2–7] imbedded in an extensive H-bond network that possesses some much stronger than usual H-bonds [12–16].

Crystallography [2–8] has provided the molecular structure of HOs that reveals the likely steric influences on stereoselectivity and observed the ordered water molecules that favor homolytic O – O bond cleavage. Solution NMR has provided some complementary structural information that identifies isomeric substrate seatings that are relevant to stereoselectivity [17–21]. The extent of the H-bond network, and the relative strengths of the individual H-bonds in the distal network, are also characterized primarily by NMR [13,14,16,22,23] where the low-field bias [12] of the *diamagnetic* contribution to the chemical shift for labile protons reflects on the strength of the H-bond. A

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difficulty common to both crystallographic and solution spectroscopic studies of HO is the instability of all but the resting state, high-spin aquo complex of substrate-bound HO [9–11]. In only one case has it been possible to generate the actual reactive species [7] and this was achieved only at cryogenic temperatures where NMR is not applicable. Thus the bulk of the crystallographic [2–8] and NMR characterization has been carried out on complexes of "model" ligands such as the ground state ferric low-spin cyanide [4,13,15,17,20,24], azide [3,24–29] and hydroxide [16,23,30], as well as ferrous NO complexes [4,6]. Effective spin relaxation for low spin ferric complexes leads to relatively narrow lines, and the magnetic anisotropy provides large chemical shift dispersion [31,32], thereby allowing particularly effective 2D NMR structural characterization. Azide and cyanide serve as limited models for the oxy species in that $Fe^{II}-O-O$ is bent [7] while the $Fe^{III}-CN$ bond is linear [4]. The azide in the $Fe^{III}-N{=}N{=}N$ unit is bent [3], like in $Fe^{II}-OO$ [7], but is larger by one atom that still allows steric interaction with the distal helix. Crystallography of the cyanide [4] and azide [3] complexes of rat HO has shown that the $Fe^{III}-CN$ unit is linear and strongly tilted towards the α-meso position while the bent azide ligand is tilted much less in the same direction.

The tilt of the axial ligand from the heme normal is readily determined by ^{1}H NMR, since the orientation of the major magnetic axis, z (Fig. 1), is determined by the axial Fe–C ($Fe^{III}-CN$) and Fe–N, ($Fe^{III}-N_3$) vectors [32]. The orientation and anisotropy of the magnetic axes can be effectively determined either from residual dipolar splittings in ^{15}N-labeled enzyme [33], or, in the absence of such isotope labeling, from dipolar shift, δ_{dip}, contributions to the observed chemical shift, δ_{DSS}(obs), of non-labile protons in the vicinity of the active site [32,34,35]. This shift is given by [32,36,37]:

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

where R, θ', Ω' locate a proton in an iron-centered reference coordinate system, x', y', z', as shown in Fig. 1. $\Delta\chi_{ax} = \chi_{zz} - 1/2(\chi_{xx}+\chi_{yy})$, $\Delta\chi_{rh} = \chi_{xx} - \chi_{yy}$, are the axial and rhombic anisotropies of the paramagnetic susceptibility tensor, χ, and Γ(α, β, γ) is the Euler rotation that converts the reference coordinate system to the magnetic coordinate system, x, y, z, where χ is diagonal. The relationship of the two coordinate systems, as relevant to axial ligand tilt/direction, is depicted in Fig. 1. The Δχs and Γ(α, β, γ) are readily determined from observed dipolar shifts, δ_{dip}(obs), by a least-square search [13,26,32,36] for the difference between calculated δ_{dip}(calc) (via Eq. (1)) as a function of Δχs and α, β, γ, and observed dipolar shift, δ_{dip}(obs), obtained via:

$$\delta_{dip}(obs) = \delta_{DSS}(obs) - \delta_{DSS}(dia), \quad (2)$$

where δ_{DSS}(obs) and δ_{DSS}(dia) are the chemical shifts of *non-labile* protons (*vide infra*) in the paramagnetic complex of interest and a suitable diamagnetic analog.

The δ_{dip}(calc) also provides valuable information relevant to H-bond strength [12]. The δ_{DSS}(obs) for a *labile* proton in HO complexes is composed of the true diamagnetic chemical shift (δ_{DSS}(dia*)) and the dipolar shifts, as given by:

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

where only the δ_{DSS}(dia*) contribution can be used to interpret relative H-bond strength.

Ligated azide is a somewhat stronger H-bond acceptor than ligated cyanide. The strong Tyr58 $O_\eta H$ H-bond to the carboxylate of Asp140 in hHO [6,13], which in turn is linked to the exogenous ligand via an ordered water molecule, serves as a particularly sensitive probe [16] of the coupling of the H-bond network to the exogenous ligand. For the extreme difference between H-bond donor, ligated water, converting to the H-bond acceptor, hydroxide, as ligand, the H-bond strength of the Tyr58 OH is dramatically increased [16]. We report here on the detailed 2D ^{1}H NMR investigation of the low-spin azide complex of human HO isozyme-1, abbreviated hHO, which addresses the following questions. Do the major magnetic axis tilts of the azide and cyanide complexes conform to the expectations consistent with the difference in tilts seen in crystals? [3,4] Can significant differences in the H-bond acceptor strength of cyanide versus azide be detected in the distal H-bond network coupled to the axial ligand? And can differences in chemical shifts for the azide and cyanide complexes be interpreted in terms of differences in molecular structure or differences in the magnetic properties of the chromophores? The azide and cyanide complexes exhibit different patterns of π-spin transfer to the substrate, with strong low-field shifts for the substituents at positions 2, 3, 6, 7 for the cyanide [13] and positions 1, 4, 5, 8 for the azide [25–27] complexes. This difference is due to an in-plane 90° rotation of the orbital hole, with the cyanide complex exhibiting the $(d_{xy})^2(d_{xz}+d_{yz})^2(d_{xz}-d_{yz})^1$ and the azide complex the $(d_{xy})^2(d_{xz}-d_{yz})^2(d_{xz}+d_{yz})^1$ configuration due to the fact that the rhombic perturbation is determined by the proximal His imidazole [31,32,38] plane in the former and the distal azide plane [27] in the latter.

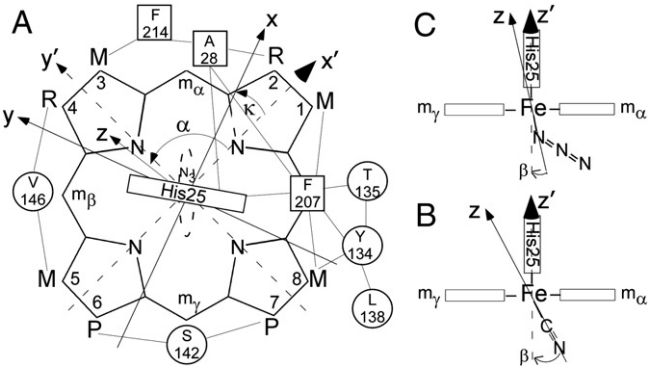

Fig. 1. Schematic depiction of the environment of the substrate in the active site of hHO, as predicted by the crystal structure [6], PDB access code 1N45, and observed herein by ^{1}H NMR. Note that the orientation (numbering) of the substrate is rotated 180° about the α,γ-meso axis relative to that in the crystal structure [6], since ^{1}H NMR has shown [13] that the substrate orientation is reversed in the dominant isomer in solution. For the native enzyme R = vinyl, while in DMDH the vinyls are replaced by methyls (R = methyl, P = propionate); the position labeling of DMDH follows the Fischer convention in the solution position of protohemin [13]. Proximal and distal residues are shown by squares and circles, respectively, and dotted lines show the expected, and observed, residue-DMDH and inter-residue contacts. The steric tilts of the: linear Fe–CN; and bent Fe–N=N=N units towards the α-meso position that is attacked are shown in (B) and (C) respectively. The reference coordinate system, x', y', z', (dashed lines) places the x', y', axes in the mean pyrroles N plane, as shown in (A), with the z' axis oriented to the proximal side as shown in (B) and (C). The magnetic coordinate system, x, y, z, shown in solid lines, has the major axis (z) tilted by an angle β from the heme normal (z'), in a direction given by the angle, α, between the projection of z on the x', y' plane and the x' axis as shown in (A). The projection of the rhombic magnetic axes, x, y, on the x', y' plane is given by the angle κ~α+β.

2. Materials and methods

2.1. Protein preparation

Solubilized, wild-type human heme-oxygenase-1, abbreviated hHO, was prepared as described previously and stored at −80 °C; enzyme assays confirm the purity and full activity of the preparation [39]. Previous studies have shown that the native protohemin substrate (R = vinyl in Fig. 1) is orientationally disordered for hHO complexes [13]. In order to obviate the protohemin orientational isomerism about the α,γ-axis that leads to degraded sensitivity and resolution, we use the synthetic two-fold symmetric substrate

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