1H NMR Investigation of the Distal Hydrogen Bonding Network and Ligand Tilt in the Cyanomet Complex of Oxygen-avid Ascaris suum Hemoglobin*

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The O2-avid hemoglobin from the parasitic nematode Ascaris suum exhibits one of the slowest known O2 off rates. Solution 1H NMR has been used to investigate the electronic and molecular structural properties of the active site for the cyano-met derivative of the recombinant first domain of this protein. Assignment of the heme, axial His, and majority of the residues in contact with the heme reveals a molecular structure that is the same as reported in the A. suum HbO2 crystal structure (Yang, J., Kloek, A., Goldberg, D. E., and Mathews, F. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4224–4228) with the exception that the heme in solution is rotated by 180° about the alpha-meso axis relative to that in the crystal. The observed dipolar shifts, together with the crystal coordinates of HbO2 provide the orientation of the magnetic axes in the molecular framework. The major magnetic axis, which correlates with the Fe-CN vector, is found oriented ~30° away from the heme normal and indicates significant steric tilt because of interaction with Tyr30(B10). The three side chain labile protons for the distal residues Tyr30(B10) and Gln64(E7) were identified, and their relaxation, dipolar shifts, and nuclear Overhauser effects to adjacent residues used to place them in the distal pocket. It is shown that these two distal residues exhibit the same orientations ideal for H bonding to the ligand and to each other, as found in the A. suum HbO2 crystal. It is concluded that the ligated cyanide participates in the same distal H bonding network as ligated O2. The combination of the strong steric tilt of the bound cyanide and slow ring reorientation of the Tyr30(B10) side chain supports a crowded and constrained distal pocket.

The O2 binding globins, myoglobin (Mb)1 and hemoglobin (Hb), despite highly varied sequences throughout phylogeny, possess a highly conserved folding topology of 7–8 helices (A–H), with the heme wedged in between the E and F helices and ligated by one, His F8 (proximal), of only two (the other is Phe CD1) completely conserved residues (1–3). Despite this strong structural homology, the O2 ligation rates and O2 affinities vary over a remarkably wide range (by ~106), depending on the exact nature of several distal residues at the key positions B10, E7, and E10 (4–8). The most important distal interaction for stabilizing bound O2 is hydrogen bonding to the ligand, for which the donor is generally His E7 (1, 7, 9, 10) and is Gln E7 in a few cases (2). In several invertebrates, such as Aplysia and Dolabella Msb that possess a Val E7, the distal H bond to the ligand is provided by an Arg at position E10 (11–13).

A particularly noteworthy class of globins is that of parasitic nematodes that possess, in addition to a H bond donor at position E7, a Tyr at position B10 that is also capable of H bonding to the ligand (4, 5, 8, 14–17). In the case of the Hb from Ascaris suum, the extraordinarily high O2 affinity and extremely low O2 off-rates have been attributed to a distal H bonding interactions for the Tyr30(B10) and Gln64(E7) side chains with bound O2. Stabilizing H bond interactions of Gln64(E7) and Tyr30(B10) with ligated O2 are supported by the observations of enhanced O2 off-rates upon mutating either residue (5, 15). The positions of these two key residues are clearly defined in the crystal structure of A. suum HbO2, in which the two residues are appropriately poised to serve as H bond donors to bound O2, with the Gln64(E7) additionally providing an H bond to the Tyr30(B10) side chain O that stabilizes the optimal dispositions of these two residues (8). Resonance Raman spectroscopy has confirmed the role of Tyr30(B10) as a H bond donor (18, 19), and flash photolysis experiments (19) have indicated that A. suum Hb possesses a very compact and constrained distal pocket when compared with other globins.

Resonance Raman spectroscopy has shown that A. suum HbCO exhibits the lowest reported CO stretching frequency (19). The strong modulation of the CO stretching frequency by globin distal environment has been discussed in the context of both steric tilt/bending of the Fe-CO unit from the heme normal and pocket dielectric effects (20–23), and the currently accepted interpretive basis is that the latter effect is the major determinant of νCO (7, 24). Nevertheless, crystal structures of myoglobin invariably find the carbonyl oxygen placed off-axis from the heme normal, indicating that the Fe-CO unit is bent/tilted from the heme normal (7, 25–27).

The cyanomet derivatives of globins can serve as valuable structural (but not functional) (28, 29) models for both O2 and CO binding, in that FeCN, like FeO2, is polar and is a good H bond acceptor (30) and, like FeCO, prefers to bind normal to...
The heme in the absence of distal steric interactions (31). In the one case where the crystal structures of both the carbonyl and cyanomet globins have been reported, there is a good correlation in the degree and direction of the off-axis placement of the terminal atoms (26). Theoretical considerations have indicated that distal ligand tilt could be modulated by tilt of the proximal His (32). In the crystallographically and NMR characterized globins to date, the axial His is essentially normal to the heme. The crystal structures of *A. suum* HbO$_2$, on the other hand, show that the axial His imidazole plane is tilted some $-8^\circ$ in the direction of pyrrole C with respect to the heme plane (8). Thus a determination of orientation of the Fe$^{3+}$CN or Fe$^{2+}$CO units relative to the heme in *A. suum* Hb would indicate whether the axial His could contribute to distal ligand tilt and provide some insight as to whether there is likely to be a large Fe-CO tilt that could contribute to the reduced value for $v_{oe}$.

Solution $^1$H NMR of the paramagnetic cyanomet Hb derivatives can provide significant structural details on the distal pocket in relation to both stabilizing H bonding and destabilizing steric interactions with the bound ligand (30, 33, 34). On the one hand, the dipolar shifts and moderate relaxation imparted to distal residues and their labile protons facilitate their detection, identification, and detailed placement relative to the bound ligand (35, 36). On the other hand, the sizable dipolar shifts for active site residues allow the quantitative determination of the orientation of the paramagnetic susceptibility tensor, for which the major magnetic axis can be correlated to the degree of Fe-CN tilt from the heme normal (34). There is generally very good agreement in the magnitude and direction of Fe-CN tilt observed in crystal structure and the orientation of the major magnetic axes determined by solution $^1$H NMR (26, 27, 38–41). Lastly, the expanded chemical shift scale for heme pocket residues because of the hyperfine interaction increases the prospect for measuring rapid dynamic processes, such as ring orientation, that can constitute probes for the constraints in the heme pocket (42).

We report herein on the solution $^1$H NMR characterization of the cyanomet complex of the D1 domain of *A. suum* methHbCN, which demonstrates that the distal H bonding network is essentially identical to that in HbO$_2$, that the Fe-CN unit appears tilted strongly away from the heme normal in the direction of the observed terminal oxygen in HbO$_2$, and that the distal pocket is sufficiently crowded to strongly tilt the Fe-CN vector and to impede the reorientation of the Tyr$^{20}$(B10) ring.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—Native protein samples were prepared as described previously (15, 43). The cyanide complexes were prepared by adding KCN to the protein solution in a molar ratio of 10:1 buffered with 50 mM phosphate, 200 mM NaCl at pH 7.2. $^2$H$_2$O sample was prepared by repeatedly washing protein with $^2$H$_2$O in the same buffer with a Centricon (Amicon Inc.), and pH was read directly from the pH meter without the isotope effect correction; the final protein concentration was $-2$ mM.

**NMR Spectra**—All $^1$H NMR spectra were recorded on a GE O $500$ spectrometer operating at $500$ MHz. Chemical shift values were referenced to 2,2-dimethyl-2-pentanone-5-sulfonate (DSS) through the residual water signal. Reference spectra were collected with $^2$H$_2$O saturation. Steady-state NOE and inversion-recovery spectra were collected at a repetition rate of $3$ s$^{-1}$ (34). The residual water signals were removed from the free induction decay by convolution difference. The nonselective spin lattice paramagnetic relaxation times for the resolved peaks were derived from two-parameter exponential least squares fits using only short ($\leq 50$ ms) delays. Estimates for distance to the iron for proton $i$, $R_{ip}$, were obtained from the nonselective paramagnetically dominated $T_1$ values using the following relation.

$$\frac{T_1}{T_1'} = \frac{R_{ip}}{R_{ip}} \quad (Eq. 1)$$

where the reference $T_1 = 150$ ms ($R_{ip} = 6.1$ Å) for a heme methyl, or $T_1 = 30$ ms ($R_{ip} = 5.1$ Å) for the His(F8) N-H, provided upper and lower limits, respectively (44). Interproton distances, $R_{ij}$, were estimated from steady-state NOEs, $\eta_{ij}$, to protons with paramagnetically dominated (nonselective) $T_1$ values, via the following two equations.

$$\eta_{ij} = \sigma_i \mathcal{T}_0 \quad (Eq. 2)$$

where

$$\sigma_i = -0.1 \times 10^{-6} \quad (Eq. 3)$$

**NOE$^2$(45) and TOCSY (46–48) spectra were collected over a temperature range of $20–35$ °C in $^2$H$_2$O. Two different spectral windows and mixing times were used for NOESY 25.0 KHz using 2048 complex points at $3$ scans/s with a mixing time of $35$ ms to observe the hyperfine shifted signals and $10.0$ KHz at $1$ scan/s with a mixing time of $100$ ms to cover the diamagnetic window at optimal digital resolution. The clean TOCSY spectra were collected over $12.0$ KHz at $2$ scans/s with a spin locking time of $35$ ms using the MLEV-17 mixing scheme (47). All the two-dimensional data sets were processed and to impede the reorientation of the Tyr$^{20}$(B10) hydroxyl proton; hence its position was determined from the $^1$H NMR spectral parameters.

$^2$ It is noted that the conventions for the $x'$, $y'$, and $z'$ differ from that previously used (33, 34, 40, 50) by a $45^\circ$ rotation (see text) in the heme plane and referencing to the $x'$ axis, rather than $-x'$ axis, so that $\beta(\text{new}) = \beta(\text{old})$, $\alpha(\text{new}) = \alpha(\text{old}) + 135^\circ$, and $\gamma(\text{new}) = \gamma(\text{old}) - 45^\circ$. (51).
The heme is labeled with the Fisher notation, and the substituents are in the heme plane and referencing that tilt of the major magnetic axes from the heme normal, most strongly relaxed resolved protons are labile. The resolved portions of the 1H NMR spectra of A. suum metHbCN in 1H2O (A) and in 2H2O (B). The assigned resolved signals are labeled by the Fisher notation for the heme and by the one-letter code for the residue and sequence position. Also shown are steady-state NOE difference spectra upon saturating the low field TyrB10(B10) OH signal (C) and upon saturating the upfield Gln(E7) NH signal (D). The intensity of the saturated signals in traces C and D are identical. The detected NOEs are assigned as presented in the text. An asterisk indicates off-resonance saturation.

**RESULTS**

A schematic representation for selected heme cavity residues on the proximal (squares) and distal (circles) sides of the heme and the heme orientation as reported in the crystal structure of A. suum Hp O2 (A) and as determined herein by NMR (B).

The heme is labeled with the Fisher notation, and the substituents are labeled M (methyl), V (vinyl), and P (propionate). The expected (on the basis of the crystal structure with the rotated heme) and observed inter-residue and residue-heme dipolar contacts are shown in B by double-sided arrows. The iron-centered, crystal structure-based coordinate system (x’, y’, z’) is shown in C, as is the magnetic coordinate system (x, y, z), where y is diagonal. The two systems are related by the Euler rotation, \( \Gamma (\alpha, \beta, \gamma) = [x’, y’, z’] \Gamma (\alpha, \beta, \gamma) \), where \( \beta \) is the tilt of the major magnetic axes from the heme normal, \( \alpha \) is the angle between the projection of the tilt on the heme plane and the \( x' \) axis, and \( \kappa = -\alpha + y \) define the projection of the rhombic axes on the \( x’, y’ \) plane. \( \phi \) is the orientation of the proximal His imidazole ring plane relative to the \( N_x-Fe-N_z \) vector (\( x’ \) axis). It is noted that the convention for \( x’, y’, z’ \) differs from that used previously by a 45° (34, 39–41, 44, 50) rotation in the heme plane and referencing \( \alpha \) to the \( +x' \) rather than \( -x’ \) axis, so that \( \kappa(\text{new}) = \kappa(\text{old}) \), \( \alpha(\text{new}) = \alpha(\text{old}) + 135° \), and \( \kappa(\text{new}) = \kappa(\text{old}) - 45° \).

**Heme Assignments**—The heme substituents could be unambiguously assigned as described in detail elsewhere (13). Two TOCSY-detected vinyl and one propionate groups exhibit NOESY cross-peaks to low field resolved methyls that pair 1-CH3, 2 vinyl and 3-CH3, 4 vinyl; NOESY cross-peaks between the remaining methyls and one propionate groups exhibit a NOE to the C9 NH fragment. A very broad and strongly relaxed (34) at 12 ppm, which is part of a nonlabile proton peak must arise from the axial His ring (34), and a NOE to the C8H upon saturating this
Fig. 3. A and B, portions of the TOCSY (15-ms mixing time) spectra showing the scalar correlation for the Phe44(CD1) and Tyr30(B10) rings. C and D, portions of the NOESY spectrum that illustrates the dipolar contact between the two heme methyls that uniquely assign 1-CH3 and 8-CH3 of the heme, which uniquely characterize the orientation of the heme in the pocket as rotated by 180° about the α,γ-meso-axis relative to that found in the HbO2 crystal structure.

| Proton | T1 ms | Slope × 10^3 | Intercept | δ2o(calc) | δo(calc) |
|--------|-------|--------------|-----------|------------|----------|
| Heme   |       |              |           |            |          |
| 1-CH3  | 16.89 (73) | 6.04 | −3.03 | −3.43 | 16.7 |
| 3-CH3  | 10.83 (105) | 0.74 | 8.40 | −3.10 | 10.3 |
| 5-CH3  | 14.64 (87) | 4.17 | 0.87 | −2.70 | 13.7 |
| 8-CH3  | 11.99 (−102) | 0.54 | 10.21 | −1.50 | 9.89 |
| 2-H    | 20.24 | 4.16 | 6.51 |          |          |
| 3-H    | −3.49 | −2.95 | 6.24 |          |          |
| 2-Hα   | −2.25 | −1.94 | 3.13 |          |          |
| 4-H    | 10.56 | −0.48 | 12.14 |          |          |
| 4-Hβ   | −2.91 | −2.66 | 5.86 |          |          |
| 4-Hγ   | 3.36 | −2.74 | 5.66 |          |          |
| 7-H    | 2.37 | 2.69 | 6.56 |          |          |
| 7-Hβ   | 0.39 | −2.50 | 4.92 |          |          |
| 5-meso-H | 3.07 | −3.24 | 14.35 | −12.30 | 5.37 |
| 6-meso-H | 2.46 | −3.35 | 15.7 | −1.34 | −6.20 |
| 6-meso-H | 6.15 | −5.56 | 20.55 | −4.17 | −3.65 |

Additional assignments (data not shown), include two upfield resolved methyls (one strongly relaxed) that are part of a five-spin system diagnostic of an ile, with a strongly relaxed C5H that exhibits the NOESY cross-peaks to 5-CH3 and 4- vinyl (as predicted by the 180° rotated heme) for ile20(E11); this residue exhibits the expected NOESY cross-peaks to the Tyr30(B10) ring (shown schematically in Fig. 1). Common NOE contacts to 4-vinyl and 5-CH3 for a TOCSY-detected Ala uniquely identifies Ala24(E14). TOCSY spectra detect three Phe rings with weak hyperfine shifts. They are assigned to Phe44(CD1), Phe40(E3), and Phe30(E15) based on their predicted dipolar contacts to Phe44(CD1) and Tyr30(B10), only to Phe44(CD1), and to 3-CH3, respectively. Two low field shifted TOCSY-detected fragments with slopes and intercepts indicative of aromatic pro-

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*Distal Pocket Residues—The extreme low field, strongly relaxed (T1 = ~11 ms) labile proton peak at 22 ppm does not participate in a NOESY map, but when it is saturated (Fig. 2C), it exhibits a strong NOESY cross-peak to a two-proton signal under the 8-CH3. This signal under the 8-CH3 in turn exhibits a TOCSY cross-peak to 9.0 ppm. The strong relaxation of the labile proton and the intercept in Curie plots for the TOCSY detected fragment uniquely identify the complete ring of Tyr30(B10). A strong NOE to the Phe44(CD1) Cβ, together with the latter 1H and Nδ, with a strongly relaxed Cβ (see below). The ratio of the steady-state NOEs to the 4 ppm Gluβ(E7) Nδ peak upon saturating the Gluβ(E7) Nδ and Tyr30(B10) OH (0.5), together with the fixed ~1.9 Å distance between Nα and Nα, leads to a 2.0 ± 0.2 Å estimate for the Tyr30(B10) OH to Gluβ(E7) Nδ distance.

Additional assignments (data not shown), include two upfield resolved methyls (one strongly relaxed) that are part of a five-spin system diagnostic of a ile, with a strongly relaxed C5H that exhibits the NOESY cross-peaks to 5-CH3 and 4-vinyl (as predicted by the 180° rotated heme) for ile20(E11); this residue exhibits the expected NOESY cross-peaks to the Tyr30(B10) ring (shown schematically in Fig. 1). Common NOESY contacts to 4-vinyl and 5-CH3 for a TOCSY-detected Ala uniquely identifies Ala24(E14). TOCSY spectra detect three Phe rings with weak hyperfine shifts. They are assigned to Phe44(CD1), Phe40(E3), and Phe30(E15) based on their predicted dipolar contacts to Phe44(CD1) and Tyr30(B10), only to Phe44(CD1), and to 3-CH3, respectively. Two low field shifted TOCSY-detected fragments with slopes and intercepts indicative of aromatic pro-

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3 Denotation of NHe of Glu44 was based on the x-ray structure (8).
tons, together with NOESY cross-peaks to Met\textsuperscript{103}(FG5) identify the C\textsubscript{5}H-C\textsubscript{6}H and C\textsubscript{c5}C\textsubscript{c6}H portions of Trp\textsuperscript{105}(G5); the remainder of the ring protons could not be located because of likely strong relaxation and near degeneracy with other protons and in position under the residual solvent peak. The observed inter-residue and residue-heme dipolar contacts are summarized in Fig. 1B. Spectral congestion precluded further assignments.

The assignments, chemical shifts, and \( T_1 \) values for the residues described in Fig. 1 are listed in Table II.

**Magnetic Axes**—The orientation of the magnetic axes\textsuperscript{2} was found to be essentially independent of the selection of input data or whether the anisotropies were also determined or held constant at the values determined for sperm whale metMbCN (50, 51). The resulting orientation of \( \chi \) is defined by \( \beta = 29.5^\circ \pm 1.0^\circ \) (tilt from the heme normal), \( \alpha = 159^\circ \pm 10^\circ \) (direction of tilt projected on the heme plane), and \( \kappa = \alpha + \gamma = 59 \pm 10^\circ \) (rhombic axes projected on the heme plane). The residual error function, \( Fm \), is small in all cases (-0.05 ppm\textsuperscript{2}), and the resulting correlation between observed and calculated dipolar shifts is very good, as illustrated in Fig. 4. The magnitude of the tilt of the major magnetic axis, \( z \), from the heme normal (\( z' \) axis), \( \beta = -30^\circ \), is nearly twice as much as that observed previously in cyamotin globins (34, 50, 51). The large magnitude of the tilt of the major magnetic axis from the heme normal indicated by the complete magnetic axes determination also reveals itself clearly in the analysis of the dipolar shift pattern for individual residues. Thus the nodal surface for the axial dipolar shift can be mapped by considering the magnitude and direction of dipolar shifts of residues near the nodal surface. The plots in Fig. 5 for the protons whose shift direction/magnitude reflect primarily the axial geometric factor node are shown as a function of tilt angle \( \beta \). The agreement with the experimental shifts is acceptable within 30 °.

**The Orientation of Tyr\textsuperscript{30}(B10) and Gln\textsuperscript{64}(E7)**—Predicted \( \delta_{\text{dip}} \) values for the Tyr\textsuperscript{30}(B10) ring and Gln\textsuperscript{64}(E7) \( N_H \)s, based on the HbO\textsubscript{2} crystal coordinates (8), are included in Fig. 4 as filled circles and filled squares, respectively. It is observed that the uniquely placed protons on the Gln(E7) \( N_C \)H result in shifts that are well predicted, indicating that this residue in met HbCN maintains the same orientation relative to the iron as in HbO\textsubscript{2}. The \( R_p \)s = 4.5 ± 0.4 Å estimated from the \( T_1 = 25 \) ms for the Gln\textsuperscript{64}(E7) \( N_C \)H is consistent with the crystallographic \( R_p \) = 4.1Å. Moreover, the predicted dipolar shifts for the Gln\textsuperscript{64}(E7) nonlabile side chain protons are small and are consistent with the likely appearance of protons in the poorly resolved and very crowded aliphatic envelope. In the case of Tyr\textsuperscript{30}(B10), the dipolar shifts are very well predicted for the ring, which is consistent with conserved \( \chi_1, \chi_2 \) angles with respect to HbO\textsubscript{2}. The placement of the proton on the hydroxyl oxygen crystal coordinates, however, unlike the \( N_H \)s of Gln\textsuperscript{64}(E7), is not unique. Hence the \( \delta_{\text{dip}} \) (Fig. 6A), distance to Phe\textsuperscript{44}(CD1) \( C_H \) (Fig. 6B), distance to Gln\textsuperscript{64}(E7) \( N_H \) (Fig. 6C), and distance to the iron, \( R_{Fe} \) (\( T_1 = 10 \) ms, \( R_{Fe} = 4.0 \pm 0.4 \) Å) (Fig. 6D) for the Tyr\textsuperscript{30}(B10) \( OH \) are calculated as a function of the dihedral angle between the H-O-C and ring planes, \( \chi_3 \), as illustrated in Fig. 6; the observed values are shown by shaded regions. It is clear that each of the four observable values are optimally predicted for the angle \(-20^\circ \) in Fig. 6, which leads us to conclude that we have uniquely spatially located the labile proton for the distal Tyr\textsuperscript{30}(B10). The orientation of Tyr\textsuperscript{30}(B10) OH is taken as the temperature is lowered. The line broadening, however, can be quantitated by observing only the steady-state

\[
 \begin{array}{|c|c|c|c|c|}
 \hline
 \text{Residue} & \text{\( N_H \)} & \text{\( C_H \)} & \text{Others} \\
 \hline
 \text{Tyr\textsuperscript{30}(B10)} & 9.17 & 5.56 & C\textsubscript{Ha} 9.17, C\textsubscript{Ha} 11.62; -OH 26.01 (10)\textsuperscript{b} \\
 \text{Phe\textsuperscript{44}(B14)} & 9.05 & 5.55 & C\textsubscript{Ha} 8.05; C\textsubscript{Ha} 8.40; C\textsubscript{Ha} 7.58 \\
 \text{Phe\textsuperscript{44}(C7)} & 9.06 & 5.56 & C\textsubscript{Ha} 5.67; C\textsubscript{Ha} 7.08 \\
 \text{Phe\textsuperscript{44}(CD1)} & 9.05 & 5.55 & C\textsubscript{Ha} 8.24; C\textsubscript{Ha} 10.28; C\textsubscript{H} 15.47 \\
 \text{Phe\textsuperscript{44}(E3)} & 9.05 & 5.56 & C\textsubscript{Ha} 8.59; C\textsubscript{Ha} 6.72; C\textsubscript{H} 6.46 \\
 \text{Gln\textsuperscript{64}(E7)} & 9.05 & 5.56 & C\textsubscript{H} 2.58; N\textsubscript{c5}H 3.70; N\textsubscript{c6}H -6.13 (24)\textsuperscript{b} \\
 \text{Ala\textsuperscript{17}(E14)} & 9.58 & 5.31 & C\textsubscript{H} -1.72; C\textsubscript{a}H -3.82; C\textsubscript{b}H -0.84; C\textsubscript{Ha} -1.26; C\textsubscript{b}H 1.23 \\
 \text{Leu\textsuperscript{22}(F4)} & 9.22 & 6.59 & C\textsubscript{Ha} 0.97 \\
 \text{Asp\textsuperscript{94}(F6)} & 9.06 & 5.37 & C\textsubscript{Ha} 2.42; CH 2.78 \\
 \text{Arg\textsuperscript{97}(F9)} & 9.03 & 4.02 & C\textsubscript{Ha} 3.59; C\textsubscript{H} 3.35 \\
 \text{Val\textsuperscript{101}(FG3)} & 7.69 & 3.11 & C\textsubscript{Ha} 3.37; C\textsubscript{H} 2.72 \\
 \text{Trp\textsuperscript{105}(G5)} & 7.12 & 3.46 & C\textsubscript{Ha} 4.13 \\
 \text{Phe\textsuperscript{140}(H15)} & 9.58 & 4.02 & C\textsubscript{Ha} 7.03; C\textsubscript{H} 6.59 \\
 \hline
 \end{array}
\]

\( <\text{Shifts at 30 °C (in ppm) referenced to DSS.} \)

\( ^b\text{T} \) \( _1 \) values (in ms) are given in parentheses.

\( ^c\text{Shifts at 35 °C.} \)

**TABLE II**

Assignment of dipolar-shifted active site residues in \( A. \) \textit{suum} cyanomet hemoglobin (domain I)

**FIG. 4**. Plot of \( \delta_{\text{dip}}(\text{obs}) \) (via Equation 6) versus the \( \delta_{\text{dip}}(\text{calc}) \) (via Equation 5) for \( A. \) \textit{suum} metMbCN at 30 °C obtained from the optimized magnetic axes with \( \Delta_{\text{Fe}} = 2.3 \times 10^{-6} \text{m}^2/\text{mol}, \)

\( \Delta_{\text{Fe}} = -0.46 \times 10^{-6} \text{m}^2/\text{mol}, \alpha = 139^\circ, \beta = 29.1^\circ, \) and \( \kappa = 59^\circ. \) The open circles represent the data used as input to determine the magnetic axes. The points for the Gln\( N_H \)s and Tyr\textsuperscript{30}(B10) ring protons, which were not used as input, are shown by closed squares and closed circles, respectively. The solid line represents unit slope for a perfect fit.
NOE for the averaged $C_\text{H}$ peak upon saturating the Tyr$^{30}(B10)$ $\text{OH}$, as shown in Fig. 7. Plotting the $\ln$ (linewidth) versus reciprocal temperature shows a plot with selective increase in slope at low temperature for the $C_\text{H}$s peak, which yields an estimated exchange contribution of 20 Hz at 30 °C. This value, together with the $\delta_{\text{ddip(calc)}}$ for the individual Tyr$^{30}(B10)\ C_\text{H}s$, results in an estimated shift difference of 4.4 ppm, which at 500 MHz, results in a reorientation rate of $5 \times 10^6$ s$^{-1}$ using the standard equation for chemical exchange in the first exchange limit (57).

**DISCUSSION**

**Heme Pocket Molecular and Electronic Structure**—The pattern of the heme methyl contact shifts has been proposed to largely reflect the orientation of the axial His imidazole ring relative to a heme pyrrole-Fe-pyrrole axis (12, 58–61). For an axial His oriented along such an $\text{N-Fe-N}$ axis, large contact shifts are predicted and observed primarily for the pyrroles normal to the His plane. Thus the contact shift patterns among different globins are modulated separately by the orientation of the His relative to the heme and the orientation of the heme about the $\alpha$, $\gamma$-meso axis. In cases where the axial His is oriented close to meso-Fe-meso vectors (11, 62), the four pyrroles exhibit comparable contact shifts (13, 63). *A. suum* metHbCN, like mammalian globins, exhibits large contact shifts for 1-CH$_3$ and 5-CH$_3$, arguing for orientation of axial His along the $\text{N_3-Fe-N_3}$ vector of the heme if the heme and the axial His$^{97}$($F8$) were orientated similarly. However, the heme methyl contact shift pattern in *A. suum* metHbCN, like mammalian globins, exhibits large contact shifts for 1-CH$_3$ and 5-CH$_3$, arguing for orientation of axial His along the $\text{N_3-Fe-N_3}$ vector of the heme if the heme and the axial His$^{97}$($F8$) were orientated similarly. However, the heme methyl contact shift pattern in *A. suum* metHbCN is achieved by completely different means than in sperm whale metMbCN. Thus, as shown in Fig. 1, the axial His ring (as viewed from the proximal side) is rotated by $\theta = 45^\circ$ ($\phi = -65^\circ$) relative to that in sperm whale Mb, which should result in larger 3-CH$_3$, 8-CH$_3$ than 1-CH$_3$, 5-CH$_3$ contact shifts, if the heme were seated in the pocket the same as in sperm whale Mb. However, the rotation of the heme by $180^\circ$ about the $\alpha$, $\gamma$-meso axis, when compared with sperm whale Mb, reverses this pattern and leads to larger 1-CH$_3$, 5-CH$_3$ contact shifts than 3-CH$_3$, 8-CH$_3$ contact shifts. Thus the fortuitous similarity in the heme contact shift pattern in *A. suum* metHbCN and mammalian globins is due to off-settting influences of the differences in the orientation of both the axial His and the heme.

The determined heme methyl contact shifts, together with the x-ray determined axial His orientation, thus independently confirm that the heme in *A. suum* metHbCN in solution is rotated by $180^\circ$ about the $\alpha$, $\gamma$-meso axis relative to that reported in the HbO$_2$ crystal structure. These results also suggest that caution should be exercised in assigning a heme
orientation based on heme methyl contact shifts in a cyanomet globin unless the orientation of the axial His is known. The re-evaluation of the x-ray diffraction data to reconcile the alternate heme orientation in the crystal and solution has shown that the heme in the crystal is, in fact, rotated by 180° about the a, γ-meso axis from that originally reported (8) and the same as found by 1H NMR in solution.

Theoretical considerations (61, 64) confirmed in model compounds (65, 66) dictate that if the orbital ground state is determined by the axial His(F8) bonding, the rhombic axes, κ, and the angle between the heme N-Fe-N and imidazole plane, φ (Fig. 1C), obey the counter-rotation rule where κ = -φ. The present results conform quite well to these predictions, as shown in Fig. 8. The temperature dependence of the heme methyl shifts reveals that the 1-CH3, 5-CH3 exhibit positive peaks that are steeper than Curie (T−1) behavior, whereas the 3-CH3, 5-CH3 exhibit slopes that are negative or exhibit anti-Curie behavior. This effect is expected on the basis of thermal population of the excited orbital state, where the lone spin on the iron becomes delocalized into pyrroles B and D (60, 67–69). Lastly, the magnetic axes reported above allow the determination of δip for the axial His, which, in turn, provides δcon for each of the positions, as shown in Table I. Thus only the C-H exhibits large contact shifts that are very similar to those reported for sperm whale metMbCN (70) and confirms an essentially conserved axial His-Fe bond in A. suum relative to sperm whale Mb.

Distal Hydrogen Bonding Network—The excellent correlation between the observed and crystal-structure predicted values for δip and T1 for the Tyr30(B10) ring and Gln 64(E7) Nε2H side chain shows that their dispositions in metHbCN are essentially quantitatively conserved relative to those in the HbO2 crystal structure (8). The position of the Tyr30(B10) hydroxyl proton, deduced from its relaxation, NOESY, and dipolar shift constraints, with an χ3 value of ~20°, is precisely in the position to make an ideal H bond to the strongly tilted cyanide ligand. The short inter proton distance between the Tyr30(B10) OH and Gln 64(E7) Nε2H, moreover, is consistent with an H bond between the latter proton and the Tyr hydroxyl O. Hence the present 1H NMR data provide strong support that both Tyr30(B10) and Gln64(E7) serve as a H bond donors to bound cyanide ligand to in a manner that is essentially the same as for the bound O2 in HbO2. It is concluded that cyanide serves as a valid model for the H bonding experienced by a ligated O2 molecule.

Distal Pocket Crowding—Flash photolysis experiments have suggested a compact and constrained heme pocket for A. suum Hb (19). Significant crowding in the distal pocket is evident in two 1H NMR spectral parameters. The major magnetic axis (Fe-CN tilt) is tilted from the heme normal by ~30°, nearly twice as much as in other globins (34, 39–41, 51, 63). This can be rationalized by the disposition of the Tyr30(B10) ring, which provides a steric barrier to ligation along the heme normal. The orientations of the Tyr30(B10) ring and the Fe-CN tilt (if only tilted and not primarily bent) determined herein place the two residues in van der Waals’ contact between the Tyr Oγ and the N of the bound cyanide. However, the tilt of the major magnetic axis (~30°) is in the same direction (toward pyrrole C) as is the tilt of the proximal His97(F8) imidazole plane (by ~10°) observed for HbO2 (8), so that the large tilt in the major magnetic axis, and hence the Fe-CN tilt, could have a significant contribution (to ~10°) from the proximal His tilt (32).

The present results suggest that the crystal structure of A. suum HbCO would find the CO off axis to a degree that is much larger than found in other carbonyl globins. The role of the tilt for the axial His(F8) in contributing to either Fe-CO (32) or Fe-CN tilt could be addressed by either the crystal structure of the carbonyl complex or the solution 1H NMR determination of the magnetic axes of the cyanomet complex, for the A. suum Hb mutant where the covalent connection between the axial imidazole and the F-helix backbone is severed in the His(F8) → Gly mutant (71, 72), allowing an exogenous imidazole to bind in the preferred normal to the heme.

Aromatic rings in the heme pocket of globins are generally found with sufficient local flexibility to yield only rotationally averaged 1H NMR signals (37, 73), despite the apparent close packing suggested by the crystal structures. Thus, Phe(CD1) is generally found packed tightly against the heme surface but nevertheless exhibits an 1H NMR spectrum that is rapidly averaged by the 180° ring flip. The Tyr30(B10) ring exhibits an averaged NMR spectrum, but the rotation contributes significantly to the linewidth, and standard analysis in the fast exchange limit (57) using the δip(calc) for the individual CαH3 results in a rotation rate of ~1 MHz. A comparison can be made to globins with Phe rather than Tyr(B10) and with a Gln(E7), i.e. elephant Mb and the sperm whale Leu30(B10) → Phe/His30(B10) → Gln and Leu30(B10) → Phe/His30(B10) → Gln/Val30(E11) → Phe Mb mutants, for which the B10 ring exhibited “normal” linewidth indicative of much faster reorientation (39, 51). Whether the constraints on the Tyr30(B10) ring in A. suum Hb result from “pinning down” the extremity via the H bond to the ligand or from the tight van der Waals’ contacts with the aromatic ring is not known but could be elucidated in a comparison of the solution 1H NMR spectra of WT and Tyr(B10) → Phe A. suum mutant Hb.

Conclusions—The present NMR data provide support that the heme pocket of A. suum Hb is highly constrained, as evidenced by larger tilts from the heme normal for Fe-CN than previously observed and slow reorientation of the Tyr30(B10) ring. The heme is shown to be rotated by 180° about the a, γ-meso axis relative to that originally reported in the crystal (8), and the pattern of heme methyl contact shifts is shown to be consistent with the deduced heme orientation. The Tyr30(B10) and Gln64(E7) side chain labile protons in met HbCN are located at essentially the same positions as found in the HbO2 crystal and hence provide H bonds to the bound cyanide and establish that the metHbCN is a valuable structural model for aspects of both HbO2 and HbCO. However, although the Fe3+-CN unit can serve as limited structural...
models for the Fe$^{2+}$-CO and Fe$^{2+}$-O$_2$ units in globins, cyanide ligation rates unfortunately are not functionally relevant to O$_2$ or CO binding. This is due to the fact that free cyanide at physiologic pH range is protonated, so that both the on- and off-rates involve protonation/deprotonation steps that are strongly influenced by local pocket polarity that modulates the cyanide pK. Thus the cyanide on- and off-rates directly relate to neither distal steric nor H bonding effects (28, 29).

REFERENCES

1. Dickerson, R. E., and Geis, I. (1983) Hemoglobin: Structure, Function, Evolution and Pathology, 65–82, Benjamin-Cummings, Menlo Park, CA
2. Kapp, O. H., Moens, L., Vanfleteren, J., Troman, C., Suzuki, T., and Vinogradov, S. (1995) Protein Sci. 4, 2179–2190
3. Moens, L., Vanfleteren, J., Van de Peer, Y., Peeters, K., Kapp, O., Casu, N., Liotta, C., Gatti, G., Ascenzi, P., and Brunori, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1324–1333
4. De Baere, I., Liu, L., Moens, L., Van Beeumen, J., Gielen, C., Richard, E., Van Loo, J. H., and Pauwels, K. (1994) J. Mol. Biol. 224, 891–897
5. Springer, B. A., Sligar, S. G., Olson, J. S., and Phillips, J. G. (1994) Chem. Rev. 94, 699–714
6. Yang, J., Klok, A., Goldberg, D. E., and Mathews, F. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4224–4228
7. Nagai, K., Luisi, R., Shih, D., Miyazaki, G., Imai, K., Poyart, C., De Young, A., Kwiatkowski, L., Noble, R. W., Lin, S. H., and Yu, N. T. (1978) Nature 278, 358–360
8. Phillips, S. E. V., and Schoenen, B. P. (1981) Nature 289, 81–82
9. Bolognesi, M., Coda, A., Frigerio, F., Gatti, G., Ascenzi, P., and Brunori, M. (1990) J. Mol. Biol. 213, 621–625
10. Yamamoto, Y., Iwafune, K., Chojo, R., Inoue, Y., Imai, K., and Suzuki, T. (1992) J. Mol. Biol. 224, 891–897
11. Bolognesi, M., Coda, A., Frigerio, F., Gatti, G., Ascenzi, P., and Brunori, M. (1990) J. Mol. Biol. 213, 621–625
12. Yamamoto, Y., Iwafune, K., Chujo, R., Inoue, Y., Imai, K., and Suzuki, T. (1992) J. Mol. Biol. 224, 891–897
13. Sherman, D. R., Kloek, A. P., Krishnan, B. R., Giuinn, B., and Goldberg, D. E. (1990) J. Biol. Chem. 265, 1504–1507
14. Klok, A. P., Yang, J., Mathews, F. S., Frieden, C., and Goldberg, D. E. (1994) J. Biol. Chem. 269, 2377–2379
15. Rashid, A. K., Van Hauwaert, M. L., Haque, M., Siddiqi, A. H., Lasters, I., De Bruyne, W., and La Mar, G. N. (1998) J. Biol. Chem. 273, 9517–9526
16. Rashid, A. K., Van Hauwaert, M. L., Haque, M., Siddiqi, A. H., Lasters, I., De Bruyne, W., and La Mar, G. N. (1998) J. Biol. Chem. 273, 9517–9526
17. Rashid, A. K., Van Hauwaert, M. L., Haque, M., Siddiqi, A. H., Lasters, I., De Bruyne, W., and La Mar, G. N. (1998) J. Biol. Chem. 273, 9517–9526