Solution $^1$H NMR Study of the Influence of Distal Hydrogen Bonding and N Terminus Acetylation on the Active Site Electronic and Molecular Structure of *Aplysia limacina* Cyanomet Myoglobin*

(Received for publication, August 18, 1999, and in revised form, October 13, 1999)

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The sea hare *Aplysia limacina* possesses a myoglobin in which a distal H-bond is provided by Arg E10 rather than the common His E7. Solution $^1$H NMR studies of the cyanomet complexes of true wild-type (WT), recombinant wild-type (rWT), and the V(E7)H/R(E10)T and V(E7)H mutants of *Aplysia* Mb designed to mimic the mammalian Mb heme pocket reveal that the distal His in the mutants is rotated out of the heme pocket and is unable to provide a stabilizing H-bond to bound ligand and that WT and rWT differ both in the thermodynamics of heme orientational disorder and in heme contact shift pattern. The mean of the four heme methyl shifts is shown to serve as a sensitive indicator of variations in distal H-bonding among a set of mutant cyanomet globins. The heme pocket perturbations in rWT relative to WT were traced to the absence of the N-terminal acetyl group in rWT that participates in an H-bond to the EF corner in WT. Analysis of dipolar contacts between heme and axial His and between heme and the protein matrix reveal a small $\sim 2^\circ$ rotation of the axial His in rWT relative to true WT and a $\sim 3^\circ$ rotation of the heme in the double mutant relative to rWT Mb. It is demonstrated that both the direction and magnitude of the rotation of the axial His relative to the heme can be determined from the change in the pattern of the contact-dominated heme methyl shift and from the dipolar-dominated heme meso-H shift. However, only NOE data can determine whether it is the His or heme that actually rotates in the protein matrix.

Myoglobin (Mb) is a member of the globin family of proteins of approximately 140–150 residues that encapsulate heme and exhibit a remarkably strongly conserved fold of seven to eight helices (A–H) despite a high variability in sequence (1–3). The heme is wedged between the E and F helices, and only the axial (proximal) His F8 (eighth residue on helix F) and Phe CD1 (first residue on the loop between the C and D helices) are completely conserved. This conserved globin fold, however, results in a very wide range of functionality, which appears to be controlled primarily by the nature of the “distal” residues at position E7, E11, E10, and B10 that line the ligand binding pocket (4). The major interaction that strongly stabilizes O$_2$ over CO binding has been shown to involve H-bonding to the bound O$_2$ by a distal residue, although destabilization of the bound CO by distal steric interaction cannot be completely discounted (4–6). Although the distal H-bond in vertebrate globins is always provided by residue E7 (which is overwhelmingly His, but occasionally Gln (2)), there is much more variation in the nature of the E7 residue and the position of the distal H-bond donor in nonvertebrate globins (3). Such alternate residue H-bond stabilization of the bound O$_2$ has been established in natural globins from sea hares (Arg E10) (7) and trematodes (Tyr B10) (8–10) and in synthetic globins at position E11 (Asn and Thr) (11).

An effective strategy for determining the role of individual residues is to perform functional and molecular structural studies of site directed mutant globins (4). X-ray crystallography is generally the most effective tool for describing both the global and heme pocket structures of globins (4, 6, 12). The description of the globin active site structure, however, is just as effectively pursued by NMR, particularly in the paramagnetic oxidation/spin states, in which structurally exquisite sensitive hyperfine shifts impart improved active site resolution over a diamagnetic analog (13–15). Hyperfine shifts reflect electronic and/or magnetic properties of the chromophore, and hence can be extraordinarily sensitive to (and hence render detectable) small structural changes that are unlikely to be detected in either a crystal structure or a solution NMR structure of a diamagnetic analog. Perhaps the best examples of the exquisite sensitivity are the observation of isotope effects on iron-porphyrin covalency due to the distal H-bond to the bound ligand (16–18) and the characterization of small populations of globins with alternate orientation of the heme about the $\alpha,\gamma$-meso axis (19, 20).

The information content is particularly rich in the cyanomet globins, in which the bound cyanide can model both the H-bond acceptor properties of bound O$_2$ (21–23) and the potential steric tilt/bend of bound CO (17, 24–28). The large dipolar shifts, moreover, guarantee that any heme pocket labile proton can be detected (usually resolved), and its role in H-bonding to the ligand elucidated directly by its placement in the distal pocket (17, 22, 23, 26, 28), and indirectly by the expected influence of such a distal H-bond on the electronic structure of the heme (16–18). The pattern of the dominant heme methyl contact shifts reflect the orientation of the axial His relative to the heme (i.e. $\phi$ in Fig. 1) (29–33), and the pattern of the dominant meso-H dipolar shift reflects the orientation of the rhombic

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* This research was supported by National Institutes of Health Grant HL 16087 (to G. N. L.) and by Grant 97.04083.CT04 from the CNR of Italy (to M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Mb, myoglobin; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; metMbCN, cyanide complex of ferric myoglobin; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; WT, wild-type; rWT, recombinant WT; V(E7)H/R(E10)T-Mb, Val$^\alpha$(E7) → His/Arg$^\alpha$(E10) → Thr-Mb; V(E7)H-Mb, Val$^\alpha$(E7) → His-Mb.
magnetic axes (κ in Fig. 1) (34, 35). The mean of the heme methyl contact shifts has been shown to be sensitive to distal H-bonding to bound cyanide in models (36), but it has not yet been assessed in globins. Lastly, the dominant dipolar shifts for nonligated residues provide information on the orientation of the paramagnetic susceptibility tensor, which can be related to the tilt/bend of the Fe-CN unit and the orientation of the axial His and thereby facilitate the determination of the orientation of mutated residues in the heme cavity (17, 21, 22, 24–26, 28).

The Msbs from the sea hare Aplysia limacina (37), like those from Dolabella auricularia (38), possess a Val E7 but still exhibit high O2 affinity and reasonably slow O2 off-rates. Both crystallography (12) and solution 1H NMR on Aplysia Mb (22, 39) and solution 1H NMR of Dolabela Mb (40) have demonstrated that Arg E10 can orient into the heme pocket and provide an H-bond to bound ligand. The sharp increase in $k_{\text{off}}$ and decrease in affinity for O2 in the Aplysia R(E10)/T-Mb mutant directly confirms (7) the H-bonding role for this residue. A question that naturally arises is whether the alternate H-bonding residues in sperm whale and Aplysia Msbs can be interchanged solely by interchanging the Val E7/Arg E10 in Aplysia with the His E7/Thr E10 of sperm whale Mb. Similar studies have shown that substitution of the key distal residues can in small or large part transfer an unusual functional property from one globin to another (17, 26, 28). Thus, although the O2 off-rate increases sharply and its O2 affinity decreases sharply upon substituting His E7 by Val in sperm whale Mb, a significant portion of the O2 affinity can be recovered by inserting Arg E10 in the sperm whale H(E7/V/T/E10)/R-Mb mutant (41).

Solution 1H NMR of sperm whale H(E7/V/T/E10)/R-MetMb CN found that Arg E10 side chains oriented to provide an H-bond to the bound cyanide (22). Hence, the distal H-bonding scheme of Aplysia Mb can be transferred, albeit less effectively, to sperm whale Mb. The successful cloning and expression of Aplysia Mb has been reported (7), although the N terminus of the recombinant protein, in contrast to true wild-type (12), is not acetylated (7). Substitution of Arg E10 for the Thr found in mammalian Mb led to very large decrease in O2 affinity and increase in O2 off-rate and autoxidation and clearly identified Arg E10 as the source of the H-bond stabilization to the O2 ligand (7). However, engineering the second residue to convert the Aplysia Mb to mimic the sperm whale Mb pocket, V(E7)/H/R/E10/T-Mb, failed to either recover a significant fraction of the O2 affinity or retard the O2 off-rates relative to WT Aplysia Mb.

We report herein on the 1H NMR spectra of the cyanomet complex of the Aplysia mutants V(E7)/H-Mb and V(E7)/H/R/E10/T-Mb that show that the individual distal His E7 residues are indeed oriented out of the heme pocket and hence cannot participate in any H-bonding interaction with the bound cyanide. On the other hand, comparison of the 1H NMR spectra of native wild-type (WT) and recombinant wild-type (rWT) Aplysia metMbCN shows that the N-acetylation missing in rWT Mb (7) leads to changes in both the relative stability of the alternate heme orientations in the heme pocket and a small reorientation of the proximal His imidazole plane relative to the F-helix, when compared with true WT.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation—**A. limacina V(E7)/H and V(E7)/H/R/E10/T mutant myoglobins were expressed and purified as described previously (7). Cyanide complex of ferric Mb (metMbCN) samples were made by exchanging the protein with a 50 mM NaCl, 10 mM KCN, 50 mM K2HPO4-KH2PO4 solution containing 50 mM NaCl, 10 mM KCN, 50 mM K2HPO4-KH2PO4 at pH 8.2 in an Amicon ultracentrifuge cell. The final solutions had a protein concentration of ~1.5 mM.

**1H NMR Spectra—**1H NMR spectra were collected on a GE Omega 500 MHz spectrometer. The strongly relaxed signals were optimally detected in WEFT spectra (42). Nonselective T1 values for the resolved strongly relaxed protons were measured via inversion-recovery experiments. Steady-state NOEs were recorded as described in detail previously (43). The phase-sensitive TOCSY (44), NOESY (45), and conventional magnitude COSY (46) employed the method described by States et al. (47) to provide quadrature detection in the t2 dimension. Solvent suppression, when required, was achieved by direct saturation in the relaxation delay period. 512 blocks were collected with 25.0 kHz spectral width to include all resonances and 10 kHz to improve resolution for the diamagnetic envelope. 128–256 scans were accumulated with a repetition rate of 0.7 or 1.2 s−1 for each block with free induction decays acquired in 512–1024 complex points. The data were processed as described previously (22); details are given in the figure legends. All two-dimensional data were processed on Silicon Graphics workstation using the software package Felix from Biosym/MSI (San Diego, CA).

**Determination of Magnetic Axes—**The magnetic axes were determined as described in detail previously (21, 22, 24–26, 28, 34, 35). Experimental dipolar shifts (α values) for the structurally conserved proximal side of the heme were used as input to search for the Euler rotation angles, $\Gamma(\alpha, \beta, \gamma)$, which transform the molecular pseudosymmetry coordinates ($x', y', z'$ or $R, \theta, \Omega$ (Fig. 1)), readily obtained from crystal coordinates (12), into magnetic axes $x, y, z$ (where $\chi$ is diagonal) by minimizing the global error function, $F_{\text{HN}}$, where $\delta_{\text{HN}}(\text{calc})$ and $\delta_{\text{HN}}(\text{obs})$ are given by

$$F_{\text{HN}} = \frac{1}{2}\pi N \sum_{n=1}^{N} \left[ \Delta \chi_n \left( 3 \cos^2 \theta_n - 1 \right) + 3 \sum_{i < j} \Delta \chi_{ij} \sin \theta_n \cos \theta_n \right]$$

and

$$\delta_{\text{HN}}(\text{obs}) - \delta_{\text{HN}}(\text{calc}) = \delta_{\text{HN}}$$

respectively. $\Delta \chi_n$ and $\Delta \chi_{ij}$ are axial and rhombic anisotropies, and $\delta_{\text{HN}}(\text{obs})$ is the observed chemical shift referenced to DSS. $\delta_{\text{HN}}$ is the shift in the isostructural diamagnetic complex that is calculated via $\delta_{\text{HN}} = \delta_{\text{HN}} + \delta_{\text{HN}}$, where $\delta_{\text{HN}}$ is the shift in an unfolded tetra peptide (48); $\delta_{\text{HN}}$ is the shift of an amino acid proton typical for α-helices, β-strands, coils, etc. (49); and $\delta_{\text{HN}}$ is the ring current shift (50). Minimizing the error function $F_{\text{HN}}$ in Equation 1 was performed over three parameters, $\alpha$, $\beta$, and $\gamma$, using the $\Delta \chi_n$ and $\Delta \chi_{ij}$ from WT metMbCN (22) or extended to all five parameters to yield both the Euler angles and anisotropies, using the A. limacina Mb crystal coordinates (12), as described in detail previously (22).

**Dipolar Shift Simulations—**The position of a substituted or perturbed residue can be determined by minimizing a local error function (22–24, 26, 28). This local error function, designated $F^p(\text{residue}/n')$ (where $n'$ is the number of protons) to distinguish it from that global error function in Equation 1 is given by the following equation,

$$F^p(\text{residue}/n') = n' \sum \delta_{\text{HN}}(\text{obs}) - \delta_{\text{HN}}(\text{calc})$$

where $\delta_{\text{HN}}(X_1, X_2, \ldots)$ represents the $\delta_{\text{HN}}(\text{calc})$ as a function of a bond rotations $\chi_1, \chi_2, \ldots$ using the magnetic axes derived from conserved structural elements. The bond angle that minimizes the residual error function $F^p(\text{residue}/n')$ defines structural changes, as described in detail previously (17, 22–24, 26, 28). The molecular modeling was carried out on a Silicon Graphics Indigo work station from available crystal coordinates using the Insight II program (Biosym/MSI).

**RESULTS**

**Heme Orientational Heterogeneity—**1H NMR spectra for both metMb (51) and metMbCN (19, 20) had shown that in solution, ~20–25% of the globin possesses a heme rotated 180° about the α2 axis with respect to the average orientation reported in the crystal structure (12) (shown in Fig. 1). The metMb spectra at pH 6.0 exhibit low field heme methyl peaks very similar to those of WT (51). However, as shown in the 1H NMR spectra for the lowest field pair of heme methyls of WT, rWT, V(E7)/H/R/E10/T-MetMb and V(E7)/H-MetMb in Fig. 2, the relative intensities of the major component (M) to minor compo-
Fig. 1. Schematic representation of the crystal structure-based reference coordinate system, x*, y*, z*, in which the paramagnetic susceptibility tensor is diagonal; and the electronic coordinate system, x, y, z, in which d*x, d*y, d*z are eigenfunctions, as determined by the axial His orientation relative to the x' axis with angle φ. The reference and magnetic coordinate systems are related by the Euler rotation G(α, β, γ) according to (x*, y*, z*) = (x', y', z')'G(α, β, γ), where β is the tilt of the major axis from the heme normal (not shown), α is the angle between the projection of the tilt on the heme plane and the x' axis (not shown), and γ = α + γ corresponds to the location of the rhombic magnetic axes relative to the x' and y' axes (shown). Theoretical considerations demand that κ = −φ. Panel A shows the effect of a counterclockwise rotation of the axial His by Δφ relative to heme and protein, with the result that the new φi = φ − Δφ, and the new κi = κ − Δφ. Panel B shows the effect of a counterclockwise rotation of the heme by Δφ relative to a stationary axial His and protein matrix. The new φi = φ + Δφ, but the new κi, still referenced to the original axial x', y', must be represented as κi = κ + 2Δφ.

Fig. 2. Low-field portion of the 500 MHz 1H NMR spectra showing the low-field pair of methyls for both the major (M) and minor (m) heme orientation for the WT (A), rWT (B), V(E7)H/R(E10)T-metMb (C), and V(E7)H-metMb (D) complexes in 2H2O at pH 6.0 and 30 °C.

Fig. 3. Upfield portion of the 500 MHz 1H NMR spectra showing the vinyl proton peak for the major (h1βc and h2βI) and minor (h1βc and h2βI) isomers of WT (A), rWT (B), V(E7)H/R(E10)T-metMbCN (C), and V(E7)H-metMbCN (D) complexes in 2H2O at pH 8.2 and 25 °C.

The resolved portions of the 500 MHz 1H NMR spectra of Aplysia native WT, rWT, V(E7)H/R(E10)T-metMbCN, and V(E7)H-metMbCN in 2H2O are illustrated in Fig. 4, A–D, respectively, and reveal equilibrium major to minor isomer ratios of 3.3:1:0, 2.1:1:0, 3.3:1:0 and 3.3:1:0, respectively; thus, the population of the minor component is clearly greater in rWT than WT metMbCN, but somewhat lower, with a 3.5 ± 0.3:1:0 ratio, for the two mutants. The upfield portion of equilibrated cyanomet Aplysia Mb (17, 23–26, 34, 35). Hence, NMR data are shown only to substantiate changes in molecular structure from that of WT metMbCN. The complete heme resonances were assigned as reported previously (20) by detecting the characteristic NOESY contacts predicted from the WT crystal structure (12), with the AMX i–Z 1-AMX j–Z k and AMX i–Z 1-AMX j–Z k–Z 1-AMX i–Z 1-AMX j–Z k–Z 1-AMX i, must be represented as AMX i–Z 1-AMX j–Z k–Z 1-AMX i–Z 1-AMX j–Z k–Z 1-AMX i–Z 1.

The residues of the 500 MHz 1H NMR spectra of Aplysia native WT, rWT, V(E7)H/R(E10)T-metMbCN, and V(E7)H-metMbCN in 2H2O at pH 8.2 and 25 °C. We consider here the 1H NMR spectral properties and heme pocket structure only for the major isomers in solution for each metMbCN complex; extensive and detailed assignments for the major isomer and some for the minor isomer of WT metMbCN have been reported previously (20, 22).

metMbCN Assignments—The procedure for obtaining complete assignment of the heme and the heme cavity residues have been presented in detail for Aplysia WT metMbCN (20) and applied to both WT and numerous mutants of sperm whale Mb (17, 23–26, 34, 35). Hence, NMR data are shown only to substantiate changes in molecular structure from that of WT metMbCN. The complete heme resonances were assigned as reported previously (20) by detecting the characteristic NOESY connections among four low field methyls, two TOCSY-detected three-spin fragments (vinyls), two TOCSY-detected four-spin systems (propionates), and the four strongly relaxed meso-H signals, each with large, temperature-dependent hyperfine shifts. The heme chemical shifts for the four complexes of interest are listed in Table I.

Standard sequential (52) assignments for Aplysia V(E7)H/R(E10)T-metMbCN locate three segments with the inter-residue NOESY contacts characteristic of three α-helical fragments, labeled I, AMX–Ala–Z 2-Ser–Z 1-AMX; II, AMX–Thr–Z 2-Thr–Z 1-He 4–Z 1-AMX–Z 1–Thr–Z 1; and III, AMX–Z 3–Ala–Z 2–Val–Z 1–Z 3 exhibiting the large, low field dipolar shifts characteristic of His58(F8), as confirmed by the NOE to the peptide NH from a strongly hyperfine-shifted and relaxed labile proton at 14 ppm readily identified as the His58(F8) N-H (20). The residues exhibit all the NOESY contacts predicted from the WT crystal structure (12), with the exception of small differences in intensity for His F8 protons as
considered in detail below. Fragment II, the TOCSY/NOESY data of which are shown in Fig. 5, is identified by the sequence as due to His^{85}(E7)–Arg^{66}(E10), with His^{68}(E7) and Ile^{67}(E11) exhibiting significant dipolar shifts, and with residues E10, E11, and E14 exhibiting the NOESY cross peak pattern for rWT connectivities that is identical to that of WT (20) and very similar but not identical (see below) for the mutants relative to rWT. The largest dipolar shifts are displayed by the His^{63}(E7) C_{\alpha} fragment of His^{68}(E7) that is identical to that of WT (20) and very similar but not identical (see below) for the mutants relative to rWT. The largest dipolar shifts are displayed by the His^{63}(E7) C_{\alpha} signals that correspond to the heme position in Fig. 1. The resolved signal for the axial His^{65}(F8) for the major isomer is also labeled.

Saturation of the heme 1-CH$_3$ peak in WT and rWT metMbCN leads to identical NOEs to Ile^{67}(E11) C_{\alpha}, as shown in Fig. 6, A and B, dictating that the heme orientation relative to the protein matrix is the same in WT and rWT. On the other hand, saturating the His^{65}(F8) N$_{\alpha}$H signal in WT and rWT reveals numerous differences in the pattern of NOEs, as shown in Fig. 7. Quantitative comparison of the NOEs is complicated by overlap of the His^{65}(F8) N$_{\alpha}$H for both the major and minor isomers in each globin and the fact that the His^{65}(F8) C$_{\alpha}$H shifts differ, but also overlap for the two isomers, rendering the interpretation of the NOEs to the C$_{\alpha}$H peaks ambiguous. Analysis of the WT crystal structure reveals that a small 2–3° rotation of His^{65}(F8) ring does not significantly alter the distance between His^{65}(F8) N$_{\alpha}$H and Phe^{91}(F4) C$_{\alpha}$H, but increases the His^{65}(F8) N$_{\alpha}$H distance to His^{68}(F8) C$_{\alpha}$H and decreases the His^{68}(F8) N$_{\alpha}$H distance to the Phe^{91}(F4) ring protons for counterclockwise change in $\phi$, shown in Fig. 1A. Comparison of panels A and B of Fig. 7 shows that the NOE to His^{65}(F8) C$_{\alpha}$H is significantly larger and to the Phe^{91}(F4) ring protons is significantly smaller, in rWT compared with WT, establishing that His^{65}(F8) rotates counterclockwise in rWT relative to WT. The magnitude of the NOEs intensity differences are consistent with a His ring rotation of $-2^\circ$.

**Heme Orientation in the Mutants—**Differences in the meso-H hyperfine shift pattern (34) between WT and H(E7)V/R(E10)T-metMbCN indicate (see under “Discussion”) that the relative rotational position of the heme and axial His differ in rWT and the double mutant metMbCN. Saturation of the His^{65}(F8) N$_{\alpha}$H signal does not shed light on whether the axial His ring has rotated because the key Phe^{91}(F4), Ala^{92}(F5), and His^{95}(F8) C$_{\alpha}$H signals are degenerate (not shown). However, saturation of the heme 1-CH$_3$ signal in the double mutant leads to a $-50^\circ$ larger NOE to Ile^{67}(E11) C$_{\alpha}$H than in rWT (Fig. 6C), and the implied decrease in the distance of 5% requires a 3° counterclockwise rotation of the heme in the mutant relative to conserved His^{68}(F8) and protein matrix, as illustrated in Fig. 1B. The heme rotational position of the single mutant was found unchanged from that of rWT on the basis of the magnitude of the 1-CH$_3$ NOE to Ile^{67}(E11) C$_{\alpha}$H (not shown).

**Determination of Magnetic Axes—**The magnetic axes for rWT and WT Aplysia metMbCN are essentially identical, as expected from the fact that the dipolar shifts for nonligated residues are largely indistinguishable (Table II), with $\alpha = 65^\circ$, $\beta = 7.0^\circ$, and $\kappa = 25^\circ$ (and with uncertainties of $\pm 10^\circ$, $\pm 1^\circ$, and $\pm 10^\circ$, respectively) with $\Delta \lambda_{ax} = 2.38 \times 10^{-8}$ m$^3$/mol and $\Delta \chi_{eq} = -0.55 \times 10^{-8}$ m$^3$/mol, as reported previously$^2$ (22). Both five-parameter and three-parameter searches based on the WT (35) $\Delta \lambda_{ax}$ and $\Delta \chi_{eq}$ using a variety of input data sets yielded highly clustered angles for each mutant with that using the same 21

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$^2$ It is noted that the convention for $x$, $y$, and $z$ differs from that used previously for $A. limacina$ metMbCN (22) by a $45^\circ$ rotation in the heme plane and referencing of the $a$ to the +$x$ rather than the $-x$ axis (35), so that $\beta$(new) = $\beta$(old), $\alpha$(new) = $\alpha$(old) + $135^\circ$, and $\kappa$(new) = $\kappa$(old) $-45^\circ$. 

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proximal side protons yields $\alpha = 75^\circ$, $\beta = 11^\circ$, and $\kappa = 35^\circ$ for V(E7)H/R(E10)T-metMbCN and $\alpha = 95^\circ$, $\beta = 8.5^\circ$, and $\kappa = 25^\circ$ for V(E7)H-metMbCN, with the optimized anisotropies in the five-parameter search inconsequentially altered from those of WT. In each case, the correlation between $\delta_{\text{dip}}$(obs) and $\delta_{\text{dip}}$(calc) was excellent not only for the input data protons but also for the majority of the distal side, excluding the mutated residues (not shown).

**DISCUSSION**

**Heme Cavity Structure of the Sperm Whale Mb Mimic**—The dipolar shifts for the His$^{63}$(E7) in the Aplysia Mb mutants show that $\chi_1$ is rotated by $-120^\circ$ from that found in sperm whale Mb (6). The magnitude and direction of the change in $\chi_1$ orients the imidazole ring out of the heme pocket and in the direction of the protein surface. The temperature dependence of the $\delta_{\text{dip}}$(obs) for the His$^{63}$(E7) C$_2$Hs correlates well with that for other dipolar shifted protons (21), indicating that the His$^{63}$(E7) orientation is relatively well defined by $\chi_1 \sim -40^\circ$ and does not represent any equilibrium between an “in” and “out” orientation, as observed in crystals of Chironomus HbIII (53). The His$^{63}$(E7) orientation deduced herein for the mutant metMbCN complexes is completely consistent with the observation (7) of low $O_2$ affinity and rapid $O_2$ off-rate indicative of the absence of significant H-bond stabilization of the bound $O_2$. Superposition of the crystallographically defined heme cavities of sperm whale (6) and Aplysia (12) Mb indicates that the His E7 C$_2$H is $\sim 1.1$ Å closer to the iron in Aplysia than sperm whale Mb, such that the His E7 “in” orientation in Aplysia Mb, like that of sperm whale, could not be accommodated in the ligated state.

**Effect of N-Acetylation on WT Mb Structure**—A logical link between the orientation of the His$^{63}$(F8) ring and N-acetylation in Aplysia Mb is found in the unique, direct interaction between the N terminus and the beginning of the F helix in the form of an H-bond between Leu$^2$ NH to the Ala$^{78}$(EF) carbonyl at the end of the E-helix, as depicted (12) in Fig. 9. Deletion of the acetyl group (7) can be expected to seriously impact this interaction. The absence of N-acetyl group in rWT metMbCN leads to $\sim 0.3$ Kcal/mol decrease in the relative stabilization of the major versus minor heme orientation and a $\sim 2^\circ$ counterclockwise rotation for the axial His plane relative to WT metMbCN. The change in the ratio of the heme orientations demands a small difference in the contacts between the protein and pyrroles A and B of the heme, and the rotation of the His suggests a small translation of the F-helix relative to the heme (rather than heme relative to the F-helix) (see below).

**Rotation of the His$^{63}$(F8) about $\chi_2$ in the Aplysia Mb crystal structure** shows that the ring N$_i$ ligated to the iron translates laterally by $\sim 0.03$ Å per $1^\circ$ counterclockwise rotation of the axial His, in the direction indicated by the arrow in Fig. 9. The conserved contact shifts for the axial His argue against such a distortion for the iron-His bond. The $\sim 2^\circ$ axial His imidazole rotation, however, would leave the axial His bond intact if the F-helix translated relative to the heme by $\sim 0.07$ Å in the direction of the $\gamma$-meso position as shown by the arrow in Fig. 9. Such a movement of the central portion of the F-helix is consistent with a $\sim 0.15$ Å movement of the N terminus of the

### Table I

$^1$H NMR chemical shifts for heme and axial His resonances of A. limacina cyanomet complexes of native WT, rWT, V(E7)H-Mb, V(E7)H/R(E10)T-Mb in $^{1}H_2$O at pH 8.2 and 25°C

| Heme | WT$^a$ | rWT | WT-rWT | V(E7)H/R(E10)T | V(E7)H |
|------|--------|-----|--------|----------------|--------|
| 1-CH$_3$ | 11.78 | 12.04 | -0.26 | 16.64 | 11.98 |
| 3-CH$_3$ | 17.80 | 17.38 | -0.42 | 16.95 | 16.58 |
| 5-CH$_3$ | 15.61 | 16.01 | -0.42 | 15.41 | 16.71 |
| 8-CH$_3$ | 9.95 | 9.62 | -0.33 | 9.13 | 8.46 |
| 2-H$_2$ | 16.42 | 16.60 | -0.18 | 15.50 | 16.14 |
| 2-H$_2$s | -4.39, -4.78 | -4.40, -4.81 | 0.03 | -4.64, -4.94 | -4.55, -4.70 |
| 4-H$_2$ | 5.17 | 5.20 | 0.03 | 4.96 | 5.29 |
| 4-H$_2$s | -0.63, -0.48 | -0.91, -0.57 | 0.28 | -1.10, -0.83 | -0.24, -0.04 |
| 6-H$_2$ | 18.05, 14.93 | 17.96, 14.38 | 0.95 | 8.43, 13.96 | 12.89, 15.87 |
| 6-H$_2$s | -1.34, 1.57 | -1.33, 1.67 | -0.05 | 1.49, 1.05 |
| 7-H$_2$ | 4.05, 4.55 | 3.84, 4.41 | 0.21 | 3.89, 4.08 | 3.73, 3.78 |
| 7-H$_2$s | -2.84, -2.11 | -2.83, -2.17 | 0.05 | -3.35, -1.97 | -2.75, -1.92 |
| $\alpha$-meso-H | 0.98 | 1.04 | -0.06 | 0.09 | 0.54 |
| $\beta$-meso-H | 5.96 | 5.82 | -0.14 | 5.60 | 5.89 |
| $\gamma$-meso-H | -0.42 | -0.34 | 0.08 | -0.28 | -0.42 |
| $\delta$-meso-H | 5.01 | 4.76 | +0.25 | 5.63 | 4.76 |
| $\Delta\delta$-meso-H | -0.51 | -4.94 | | -6.16 | -5.26 |

| His$^{63}$(F8) | NH | C$_2$H | C$_3$H | C$_4$H | C$_5$H | C$_6$H | C$_7$H | C$_8$H | C$_9$H | N$_i$H |
|----------------|------|------|------|------|------|------|------|------|------|-------|
| 11.32 | 7.62 | 10.55 | 10.72 | 18.35 | -2.35 | 14.30 | 7.72 | 10.46 | 10.84 | 18.86 |
| 11.41 | 7.72 | 10.46 | 10.84 | 18.86 | -3.01 | 14.27 | -10.10 | 9.85 | 10.12 | 12.90 |
| -0.09 | -0.10 | -0.09 | -0.12 | -0.51 | -0.66 | -0.13 | 7.55 | 9.95 | 10.40 | 12.29 |
| 11.42 | 7.55 | 9.85 | 10.40 | 12.29 | -5.5 | -1.80 | 6.57 | 9.79 | 10.04 | 12.29 |

$^a$ Shifts in ppm from DSS.

$^b$ Taken from Ref. 20.

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Taken from Ref. 20
F-helix in the same direction. The direction of the movement of the F-helix terminus would suggest that the deletion of the N-acetyl group either strongly destabilizes or abolishes the H-bond between the N terminus and the EF corner in rWT. It is noted that such a small translation of the F-helix could account for the altered stabilities for the two heme orientations, inasmuch as Phe91(F4) represents an important contact with pyrrole A.

Heme Methyl Shifts as Indicator of Distal H-Bonding—It has been observed that the heme mean methyl hyperfine shift in low spin hemin models with ligated cyanide are systematically dependent on the H-bond strength of the solvent, with decreasing H-bond donation leading to increasingly upfield shifted heme methyl shifts (36). Comprehensive published assignments of sperm whale WT (43) and mutants (17, 23, 24, 26), in fact, support a correlation between the heme mean methyl shift and presence of labile protons in contact with the bound ligand.

Similarly, the addition of second H-bond (albeit weaker) via Tyr29(B10) results in a ~0.8 ppm low field bias to the shift. The \( \delta_{\text{CCH}_{3}} \) in Aplysia V(E7)H/R(E10/T-metMbCN is found 0.8 ppm to upfield of WT Aplysia metMbCN, confirming the absence of a distal H-bond. Interestingly, Aplysia V(E7)H-metMbCN exhibits a \( \delta_{\text{CCH}_{3}} \) intermediate between WT and the double mutant, suggesting the possibility that the Arg66(E10) may have a weak interaction with the distal ligand.

The labile proton for Arg66(E10), observed clearly in both WT (22) and rWT Aplysia metMbCN, was not detected in the V(E7)H-metMbCN NMR spectrum. However, even a weak H-bond or only a fractional populated, dynamic H-bond would lead to rapid exchange with solvent. Indeed the O2 off-rates were found to be very similar for both the single and double mutants (7).

The present data confirm a valuable role of the variable heme mean methyl shift as an indicator of differentiation distal H-bonding among a series of mutant metMbCN complexes. However, because it has been shown that the heme mean methyl shift varies with the axial His orientation (33), as defined by \( \phi \) in Fig. 1, it may serve as an indicator of distal H-bonding among a series of point mutants, but not among a...
**TABLE II**

$^1$H NMR chemical shifts of nonligated amino acid residues in native WT, recombinant WT, and the V(E7)H/R(E10)T- and V(E7)H/mutant A. limacina metMbCN in $^2$H$_2$O at pH 8.2 and 25 °C

| Residue | Proton | WT$^b$ | rWT | V(E7)/H/R(E10)T Mb | V(E7)/H Mb |
|---------|--------|--------|-----|------------------|------------|
| Phe$^{43}$(CD1) | C, Hs | 8.87 | 8.86 | 8.67 | 8.68 |
|      | C, Hs | 11.42 | 11.38 | 12.77 | 11.75 |
|      | C, H | 6.19 | 6.15 | 7.78 |
| Val/His$^{65}$(E7) | N, H | 9.60 | 9.59 | 9.37 | 9.22 |
|      | C, H | 7.87 | 7.65 | 8.42 | 7.76 |
|      | C, H | 4.83 | 4.75 | 10.15 | 8.77 |
|      | C$_2$H$_5$/C$_2$H$_3$ | 8.63 | 8.40 | 6.21 | 5.84 |
|      | C, H | 3.50 | 3.79 |
| Ser$^{66}$(E8) | N, H | 10.63 | 10.96 | 11.22 | 10.98 |
|      | C, H | 5.41 | 5.64 | 5.64 | 5.73 |
| Arg/Thr$^{66}$(E10) | N$_2$ | 8.94 | 8.91 | 8.54 |
|      | C, H | 4.26 | 4.27 | 3.81 |
|      | C, H | 2.27 | 2.23 | 6.49 |
|      | C$_2$H$_5$/C$_2$H$_3$ | 3.26 | 3.24 | 0.23 |
|      | C, H | 6.05 | 6.05 |
|      | C, H' | 3.41 | 3.41 |
|      | C, H | 2.95 | 2.90 |
|      | C, H' | 5.80 | 5.89 |
|      | N, H | 16.20 | 16.34 |
| Ile$^{77}$(E11) | N, H | 9.76 | 9.74 | 9.48 |
|      | C, H | 2.54 | 2.46 | 2.74 | 2.39 |
|      | C, H | 3.04 | 3.03 | 3.08 | 2.99 |
|      | C, H | 9.49 | 9.47 | 7.90 | 9.12 |
|      | C, H' | 3.61 | 3.57 | 0.25 | 2.06 |
|      | C, H$_2$ | −0.35 | −0.38 | −0.90 | −0.16 |
|      | C, H$_3$ | 1.30 | 1.25 |
| Arg$^{79}$(E14) | N, H | 7.04 | 6.73 |
|      | C, H | 3.95 | 3.76 |
|      | C, H | 1.19 | 1.02 |
|      | C, H | 0.96 | 0.87 |
|      | C, H' | 5.80 | 5.89 |
| Phe$^{91}$(F4) | N, H | 9.12 | 9.08 | 9.38 | 9.27 |
|      | C, H | 6.02 | 5.99 | 6.57 | 6.43 |
|      | C, H | 4.55 | 2.26 |
|      | C, H' | 4.45 | 2.44 |
|      | C, H | 8.63 | 8.75 | 9.55 | 9.13 |
|      | C, H | 8.87 | 8.92 | 10.26 | 9.20 |
|      | C, H | 10.73 | 10.91 | 12.39 | 11.99 |
| Ala$^{92}$(F5) | N, H | 10.01 | 10.00 | 10.14 | 10.05 |
|      | C, H | 6.44 | 6.38 | 6.57 | 6.46 |
|      | C, H$_2$ | 2.52 | 2.53 | 2.61 | 2.56 |
| Lys$^{93}$(F6) | N, H | 8.68 | 8.85 | 8.89 | 8.93 |
|      | C, H | 3.71 | 3.73 | 3.62 | 3.78 |
| Glu$^{94}$(F7) | N, H | 9.12 | 9.32 | 9.47 | 9.55 |
|      | C, H | 3.90 | 3.91 | 3.79 | 4.13 |
|      | C, H | 3.09 | 3.53 |
|      | C, H | 1.38 | 1.13 |
|      | C, H | 1.90 | 1.89 |
| His$^{95}$(F8) | N, H | 11.32 | 11.41 | 11.25 | 11.42 |
|      | C, H | 7.62 | 7.72 | 6.57 | 7.55 |
|      | C, H | 10.55 | 10.46 | 9.79 | 9.85 |
|      | C, H | 10.72 | 10.84 | 10.04 | 10.40 |
|      | C, H | 18.35 | 18.86 | 12.29 | 12.90 |
|      | C, H | −2.35 | −3.01 | −1.80 | −4–6 |
|      | N, H | 14.30 | 14.27 | 16.13 | 15.57 |
| Val$^{96}$(F9) | N, H | 10.77 | 10.76 | 10.63 | 10.77 |
|      | C, H | 5.21 | 5.22 | 4.91 | 5.03 |
|      | C, H | 2.91 | 2.92 | 2.81 | 2.91 |
|      | C$_2$H$_3$ | 1.58 | 1.58 | 1.49 | 1.57 |
|      | C$_2$H$_3$ | 2.01 | 2.02 | 1.89 | 1.94 |
| Phe$^{98}$(FG2) | C, H | 4.12 | 4.00 | 4.14 |
|      | C, H | 2.96 | 2.37 | 2.41 | 2.76 |
|      | C, H | 2.40 | 2.41 |
|      | C, H | 5.78 | 5.67 |
|      | C, H | 3.96 | 3.76 |
|      | C, H | 4.93 |
| Val$^{100}$(FG4) | C, H | 3.76 | 3.55 | 3.76 | 3.16 |
|      | C, H | 3.60 | 3.52 | 3.42 | 2.94 |
|      | C$_2$H$_3$ | 0.86 | 0.85 | 0.72 | 0.09 |
|      | C$_2$H$_3$ | −0.35 | −0.34 | 0.17 | −1.03 |
| Phe$^{102}$(G5) | N, H | 7.34 | 7.62 |
|      | C, H | 3.22 | 3.21 | 3.37 | 3.03 |
|      | C, H | 2.79 | 2.79 | 2.85 |
|      | C, H | 2.98 | 2.97 | 2.98 |
|      | C, H | 7.18 | 7.16 | 7.10 | 6.85 |
|      | C, H | 7.94 | 7.98 | 7.87 | 7.21 |
|      | C, H | 10.54 | 10.80 | 12.31 | 9.00 |
series of natural genetic variants that exhibit significantly different values, such as Aplysia \( f_{52}^{22°} \) and sperm whale \( f_{5}^{25°} \) metMbCN.

Effect of His/Heme Rotation on 1H NMR Spectral Parameters—The perturbed NOE pattern between heme and the protein matrix or axial His and protein matrix indicate that \( f \) differs slightly between WT and rWT metMbCN and between rWT and V(E7)H/R(E10)T-metMbCN (C). The NOE difference spectra reflect identical 1-CH₃ intensity, allowing comparison of NOE intensity among the three complexes. Note significantly enhanced intensity to Ile67(E11) in the double mutant relative to either WT or rWT metMbCN.

Effect of His/Heme Rotation on 1H NMR Spectral Parameters—The perturbed NOE pattern between heme and the protein matrix or axial His and protein matrix indicate that \( \phi \) such as Aplysia \( \phi = -22° \) and sperm whale \( \phi = -5° \) metMbCN.

Effect of His/Heme Rotation on 1H NMR Spectral Parameters—The perturbed NOE pattern between heme and the protein matrix or axial His and protein matrix indicate that \( \phi \) differs slightly between WT and rWT metMbCN and between rWT and V(E7)H/R(E10)T-metMbCN (C). The values of \( \kappa \) obtained from the magnetic axes, however, have \( \pm 10° \) uncertainties (35) and indicate that \( \kappa \) for the four complexes of interest is the same within the uncertainties. Nevertheless, changes in \( \phi \) and \( \kappa \) manifest themselves in extraordinarily sensitive manners in the heme hyperfine shift patterns (30–34). It is recognized that the asymmetry in the heme methyl hyperfine shift pattern is dominated by the contact interaction that imposes strong heme methyl shifts dependence of the on the His/heme orientation defined by \( \phi \) in Fig. 1. In contrast, it has been shown (34, 55) that the asymmetry of the heme meso-H shifts is dominated by the rhombic term of the dipolar shift, which reflects \( \kappa \) in Fig. 1. Theoretical considerations (54) lead to the expectation that \( \kappa = -\phi \). The changes in heme methyl shifts going from WT \( \rightarrow \) rWT metMbCN, based on published modeling of the contact shift (33), estimate a \( -2° \) counterclockwise rotation of \( \phi \).

For the meso-H, the hyperfine shift asymmetry can be cast in the form,

\[
\Delta \delta_{\text{meso-H}}(\text{obs}) = \frac{1}{2} \delta_{\text{obs}}(\alpha\text{-meso-H}) - \delta_{\text{obs}}(\beta\text{-meso-H}) + \delta_{\text{obs}}(\gamma\text{-meso-H}) - \delta_{\text{obs}}(\delta\text{-meso-H}) \quad (\text{Eq. 5})
\]

as discussed previously (34). The observed values for the four metMbCN complexes are included in Table I. The calculated
value, $\Delta \delta_{\text{meso-H}}^{\text{calc}}$, is obtained using the dipolar shifts predicted by the magnetic axes (34), as follows:

$$\Delta \delta_{\text{meso-H}}^{\text{calc}} = \frac{1}{2} \delta_{\text{dip}(a\text{-meso-H})} - \delta_{\text{dip}(\beta\text{-meso-H})} + \delta_{\text{dip}(\gamma\text{-meso-H})} - \delta_{\text{dip}(\delta\text{-meso-H})} \text{ (Eq. 6)}$$

For WT metMbCN, $\Delta \delta_{\text{meso-H}}^{\text{calc}} = -5.26$ ppm, which is in excellent agreement with the observed value of $-5.21$ ppm in Table I. It is noted, however, that in rWT metMbCN, $\Delta \delta_{\text{meso-H}}^{\text{calc}}$ becomes more positive to $-4.94$, which corresponds to a calculated $\Delta \delta_{\text{meso-H}}$ with $\kappa$ decreased by $-2^\circ$ ($\Delta \phi$ in Fig. 1A).

Hence, the change in pattern of methyl contact shifts and meso-H dipolar shifts independently confirm both the $2^\circ$ decrease in $\phi$ detected by altered NOEs of His$^{\text{E7}}$(F8) N$\text{H}$ to the F helix and the counter-rotation rule (54) that demands that $\kappa = -\phi$. With the exception of the heme and axial His, only very minor shift differences are observed between WT and rWT metMbCN (see Table II). The observed shift changes, moreover, correlate well in the direction and, at least qualitatively, in magnitude with changes in predicted dipolar shifts due to a $2^\circ$ difference in rhombic magnetic axes that result from the rotation of the axial His plane in rWT relative to WT Mb. The solid symbols represent the His$^{\text{E7}}$(F8), and the open markers are for nonligated residues.

For a change in $\delta$ in comparing rWT and V(E7)H/R(E10)T-metMbCN is difficult to detect in the heme methyl contact shift pattern, because the mean shift decreases by $-0.8$ ppm on abolishing the distal H-bond (see above). The $\Delta \delta_{\text{meso-H}}^{\text{obs}}$, nonetheless, is much more negative in the double mutant ($-6.16$ ppm) than in rWT ($-4.94$ ppm), and this difference is accounted for in Equation 6 by a $-4$ to $-5^\circ$ increase in $\kappa$. Again, the changes in $\kappa$ are in opposite direction to the changes in $\phi$, and both confirm the direction of the rotation of the heme. It is noted that $\Delta \kappa = -4$ to $-5^\circ$ is larger than the $\Delta \phi = 3^\circ$ deduced from the NOE data. However, as detailed consideration shows in Fig. 1B, if the heme rather than the axial His rotates, the
experimental $k_\alpha$, defined with respect to the original reference coordinates $x',y'$, must change by $2\Delta \phi$ for a net heme rotation by $\phi$.

Conclusions—Solution $^1$H NMR spectra of Aplysia metM-bCN show that the insertion by mutagenesis of His E7 in both V(E7)H-Mb and V(E7)H/R(E10)T-Mb mutants is oriented out of the heme pocket and not able to provide a stabilizing H-bond to bound ligands. Comparison of WT and rWT metMbCN, moreover, shows that the abolished N-acetylation in rWT leads to both a change in the relative stabilities of the alternate heme orientations and a small change in the orientation of the axial His ring in each isomer. It is shown that the rotation of axial His in rWT and of the heme in the double mutant relative to WT lead to a series of changes in the asymmetry of the heme methyl dominant contact shifts and heme meso-H dominant dipolar shifts that are completely consistent with the currently accepted relationship between the active site molecular and electronic structures. Lastly, analysis of the heme mean methyl shift among sperm whale and the present Aplysia metMbCN mutants reveals that it serves as a valuable empirical indicator of distal H-bonding to cyanide. The facility with which the electronic structure changes can be related to small, but possibly functionally relevant, changes in molecular structure, is a testament to the exquisite sensitivity of hyperfine shifts to molecular structure.

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