A $^1$H NMR Comparison of the Met-Cyano Complexes of Elephant and Sperm Whale Myoglobin

ASSIGNMENT OF LABILE PROTON RESONANCES IN THE HEME CAVITY AND DETERMINATION OF THE DISTAL GLUTAMINE ORIENTATION FROM RELAXATION DATA*

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R. Krishnamoorthi and Gerd N. La Mar‡
From the Department of Chemistry, University of California, Davis, California 95616

Hiroshi Mizukami§ and Alex Romero¶
From the §Division of Regulatory Biology and Biophysics, Department of Biological Sciences and the ¶Department of Anatomy, School of Medicine, Wayne State University, Detroit, Michigan 48202

The met-cyano complex of elephant myoglobin has been investigated by high field $^1$H NMR spectroscopy, with special emphasis on the use of exchangeable proton resonances in the heme cavity to obtain structural information on the distal glutamine. Analysis of the distance dependence of relaxation rates and the exchange behavior of the four hyperfine shifted labile proton resonances has led to the assignment of the proximal His-F8 ring and peptide NHs and the His-G3 ring NH and the distal Gln-E7 amide NH. The similar hyperfine shift patterns for both the apparent heme resonances as well as the labile proton peaks of conserved resonances in elephant and sperm whale met-cyano myoglobins support very similar electronic/molecular structures for their heme cavities. The essentially identical dipolar shifts and dipolar relaxation times for the distal Gln-E7 side chain NH and the distal His-E7 ring NH in sperm whale myoglobin indicate that those labile protons occupy the same geometrical position relative to the iron and heme plane. This geometry is consistent with the distal residue hydrogen bonding to the coordinated ligand. The similar rates and identical mechanisms of exchange with bulk water of the labile protons for the three conserved residues in the elephant and sperm whale heme cavity indicate that the dynamic stability of the proximal side of the heme pocket is unaltered upon the substitution (His→Gln). The much slower exchange rate (by $>10^4$) of the distal NH in elephant relative to sperm whale myoglobin supports the assignment of the resonance to the intrinsically less labile amide side chain.

The apparent ubiquitous distal histidine in heme proteins has been assigned a focal role in modulating the reactivity of the iron center (1–10). It has been proposed that the distal histidine acts as hydrogen bond donor to stabilize the bound ligand in oxy-(11) and met-cyano-(12) myoglobin (Fig. 1A), as a hydrogen bond acceptor in met-aquo-myoglobin (13), and, in general, as a dynamic trap door controlling the binding of ligands to the heme (14). Elephant myoglobin has the interesting replacement of the distal histidine by a glutamine residue, with only three functionally insignificant nonconserved amino acids at the protein surface (15, 16). It has been concluded on the basis of indirect observation of both elephant met-aquo- (17) and nitrosyl- (18) Mb that the distal glutamine does interact with the coordinated ligand. In the NMR study of high spin met-aquo-Mb, however, the absence of resolved resonances from the distal residue precluded direct determination of its orientation in the heme cavity (17).

A much more suitable protein form for direct investigation of the distal amino acid is the low spin ferric form such as metMbCN, for which the efficient electron spin relaxation and large magnetic anisotropy generally lead to resolvable peaks for the distal residue due to the dipolar interaction (12, 19–21). The resonances of noncoordinated amino acid side chains close to the iron relax solely by the dipolar mechanism, and hence structural information leading to assignments can be obtained by an analysis of the differential relaxation behavior of these as well as heme methyl resonances in terms of their relative $r^{-4}$ distance dependence (12, 22).

We have shown elsewhere (12, 23) that the heme resonances

The abbreviations used are: Mb, myoglobin; metMbCN, metmyoglobin cyanide.

![Fig. 1. Schematic representation of distal amino acid side chain interaction in met-cyano-myoglobin complexes. A, HisE7 of sperm whale Mb as deduced from $^1$H NMR relaxation data (12); B and C, two possible orientations of the amide side chain of Gln-E7 of elephant Mb.](image-url)
most readily assigned unambiguously by relaxation methods are the exchangeable protons found within 7 A of the iron center. There are only four such protons in sperm whale Mb, and a combination of relaxation data and exchange behavior has lead to their direct assignment to the ring NH of His-F8 (proximal), His-E7 (distal), and His-FG3 and the peptide NH of His-F8 (12). In particular, the ring NH of His-E7 was located (12) at 4.2 A from the iron in a geometry consistent with its participation in a hydrogen bond to the coordinated cyanide, as found in a neutron diffraction study of oxymyoglobin (11).

We report herein on a 1H NMR study of the relaxation and exchange behavior of labile proton resonances of elephant metMbCN and show that the distal glutamine permits an interaction with the bound ligand very similar to that found for histidine in sperm whale metMbCN.

EXPERIMENTAL PROCEDURES

Myoglobin from Asian elephant was isolated and purified as described earlier (16). The met-cyano complex of this protein was prepared by dissolving about 25 mg of the protein in 0.5 ml of 99.9% H2O or 90% H2O and 10% D2O, containing 0.2 M NaCl, centrifuging out any precipitate, and adjusting the pH to the desired value by the addition of 0.2 M HCl or 0.2 M NaOH. The pH was measured in a NMR tube by using an Ingold microcombination electrode and a Beckman 3550 pH meter. The met-cyano complex was prepared by dissolving about 25 mg of the protein in 0.5 ml of 99.9% H2O or 90% H2O and 10% D2O, containing 0.2 M NaCl, centrifuging out any precipitate, and adjusting the pH to the desired value by the addition of 0.2 M HCl or 0.2 M NaOH. The pH was measured in a NMR tube by using an Ingold microcombination electrode and a Beckman 3550 pH meter. The met-cyano complex was prepared by dissolving about 25 mg of the protein in 0.5 ml of 99.9% H2O or 90% H2O and 10% D2O, containing 0.2 M NaCl, centrifuging out any precipitate, and adjusting the pH to the desired value by the addition of 0.2 M HCl or 0.2 M NaOH. The pH was measured in a NMR tube by using an Ingold microcombination electrode and a Beckman 3550 pH meter. The met-cyano complex was prepared by dissolving about 25 mg of the protein in 0.5 ml of 99.9% H2O or 90% H2O and 10% D2O, containing 0.2 M NaCl, centrifuging out any precipitate, and adjusting the pH to the desired value by the addition of 0.2 M HCl or 0.2 M NaOH. The pH was measured in a NMR tube by using an Ingold microcombination electrode and a Beckman 3550 pH meter.

For the measurement of intrinsinc spin-lattice relaxation times (TI) of exchangeable and nonexchangeable resonances, the conventional 180°-90° pulse sequence (24) was used and the repetition time was >5T1 of the slower relaxing resonance measured. The pH was so chosen that three of the exchangeable resonances exhibited no saturation transfer. The water signal was presaturated using a 300-ms decoupler pulse. Relaxation times were calculated via a linear least squares fit to the equation

$$I(\tau) = I_0 \exp(-\tau/T_1)$$

where I(\tau) and I0 are the intensities of the resonances at \tau = 0 and \tau = T1, respectively, after the 180° pulse and \tau is the delay time between pulses. The exchange rates were calculated for the water exchangeable resonances by using the method of saturation transfer employing the Redfield 2-1-4 pulse sequence (25, 26). The spectra were collected as described earlier (12).

The degree of saturation transfer, namely the saturation factor SF, is given by (12, 26).

$$SF = I/I_0 = T_1/(T_1 - \tau_S)$$

where I and I0 are the peak heights with and without H2O saturation, respectively, \tau_S is the lifetime of the exchangeable proton in the protein environment, and T1 is the intrinsic spin lattice relaxation time. Overhauser effects are assumed negligible (12, 26).

RESULTS

A typical spectrum of the met-cyano complex of elephant myoglobin in H2O at 25°C and pH 8.6 is illustrated in Fig. 2A. The pattern of hyperfine shifted resonances closely resembles that found for sperm whale metMbCN (12, 18, 27). Prominently observed are the three heme methyl resonances (e, h, and l) which have very similar shifts to those previously reported for methyls 5, 1 and 8, respectively. Also present with similar shift is a CH (y) of Ile-99 (28) and the vinyl Hα (g) and Hα (w and x) of the presumed 2-vinyl group. The connectivity of the three vinyl resonances is established by irradiating g which leads to a difference spectrum for w and x (Fig. 2A, inset, clearly identifying w as Hα(cis) and x as Hα(trans). Additional nonlabile single proton peaks observed in the downfield window (f and e) of elephant metMbCN may arise from either the distal glutamine or from residues whose position moves (i.e. Val-Ell) when the bulkier His-E7 is replaced by glutamine. Signals labeled z arise from a minor component which presumably originates from the heme orientation rotated by 180° about the a-y-meso axis, as demonstrated for sperm whale metMbCN (29-31). The exchangeable resonances with large hyperfine shifts are identified by comparison of the spectra in H2O and D2O in B and A of Fig. 2, respectively. Three new resonances are observed when the water signal is saturated: a, b, and c, of which a and b are degenerate with nonlabile peak f. Raising the temperature to 40°C leads to partial resolution of a and b peaks (Fig. 2, C). Also, a spectrum obtained by the Redfield method (25) without saturating the solvent realizes an additional peak (d), as shown in Fig. 2C, inset. The chemical shifts of these labile protons are listed in Table I.

Fig. 3 displays the temperature dependence of the resolved heme methyl e, vinyl Hα, and the exchangeable peaks a and b of elephant metMbCN. Also included in Fig. 3 for comparison are similar data for the hemes 5-CHα and 2-Hα, and the proximal and distal histidyl labile ring protons of sperm whale metMbCN. The partially relaxed 1H NMR spectra of the resonances in the window at 13–20 ppm are illustrated in Fig. 4. The resolved heme methyl peak h and labile proton b yield T1 values from the slopes of the plots of Equation 1 as shown in Fig. 5. The broader labile proton peak, a, heavily overlaps peak f. However, good estimates of the T1b, values are obtained in two ways. First, deconvolution of the composite peak f/a leads to an apparent T1f/a = 10.5 ± 1.0 ms as shown in the plot in Fig. 5. An alternate method is to estimate the T1 from the null (8 ms) in Fig. 4, where T1 = τnull/in 2. This yields T1 = 11.2 ms for a, in excellent agreement with the result in Fig. 5. In fact, in each case, the T1 values from the null points (~18 ms for b and ~12 ms for g) are within the uncertainties obtained from the plots in Fig. 5 (see Table I). The resulting T1 values for the labile proton peaks a, b, and c, as well as the heme methyl peak, h, are listed in Table I.
Assignment of labile proton resonances in elephant met-cyano-myoglobin based on relative dipolar relaxation times

All data were obtained at 40 °C and pH 8.6 in H2O solution.

| Peak | Assignment * | Shift ** | T1(CH3) ms | rCHD = (T1(H))/T1(CH3) ** | Predicted r ** | X-ray r ** |
|------|-------------|----------|-------------|-----------------------------|----------------|-------------|
| a    | Distal Gln-E7 amide NH (His-E7 ring NH) | 18.7 (22.0) | 10.5 ± 1.0 (9.9 ± 0.2) | 0.66 ± 0.01 (0.68 ± 0.02) | 4.1-4.3 | 4.2 |
| b    | Proximal His-F8 ring NH | 19.7 (20.5) | 25.9 ± 2.0 (24.8 ± 0.5) | 0.76 ± 0.06 (0.79 ± 0.06) | 4.6-5.3 | 5.1 |
| c    | Proximal His-F8 peptide NH | 13.1 (13.2) | 145 ± 6 (114 ± 6) | 1.02 ± 0.03 (1.01 ± 0.03) | 6.1-6.5 | 6.9 |
| d    | His-FG3 ring NH | 13.7 (14.0) | 131 ± 4 (126 ± 2) | 1.00 (1.00) | 6.2 | 6.2 |
| b    | Heme methyl (1-CH3) | 16.2 (17.8) | 131 ± 4 (126 ± 2) | 1.00 (1.00) | 6.2 | 6.2 |

* Data in parentheses are for analogous peaks found in sperm whale metMbCN (12).

** Obtained from r1 = rCHD(T1(H)/T1(CH3)) using rCHD = 6.2 Å (12).

** Obtained from x-ray crystallographic coordinates of sperm whale Mb (Ref. 11).

Relaxation data and, hence, distance were not obtainable since the saturation factor was <1.0 at all pH values.

![Curie plot](image)

**Fig. 3.** Curie plot for selected methyl, the vinyl H, the exchangeable proton peaks a and b of elephant metMbCN, and the labile ring protons of the proximal and distal histidyl imidazole of sperm whale metMbCN. The apparent intercepts at T⁻¹ = 0 are given in parentheses at the left of each set of points. DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

A typical pair of Redfield 2-1-4 spectra (12, 25) with (upper) and without (lower) H2O saturation is shown in Fig. 6A. The intensities of nonexchangeable protons remain unaltered upon H2O saturation. The saturation factors, as defined in Equation 2, for peaks a, b, and c at various pH values are given in Table II where they can be compared with similar data for analogous resonances in sperm whale metMbCN (12). It is observed that peaks b, c, and d exhibit the same pH behavior and essentially the same exchange rates in elephant metMbCN in H2O at 40 °C and pH 8.6 showing the region containing a heme methyl (b), the vinyl H, and labile proton peaks a and b. The delay time, τ, in the conventional 180°-τ-90° pulse sequence is given in milliseconds.

![NMR spectra](image)
as in sperm whale metMbCN (12). Proton peak a, on the other hand, fails to exhibit any saturation transfer even at low pH (Fig. 6, B), where the corresponding peak in sperm whale metMbCN has disappeared due to exchange broadening (12, 19).

**DISCUSSION**

**Electronic Structure of the Heme**—The very similar shifts and spread for the heme methyl in elephant metMbCN and sperm whale metMbCN (12, 18, 27) indicate that the electronic/molecular structures in the two heme pockets closely resemble each other (21). This is supported by the essentially identical Curie behavior observed for the farthest downfield shifted heme methyl in elephant and 5-CH₃ of sperm whale metMbCN. The Curie plots for the vinyl H, for both proteins, have essentially the same shifts near 14 ppm (Table I). Assignments can be deduced from the relative $R = (T_1)_{hv}/T_1$ values by comparison to similar data for sperm whale metMbCN (12) using the known heme methyl distance (6.2 Å) for peak h. Peak b has a distance which uniquely corresponds to the proximal histidyl ring NH; its shift (Table I) is also very similar to that in sperm whale metMbCN (12), as is its temperature dependence (Fig. 3). Similarly, peak c must arise from the His-F8 peptide NH (12, 19). Resonance d did not permit a determination of its intrinsic $T_1$, due to extensive saturation transfer at all pH values. However, its pH-dependent linewidth profile is essentially identical to the analogous resonance in sperm whale metMbCN where it could be assigned to His-FG3 ring NH (12, 19). The assignment of peaks b, c, and d is strongly supported by the exchange properties with pH (see below).

Peak a exhibits the shortest $T_1$ and hence is closest to the iron. Although its $T_1$ is very similar to that found for the His-E7 ring NH in sperm whale metMbCN (12), its resonance position is at appreciably higher field (Table I). However, the temperature dependence of peak a in elephant metMbCN is much steeper than for the His-E7 ring NH of sperm whale metMbCN, indicative of a diamagnetic shift about 5 ppm further upfield in the former protein. Thus, the peak a is further upfield in elephant than sperm whale metMbCN primarily because of the diamagnetic origin and does not indicate smaller hyperfine interaction in the former protein.

![Fig. 5. Semilogarithmic $T_1$ plots of Equation 1 for a heme methyl, h (O), and labile protons a (□) and b (Δ) for elephant metMbCN in H₂O at 40 °C and pH 8.6.](image-url)
The only labile protons which can be closer than 5.0 Å in elephant Mb, assuming an otherwise similar structure for the heme pocket (33), are the Glu NHs. The relaxation data already establish a very similar distance from the iron. The similar hyperfine shifts, which depend on the geometric factor (3 cosθ^2 − 1)−3 (θ is the angle between the heme normal and r), require nearly the same θ. Thus, one Glu NH must occupy essentially the same position relative to the iron and bound CN as previously found for the His-E7 ring NH in sperm whale metMbCN (12). The higher field intercept for peak a for Glu-E7 than His-E7 is expected on the basis of the higher field diamagnetic shift for an amide proton relative to an imidazole ring NH (34).

No additional labile proton signal could be attributed to the second NH of the Glu-E7 side chain. Two possible geometries of the side chain could account for this. On the one hand, the orientation of the amide group could be as in Fig. 1C, where the second NH is also close enough to the iron to be significantly relaxed, but the angle, θ, would be near the magic angle, making 3cosθ^2 − 1, and, hence, the dipolar shift too small to shift its peak out of the diamagnetic envelope. The alternate orientation could be that depicted in Fig. 1B, where the second NH is simply 1.9 Å further away from the iron. Even if θ were unchanged, the r−3 dependence in the geometric factor would reduce it by a factor of 3 relative to the closer NH. Such a diminished dipolar shift would make resolution of the signal outside the diamagnetic envelope unlikely. Since the detection of two interconvertible species in the 1H NMR spectrum of sperm whale met-aquo-Mb indicated that the distal Glu can interact with both protons of the coordinated water using both the amine and carboxyl groups (17), the latter orientation (Fig. 1B) is concluded to be more likely in metMbCN.

**Dynamics of Labile Proton Exchange**—Using the observed saturation factors and the intrinsic T1 values for peaks b and c, exchange rates could be calculated using Equation 2 and are listed in Table II. Similarly, the pH-dependent line width of d can be analyzed via the relation τ−1 = π(exchange line width), where the line width in the absence of exchange is assumed to be the same as for the peak in sperm whale metMbCN (12). It is clear from the data in Table II that these three labile protons exhibit the same mechanisms and exchange rates as for the analogous protons in sperm whale metMbCN (12) (peaks b and c, base catalysis; peak d, both base and acid catalysis). Thus, the dynamic stability of the proximal side of the heme pocket is essentially the same in sperm whale and elephant myoglobins.

Peak a, on the other hand, which is assigned to the distal Glu-E7 NH, exhibits no saturation transfer at either basic or very acid pH. At pH 5.0, where the extrapolated exchange rate for the distal His-E7 ring NH is 3 × 10^8 s⁻¹ for sperm whale metMbCN (12), the T₁ ~ 11 ms and unity saturation factor for the Glu-E7 amide NH require an exchange rate ≈ 3 × 10^7 s⁻¹. This dramatic decrease in lability of proton a in elephant relative to sperm whale metMbCN is consistent with the known highly reduced intrinsic lability of an amide NH relative to an imidazole ring NH (35) and supports the assignment of peak a to the distal Glu-E7 side chain.

**CONCLUSION**

The hyperfine shifts for both nonlabile heme proton resonances as well as the labile proton resonances of conserved amino acids in the heme pocket of met-cyano-myoglobin support a very similar electronic/molecular structure of the heme pocket in elephant and sperm whale myoglobin. One of the distal glutamine side chain NH resonances in elephant metMbCN has been located and assigned. The glutamine NH interacts with the coordinated cyanide in a fashion very similar to that of the distal histidyl labile ring proton in sperm whale Mb. The dynamic stability of the heme cavity appears to be unaffected by the replacement of the distal His→Glu.

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