A Proton NMR Investigation of the Influence of Distal Glutamine on Structural and Dynamic Properties of Elephant Metmyoglobin*

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The proton NMR spectra of metmyoglobin from the Asian elephant, which has the replacement of glutamine for the usual distal histidine, are reported and analyzed. In the low pH region, we detect two interconvertible forms of the met-aquo-protein whose relative stabilities are independent of pH, but depend strongly on both temperature and solvent isotope composition. As the pH is raised, both species convert to the met-hydroxy form, as found for other myoglobins. The temperature dependence of the heme methyl shifts for both acidic protein forms indicates essentially high spin character for the iron, and the mean heme methyl shifts are interpreted as indicating one form with a very slightly weaker hydrogen bond acceptor, but also the amine group. We conclude that a distal glutamine can act both as a stronger and as a weaker hydrogen bond acceptor towards coordinated water than the usual distal histidine. The relative rates of conversion of the two met-aquo-myoglobin forms to MetMbOH is found to be consistent with the proposed structures for the two forms.

Current hypotheses regarding the structure-function relationships in myoglobin and hemoglobin assign an important role to the distal residue in stabilizing the iron-bound ligand (1–10). The vast majority of myoglobins and hemoglobins have a histidine as the distal residue which is thought to be crucial for proper function for this class of proteins. The various roles assigned to the distal histidyl imidazole include acting as a hydrogen-bond donor to the bound ligand in oxy- and met-cyano proteins (Refs. 11 and 12, respectively), a hydrogen-bond acceptor towards coordinated water in met-aquo-systems (13), (i.e. Fig. 1, C), an electron donor towards heme bound carbonyls (14), and as a dynamic trap door (15) which controls entry and exit into the isolated heme pocket in myoglobin (16) and hemoglobin (3).

While there exists a number of monomeric oxygen binding hemoproteins without a distal histidine (e.g. aplysia (17), Chironomus thummi thummi (18), Olycera (6)), comparison of their structure and properties with the more common distal histidine containing myoglobin and hemoglobin, in terms of difference in distal interactions, is complicated by the large number of important nonconserved amino acid substitutions, additions and/or deletions. It has been shown (19–21) recently, however, that Mb1 from the Asian elephant has a distal glutamine substituted for the more common histidine, and exhibits only three other nonconserved amino acid substitutions which are all on the protein surface and hence likely functionally irrelevant. Elephant Mb has been shown to exhibit an oxygen affinity very similar to that of other myoglobins but a significantly reduced susceptibility to autoxidation (20). An acid = alkaline transition characteristic of distal histidine containing Mbs has also been found for elephant MetMb using optical spectroscopy (21), although with a reduced pK value. While structural consideration of a glutamine substituted for a histidine with a Cα at the same position as in the x-ray structure of sperm whale Mb indicates that glutamine can approach the iron closer than histidine (20), ESR studies on elephant nitrosyl Mb indicate (21) a diminished interaction of the NO with the distal residue as compared to the same form of sperm whale Mb (21). Proton NMR spectroscopy provides a particularly sensitive technique for monitoring interaction between heme and distal amino acid residues (22–28). In paramagnetic forms of the proteins such interactions modify the hyperfine shifts (29) experienced by the heme substituents, and in certain protein forms such as met-cyano-Mb, it is possible to resolve signals from the distal residue whose hyperfine shift and relaxation properties give direct information on the position of the distal residue (12, 23). In this report we consider the 1H NMR properties of elephant met-aquo-Mb and compare them with those of the well characterized NMR properties of the analogous form of sperm whale Mb (24, 26). We present evidence that elephant MetMb at low pH exists in two distinct but interconvertible forms previously undetected by visible spectroscopy (21), and show that the spectral parameters suggest that the two species differ in the extent of interaction of the coordinated water with the distal glutamine, showing both weaker and stronger interactions than in sperm whale MetMb.

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‡ The abbreviations used: Mb, myoglobin; MetMb, metmyoglobin; MetMbH₂O, met-aquo-myoglobin.
Experimental Procedures

Asian elephantMb was isolated from skeletal muscle under CO as described earlier (20). The Met form was prepared by flash photolysing off the CO and oxidizing the protein with 2 eq of potassium ferricyanide and passing through a Sephadex G-25 column equilibrated with 0.02 M NaOH, and the eluent was concentrated in an Amicon micro cell, and lyophilized. A typical NMR sample consisted of dissolving 30 mg of the lyophilized protein in 0.5 ml of 'H20 or 90% 'H2O, 10% 'HCl solution containing 0.2 M NaCl. The pH was adjusted using 0.2 M HCl or 0.2 M NaOH. The pH was measured in the NMR tube using an Ingold micro combination electrode and a Beckman Model 3500 pH meter, and is uncorrected for the isotope effect.

'1H NMR spectra were collected on a Nicolet 360 instrument operating at a proton frequency of 360.067 MHz using quadrature phase detection. A typical spectrum was obtained by presaturating water with a 150-ms decoupler nonphase selective pulse and computer averaging 5000 to 8000 transients of 4096 points over a band width of 40 kHz. The signal/noise was improved by apodizing the free induction decays which introduced 60 Hz line broadening; account was taken of the artificial line broadening in all calculations. The line widths and areas of peaks were measured by using the NTCCAP curve fitting program available on the Nicolet 1180 Data System.

Results

The influence of pH on the 360 MHz '1H NMR spectra of elephant MetMbH2O is illustrated in Fig. 2, B–E. In the low pH region, the spectrum exhibits resonances in the same spectral window (24, 26) as sperm whale MetMbH2O (Fig. 24), but there are many more resonances suggestive of more than one species in solution. Raising the pH has no effect on the position, relative intensities, and line widths of the MetMb peaks, although all resonances lose intensity while a new set of resonances (labeled C) appear. This species, identified as MetMbOH, has a spectrum very similar to that of sperm whale MetMbOH at pH 10.5 is included in F for comparison.

Two subsets of resonances in elephant acidic MetMb are readily identified, A1, B1, on the basis of altering the relative population of the two species. The spectra of MetMbH2O in H2O and 'H2O at 5°C are compared in Fig. 3, A and B. It is clear that, while the relative intensities of the various signals within a subset (A or B) remain unchanged, the A peaks are more intense relative to the B peak in H2O than in 'H2O. Moreover, when the temperature is raised for either sample, the A peaks gain intensity at the expense of the B subset, as illustrated for the 'H2O solution in Fig. 3, B–D. Among the resonances of each subset there are four peaks (A1–A4 and B1–B4) with three times the intensity of any of the other resonances, and hence can be assigned to the four heme methyls (26) for the two species in equilibrium in solution. The relative intensities of the resolved methyl peak A and B, allow determination of the equilibrium constant for the reaction:

\[
A \rightleftharpoons B
\]

\[
K = \frac{[B]}{[A]}
\]

Fig. 1. Potential interactions between coordinated water and the distal residue in met-aquo-myooglobin. A, glutamine acting solely as a hydrogen-bond acceptor via its carbonyl group; B, glutamine acting as a hydrogen-bond acceptor with its carbonyl group as well as its amine nitrogen; C, histidine serving as a hydrogen-bond acceptor with the ring nitrogen.

Fig. 2. The hyperfine shifted portion of the 360 MHz '1H NMR spectra of MetMb in 'H2O at 25°C. A, sperm whale MetMbH2O at pH 6.2; the four peaks with area for three protons at 92, 85, 73, and 53 ppm have been shown to originate from the heme methyls 8, 5, 3, and 1, respectively. B–E, elephant MetMb at pH 6.5 B, pH 8.6 C, pH 9.15 D, and pH 10.5 E. At low pH (A) two sets of signals are observed A1–A4 and B1–B4 which originate from heme methyls from two distinct species. As the pH is raised, the A1 and B1 set of signals decreases in intensity and a new set, C1, appears (traces B–E). At pH 10.5 only the species with peaks C1 exists, and we assign it to elephant MetMbOH. The trace for sperm whale MetMbOH at pH 10.5 is included in F for comparison.
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**Fig. 3. Low field portion of the 360 MHz \(^1\)H NMR spectra of elephant MetMb at pH 6.5.**

A, in H\(_2\)O at 5 °C; B, in \(^2\)H\(_2\)O at 5 °C (note the decrease in the intensities at peak A\(_1\)-A\(_4\) upon replacing H\(_2\)O with \(^2\)H\(_2\)O); C, in \(^2\)H\(_2\)O at 25 °C, and D, in \(^2\)H\(_2\)O at 45 °C. In the three traces for MetMb in \(^2\)H\(_2\)O, note the increase as the temperature is raised of the intensities of peaks A\(_1\)-A\(_4\) relative to those at peaks B\(_1\)-B\(_4\).

with \( K = [B]/[A] = k_{AB}/k_{BA} \), where \( k_{AB} \) and \( k_{BA} \) are the first order interconversion rates. The van't Hoff plots for \( K \) in H\(_2\)O and \(^2\)H\(_2\)O are given in Fig. 4 and the thermodynamic parameters are listed in Table I.

The variable temperature data for the heme methyl resonance of each species are presented in Fig. 5 in the form of a Curie plot (29). The data lie on straight lines with apparent intercepts at \( T^{-1} = 0 \), similar to those found for sperm whale MetMb\(_2\)O\(_2\) (26). The shifts at 25 °C for the two sets of methyl peaks for elephant MetMb\(_2\)O are compared in Table II with similar data for sperm whale MetMb\(_2\)O\(_2\) (26).

In addition to the shifts presented in Fig. 5, the resonances of both A, and B, exhibit line broadening as the temperature is raised. The influence of temperature on the line width of peak A\(_1\) is shown in Fig. 6. The decrease in line width with increasing temperature on the right is indicative of normal paramagnetic relaxation (30). The broadening experienced at higher temperatures reflects exchange contributions in the NMR slow exchange limit, which can be analyzed via the standard equation (30):

\[ x(\delta_{A\text{ex}} - \delta_e) = k_{AB}, \]  

where \( \delta_{A\text{ex}} \) and \( \delta_e \) are the line width in the presence and absence of exchange for peak A, respectively; a similar equation holds for resonance B. Although similar line broadening is also detected for the B, peaks at elevated temperatures, the lower intensities and poorer resolution precluded a quantitative analysis. The value of \( k_{BA} \) derived from Equation 2, together with the estimated \( k_{AB} \) using the relation \( K = k_{AB}/k_{BA} \) are included in Table I. Thus, the exchange broadening at higher temperature must involve the interconversion between the A and B species.

The exchange broadening of A\(_1\) is found to be essentially independent of pH in the range 5–8. However, as species C (MetMbOH), is appreciably populated, the A\(_1\) resonances selectively experience additional line broadening, as illustrated in the variable temperature NMR traces at pH 9.0 in Fig. 7. Peaks A\(_1\) clearly broaden much more extensively than peaks B\(_1\); in fact, peak B\(_1\) exhibits essentially the same line width at high temperature in the absence of C (i.e. pH 6). A plot of the line width of peak A\(_1\) in the presence of species C is included in Fig. 6. This excess line broadening of the A\(_1\) resonances at alkaline pH in the presence of MetMbOH must represent the additional slow exchange contribution (31) due to the interconversion between A\(_1\) and C as in reaction 3.

\[ A \overset{k_{AB}}{\leftrightarrow} B \overset{k_{BC}}{\leftrightarrow} C \]

The absence of such excess broadening for B, peaks dictates that the A \( \rightarrow \) C interconversion is appreciably faster than the B \( \rightarrow \) C interconversion (with the A \( \rightarrow \) C rate also faster than the A \( \rightarrow \) B rate). The values estimated at 35 °C are \( k_{AC} \sim (16 \pm 5) \times 10^8 \text{ s}^{-1} \), \( k_{BC} \sim (0.5 \pm 0.1) \times 10^8 \text{ s}^{-1} \), \( k_{CA} \) and \( k_{CB} \) are not determinable due to the poor resolution of peaks for species C.

**DISCUSSION**

Equilibrium between Two Met-aquo-species—The optical spectrum of elephant metmyoglobin has been studied (21) as a function of pH and shown to be consistent with a simple equilibrium between acidic (met-aquo) and alkaline (met-hydroxy) forms, as is the case of sperm whale or human metmyoglobin, except that the pK (8.5) is lower by 0.4 unit in the elephant protein. The present NMR data yield the first direct evidence for two distinct species in the acidic pH region. The failure to detect both species by optical spectroscopy can probably be traced to the fact that the equilibrium between the two acidic forms is independent of pH.

The two sets of resonances A\(_1\) and B\(_1\), observed at low pH arise from two interconvertible forms of elephant MetMbH\(_2\)O.
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The temperature dependence of the methyl hyperfine shifts (Fig. 3) exhibit straight lines with near zero intercepts similar to those observed for sperm whale MetMbH₂O (26). Single proton peaks (vinyl H₂, propionate H₃) when resolved, exhibit similar properties. Thus, both the A and B species have to be assumed to be predominantly high spin (32). The average heme-methyl shifts for species A is slightly larger than that reported for sperm whale MetMbH₂O (Table I), while the average shift for species B is quite a bit smaller. Five-coordinate high spin iron(II) is known to exhibit significantly smaller average heme methyl shifts in models (33). However, such five-coordinate models also exhibit a characteristic upfield (~40 ppm) meso-H shift (33) which cannot be detected for elephant MetMbH₂O under conditions where the same resonance can be resolved downfield in sperm whale MetMbH₂O (26). Hence, we conclude that both A and B must be six coordinate. In such species the average heme methyl shift can be expected to reflect the strength of the axial field, where the cis effect (34) predicts a decrease in iron-porphyrin σ bonding (and hence, decreased heme methyl shifts), as the axial field strength increases (35).

On the basis of the closer similarity of the average as well as the spread of the heme methyl shifts to those of sperm whale MetMbH₂O, we assign A the structure A in Fig. 1, where the glutamine NH acts as a hydrogen-bond donor similar to the histidyl imidazole in sperm whale MetMbH₂O (Fig. 1C). On the basis of the slightly larger average heme methyl shifts for A, we conclude (34, 35) that the hydrogen-bonding interaction with glutamine in A is slightly weaker than in sperm whale MetMbH₂O. A similar decrease in the interaction between coordinated NO and distal hydrogen-bond donor for elephant relative to sperm whale myoglobin has been deduced for nitrosyl myoglobins (21).

The large isotope effect (factor of ~1.5) on the A → B...
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Fig. 6. Semilog plot of observed line width of resonance A1 as a function of reciprocal temperature. ○, pH 6.5 when only species A and B are present in solution; ●, pH 9.3 when there is an appreciable amount of species C (MetMbOH) present. The dashed line represents the line width behavior expected in the absence of any exchange effects.

Fig. 7. Low field portion of the 360 MHz 1H NMR spectrum of elephant metmyoglobin in H2O at pH 9.1 as a function of temperature. Note the simultaneous presence of all three species A, B (two forms of MetMbH2O), and C (MetMbOH) which have resonances labeled A1, B1, and C1, respectively. As the temperature is raised, the A1 peaks (particularly note A1b) broaden considerably more than the B1 peaks (note B1) when C is present, reflecting additional exchange contribution which dictates that the A1 → C1 rate is much faster than the B1 → C1 rate.

The equilibrium constant observed in H2O and 2H2O suggests a structural difference between the two species involving a hydrogen bond with the coordinated water (36). The larger K in 2H2O indicates stronger or more extensive hydrogen bonding in species B. The difference in ΔG for the process A → B in both solvents is also consistent with the presence of one additional hydrogen bonding species B (36). The negative ΔS indicates more restricted rotation or motion in species B.

One difference between glutamine and histidine as distal residues is that the former has two potential sites for simultaneous interaction with the coordinated water. While the resonance stabilization usually makes the O=C—NH2 fragment planar, (Fig. 1A), it seems plausible that hydrogen bond donation towards the amine portion could stabilize a structure such as that depicted in Fig. 1B. The loss of the partial resonance would be compensated by the newly formed hydrogen bond, and this hydrogen bond would be stronger in 2H2O than H2O. The additional hydrogen bond in Fig. 1B would also cause the coordinated water to act as a stronger σ donor. This increased axial field would account for the smaller heme methyl hyperfine shift via the well known cis effect (34, 35). Thus, species B indicates that the coordinated water is capable of interacting more strongly with the distal glutamine than a distal histidine. We propose that this species can be represented by the structure in Fig. 1B. The ability of glutamine to provide two sites for interaction with a coordinated ligand has also been invoked to explain the lower pK for the acid = alkaline transition via preferential stabilization of the coordinated hydroxy ligand (21).

The absence of influence of pH on the A/B equilibrium is also consistent with an "intermolecular" equilibrium between structures involving solely the coordinated water and the distal glutamine. Increasing the pH leads to conversion to the MetMbOH form (peaks C1), which appears to be very similar to sperm whale MetMbOH. The apparent pK ~8.7 in 2H2O is consistent with that reported for elephant MetMbH2O in H2O on the basis of optical data (21). The optical data, however, failed to discriminate between the two contributing acidic forms of the protein.

The Dynamics of Interconversion—The pH-dependent line broadening of both the A1 and B1 resonances at low pH is indicative of kAB and kBA both being first order rate constants. The additional line broadening at alkaline pH in the presence of species C for the A1 but not the B1 resonances dictates kAC is much larger than kAC. In considering the proposed structures of species A and B (Fig. 1, A and B), it is clear that one water proton is exposed for attack by hydroxide for the proposed structure of species A, while both protons are involved in interacting with glutamine in the structure of B. Thus, the proposed structure of A and B provide a rationalization for the reduced liability of the water proton in species B relative to that in species A.

Thus, we conclude glutamine is capable of interacting strongly with the coordinated ligand in MetMb. Similar conclusions are indicated by the relaxation properties of exchangeable protons in the heme cavity of elephant met-cyanometMb. Further NMR investigation of ligated forms of elephant myoglobin designed to provide further characterization of distal interactions are in progress in these laboratories.

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