Interaction of Ligands with the Distal Glutamine in Elephant Myoglobin*

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The effects of distal glutamine (E7) replacement in elephant myoglobin were studied by comparing the temperature-dependent nitrosyl electron spin resonance spectra, redox potentials, and the acid-alkaline equilibria of elephant and human myoglobins. For myoglobins containing a distal histidine, the nitrosyl ESR spectra do not exhibit superhyperfine splitting until near liquid helium temperatures (Yoshimura, T., Ozaki, T., Shintani, Y., and Watanabe, H. (1979) Arch. Biochem. Biophys. 193, 361-313). Studies presented here show that the ESR spectra of nitrosyl elephant myoglobin exhibit 9-line superhyperfine splitting well above liquid nitrogen temperatures, similar to the temperature profiles of isolated heme complexes (Morse, R. H. (1980) Fed. Proc. 39, 2006). It is concluded that the shift in the spectral equilibrium to higher temperature indicates a diminished interaction between NO and the distal position in elephant myoglobin. In addition, the redox potential of elephant myoglobin was found to be nearly 100 mV greater than that of human myoglobin, and the pKₐ of the acid-alkaline equilibrium (oxidized myoglobin) was 8.5, being 0.4 unit less than that of other vertebrate myoglobins. These different reactivities between elephant and human myoglobins are discussed based on the nature of charge interactions between polar ligands and distal glutamine and histidine.

Distal histidines of hemoproteins are evolutionarily conserved and their replacement with other amino acids often produces impaired molecular function. However, recent analysis reveals that glutamine could be a successful replacement for distal histidine (1-3). For example, Romero-Herrera et al. (4) have shown that the distal histidine of Asian elephant myoglobin is replaced with glutamine, yet its interaction with oxygen is similar to that of human or whale myoglobin. Also, the replacement reduced the rates of heme autoxidation and CO dissociation, indicating that glutamine can interact strongly with polar ligands. These observations are consistent with the reports that the polar nature of glutamine allows for various types of charge interactions with the distal histidine (5-7), while O₂ has been shown to form a hydrogen bond with distal histidine (8). In opposum hemoglobin, which contains distal glutamines in the α-chains and has slightly reduced oxygen affinity compared with that of human hemoglobin (9, 10), a weakened interaction between both NO and O₂ with distal glutamine has been observed using ESR (10, 11). Other ESR experiments (12, 13) have revealed that the nitrosyl heme complexes exhibit two distinct spectral states in thermal equilibrium, being dependent upon the nature of the nitrogenous base, while in heme proteins, this equilibrium has been shown to be subject to the specific environment surrounding the heme complex.

In this paper, the role of glutamine as a distal amino acid to the heme is examined by comparing the temperature-dependent ESR spectra of elephant nitrosyl myoglobin with those of whale and human nitrosyl myoglobins. Since both NO and O₂ form similar bent configurations with the heme (14), the information obtained for the NO-glutamine interaction may offer insight to the interaction between O₂ and the distal amino acid. Furthermore, the effect of glutamine substitution upon the heme redox potential and the pKₐ for the acid-alkaline equilibrium measured from the titration of elephant ferric myoglobin have been determined. Since the ferrous state is required for the physiological function of myoglobin, any successful substitutions at the distal site must maintain the proper steric and electron orbital configurations near the heme iron. The specific interactions between various ferrous ligands and the aqua-ligands of the ferric myoglobin to glutamine are discussed and compared to that of distal histidine.

MATERIALS AND METHODS
Asian elephant myoglobin and human myoglobin were isolated from skeletal muscles under CO as described by Romero-Herrera et al. (4). Whale myoglobin from skeletal muscle (type 2) was obtained from Sigma in ferric form. Ferric whale myoglobin was reduced in 0.1 M phosphate buffer at pH 7.0 in the presence of CO by adding a 2-fold molar excess of sodium dithionite and then quickly passing the sample through a Sephadex G-25 column equilibrated with the same phosphate buffer under nitrogen. Ferric myoglobin was prepared by the addition of ferricyanide (15) and excess ferricyanide was removed by gel filtration (G-25). Nitrosyl myoglobins were prepared from carbon monoxymyoglobins in a sealed chamber coupled to a 3-mm quartz ESR tube. After repeated evacuation and flushing with oxygen-free nitrogen (Air Products), NO gas, which had been passed through a column of solid KOH, was added to the chamber at 1 atm at 0 °C for 10 min. Excess NO was then removed by repeated evacuation and flushing with N₂, and the samples were frozen immediately in liquid nitrogen. The concentration of myoglobin was determined spectrophotometrically at 540 nm (ε = 11.3) as described by van Kampen and Zijlstra (16).

The x-band ESR spectra of nitrosyl myoglobins were obtained with a Varian E-9 spectrometer equipped with a variable temperature accessory at a modulation amplitude of 2 gauss with a microwave power of 20 milliwatts.

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Redox titrations of myoglobin were performed as described by Brunori et al. (17) at 30 °C. Five ml of 1 mM ferric myoglobin in 0.1 M phosphate buffer at pH 7.0 were successively reduced with anthraquinone-2-sulfonate. Prior to use, the titrant was recrystallized twice from ethanol and oxidized by titration with NaBH₄. Toluuidine blue and thionine were present in 1-2 mol % as redox mediators. Potentials were recorded using Radiometer calomel and platinum electrodes on a Corning Research Model 112 pH meter. The acid-alkaline equilibrium of ferric elephant myoglobin was determined spectrophotometrically by adding 0.1 ml of an unbuffered ferric myoglobin (0.6 mM) to 1.0 ml of phosphate buffer (pH 6.5-8.0) or glycyl/glycine NaOH buffer (pH 8-11.2) with the final ionic strength of 0.1. The pH of each mixture was determined and the absorption spectrum was obtained with a Cary 118C (Varian) spectrophotometer at 20 °C.

RESULTS

Temperature-dependent ESR Spectra of Nitrosyl Myoglobin—Fig. 1 shows the x-band ESR spectra of elephant and whale nitrosyl myoglobins taken between −196 and 22 °C. In contrast to the relatively smooth spectrum of whale nitrosyl myoglobin at −196 °C, the asymmetric line shape of elephant nitrosyl myoglobin is characterized by a narrower g, absorption and a 9-line superhyperfine splitting superimposed over the midpoint region having coupling constants of 21 and 6.5 gauss. Whereas both myoglobins exhibit spectra that are nearly indistinguishable at room temperature (22 °C), the superhyperfine splitting in elephant nitrosyl myoglobin at low temperature is attributed to nuclear coupling with the ε-nitrogen of the proximal histidine and the nitrogen of the NO ligand (13). A nearly identical ESR spectrum to that of elephant nitrosyl myoglobin has been observed for nitrosyl cytochrome c (13, 18) at −196 °C, while a similar temperature profile for the spectral equilibrium is seen for isolated nitrosyl hemes complexed with unhindered nitrogenous bases (12).

Most hemoproteins exhibit the highly asymmetric nitrosyl ESR spectrum only near liquid helium temperatures, suggesting that distal glutamine interacts only weakly or not at all with the nitrosyl ligand.

Redox Titrations—In most hemoproteins having distal substitutions, the heme iron is subject to accelerated oxidation. This generality does not apply to elephant myoglobin, as it exhibits a resistance to heme oxidation during extraction procedures. To determine if an increase in the redox potential was in part responsible for this unusual property, the potentiometric titrations of elephant and human myoglobins were compared under identical buffer conditions at 30 °C, and the results are plotted in Fig. 2. As expected, both myoglobins exhibited a simple redox titration curve involving the transfer of a single electron and having an n value of 1.07 for human myoglobin and 0.94 for elephant myoglobin. The n value was determined by best fit to the following equation

\[ n = \frac{RT}{F} \times \frac{d \ln ([\text{myoglobin} - \text{Fe}^{3+}]/[\text{myoglobin} - \text{Fe}^{2+}])}{dE} \]

**Fig. 1.** The temperature dependence for the ESR spectra of nitrosyl myoglobin in 0.1 M phosphate buffer at pH 7.0 and comparative spectra of whale and elephant myoglobins obtained at liquid nitrogen and room temperature. ESR spectra were obtained at a microwave power of 20 milliwatts with 2 gauss modulation and are not normalized with respect to signal intensity. The hyperfine splitting values for elephant myoglobin (Mb) are 21 and 6.5 gauss (G).

**Fig. 2.** The comparative redox titrations of elephant and human myoglobins at 30 °C in 0.1 M phosphate buffer at pH 7.0. Potentials were recorded after progressive reduction of ferric myoglobin (Mb) with anthraquinone-2-sulfonate using a saturated calomel reference electrode and a platinum electrode (Radiometer). The titration curves exhibit a one-electron transfer having n values of 1.07 for human myoglobin and 0.94 for elephant myoglobin.

**Fig. 3.** The absorption spectra and titration curve for the acid-alkaline equilibrium of ferric elephant myoglobin (0.05 mM) obtained at 20 °C with an ionic strength of 0.1. Samples below pH 8.0 are in 0.1 M phosphate buffer, and samples above pH 8.0 are in 0.1 M glycyl/glycine NaOH buffer. Isosbestic points are located at 625.5, 625.0, and 493.3 nm.
The $E_{1/2}$ corrected to the hydrogen standard was 60.2 mV for human myoglobin and 156.4 mV for elephant myoglobin. The $E_{1/2}$ for human myoglobin is in close agreement to that of Antonini and Brunori (15), while that of elephant myoglobin is nearer the value obtained for hemoglobin at neutral pH. This relatively high value for $E_{1/2}$ of elephant myoglobin, being nearly 100 mV greater than that of human myoglobin, reveals a physiologically important effect of glutamine replacement.

**Acid-Alkaline Equilibrium**—Differences between the acid base properties of oxidized elephant and human myoglobins, particularly the ionization of the aqua-ligand, may contribute to the elevated redox potential observed for elephant myoglobin. In Fig. 3, the results of the spectrophotometric titration between pH 6.5 and 11.2 are shown for elephant myoglobin. The spectra exhibit several well defined isosbestic points at 625.5, 552, and 493.3 nm, indicating the presence of a typical thermal mixture of single high and low spin forms (19, 20). The acidic spectrum of elephant myoglobin is red-shifted, while the alkaline spectrum is blue-shifted as compared to the corresponding whale spectrum (data not shown). The titration curve generated from the absorbance differences at 595 nm gives a single $pK_a$ at pH 8.50, being approximately 0.40 unit less than that of human or whale myoglobin (12).

**DISCUSSION**

Our ESR results show that the two-state spectral equilibrium of elephant nitrosyl myoglobin resembles that of nitrosyl heme complexes coordinated with unhindered nitrogenous bases (12). Morse (13) has shown that the temperature-dependent ESR spectra arise from a thermal equilibrium between two hexacoordinated complexes separated by only 2-3 kcal/mol. This hypothesis attributes the highly asymmetric ESR spectrum to the displacement of the heme iron toward the proximal histidine. If this mechanism is valid, it suggests that the interaction of NO-glutamine in elephant myoglobin is weak compared to that of the distal histidine, thus enabling the iron displacement to occur only at higher temperatures.

Evidence indicating a weakened NO-glutamine interaction has also been observed from ESR studies using opposum nitrosyl hemoglobin (9, 11). In this hemoglobin, the $\alpha$-chain contains the pentacoordinated nitrosyl heme complex at low pH as judged from the $T$ state ESR spectrum. It has been suggested that a weakened donor-acceptor interaction between the substituted glutamine and the NO ligand contributes to the disruption of the proximal iron bond due to the strong “trans” effect of NO. However, above pH 8, the ESR spectrum of opposum nitrosyl hemoglobin exhibits a hexacoordinate spectrum with the “elephant type” line shape visible in conjunction with the $\beta$-chain spectrum. Because NO preferentially forms a bent complex with the heme iron (14) and is directed away from the distal residue, it is conceivable that the more nucleophilic histidine forms a stronger interaction with the relatively distant NO ligand than does glutamine.

In contrast to the weakened NO-glutamine interaction described above, a strong glutamine-OH$^-$ interaction is evident from the comparatively low $pK_a$ found for the acid-alkaline equilibrium of elephant ferric myoglobin. The glutamine-OH$^-$ interaction may involve hydrogen bonding as illustrated in Fig. 4. For hemoproteins having a distal histidine, the spin state transition, as suggested by Caughey (21), may involve an equilibrium between a weak and strong hydrogen bond from the $N_ε$-hydrogen of the histidine to the ferric bound hydroxyl oxygen. The strength of this hydrogen bond is determined by the environment surrounding the $N_ε$ of histidine, which is exposed to the external solvent. However, with glutamine, little change in the strength of the hydrogen bond to the hydroxyl oxygen is expected by changes in external pH. Therefore, for this myoglobin at low pH, protonation of the hydroxyl ligand must originate from the solvent, accompanied by the breakage of the hydrogen bond from the distal amide. In addition to the proposed stability of the glutamine-OH$^-$ interaction and the requirement that an external proton enter the heme pocket, changes in the heme pocket hydrophobicity and charge distribution near the sixth coordination position (22) may also contribute to the lower $pK_a$ found for the spin state equilibrium of elephant ferric myoglobin.

Perhaps the most significant difference observed between elephant and human myoglobins is the heme redox potential. In elephant myoglobin, it is nearly equal to that of hemoglobin, while the binding of ligands indicates that glutamine replacement does not sterically affect heme accessibility any more than histidine. This conclusion is also supported by a computer fitting of glutamine at the E7 position (4), suggesting that the heme in elephant myoglobin does not experience changes in solvent exposure which could account for the resistance to oxidation.

The question of acid-alkaline differences between oxidized and reduced myoglobins and their linkages to the redox potential has been studied by several investigators (17, 23, 24). In myoglobin, several ionizable linkage groups are evident, including the aqua-ligand ($pK_a$ 9.0). At pH 7.0, the effect of the $pK_a$ shifting from 9.0 in human myoglobin to 8.5 in elephant myoglobin only changes the $H_2O$-OH$^-$ equilibrium by several percent. This change in the acid-alkaline equilibrium, however, would be expected to slightly lower the redox potential as judged from previous pH profile studies (23, 24) instead of increasing the $E_{1/2}$. Alternatively, $pK_a$ shifts in the ionizaion or the substitution of redox linked residues active near neutral pH might also contribute to the unusually high redox potential for elephant myoglobin.

While acid base and minor steric differences may exist between elephant and human myoglobins, the important consideration in regard to the redox potential probably lies in the electronic differences between histidine and glutamine that result in reducing the free energy of the ferric derivative. The free energy difference between the ferric states of elephant and human myoglobins may conceivably result from a greater charge interaction between the nucleophilic imidazole and ferric iron acting through the aqua-ligand. Although the amide group in elephant myoglobin may form significant interactions with the aqua-ligand as illustrated (Fig. 4), the weaker electron

![Fig. 4. The proposed structures for hydroxide bonded to heme in ferric myoglobin having a distal histidine as proposed by Caughey (21) or distal glutamine. The possible protonation site associated with the spin state transition shows a solvent proton acting at the surface of the exposed $N_ε$ of imidazole resulting in the formation of a strong hydrogen bond to the hydroxide ligand. In the case of glutamine, the hydroxide ligand is shown protonated directly from the solvent.](http://www.jbc.org/)

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density of the amide group affords less charge stabilization for the ferric ion. On the other hand, the free energy of deoxy-myoglobin with either glutamine or histidine is expected to be similar since the distance between the iron and distal residues is not bridged by solvent or ligand (14) which can actively transmit polar interactions.

As we have demonstrated, glutamine is a particularly valuable distal substitution for investigating the role of the distal position (E7) in the functional and physicochemical properties of hemoproteins. Unlike hemoglobin, the comparative studies using myoglobin can be interpreted without regard to quaternary effects, as in the case with opposum hemoglobin. Furthermore, in myoglobin, spectral dilution from the complementary chain is absent. Although our previous report (4) shows that elephant myoglobin has similar O₂-binding properties to that of human myoglobin, heme autoxidation was noted to be markedly different. The results presented here suggest the predominant physiological effect of glutamine substitution is in maintenance of the reduced state for the iron. The mechanism by which glutamine replacement protects against heme autoxidation will be examined in the future.

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