The Mechanism of Water Proton Nuclear Magnetic Resonance Relaxation in the Presence of Mammalian and Aplysia Metmyoglobin Fluoride*

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SUMMARY

A mechanism of proton exchange leading to water proton relaxation is proposed for horse heart metmyoglobin fluoride which is mediated by H2O molecules and involves the distal histidine. The mechanism is based on a comparison of water proton NMR relaxation (T1 (H)) in the presence of horse and Aplysia metmyoglobin fluoride; Aplysia metmyoglobin fluoride was chosen for comparison because it lacks the distal histidine. Proton relaxation for horse heart metmyoglobin fluoride has been observed over a pH range of 5.0 to 11.5 in H2O and 95% D2O solutions while simultaneously monitoring the optical absorbance at 542 nm. For horse heart metmyoglobin fluoride, proton relaxation shows three pK values, at pH 6.2, 8, and one at a higher pH, which varies with the fluoride ion concentration due to the equilibrium metmyoglobin fluoride + OH− → MbOH + F−. Optical spectroscopy shows two pK values, one at pH 6.2 and one above 9. The latter pK has a large deuterium isotope effect in the optical spectrum, but none for proton relaxation. Theorell and Ehrenberg (1951) Acta Chem. Scand. 5, 823) reported changes in the magnetic susceptibility of metmyoglobin fluoride with pK values of 6.3 and 8.11; however, the magnetic susceptibility increases when T1 (H) decreases and vice versa. We therefore conclude that the proton-iron distance changes twice during the titration.

In the presence of sufficient fluoride to convert metmyoglobin to metmyoglobin fluoride, the water proton relaxation rate, T1 (H), is enhanced by approximately a factor of 215 over T1 (H) of the aquo complex (1). We will discuss three types of results in this paper. First, the effect of using D2O as a solvent on the titration behavior of the proton relaxation and the absorption at 542 nm. The NMR experiment is sensitive only to protons and therefore can potentially measure the behavior of the protonated ligand, while the optical spectrum measures the behavior of the predominant species, which in 95% D2O is Mb2 with a deuterated ligand. Thus by comparing the NMR and optical pH values in D2O it should be possible to learn something about the groups which are titrated. The second aspect is the comparison of the values of T1 (H) for MbF as a function of pH with the magnetic susceptibility data of Theorell and Ehrenberg (2). From these data we can draw conclusions about the relative proton distance during the course of the titration. Third, we will propose a mechanism for proton exchange in MbF solutions based on a comparison of the T1 (H) behavior of Aplysia Mb, which lacks a distal histidine, with that of horse Mb.

EXPERIMENTAL PROCEDURE

Commercial horse heart myoglobin preparations were used throughout. Measurements were made on Seravac Lots 22L, 50, 52C, and 57A. Lyophilized material was dissolved in buffer and centrifuged. Myoglobin was prepared from the buccal muscle of Aplysia californica as described by Rossi-Fanelli and Antonini (3). trans-1,2-Diaminocyclohexane tetraacetic acid (CDTA) was added to the buffer used for grinding to a concentration of 10−3 M in an attempt to avoid metal contamination. The crude protein was then concentrated and run in 0.02 2-amino-2-methyl-1,3-propanediol at pH 8.7 on a 25-cm column containing 5 cm of DEAE-Sephadex on the top and 20 cm of Sephadex G-50 (fine). Titrations were performed by making a high pH preparation and a low pH preparation and mixing the two to obtain intermediate points. This procedure avoids changes in Mb, F−, and ionic strength due to dilution or addition of reagents. Samples in D2O were prepared by dissolving lyophilized material directly in D2O phosphate buffer. All experiments were carried out in 0.1 M phosphate buffer. Background relaxation (T1 (H) due to the intrinsic relaxation rate of protons in water and to the contribution of the globin or protein part) was determined by the addition of sufficient cyanide to saturate the heme site. The value of T1 (H) in the presence of MbCN was then measured and subtracted from the value of T1 (H) obtained under the same conditions in the absence of cyanide. That this is a valid procedure has been shown by demonstrating that T1 (H) due to MbCN is equal to that due to dinuclear MbO2 or MbCO (4). Using this approach it is possible to separate the paramagnetic contribution, T2ρ (H), from all other contributions to T1 (H).

NMR Measurements—Relaxation times were measured with a coherent pulsed spectrometer used previously (5). Frequencies of 10 and 25 MHz were used, but no frequency dependence of the

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1 C. Simo, W. E. Schillinger, and T. L. Fabry, unpublished manuscript.

2 The abbreviation used is: Mb, metmyoglobin.
RESULTS

Titration Behavior in D_{2}O and H_{2}O—The pH dependence of T_{1}^{-1}(H) and the optical density at 542 nm are shown in Fig. 1. Results are shown for data taken in H_{2}O and 95% D_{2}O. The NMR data have three pK values, at pH 6.2, pH 8, and above pH 9. When the optical data are taken at 542 nm no optical transition is detected at pH 8; however, pK values are observed at 6.1 and above 9. This is in agreement with the data of Theorell and Ehrenberg (2). The data in Fig. 1 were plotted in terms of pH meter readings and the scale for D_{2}O was then shifted 0.4 pH unit on the horizontal axis (6). The graphs were then shifted vertically until the T_{1}^{-1}(H) values of MbCN solutions of the samples used for the titrations in D_{2}O and H_{2}O occurred at the same point. This procedure compensates experimentally for the theoretically anticipated reduction in the background relaxation observed in D_{2}O (7). The resulting overlap of T_{1}^{-1}(H) for MbF in D_{2}O and H_{2}O represents a true equality of T_{1} in the two solvents. Note that the optical density scale is not shifted vertically.

Fluorine NMR Relaxation—^{19}F NMR relaxation was observed for commercial myoglobin samples. The values of molar relaxivity for fluorine, T_{1}^{-1}(F), showed great variation from one commercial lot to the next, while the molar relaxivity for protons remained constant from lot to lot. A titration with sodium azide at pH 5.3 while monitoring T_{1}^{-1}(H), T_{1}^{-1}(F), and absorbance at 542 nm, which is characteristic of the fluoride complex, showed no correlation between fluorine relaxation and T_{1}^{-1}(H) or absorbance, while the latter two quantities were closed parallel, as shown in Fig. 2. A subsequent analysis for copper revealed concentrations ranging from 0.8 μg/100 mg of Mb for Lot 22L to 3.8 μg/100 mg of Mb for Lot 52C, which correlates with the relative magnitude of T_{1}^{-1}(F) observed for each lot. The extreme sensitivity of T_{1}^{-1}(F) to Cu(II) is well known (5), and we attribute the measured relaxation to copper impurities. Removal of Cu(II) led to T_{1} values of the order of background relaxation, so we may place an upper limit on the heme site of 1 x 10^{-9} s^{-1}.

Aplysia Metmyoglobin—The aquo and fluoride complexes of Aplysia metmyoglobin were studied as a function of temperature and pH. For both complexes, only slight decreases in T_{1}^{-1}(H), less than 5% of the total relaxivity, were observed when cyanide or azide was used to saturate the heme site, as determined by visible spectroscopy. This is equivalent to a molar relaxivity of 0.1 ± 0.05 x 10^{-3} M^{-1} s^{-1} for the difference between the acid aquo complex and the cyanide complex of horse metmyoglobin at 25°C. Likewise, the transitions from MbHOH to MbOH and from MbF to MbOH, detected by observation of the visible spectrum, were not accompanied by changes in T_{1}^{-1}(H) as observed for mammalian myoglobins; nor did increasing the temperature to 60°C result in an increase in T_{1}^{-1} for either complex. In contrast, increasing the temperature to 40°C nearly doubles the relaxivity of the mammalian acid aquo complex. We therefore conclude that there is no relaxation attributable to protons in the first coordination sphere of the heme iron. It is possible that the
small decrease in $T_{1P}^{-1}$ (H) observed on the addition of cyanide or azide is due to outer sphere relaxation.

**Discussion**

Theory and Preliminary Observations—The paramagnetic contribution to the relaxation rate, $T_{1P}$, is related to the lifetime in the complex, $\tau_M$, and the relaxation time in the complex, $T_{1M}$, by the formula (8)

$$T_{1P}^{-1} = \frac{P_M}{\tau_M + T_{1M}}$$

where $P_M$ is the ratio of the number of proton sites in the complex to sites in solution. As a system approaches the NMR fast exchange limit, $\tau_M$ becomes smaller and $T_{1P}$ increases until it reaches the limiting value of $P_M/\tau_M$. Since $\tau_M$, the lifetime in the complex, usually decreases as the temperature increases, while $T_{1M}$ is constant or increases inversely with viscosity, we regard the absence of temperature dependence in our data as evidence of NMR fast exchange (9).

The formula used for calculating $T_{1M}^{-1}$ (H), the relaxation rate in the complex, is (10, 11)

$$T_{1M}^{-1}(H) = \frac{2}{15} \frac{h}{\mu_{eff}^2} \beta^2 \rho \left( \frac{\gamma H}{\gamma^2} \right)^2 \left( \frac{\tau c}{\gamma} \right)$$

where $\mu_{eff}$ is the effective magnetic moment calculated from $\chi_M$; the magnetic susceptibility, $\tilde{H}$, $\gamma$, and $\beta$ are physical constants in the appropriate units listed under Table 1; $r$ is the Fe(III) to proton distance in angstroms; and $\tau_c$ is the correlation time. If reasonable values of $\tau_c$ and $\chi_M$ (see Table 1), and $r$ (2.83 to 3.35 Å for H-bonded Fe–F complexes (1)) are substituted into Equation 2, then $T_{1M}$ calculated for MbF is equal to the value actually observed for $T_{1M}$. If the system were in NMR slow exchange, the observed value would be less than the calculated value by the amount predicted from Equation 1. This is further evidence that the protons in MbF solutions are in NMR fast exchange (9). A similar conclusion has been drawn by Mldvan et al. (1). If changes in $T_{1P}^{-1}$ (H) do not reflect changes in the kinetic rate of proton exchange with the paramagnetic center, then they reflect changes in either the magnetic susceptibility of the complex or changes in the Fe(III) to proton distance.

$T_{1P}^{-1}$ is calculated from the experimental results by subtracting out the contribution of the solvent water and the diamagnetic contribution of the protein to the measured $T_{1P}$ as described under “Experimental Procedure.” To evaluate $T_{1M}$, when proton relaxation is measured in water, the appropriate value to use for the “sites in solution” is simply two times 55.5 m, the concentration of protons in pure water. For the number of sites in the complex, we assume there is one proton site per myoglobin fluoride molecule. Due to the small size of the heme cavity, the only other likely possibility is that there may be two sites, in the case of “outer sphere” relaxation via a water molecule in the heme cavity. Outer sphere relaxation is also a fast exchange phenomenon and therefore one must distinguish between inner and outer sphere fast exchange by some means other than temperature dependence. One can, however, eliminate the second mechanism by comparing MbF with MbHOH relaxation, since the products of the magnetic susceptibility (2) and the correlation time are about the same. The temperature dependence of MbHOH (4) indicates that protons in MbHOH are in NMR slow exchange, in contrast to protons in MbF solutions. It seems reasonable to assume that the same outer sphere mechanism would be available to MbHOH as is available to MbF. If the relaxation by MbF were completely outer sphere, we would expect MbHOH to have the greater relaxivity, since it would have both an outer sphere and a kinetically limited contribution. Since it is smaller, we conclude that the single site model is valid.

**Titration Behavior in D$_2$O and H$_2$O**—When the NMR data in D$_2$O are compared to the data in H$_2$O, the most striking feature is how little the pK values change, and how little change occurs in the magnitude of $T_{1P}^{-1}$ (H) after the background is compensated for. In the MbF portion of the titration, the lack of change in $T_{1P}^{-1}$ (H) indicates that no substantial changes have occurred in the geometry or magnetic susceptibility of the heme site when Mb is dissolved in D$_2$O. When the optical spectrum is observed in D$_2$O, the pK of the low pH titration (6.2) does not change appreciably, but the pK of the high pH titration (above 9) is shifted to a much higher pH. Table II lists the NMR and optical pK values for two different fluoride concentrations.

Qualitatively, it is clear that the high pH titration represents a transition from MbF to MbOH. Both $T_{1P}^{-1}$ (H) and the optical spectrum change from values characteristic of the fluoride complex to values characteristic of the hydroxide complex (1). Quantitatively, the value of pK$_{spp}$, the pH at which the conversion from MbF to MbOH is half-complete, also increases when the fluoride concentration is increased, as expected for a simple displacement of fluoride by hydroxide. Calculated values of pK$_{spp}$ are presented in Table II. We will now consider the following model of the events occurring at the heme site.

1. To account for the magnitude of the relaxivity such a hypothetical water site would have to be occupied at all times and the oxygen of the water, if it were present, should be visible by x-ray crystallography.

2. It can be seen from Equation 1 that these are the only variables that need to be considered. $\mu_{eff}$ is related to $\chi_M$ by $\mu_{eff} = 2.85 (\chi_M, T)^{1/2}$ and since it is squared in Equation 1, $\chi_M$ enters linearly. For MbHOH $\chi_M$ is (33.3 X $10^{-4}$) (2.77 X $10^{-16}$) or 3.68 X $10^{-12}$, while for MbF $\chi_M$ is (14.4 X $10^{-4}$) (2.17 X $10^{-16}$) or 3.13 X $10^{-12}$. The values used for $\tau_c$ were obtained from EPR and NMR dispersion. The paramagnetic contribution to the molar relaxivity, $T_{1P}^{-1}$, at 25°C is 0.8 X $10^{-12}$ s m$^{-1}$ for the mammalian acid aquo complex and is 2.1 X $10^{-12}$ s m$^{-1}$ for the mammalian fluoride complex.

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**Table I**

| pH Range | $\chi_M$ | $T_{1P}$ | $r^2$ |
|----------|----------|----------|-------|
| Below pH 6 | | | |
| Between pH 6 and pH 8 | | | |
| Above pH 8 | | | |

*a From Theorell et al. (2).
*b Calculated for 1 mM MbF.
*c Calculated using Equation 1 with $r = 2.0 \times 10^{-10}$ m, $\gamma = 8.81 \times 10^4$ rad/gauss-s, $(\gamma H)^{-1} A^3 = 7.95 \times 10^6$ gauss, $\mu_{eff} = 2.83 (\chi_M, T)^{1/2}$ BM, where $T$ is in degrees Kelvin.

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3.2
3.0
3.2

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The assumption of H bonding to the distal histidine is consistent with the x-ray structure (12). When $T_{1}^{-1}(H)$ is observed, the $pK$ for the transition from the fluoride to the hydroxide complex is about 0.4 pH unit lower than the optical $pK$ (see Table II). The lack of deuteron isotopic effect also results in a decrease of electron density at the iron (10), which could account for the increase in $K_F$ which accompanies the pH 6.2 ionization (14, 15). It is possible that the group with a $pK$ of 8 is the $N$ nitrogen of the distal histidine.

It is interesting to note that the observed pH dependence of the relaxation implies that the fluorine in myoglobin is protonated up to pH 10 and above, in contrast to HF which has a $pK$ of 2.9. However, this is not surprising if one considers this proton as participating in a complex somewhat analogous to a metal chelate complex, that is, Fe(III)-F-distal histidine. Then a conservative estimate (16) of the equilibrium constant for the dissociation of the proton from the complex is $K_{HF}$; if one chooses values of $pK_{HF} = 2.9$ and $pK_{His} = 6.5$ and 7.5 this would result in a $pK$ for the H-bonded proton between 9.5 and 10.5.

Dependence of $T_{1}^{-1}(H)$ and $\chi_M$, and the Fe(III)-Proton Distance—The second aspect of the pH dependence of MbF to be discussed is the origin of the two small changes in $T_{1}^{-1}(H)$ at pH 6.2 and pH 8, and their relation to the magnetic susceptibility of myoglobin fluoride measured by Theorell and Ehrenberg (2), who also found two transitions at pH 6.03 and pH 8.11, in addition to the major one from MbF and MbOH. Equation 2 shows that these transitions could be due to either changes in $\chi_M$, the magnetic susceptibility, or in $r$, the iron-proton distance.

Table II

Titrations of metmyoglobin fluoride in H₂O and D₂O

| pH | $pK_{obs}$ (H₂O) | $pK_{obs}$ (D₂O) | $pK_{obs}$ (D₂O) |
|----|-----------------|-----------------|-----------------|
| 6.2 | 9.89 ± 0.1 | 10.2 ± 0.1 | 10.4 ± 0.1 |
| 8.0 | 10.25 ± 0.1 | 10.0 ± 0.1 | 10.1 ± 0.1 |

* $pK_{app}$ is the pH at which the titration MbF $\leftrightarrow$ MbOH is half-complete for a given fluoride concentration. It is calculated using the equation

$$H^{+} \text{ at } pK_{app} = (K_F/K_F + (F^-))K_F$$

where $K_F$ is $1.26 \times 10^{-4}$ moles per liter corresponding to a $pK$ of 8.9 for the acid-alkaline transition.

* $pK_{app}$ calculated for $K_F = 0.023$ (14).

* $pK_{app}$ calculated for $K_F = 0.01$ (15).†

* Average of several measurements, the $pK_{obs}$ (D₂O) corresponds to the pH meter reading at the midpoint of the titration $+0.4$ pH unit as discussed in the text.
(H) decreases, and vice versa, contrary to what would be expected from Equation 2 if $r_e$ and $r$ do not change appreciably. It seems reasonable to assume that changes in $r_e$ large enough to account for the observed results (nearly 50% of $T_1$ in the pK 6.2 transition) do not occur. A value of $2 \times 10^{-10}$ s was used in the calculation for $r_e$; values of $2.2 \times 10^{-10}$ and $1.7 \times 10^{-10}$ s were measured by EPR and NMR dispersion (4), respectively.

This is the value of the electron relaxation time, $T_1$; it is the relevant quantity, since the tumbling time of the protein and the lifetime of a nucleus at the heme site are much longer. The absolute in the iron-proton distance. These distances were calculated using Equation 2 and are presented in Table 1. The absolute values of $r$ are of course affected by the choice of $r_e$, the accuracy of the measurements of $T_1$ (H) and $\chi_m$, as well as the approximations involved in the theory from which $T_{1M}$ is calculated. The relative values of $r$ are not as subject to these uncertainties, however, and indicate the potential sensitivity of proton relaxation to structural changes. The actual numbers obtained are in the range (2.85 to 3.35 Å) determined by x-ray crystallography for H-bonded metal fluoride complexes, rather than in the range for covalently bonded metal fluoride complexes (2.68 to 2.74 Å) (1).

**Mechanism of Proton Exchange**—There are two major justifications for the assertion that the proton exchange observed in MbF solutions is due to a single proton bonded to the fluorine. The first is the separation of the optical and NMR pK values observed in D$_2$O, as discussed under the section on titration behavior in D$_2$O and H$_2$O. The second is the argument presented under the section on theory and preliminary observations based on a comparison of the magnitude of $T_1$ in MbF and MbHOH solutions.

Since the proton relaxation due to MbF is in the NMR fast exchange region we can use Equation 1 and the data in Table 1 to place a lower limit of about $2 \times 10^8$ s$^{-1}$ on the exchange rate. From the study of fluorine relaxation we can put an upper limit on the rate of fluorine exchange at $1 \times 10^8$ s$^{-1}$. Since the rate of proton exchange is so different from that of fluorine exchange, it is clear that exchange of HF does not occur; this conclusion is in agreement with that of Mildvan et al. (1). At pH 7 and 8, the proton concentration is so low that to observe an exchange rate of $2 \times 10^9$ s$^{-1}$, a kinetic rate constant of $2 \times 10^9$ s$^{-1}$ or greater would be required, exceeding even diffusion limited rate constants. It is thus improbable that we are observing simple proton exchange. We would take this fact into account when preparing the solution.

We can reconcile these facts by postulating that (a) the species entering the heme cavity is an intact water molecule which either ionizes before reaching the iron and recombines before leaving, or (b) that the water molecule exchanges its protons in a concerted reaction with the proton bound to fluorine. A mechanism similar to Case b has been proposed by Igenfrizt and Schuster (17) for the transition from MbHOH to MbOH observed by T-jump.

Proton relaxation was also investigated in solutions of Aplysia metmyoglobin for both the acid and alkaline aquo complex and the fluoride complex. Aplysia myoglobin lacks the distal histidine and yet the visible spectrum of the complexes studied are quite similar to the spectra of mammalian myoglobins. No paramagnetic contribution was observed for any of these complexes, even at high temperatures. There are two possible explanations: (a) there are no protons to exchange, and (b) the exchange is too slow to be observed. One might accept Case a for MbF; however, in view of the spectral similarities between Aplysia and mammalian myoglobins, it is an unlikely explanation for the aquo complexes. Case b suggests that the distal histidine plays a role in catalyzing proton exchange or ionization.

To summarize, we believe that proton relaxation in MbF solutions is a case where the primary contribution to relaxation may be attributed to a single site and yet the exchange with the solution is mediated by intact water molecules, not protons or hydronium ions. Furthermore, the exchange between the site and the water molecules is catalyzed by the distal histidine.

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