Magnetic Field and Temperature Induced Line Broadening in the Hyperfine-Shifted Proton Resonances of Myoglobin and Hemoglobin[1]

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Abstract: The nuclear magnetic resonance line widths of the hyperfine shifted proton resonances of deoxymyoglobin, deoxyhemoglobin, and azidometmyoglobin have been observed to show a strong increase upon decreasing the temperature or increasing the applied magnetic field, whereas those of cyanometmyoglobin exhibit no field-induced broadening and only a mild temperature dependence. The relaxation mechanism producing these effects appears to be a modulation, by rotational diffusion, of the dipole-dipole interaction between the heme methyl proton nuclear spins and the equilibrium magnetization of the heme iron electronic spin system. The longitudinal electron relaxation time for deoxymyoglobin and deoxyhemoglobin can be estimated from the magnetic field dependence of the line widths of the hyperfine shifted proton resonances. Approximate rotational correlation times for these two molecules are also calculated at various temperatures. The present study demonstrates that a knowledge of the frequency and temperature dependence of the line width is an essential element in relating line width measurements to the properties of systems containing paramagnetic centers.

In deoxyhemoglobin (Hb) there are several proton nuclear magnetic resonances (NMR) located in the region −11 to −23 ppm downfield from the proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (or approximately −6 to −18 ppm downfield from the proton resonance of the residual water at room temperature), well below the normal aromatic proton resonance envelope. These lines have been assigned to the heme protons and/or to the protons of the amino acid residues in the vicinity of the heme groups.[3] They are shifted downfield because of contact and pseudocontact interactions with the unpaired electrons of the iron atom[4-6] and hence are called hyperfine-shifted (hfs) proton resonances. During a recent study of the ligation process in Hb, it was observed that the intrinsic line widths of these resonances were strongly field dependent, increasing from ~100 Hz at a proton frequency of 90 MHz to ~270 Hz at 250 MHz in the absence of oxygen.[7,8]

Considering here the field dependence of the intrinsic deoxy-Hb line width, there are several possible mechanisms which could produce such an effect. An inequivalence between the heme groups of the two β chains or the two α chains in the Hb tetramer, reduced methyl group rotation rates of the heme methyl groups which produce the major hfs resonances, or a dipolar interaction with the electron spin initially all appeared to be plausible mechanisms for the production of this field dependence.

More recently, however, a similar field dependence of the hfs proton line widths has been observed in myoglobin (Mb) from the mollusc *Aplysia limacina*.[9] Since Mb has only a single peptide chain and a single heme group per molecule, this result would appear to rule out the possibility of chain inequivalence as a mechanism for the field dependence. An interpretation of this field dependence in terms of relaxation induced by rotational modulation of the field due to the iron electronic spin polarization has also recently been proposed by Gueron[10] and by Vega and Fiat.[11] This communication reports the results of more detailed studies on the magnetic field and temperature dependence of the hfs proton resonance line widths in deoxygenated Mb and Hb and provides a partial test of the theory proposed by Gueron and by Vega and Fiat. The terminology and notation used in this paper will follow the work of Gueron.[10]

Experimental Section

Sperm whale myoglobin was purchased from Sigma. For use in ferric form, the myoglobin was dissolved in a solution with a twofold excess of potassium ferricyanide to ensure complete oxidation of the heme iron. After oxidation, both potassium ferricyanide and ferrocyanide were removed by passage through a Sephadex G-25 column. H_2O was exchanged with D_2O by repeated ultrafiltration of the Mb solution with phosphate buffered D_2O; residual H_2O concentration was 4-6%. Azidometmyoglobin ($MetMbN_3$) was prepared by adding NaN_3 in phosphate buffer to give a final concentration for the sample of 0.5 M NaN_3 in 0.1 M phosphate buffer at pD 7.0. The pD of D_2O solutions was obtained by adding 0.4 pH units to the pH meter reading.[12] Cyanometmyoglobin (MetMbCN) was prepared by adding a fourfold excess of KCN to the sample in 0.1 M phosphate buffer at pD 7.0. For use in the ferrous form, Mb was dissolved in 0.1 M phosphate buffer and sodium dithionite (Mannox Brand, Holdman and Hardman, Miles Plotting, Manchester, England) added in the presence of a CO atmosphere to reduce the heme iron to the ferrous state. Excess dithionite was removed by passage over a CO-gassed Sephadex G-25 column. The solution was then concentrated and H_2O was exchanged with phosphate buffered D_2O (pD 7.0) by ultrafiltration. CO was replaced with O_2 by flushing the solution in a rotary evaporator in an ice-water bath under a Sylvania 150-W flood lamp. Oxygen was removed by flushing with purified nitrogen. Just before running the NMR spectra, a small amount of sodium dithionite was added to ensure that the Mb sample was completely in the ferrous deoxy state. Final protein concentration for both met- and deoxy-Mb samples was approximately 10-14%.

Normal human adult hemoglobin (Hb A) was prepared from fresh whole blood samples obtained from the local blood bank by the standard procedure used in this laboratory.[13] Deoxyhemoglobin solutions were prepared in a 0.1 M buffer solution of [bis(2-hydroxyethyl)-imino]tris(hydroxymethyl)methane (Bis-Tris) containing 10 mM inositol hexaphosphate (Ins-P_6) (Sigma) at pD 7.0 with protein concentration 12-15% by methods described previously.[7]

^1H NMR spectra were obtained on Varian T-60, Bruker HFX-90, Varian HR-100, Varian HR-220, and Bruker HX-360 spectrometers operating in the Fourier transform mode and on the MPC-HF 250-MHz spectrometer using the NMR correlation spectroscopy technique developed by Dadok and Sprecher.[14] Uncertainty in the line width measurements is estimated to be 5-10% of the measured width, depending on the baseline and the separation of the resonance in question from other resonances. Chemical shifts are measured with respect to the residual water (HDO) signal and then calculated with respect to DSS. For example, the proton chemical shift of H_2O is 4.83 ppm downfield from the proton signal of DSS at 27 °C.

Results and Discussion

According to the theory proposed by Gueron,[10] there will be a net polarization of the iron electronic spin magnetic mo-

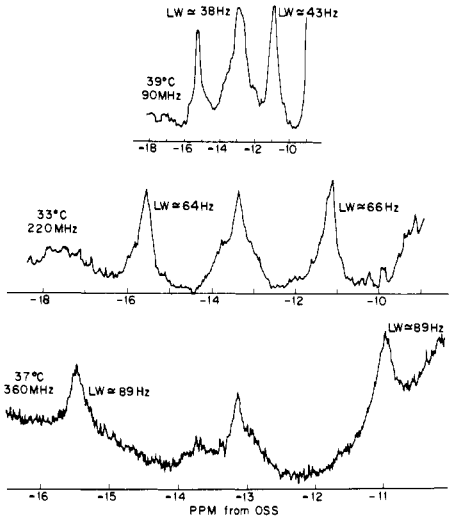

Figure 1. Hyperfine-shifted proton resonance region of deoxymyoglobin at the three frequencies 90, 220, and 360 MHz. Temperatures as shown are approximately equivalent. Samples are at pD 7.0 in 0.1 M phosphate buffer. The horizontal frequency scales are equal for the three spectra. LW represents the full line width at half-height of the resonance under consideration.

ment which will be oriented along the direction of the magnetic field. Modulation of this dipolar field due to the spin polarization (known as the "Curie spin") by rotational diffusion will then introduce an extra field-dependent term into the expression for transverse relaxation, giving

$$1/T_2 = {}^4/_5\Delta^2 S_c^2 \tau_R + {}^7/_{15}\Delta^2 S(S+1)T_{1e} + 1/T_2^{\text{diamag}} \quad (1)$$

where $\Delta = \gamma_1 g\beta/r^3$, $S_c = g\beta S(S+1)H_0/3kT$, γ_1 is the proton gyromagnetic ratio, g is the electronic g factor, β is the Bohr magneton $eh/2mc$, S is the electronic spin, τ_R is the rotational correlation time of the molecule, T_{1e} is the longitudinal electron spin relaxation time,[10] and T_2^{diamag} is the diamagnetic contribution to the transverse relaxation (M. Gueron, private communication). The first term in eq 1 is the Curie spin contribution, and the second term is the standard dipolar term. From this expression we would expect the Curie spin line width contribution to show a field dependence of H_0^2 and a temperature dependence of τ_R/T^2. For Brownian diffusion the rotational correlation time can be expressed as

$$\tau_R = (4\pi a^3/3kT)\eta \quad (2)$$

where a is the molecular radius and η is the solvent viscosity. This then gives a temperature dependence of η/T^3 for the Curie spin term. In the standard dipolar term, T_{1e} would be expected to show a much smaller temperature and field dependence than τ_R, thus making this term essentially field and temperature independent in comparison to the Curie spin term.

Deoxymyoglobin. The hfs proton resonances of deoxy-Mb at proton frequencies of 90, 220, and 360 MHz are shown in Figure 1. From these spectra it is quite clear that the line widths exhibit a substantial field dependence. The temperature dependence of the line widths at 360 MHz is shown in the spectra of Figure 2 where it can be seen that the lines broaden substantially as the temperature is decreased. The temperature dependence of the line widths at the three frequencies is shown in more detail in Figure 3. From this figure, it can be seen that there is a somewhat stronger temperature dependence than the η/T^3 predicted by the Curie spin relaxation theory.[10] This may, however, be due to the protein concentration being sufficiently high that protein-protein interactions are also affecting the rotational correlation time (i.e., an increase in the effective viscosity). (For a discussion of the effect of protein concentration on the viscosity, see the caption of Figure 3.) Support for this hypothesis is provided by the observation that going from a 13% deoxy-Mb solution to a 6% solution at 250 MHz gives a 10-Hz decrease in line width (data not shown).

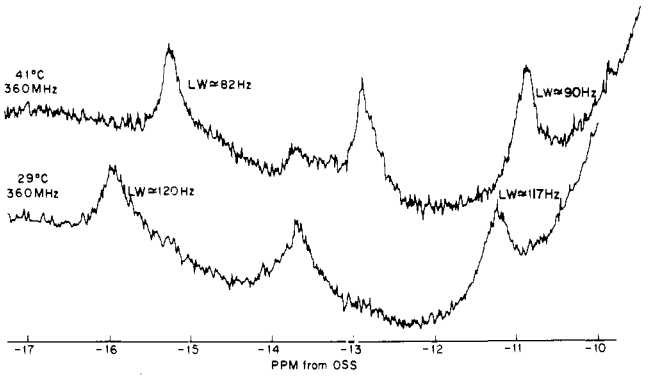

Figure 2. 360-MHz ^{1}H NMR spectra of deoxymyoglobin at 29 and 41 °C. Samples are in 0.1 M phosphate buffer at pD 7.0 with the chemical shift referenced to DSS.

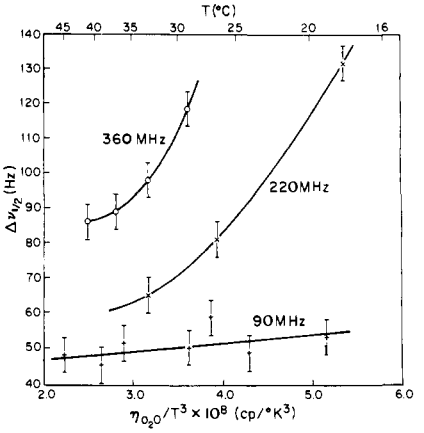

Figure 3. Temperature dependence of deoxymyoglobin line widths at 90, 220, and 360 MHz. At all fields and temperatures the proton resonances at approximately −15 and −11 ppm exhibit nearly identical line widths. Thus, the mean of these two values is plotted here to decrease the statistical uncertainty in single measurements. Estimated accuracy in the line width measurements is approximately ±5 Hz with an estimated uncertainty of ±2 °C in the temperature measurement. The scatter in the 90-MHz data is apparently due to a baseline problem. The viscosity is that of D_2O as calculated from the equation given by Hardy and Cottington.[26] It might be argued that the viscosity of the protein solution would be a more appropriate choice for η than that of the solvent. However, the variable of interest for this study is the effective (microscopic) viscosity as experienced by the Mb molecules in solution rather than the macroscopic (solution) viscosity of the protein solution as a whole. Thus the solvent viscosity is probably a more appropriate choice for η, although it should be realized that the high protein concentrations required for this study will also undoubtedly introduce significant perturbations to the microscopic solvent viscosity in the vicinity of the Mb molecules.

By picking specific temperatures and obtaining line width values from the curves in Figure 3 at these temperatures, the line width at constant temperature can be obtained as a function of frequency (ν_0). This frequency (or field) dependence is shown at the three temperatures 29, 33, and 37° in Figure 4. As predicted by the theory, the line width exhibits a ν_0^2 (or H_0^2) field dependence to an accuracy well within experimental uncertainty. Likewise, there is a zero-field intercept of approximately 45 Hz, with all three curves giving approximately the same intercept, as would be expected. The significance of this value will be discussed below in conjunction with the value derived from the hemoglobin measurements.

The slopes of the curves in Figure 4 can be used to calculate an effective correlation time from the first term in eq 1 as

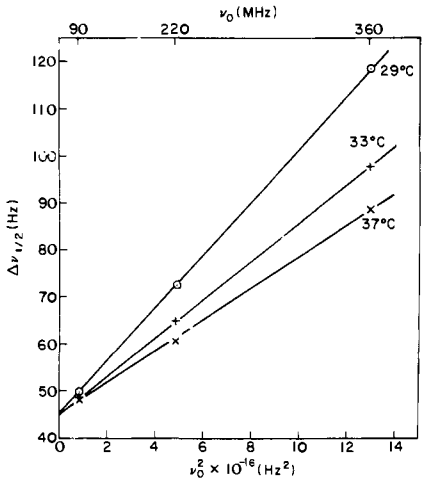

Figure 4. Field dependence of the deoxymyoglobin line width at constant temperature. Values are taken from the curves in Figure 3 at the three temperatures shown here. Error bars are omitted due to overlap; probable errors in the line width are approximately ±5 Hz.

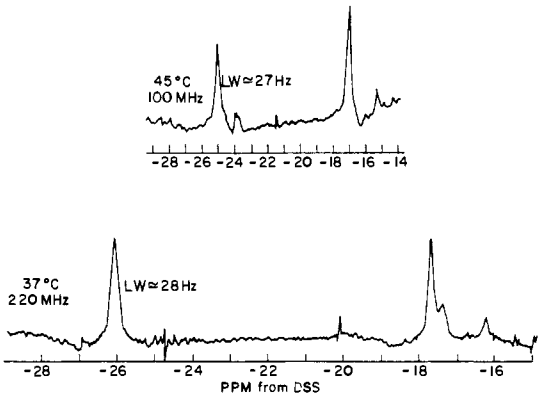

Figure 5. Hyperfine-shifted proton resonances of cyanometmyoglobin at two frequencies, 100 and 220 MHz. The temperatures are 45 °C at 100 MHz and 37 °C at 220 MHz. Samples are at pD 7.0 in 0.1 M phosphate buffer. The horizontal frequency scales are approximately equal between the two spectra. Exponential multiplication of the free induction decay before Fourier transform has introduced 5-Hz line broadening into the 100-MHz spectrmm and 4 Hz into the 220-MHz spectrum. The values, shown here and in Table I are corrected for this broadening.

$$M = 4\gamma_1^2 g^4 \beta^4 S^2 (S + 1)^2 \tau_R / 45\pi k^2 T^2 r^6 \qquad (3)$$

where M is the slope in Hz G^{-2}. Assuming that the observed resonances are due to the heme methyl protons,[15] the distance between the heme iron and the methyl protons is estimated to be 6 Å.[10,16] The product $g^2\beta^2 S(S + 1)$ can be replaced by μ^2 where is the measured magnetic moment of 5.4β.[10,17] From these values we can calculate τ_R from eq 3; the values are $\tau_R = 4.3 \times 10^{-9}$ s at 37 °C, 4.9×10^{-9} s at 33 °C, and 6.5×10^{-9} s at 29 °C. The magnitude of these values is approximately that expected from the Stokes relation, i.e., eq 2, for a molecule the size of myoglobin. This then again suggests that the field-dependent portion of the line width is indeed dictated by the correlation time. The somewhat strong temperature dependence, however, suggests that the correlation time itself is affected by protein concentration in addition to solvent viscosity and temperature. Unfortunately, due to spectrometer sensitivity limitations, it is difficult to study this effect at concentrations below ~10-12%. Thus, for consistency, a concentration of ~12% was used in the Mb studies.

Cyanometmyoglobin. A useful test of the Curie spin relaxation formalism proposed by Gueron[10] is MetMbCN which is in the low-spin ferric state ($S = \frac{1}{2}$). Since the Curie spin term in eq 1 has a spin dependence of $S^2(S + 1)^2$, we would expect the field dependence of MetMbCN to be reduced by a factor of approximately 64 when compared to the high-spin ferrous state ($S = 2$) of the deoxy derivative. As can be seen from the spectra in Figure 5, the line widths of the low-field resonance at 100 and 220 MHz appear to be virtually identical. The actual values of the line widths of the −26 ppm resonance at various frequencies and temperatures are compared in more detail in Table I. It can be seen that within experimental accuracy, the line widths of MetMbCN exhibit no field dependence. The calculated Curie spin broadening from eq 1 would be less than 1 Hz, indicating that the experimental observations are in good agreement with theory. The line widths at 220 MHz exhibit a mild temperature dependence, increasing approximately 15 Hz in going from 37 to 18 °C. At 90 MHz there is an increase of approximately 5 Hz in going from 45 to 9 °C. One could perhaps try to relate this variation to a temperature dependence of the electron relaxation time for this derivative. However, considering that the line width measurement itself is only accurate to ~5 Hz, such a discussion is of questionable value.

Azidometmyoglobin. Another application of interest in evaluating this theory is the azide derivative of met-Mb. Although nominally in the low spin state, the MetMbN$_3$ system actually appears to be a mixture of the low and high spin states ($S = \frac{1}{2}$ and $S = \frac{5}{2}$), with approximately 21% in the high spin state at 33 °C.[18] Thus, it would be expected that the hyperfine-shifted proton resonances of MetMbN$_3$ would also exhibit a strong field dependence. And, as can be seen from the spectra of Figure 6, these resonances do indeed show a marked field

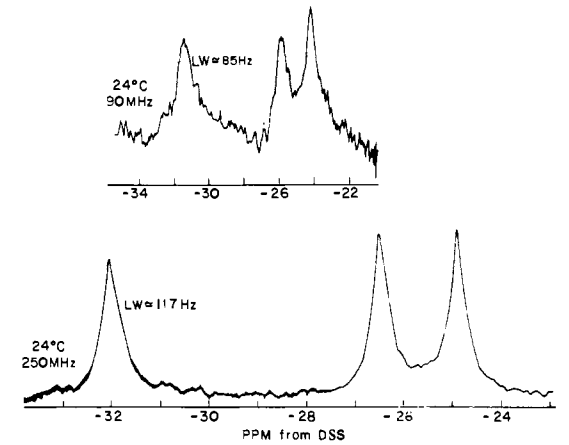

Figure 6. Hyperfine-shifted proton resonances of azidometmyoglobin at 90 and 250 MHz and at 24 °C. Samples are at pD 7.0 and 0.1 M phosphate buffer with 0.5 M NaN_3. The horizontal frequency scales are approximately equal between the two spectra. The 90-MHz spectrum has a 5-Hz additional increment to the line width due to exponential multiplication of the free induction decay before Fourier transformation. The values quoted are corrected for this broadening.

Table I. Line Width Measurements of the Cyanometmyoglobin Resonance at −26 ppm[a]

ν_0, MHz[b]	T, °C[c]	$\Delta\nu_{1/2}$, Hz[d]
60	35	28
100	32	28
100	45	27
220	37	28

[a] This resonance was chosen as a reference due to the fact that it is well separated from other resonances and an accurate baseline can thus be obtained. [b] ν_0 is the resonance frequency of the spectrometer. [c] T is the temperature of the measurement. [d] $\Delta\nu_{1/2}$ is the full resonance line width at half-height.

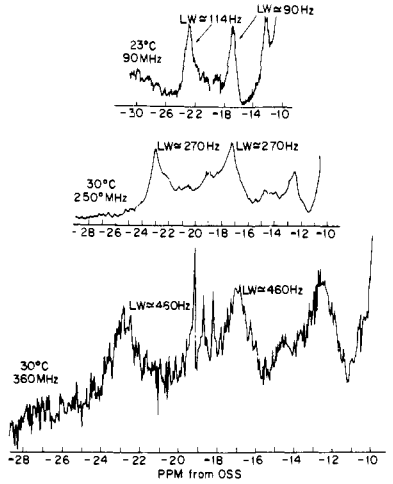

Figure 7. Hyperfine-shifted proton resonances of deoxyhemoglobin at 90, 250, and 360 MHz. Temperatures are approximately equal for the three frequencies. Samples are 14% concentration at pD 7.0 in 0.1 M Bis-Tris with 10 mM inositol hexaphosphate. The horizontal frequency scales are approximately equal for the three spectra. From previous work the resonances at −23 and −17 ppm from DSS have been respectively assigned to heme methyls of the β and α chains.[3,20] Line width values shown are corrected for the broadening added in the exponential multiplication of the free induction decay before Fourier transformation (90 and 360 MHz). The spikes near the center of the 360-MHz spectrum are due to receiver nonlinearities which permit off-frequency "ringing" from residual water in the Fourier transform mode.

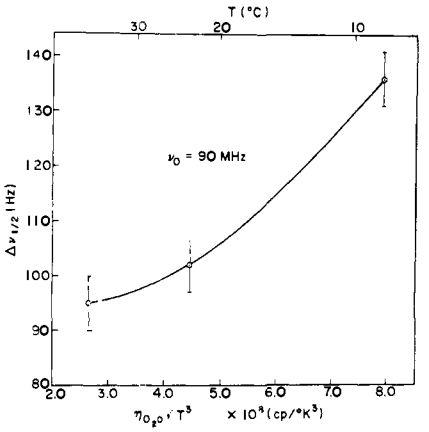

Figure 8. Temperature dependence of the deoxyhemoglobin line width at 90 MHz. The line widths of the α and β resonances have been observed to be approximately equal under all experimental conditions in deoxyhemoglobin; thus they have been averaged to reduce statistical uncertainty; the average values are plotted here. The viscosity is that of D_2O as calculated from the equations given by Hardy and Cottington.[26] See caption to Figure 3 for comments regarding the choice of η_{D_2O} for the viscosity.

dependence. The farthest downfield resonance, at −32 ppm from DSS, for example, increases in width from ~80 Hz at 90 MHz to ~117 Hz at 250 MHz. Likewise, even at 90 MHz, the line width of this resonance shows a significant temperature dependence, increasing from ~75 Hz at 39 °C to ~100 Hz at 8 °C. An analysis of line widths in this system will undoubtedly prove of interest for understanding spin state equilibrium and electronic relaxation in the two spin states. However, in addition to the Curie spin contribution, the line width will also be a function of the spin equilibrium, the spin exchange rate (both of which are temperature dependent), and the separation between the hfs resonance positions in the high and low spin states. Such an analysis is beyond the scope of the present communication.

Deoxyhemoglobin. Turning to the protein of our original interest, we observe from the spectra in Figure 7 that the line

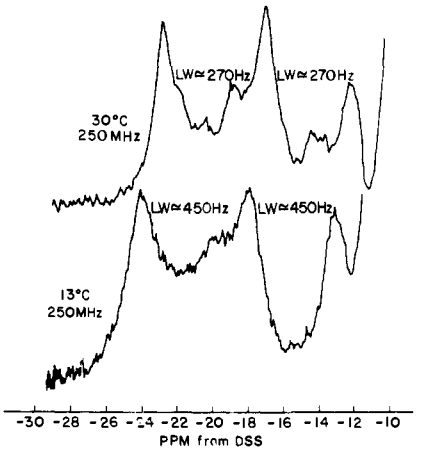

Figure 9. 250-MHz hyperfine shifted proton resonance spectra of 15% deoxyhemoglobin at 30 and 13 °C. Sample is in 0.1 M Bis-Tris buffer and 10 mM inositol hexaphosphate at pD 7.0. Chemical shift is referenced to DSS. The line width can be seen to increase by ~180 Hz in decreasing the temperature.

widths of the hfs proton resonances in deoxy-Hb exhibit an even stronger magnetic field dependence than in deoxy-Mb. In this figure the line widths of the two major resonances at −23 and −17 ppm from DSS increase by approximately a factor of 4 in going from 90 to 360 MHz. There is also a substantial temperature dependence even at 90 MHz as is shown in Figure 8, where the line width increases by approximately 40 Hz in going from 39 to 8 °C. Again, as was observed for deoxy-Mb, the rate of line width increase is slightly faster than the η/T^3 rate predicted by the theory. (For a discussion of the effect of protein concentration on the viscosity, see the caption to Figure 3.) At 250 MHz the increase in line width as the temperature is lowered is substantially larger than at 90 MHz, as can be seen from the two spectra of Figure 9, where the line width increases by ~180 Hz in going from 30 to 13 °C. The field dependence of the line width at constant temperature is shown in Figure 10. The zero-field intercept for the residual line width is approximately 75 Hz, with the intercept essentially independent of temperature as was previously observed for deoxy-Mb. This value of 75 Hz is nearly twice as large as was observed for deoxy-Mb.

The zero-field line width is the result of the second and third terms in eq 1, or

$$\Delta\nu_{1/2}{}^0 = {}^7/_{15}\frac{\gamma_1{}^2 g^2\beta^2 S(S+1)T_{1e}}{\pi r^6} + \Delta\nu_{1/2}{}^{\text{diamag}} \qquad (4)$$

where $\Delta\nu_{1/2}{}^{\text{diamag}} = 1/\pi T_2{}^{\text{diamag}}$. The most plausible source for different zero-field line widths between Mb and Hb would appear to be incomplete averaging of the heme methyl proton-proton dipolar interaction through methyl group rotation. The remainder would be modulated only by the protein rotation and would thus make $\Delta\nu_{1/2}{}^{\text{diamag}}$ proportional to τ_R. Since the Hb correlation time has been calculated as being approximately four times that of Mb, the pair of simultaneous equations (4) for deoxy-Mb and Hb may be solved to obtain the relative contributions of the two terms in eq 4 to the zero-field line widths of Mb and Hb. The results are shown in the first two lines of Table II. The values calculated for the diamagnetic component through this approach appear to be quite reasonable in comparison to the values found for methyl groups in diamagnetic Hb species. For example, the γ_1CH_3 of $E11$ valine resonance of HbCO exhibits a line width of 40-45 Hz.[19]

The decomposition of the zero-field line width into paramagnetic and diamagnetic components in Table II now also permits a calculation of the electronic relaxation time for

Table II. Decomposition of the Zero-Field Line Width into Paramagnetic and Diamagnetic Components

Protein	τ_R, s[a]	$\Delta\nu_{1/2}^0$, Hz[c]	$\Delta\nu_{1/2}^{0,\text{electron}}$, Hz[c]	$\Delta\nu_{1/2}^{\text{diamag}}$, Hz[c]	S	T_{1e}, s[d]
Deoxy-Hb	25×10^{-9}	75	35	40	2	6.1×10^{-12}
Deoxy-Mb	$5\text{-}6 \times 10^{-9}$	45	35	10	2	6.1×10^{-12}
MetMbCN	$(5\text{-}6 \times 10^{-9})$[b]	28	18	10	½	2.5×10^{-11}

[a] τ_R is the rotational correlation time at a temperature of 30 °C. [b] This value is assumed to be the same as for deoxy-Mb. [c] Line width decomposition is of the form $\Delta\nu_{1/2}^0 = \Delta\nu_{1/2}^{0,\text{electron}} + \Delta\nu_{1/2}^{\text{diamag}}$ where $\Delta\nu_{1/2}^{0,\text{electron}}$ is from the second term in eq 1 and the first term in eq 4. [d] T_{1e} is calculated from $\Delta\nu_{1/2}^{0,\text{electron}} = 7\gamma_1^2 g^2 \beta^2 S(S+1)T_{1e}/15\pi r^6$.

deoxy-Mb and Hb. Equating the value of 35 Hz to the first term in eq 4 gives $T_{1e} \simeq 6.1 \times 10^{-12}$ s. Assuming that the diamagnetic contribution to $\Delta\nu_{1/2}^0$ is unchanged in going from deoxy-Mb to MetMbCN permits the line width decomposition and calculation of MetMbCN electronic relaxation time as shown in the last line of Table II. The relative magnitude of the T_{1e} of MetMbCN being about four times longer than that of deoxy-Mb and deoxy-Hb would appear relatively reasonable since MetMbCN, a Kramers doublet, should have the longer relaxation time (M. Gueron, private communication).

Based on the calculated magnitude of the diamagnetic contribution of the zero-field line width and on the relative magnitudes of the electronic relaxation times between MetMbCN and deoxy-Mb as well as deoxy-Hb, the interpretation of the differing zero-field line widths between deoxy-Mb and deoxy-Hb appears quite reasonable. There are, however, two assumptions in this calculation which should be examined further; namely, that there is no substantial difference between the electronic relaxation times of deoxy-Mb and deoxy-Hb and that the heme methyl to heme iron distance does not differ significantly between deoxy-Mb and deoxy-Hb. Considering first the question of relaxation times, the calculations above indicate that the relaxation time only changes by a factor of 4 in going from deoxy-Mb to MetMbCN, a change in which the oxidation state, spin state, and heme ligation state have all changed. Thus it would appear rather unlikely that merely changing the protein composition around the heme would have a significant effect on the iron relaxation within an unaltered heme.

Likewise, there are several observations which suggest that the heme methyl to heme iron distance is not significantly altered in going from Mb to Hb. Specifically, it is observed that the two resonances at ~−15 and ~−11 ppm from DSS in deoxy-Mb exhibit nearly identical line widths. Since the two resonances arise from different methyl groups on the heme,[15] the observation of equivalent line widths implies nearly equal distances from the heme iron for the two different methyl groups. Thus, the heme iron to methyl group distance must be relatively rigid. Further confirmation of this assumption is derived from Hb where the two methyl resonances at ~−23 and ~−17 ppm from DSS exhibit nearly equal line widths. Since these resonances have been respectively assigned to the β and α heme methyls,[3,20] this is further evidence that different globin stresses up on the heme are insufficient to change the iron to methyl distance. There are some slight differences in the distance of the heme iron from the plane of the porphyrin between these two proteins, with the porphyrin to iron distance being 0.3–0.4 Å for deoxy-Mb (M. F. Perutz, private communication) and ~0.62 Å for deoxy-Hb.[21] However, assuming that to a first approximation the porphyrin geometry is planar, it would require a movement of slightly over 1 Å out of the plane to produce a 10% change in line width. Even assuming the largest possible change in the porphyrin to iron distance in going from Mb to Hb (of 0.3 → 0.6 Å), a change of ~4% in the zero-field line width would be produced. Thus it appears quite probable that the difference in the zero-field line widths between Mb and Hb is due to an incomplete averaging of the methyl proton-proton dipolar interactions as discussed above.

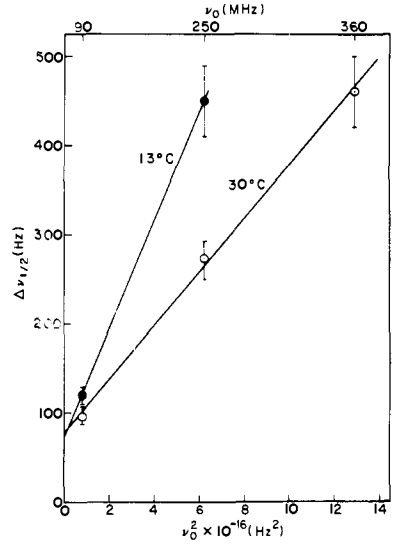

Figure 10. Field dependence of the deoxyhemoglobin line width at constant temperature. Values for the 30 °C line are taken from the spectra of Figure 7 at 360 and 250 MHz and from the curve in Figure 8 at 90 MHz. For the 13 °C line, the value from the 250-MHz spectrum in Figure 9 is used along with the 13 °C value taken from the curve in Figure 8. Although only two points are available at 13 °C, a straight line (ν_0^2) dependence is assumed due to the observation of ν_0^2 dependence at higher temperature. Electron relaxation times and correlation times are respectively calculated from the zero-field intercept and slopes of these plots.

The rotational correlation time of Hb can also be calculated from eq 4 and the slopes in Figure 10, giving values of 3.5×10^{-8} s at 30 °C and 6.3×10^{-8} s at 13 °C. The ratio of the correlation time at 13 °C to that at 30 °C is ~1.8. The value derived from spin label measurements in the limit of dilute solution (0.1 mM) at 20 °C in H_2O is 2.6×10^{-8} s.[22] Adjusting this value for the difference in temperatures and D_2O viscosity gives predicted values of 2.4×10^{-8} s at 30 °C and 4.6×10^{-8} s at 13 °C. The ratio of these correlation times at 13 and 30 °C is ~1.9. Although the magnitudes of the values calculated from the slopes in Figure 10 are somewhat larger than those derived from spin label measurements, the *ratios* of the correlation times are virtually identical. Thus, it is possible that the larger magnitude of the correlation time as calculated from NMR measurements as compared to the spin label measurement comes from either of two sources: protein-protein interactions due to high protein concentration, or small inaccuracies in either the magnetic moment or the methyl-iron distances, or both, which lead to overestimates of the correlation times.

Conclusion

In high-spin ($S = 2$) deoxymyoglobin and deoxyhemoglobin, and in azidometmyoglobin which is a mixture of high-spin ($S = 5/2$) and low-spin ($S = 1/2$) states, the line widths of the hyperfine-shifted proton resonances exhibit a complex temperature and magnetic field dependence. The hfs proton reso-

nances of low-spin ($S = \frac{1}{2}$) cyanomethemoglobin, on the other hand, exhibit no detectable field dependence and only a very mild temperature dependence. These effects appear to be adequately explained by the Curie spin relaxation formalism proposed by Gueron.[10] Using this formalism, the electron spin–lattice relaxation time, T_{1e}, have been calculated to be $\sim 6.1 \times 10^{-12}$ s for deoxy-Mb and deoxy-Hb. The Curie spin formalism has also been used to calculate correlation times for deoxy-Mb and deoxy-Hb at various temperatures. The calculated values are reasonably close to those expected, though again the values should probably be regarded as approximate.

It has been reported in a number of recent publications that chemical shift anisotropy is an important relaxation mechanism at high magnetic fields for nonproton NMR studies of both small molecules and macromolecular systems of biological interest. For example, the ^{31}P NMR studies of phospholipid vesicles,[23] the ^{19}F NMR studies of fluorotyrosine alkaline phosphatase,[24] and the ^{13}C NMR studies of cholesterol chloride[25] all indicate that the chemical shift anisotropy effect is significant. These results imply that the relaxation mechanism which determines the NMR line widths of ^{31}P, ^{19}F, or ^{13}C resonances is field (or frequency) dependent. An important consequence of this implication is that for each system under investigation there is a frequency at which both optimal sensitivity and resolution are obtained. Above this frequency, the line width increases as the square of the frequency, while the resolution between the resonances only increases linearly with frequency. For some macromolecules with large values of τ_R (the rotational correlation time), the contribution of chemical shift anisotropy to the line width becomes quite significant and the optimal frequency is lowered considerably. On the other hand, for the proton resonances it has generally been considered that both resolution and sensitivity will be improved by increasing the magnetic field strength due to a negligible contribution of the chemical shift anisotropy to line widths. This, however, is true only for systems not subject to paramagnetic interactions. The present study clearly shows that with certain paramagnetic systems, there is another relaxation mechanism which can produce line broadening of the hyperfine-shifted proton resonances upon increasing the magnetic field. With the increasing availability of high-frequency NMR spectrometers for studies of biological macromolecules, the present results emphasize that it is essential to measure ^1H NMR parameters of systems containing paramagnetic centers at more than one frequency and temperature before interpreting the meaning of the line width of a given signal.

Acknowledgments. We thank Dr. Richard Egan of Abbott Laboratories for the use of their Varian HR-100 NMR spectrometer, Dr. Steven Danyluk of Argonne National Laboratory for use of their Varian HR-220 NMR spectrometer, and Dr. Oleg Jardetzky and Dr. Norma Jardetzky of Stanford Magnetic Resonance Laboratory for the use of their Bruker HXS-360 NMR spectrometer. We also thank Miss Dolores Bertoli, Mrs. Allison C. Lin, Miss Judy A. Flowers, and Dr. Karen J. Wiechelman for their excellent technical assistance during various phases of this project. In addition, we extend our gratitude to Dr. Maurice Gueron, Dr. Sunney I. Chan, and Dr. David W. Pratt for helpful and stimulating discussions. We are especially grateful to Dr. Gueron for pointing out the significance of the diamagnetic contributions to the line width. The NMR facility at Stanford Magnetic Resonance Laboratory is supported by research grants from the National Science Foundation (GR-23633) and from the National Institutes of Health (RR-00711). This work is supported by research grants from the National Science Foundation (PCM 76-21469) and the National Institutes of Health (HL-10383 and RR-00292). Part of the work done by M.E.J. was carried out during the tenure of a Postdoctoral Fellowship of the National Institutes of Health (HL-03005) at the University of Pittsburgh. L.W.-M.F. is a recipient of a National Research Service Award (GM-05164).

References and Notes

(1) Part of the work was presented at the 19th Annual Meeting of the Biophysical Society, Philadelphia, Pa., Feb 18–21, 1975.
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(8) It should be noted that the present finding on the magnetic field dependence of the line widths of the hyperfine-shifted proton resonances of hemoglobin does not alter the main conclusion reached by Johnson and Ho (ref 7); namely, that in the presence of organic phosphates the α chains have a higher affinity for oxygen than the β chains in a normal human adult intact tetrameric hemoglobin molecule.
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