Librational Motion of an "Immobilized" Spin Label: Hemoglobin Spin Labeled by a Maleimide Derivative†

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ABSTRACT: The spin label Tempo-maleimide, when "immobilized" in hemoglobin, is shown to exhibit motional fluctuations whose amplitude and/or frequency depend on temperature and solution conditions. These motional fluctuations are observable by several electron spin resonance techniques. For desalted hemoglobin the fluctuations are detectable at approximately −15 °C using saturation transfer techniques and at approximately +25 °C using line-width measurements of normal absorption spectra. In ammonium sulfate precipitated hemoglobin, however, motional fluctuations are not detectable by either technique up to at least 40 °C. The most probable mechanism for spin-label motion appears to be either fluctuations in protein conformation which affect the label binding site or conformational transitions of the nitroxide ring itself. These motional fluctuations are shown to introduce a librational character to the overall label motion during hemoglobin rotational diffusion, with the librational motion significantly affecting the use of spin-label spectral shapes to calculate hemoglobin rotational correlation times.

Over the last few years several methods have been devised for determining the rotational motion of macromolecules by monitoring the electron spin resonance (ESR)[1] spectra of spin labels which are rigidly bound to the macromolecule. McCalley et al. (1972) and Goldman et al. (1972) have shown that the rotational correlation time of a spin-labeled macromolecule may be determined from the apparent splitting of the hyperfine extrema provided that the motion is slow enough to yield an "immobilized" spectral shape. Mason and Freed (1974) have also shown that the line widths of the hyperfine extrema are sensitive to the rate of rotational motion, with both the apparent hyperfine splitting and the hyperfine line widths being sensitive to rotational motion over the approximate range 7×10^{-9} to 3×10^{-7} s. More recently, Hyde, Dalton, and coworkers have shown that much slower rotational motion (10^{-7} to 10^{-3} s) may be monitored through saturation transfer techniques (Hyde and Dalton, 1972; Hyde and Thomas, 1973; Dalton et al., 1976; Thomas et al., 1976).

One assumption implicit in the use of these techniques to monitor macromolecular motion is that the spin label is rigidly bound to the macromolecule and exhibits only the motion of the macromolecule itself. From thermodynamic considerations alone, however, one would expect some residual motion of the label with respect to the total macromolecular matrix. If, for example, the label is bound by van der Waals interactions within a hydrophobic pocket in a protein, the label might be expected to exhibit oscillations about the minimum energy conformation with the amplitude of motion dictated by the Boltzmann relation, similar, for example, to the motion exhibited by the aromatic rings of tyrosine and phenylalanine residues within a protein interior (Gelin and Karplus, 1975; Wagner et al., 1976; Hetzel et al., 1976). For flexible nitroxide ring systems, the transition between different ring conformations would also be expected to produce effective motion of the label magnetic axes with respect to the macromolecular orientation. Furthermore, the macromolecule itself is expected to undergo substantial thermal fluctuations in conformation (Cooper, 1976) which would probably induce transient reorientations of the label axes with respect to the macromolecular axes. These transient motions of the label, when superimposed on the rotational diffusion of the macromolecule itself, would be expected to introduce a librational[2] character to the total spin-label rotational behavior.

Thus far, however, the question of spin-label librational motion appears to have been considered only very briefly in the literature. In discussing the saturation transfer spectral behavior of Tempo-maleimide spin-labeled hemoglobin in glycerol, Hyde and Thomas (1973) have suggested the possibility of the label exhibiting torsional oscillations about its central C-N bond. Using ELDOR techniques, Hyde et al. (1975) have more recently investigated the saturation transfer behavior of Tempo-maleimide labeled Hb*O_2 frozen in ice and have observed a spectral diffusion width of approximately 1.8 G for the high-field ($m_I = -1$) line (see Figure 1). Since this line arises primarily from nitroxides whose principal z axes are oriented along the direction of the magnetic field, the apparent interpretation of this spectral width is that the label exhibits significant motional fluctuation or librational motion about its average orientation within the immobilized protein matrix. An understanding of the magnitude and frequency of such librational motion will be quite important in the quantitative use of spin-label techniques to monitor macromolecular motion.

The present communication is an investigation of librational motion within Tempo-maleimide spin-labeled hemoglobin (Hb). This system has often been considered as a prime example of a protein with an immobilized spin label (McCalley et al., 1972), and thus was chosen for the present study. The results of this work indicate the existence of significant librational motion for the label at physiological temperature, with the magnitude and/or frequency of librational motion depending on both the temperature and the ionic strength of the

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[1] Abbreviations used are: ESR, electron spin resonance; Tempo-maleimide, 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy; HbCO, carboxyhemoglobin; Hb*CO, Tempo-maleimide labeled carboxyhemoglobin; ELDOR, electron-electron double resonance.

[2] The term "librational motion" is derived from astronomical usage and basically describes the motion of a system where harmonic or oscillatory motion may be superimposed on rotational motion.

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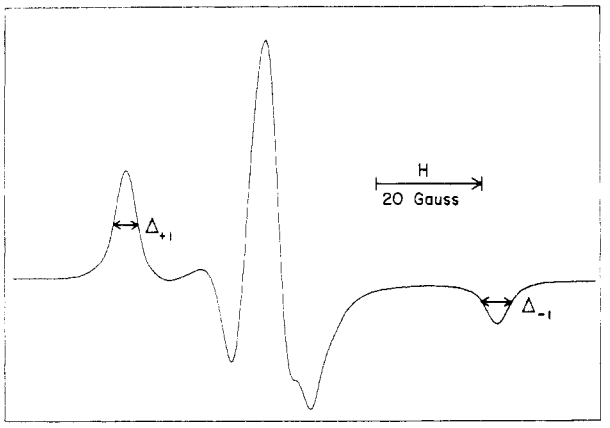

FIGURE 1: Hb*CO frozen at −9 °C. Line widths shown are the full line widths at half height with respect to the true base line.

Hb solution. The inhomogeneous broadening due to unresolved proton hyperfine structure is also shown to be of importance for interpreting line-width changes in terms of rotational motion.

Materials and Methods

Blood samples were obtained from the University of Illinois Hospital Blood Bank; hemoglobin was prepared from the pooled blood samples by the methods of Abraham et al. (1975) and kept under CO atmosphere until use. Tempo-maleimide (Syva no. 100) labeling of the Hb followed the procedures of McCalley et al. (1972) with the exception that all procedures were done at 4 °C. Hb was desalted either by running through a column of Rexyn-I-300 (Fisher) or Sephadex G-25 (Pharmacia) with glass-distilled water. Sephadex resins contain a small number of carboxyl groups which act to slow down and spread out the protein band in the absence of salt; thus, the Rexyn procedure was considered preferable. After desalting, Hb solutions were concentrated by ultrafiltration (Amicon). Hb concentrations were determined from the visible absorption bands at 540 and 569 nm (Antonini and Brunori, 1971).

Sucrose solutions were prepared at 5% (w/v) intervals from 5 to 55% using reagent grade sucrose and glass-distilled water. Hemoglobin–sucrose solutions for variable viscosity studies were then prepared by adding desalted 30–35% Hb to the sucrose solutions in a ratio of 1:10 to give a final Hb concentration of approximately 3%. Solution viscosities were calculated from the empirical relations of Barber (1966). Hb was immobilized at low temperature by plunging samples of desalted 22% Hb into liquid nitrogen and then inserting the sample into the EPR variable-temperature dewar and allowing it to warm up to the appropriate temperature. Immobilized Hb samples at high temperature were prepared by mixing labeled and unlabeled Hb in a molar ratio of 1:2 and precipitating the resulting solution with saturated (at 4 °C) ammonium sulfate at pH 7.

Spin-label EPR spectra were measured on a Varian E-112 spectrometer equipped with an E-257 variable-temperature accessory. A microwave power of 5 mW was found to introduce no observable saturation line broadening at temperatures above 0 °C and was used for all measurements above 0 °C. Below 0 °C, a power of 0.1 mW was used. No perceptible line broadening was observed at a modulation level of 1 G; thus, this modulation amplitude was used at all temperatures. A 40-G sweep width was used for all line-width measurements. The sweep width was calibrated from the tetracyanoethylene anion in dimethoxyethane (Polnaszek, 1976). Second harmonic saturation transfer spectra were measured by adjusting for null at 1-mW power and 5-G modulation and then increasing the power to 63 mW (5 dB) and measuring the out-of-phase signal (Thomas et al., 1976). The instrumental settings for modulation and power appeared reasonably accurate and were not further calibrated. Temperature was measured using a copper–constantin thermocouple and a Honeywell Model 2746 potentiometer. The relative precision of individual temperature measurements was approximately ±0.5 °C, with an estimated overall accuracy of ±1 °C. Samples were contained in 50- or 100-μL sealed capillary pipets and were held in the cavity by a specially constructed holder.

Line widths were measured by shifting the field off resonance, determining the baseline, and then measuring the full line width at half maximum with respect to the baseline. Plotted values are the average of two measurements.

Results

Conceptually, determining whether a spin label exhibits any residual mobility in relation to its protein matrix is fairly simple. By immobilizing the protein matrix, the only motional freedom remaining for the label will be that within the protein matrix itself. By lowering the temperature one can then eventually "freeze out" the various vibrational modes of the label. From this "rigid limit" spectral shape and that of the spectra at higher temperature, the mobility of the label at higher temperature can be determined.

In practice, the situation is somewhat more complex. At temperatures below 0 °C, the protein matrix may be immobilized simply by freezing the solution. At temperatures above 0 °C, the protein matrix may be immobilized by precipitating it out of solution or by lyophilization (Thomas et al., 1976). However, both of these techniques would be expected to induce substantial stresses on the Hb structure (and thus the label–Hb interaction) due to the changed environment of the Hb surface (Johnson et al., 1977). Thus far, no usable technique has been developed for immobilizing the protein matrix on a long enough time scale under physiological conditions. An indirect procedure for obtaining this information is, however, available from the methods of Mason and Freed (1974). From this work it has been shown that the correlation time of a nitroxide label can be calculated to a high degree of accuracy from the expression

$$\tau_R = a_m \left(\frac{\Delta_m}{\Delta_m^\circ} - 1\right)^{-b_m} \quad (1)$$

where τ_R is the rotational correlation time, a_m and b_m are parameters derived by spectral simulation, and Δ_ms are the full line widths at half height of the hyperfine extrema as shown in Figure 1. Δ_m° is the line width in the rigid limit, while Δ_m is the line width in the presence of motion. If one assumes the Stokes relation for rotational diffusion of Hb, that

$$\tau_R = \frac{4\pi r^3 \eta}{3kT} \quad (2)$$

where r is the effective Hb radius, η is the solvent viscosity, k is Boltzmann's constant, and T is the absolute temperature, then eq 1 can be rewritten in the form

$$\Delta_m = \Delta_m^\circ + a_m' \left(\frac{T}{\eta}\right)^{b_m'} \quad (3)$$

where $b_m' = 1/b_m$ and $a_m' = \Delta_m^\circ (3a_m k/4\pi r^3)^{b_m'}$. Dissolving the labeled Hb in sucrose solutions of varying concentrations (and thus viscosities) and measuring the line widths at constant temperature for several different temperatures will then generate families of curves for the different temperatures. The

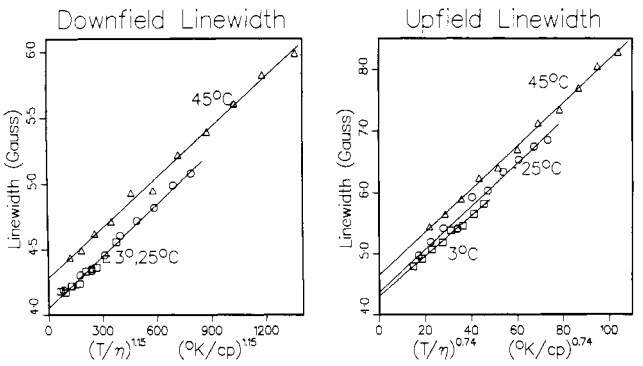

FIGURE 2: Determination of the rigid limit line widths. The symbols □, ○, and △ denote the line widths as measured at 3, 25, and 45 °C, respectively. The straight lines shown are a parameterization of eq 3, with the parameters derived by least squares fitting as described in the text. Linewidth precision is approximately ±0.05 G for the low-field lines and approximately ±0.1 G for the high-field lines. Error bars are omitted due to the high density of data.

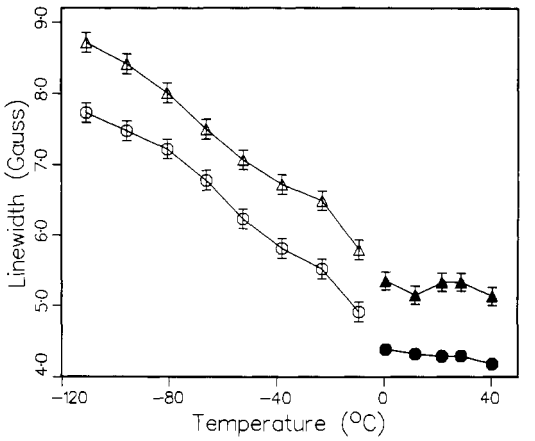

FIGURE 3: Full line widths at half height for the hyperfine lines, m_I = +1 (○ and ●) and m_I = −1 (△ and ▲) of frozen Hb*CO and precipitated Hb*CO. Data below 0 °C are from frozen Hb*CO (○ and △). Data above 0 °C are from precipitated Hb*CO (● and ▲).

TABLE I: Temperature Dependence of the Hyperfine Extremal Line Widths in the Limit of Infinite Viscosity.

T (°C)[a]	m_I[b]	$\Delta_m°$ [c]
3.2 ± 0.3	+1	4.05 ± 0.01
24.8 ± 0.1	+1	4.05 ± 0.01
44.6 ± 0.2	+1	4.28 ± 0.02
3.2 ± 0.3	−1	4.31 ± 0.03
24.8 ± 0.1	−1	4.37 ± 0.08
44.6 ± 0.2	−1	4.66 ± 0.04

[a] Mean temperature and standard deviation for each of the lines in Figure 2. [b] Lines corresponding to the nitrogen spin states m_I = ±1; m_I = +1 is the low-field line, m_I = −1 is the high-field line. [c] Rigid limit line widths are calculated by the least-squares procedures discussed in the text; quoted uncertainties are the standard errors of estimate.

rigid limit line widths can then be obtained under approximately physiological solution conditions by extrapolation of these curves to infinite viscosity ($T/\eta = 0$).

The results of these measurements are shown in Figure 2. In parameterizing these curves, a nonlinear least-squares fit was first performed, allowing all three parameters, $\Delta_m°$, a_m', and b_m', to vary. For each of the hyperfine lines, m_I = +1 and m_I = −1, the exponent b_m' was found to be independent of temperature within the error of estimate. Thus, the values were averaged to give b_{+1} = 1.15 and b_{-1} = 0.74 as shown in Figure 2. A linear least-squares fit of eq 3 was then performed to determine the values of $\Delta_m°$ and a_m' at each temperature. The results are shown as the straight lines in Figure 2 and are given in numerical form in Table I. From Figure 2 (left-hand graph) it can be seen that the low-field line widths (m_I = +1) appear completely equivalent for the 3 and 25 °C measurements; if individual least-square fits are made for these two temperatures, the difference between the two curves is not statistically significant. Hence, the data were combined and a single least-squares line is shown. For the high-field lines (m_I = −1), however, the two measurements (3 and 25 °C) appear to be distinguishable ($p \simeq 0.2$), and individual regression lines are thus shown in Figure 2 (right-hand graph).

Although the high-field line-width (Δ_{-1}) curves show significant separation between 3 and 25 °C, the difference in their rigid limit extrapolated line widths is not statistically significant. Thus, neither of the rigid limit hyperfine extremal line widths show any significant change in raising the temperature from 3 to 25 °C. In going from 25 °C up to 45 °C, however, there are significant increases in the rigid limit line widths of both the m_I = +1 and m_I = −1 lines, with the low-field line increasing by approximately 0.2 G and the high-field line increasing by approximately 0.3 G in the limit of an immobilized protein matrix.

The obvious interpretation of this temperature-induced increase in the infinite viscosity line width is a fluctuation of the label orientation within the protein matrix, with the amplitude or frequency of motion increasing with temperature. However, it might instead by argued that the T_2 electronic relaxation or the inhomogeneous broadening due to the unresolved proton hyperfine interactions could exhibit an anomalous temperature dependence, acting to increase the line width as temperature increases. From Figure 3, however, it can be seen that when the protein matrix is directly immobilized by freezing the solution both of the hyperfine lines show a steady monotonic decrease in width as the temperature is increased from approximately −110 °C up to approximately −10 °C. This behavior likewise appears to continue in a qualitatively similar fashion above 0 °C, as can be seen from the ammonium sulfate precipitated Hb*CO data also shown in Figure 3. From these data it is clear that the m_I = +1 line of precipitated Hb*CO exhibits a slow but steady decrease in width as the temperature is raised from 1 to 40 °C. The m_I = −1 line exhibits an essentially constant width over this temperature range.

From the behavior of both the frozen and precipitated Hb samples, then, it is clear that over the full temperature range of this study the effect of increasing temperature is to reduce the net line-width contributions from inhomogeneous broadening and T_2 relaxation. This in turn indicates that a thermally induced increase in the fluctuation of the label within the protein matrix is the only plausible explanation for the increase in line width observed in Figure 2. (The apparent suppression of such fluctuations in ammonium sulfate precipitated Hb*CO is not particularly surprising, since the binding of the nitroxide ring to Hb appears to be primarily hydrophobic in character, and ionic strengths as low as 0.5 M have previously been shown to substantially increase the strength of this binding (Johnson et al., 1977). Hence, the extremely high ionic strength of saturated ammonium sulfate apparently reduces either the amplitude or the frequency of librational motion to the point where it is no longer observable in the precipitated Hb*CO

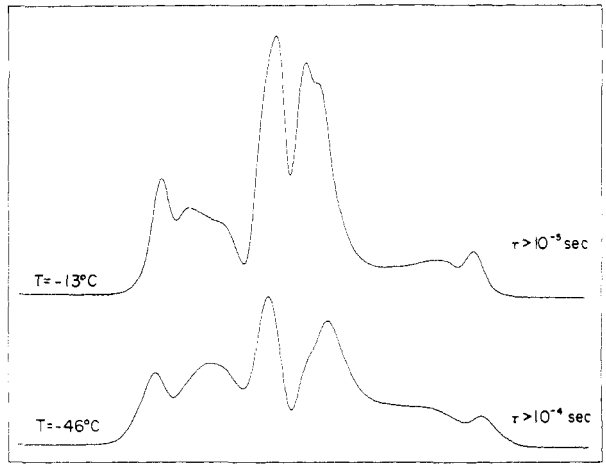

FIGURE 4: Second harmonic saturation transfer spectra of frozen Hb*CO. Modulation frequency and amplitude are 50 kHz and 5 G, respectively. Microwave power is 63 mW (5 dB). The temperatures listed are from thermocouple measurements; no corrections are made for microwave heating of the sample. Correlation times are taken from the calibration curves of Thomas et al. (1976). There are significant differences in the spectral shape between −46 and −13 °C; however, part of these differences may be due to differing line widths and differing degrees of spin saturation.

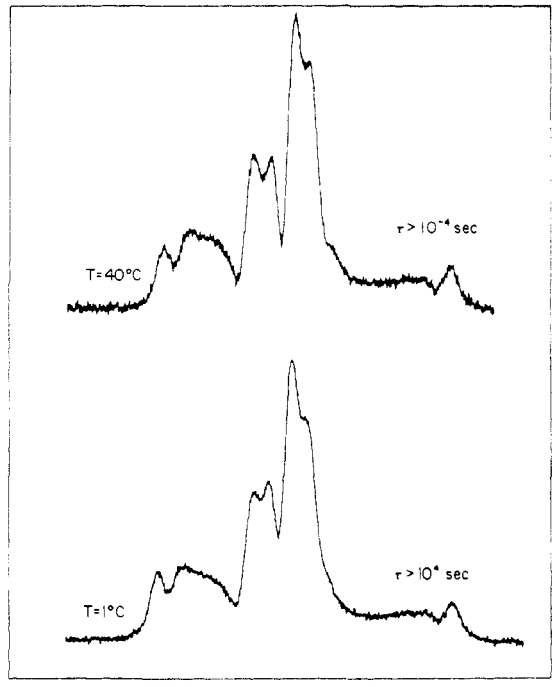

FIGURE 5: Second harmonic saturation transfer spectra of ammonium sulfate precipitated Hb*CO. Experimental conditions are the same as those of Figure 4. The central region of the 40 °C spectrum is suggestive of a slight increase in motional freedom relative to that at 1 °C; however, this is not reflected in the outer spectral "turning points". The listed correlation times are taken from the outer spectral regions (Thomas et al., 1976).

hyperfine extremal line widths.)

It is also of interest to study the behavior of the label in the immobilized Hb*CO systems using saturation transfer techniques which are sensitive to much slower motions than the normal absorption methods employed above. Second harmonic saturation transfer spectra of frozen Hb*CO at −46 and −13 °C are shown in Figure 4. From a comparison of these spectra with calibration curves (Thomas et al., 1976), there appears to be approximately an order of magnitude decrease in the correlation time as the temperature is raised to −13 °C. The presence of label motion at this temperature would agree with the previous ELDOR measurements (Hyde et al., 1975). A more detailed quantitative interpretation of the spectra, however, is probably not appropriate, since the calibration curves of Thomas et al. (1976) were obtained for Brownian rotational diffusion rather than for small amplitude librational motion in a fixed protein matrix and since there are also significant line-shape changes over the temperature range −46 to −13 °C (see Figure 3).

The label in ammonium sulfate precipitated Hb*CO, however, appears to exhibit somewhat different behavior under saturation transfer conditions. From Figure 5 it can be seen that there is virtually no change in spectral shape as the temperature is raised from 1 to 40 °C. However, the correlation time, as measured from calibration curves, appears to be longer at both temperatures than is measurable by this technique. Since these temperatures are substantially higher than those for the frozen Hb*CO samples, this is then further confirmation that the high ionic strength of saturated ammonium sulfate acts to suppress either the amplitude or the frequency of librational motion.

Discussion

Although the thermally induced increase in line width (Figure 2) is only 5–8% of the line width, this contribution is sufficient to introduce substantial errors into correlation-time calculations (eq 1), particularly for long correlation times. For example, if the increase in the infinite viscosity line width in going from 25 to 45 °C were to be attributed to Hb*CO rotational motion, the correlation time for the immobilized

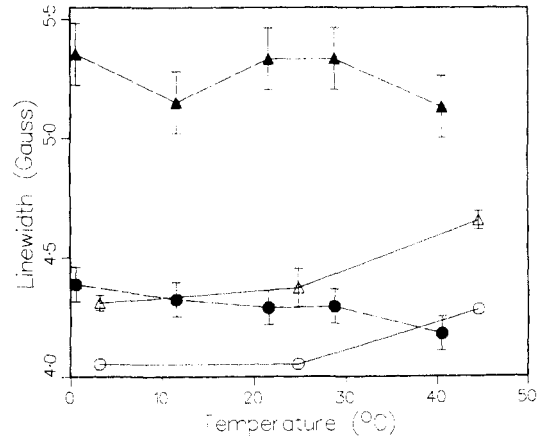

FIGURE 6: Comparison between ammonium sulfate precipitated Hb*CO and infinite viscosity extrapolated line widths. Precipitated Hb*CO values are, respectively, ● and ▲ for the $m_I = +1$ and $m_I = -1$ lines while the infinite viscosity line widths are ○ and △ for the $m_I = +1$ and $m_I = -1$ lines, respectively. Error bars are not shown for the infinite viscosity $m_I = +1$ lines (○), since the estimated uncertainties are smaller than the data points.

Hb*CO would be calculated as approximately 2×10^{-7} s, which can hardly be considered as "immobilized". Obviously, then, the librational motion introduces a rather substantial error into the correlation-time calculations. This effect will be especially important in macromolecular binding studies, particularly if attempts are made to study the thermodynamics of the binding interaction.

From Figure 6 it is also apparent that the values of the rigid limit line widths are strongly dependent on the method used to obtain them. For the $m_I = +1$ line the infinite viscosity extrapolated line width is initially about 0.4 G less than that from

the precipitated Hb*CO, with the values derived from the two methods crossing at about 38 °C and the infinite viscosity line width becoming larger than that from the precipitated Hb*CO. The difference is more extreme for the $m_I = -1$ line where, at low temperature, the precipitated Hb*CO value starts out about 1 G higher than that from the infinite viscosity extrapolation and the two curves move to within approximately 0.5 G of each other at high temperature but never cross. Since both procedures are measuring the line widths of a label which is essentially "motionless" (on the normal absorption time scale) at temperatures below 25 °C, the discrepancy between the results is significant. The source of the difference between these two methods is apparently the inhomogeneous broadening due to unresolved proton hyperfine coupling. Freed (1976) has noted that much of this inhomogeneous broadening will be averaged out with the onset of the molecular motion, suggesting that the viscosity extrapolation effectively permits averaging of the inhomogeneous broadening interactions while the precipitation procedure provides a truly immobilized protein with all of the inhomogeneous broadening mechanisms present. Deuteration of the label nitroxide ring would be expected to reduce the difference between these two methods.

One question which the present study does not entirely resolve is that of the mechanism of label motion within the protein matrix. Plausible mechanisms include (a) ring conformational transitions between "boat" and "chair" conformations or between two possible "twisted" ring conformations, (b) inversion of the N-O bond with respect to the C-N-C plane within the nitroxide ring, (c) torsional oscillation of the nitroxide ring about the C-N bond between the Tempo and maleimide moieties of the label, and (d) protein structural fluctuations which affect the label conformation and/or orientation within the protein. Each of these mechanisms will produce transient reorientation of the nitroxide principal z axis with respect to the external magnetic field. Since the resonance positions of the outer hyperfine lines depend on the orientation of the nitroxide principal axis with respect to the magnetic field (McConnell and Gaffney-McFarland, 1970), the effect of this orientational fluctuation will be to partially average the magnetic environment of the nitroxide over the solid angle covered by its angular fluctuation. Considering the end points of fluctuation as being two separate magnetic environments, this can be very roughly regarded as a two-site "exchange" system, similar to chemical exchange in NMR, but where the "exchange" is now caused by motion of the nitroxide between two different orientations in the magnetic field. If the librational motion of the nitroxide is in the fast motion limit, then the motional broadening would be expected to follow the form (Pople et al., 1959)

$$\delta(\Delta\nu_{1/2}) = \frac{\pi(\nu_A - \nu_B)^2}{4f} \tag{4}$$

where $\delta(\Delta\nu_{1/2})$ is the motional broadening in hertz, f is the frequency of librational motion in hertz, and $(\nu_A - \nu_B)$ is the separation in hertz of the nitroxide resonance positions during librational motion. If, instead, the librational motion is in the slow motion limit, then the motional broadening is related to the conformational lifetime, τ, by

$$\delta(\Delta\nu_{1/2}) = \frac{1}{\pi\tau} \tag{5}$$

Using this formalism, we now consider in detail the probable effects of each of the librational mechanisms listed above.

(a) The effect of a ring conformational transformation between the "boat" and "chair" forms would be to reorient the principal z and x axes by 104° while the transformation between the two "twisted" forms would produce a 50° reorientation of the axes. [For structural details of the conformations, see Dwek et al. (1975)]. If these transitions were to occur on the rapid-motion time scale, the hyperfine constants would be partially averaged, resulting in much larger shifts of the line positions than are actually observed (M. Johnson, unpublished). Likewise, in the rapid-motion limit one would expect to observe motional narrowing rather than motional broadening as the temperature is raised. Thus, any conformational transitions of this nature must occur in the slow-motion limit. If the values from Table I are substituted into eq 5, we would find a transition rate of $1/\tau \simeq 2\text{-}3 \times 10^6\ s^{-1}$. Briere et al. (1967) have suggested that conformational transitions $\sim 10^3\ s^{-1}$ may occur for nitroxide radicals free in solution. It appears rather doubtful that a nitroxide which is "immobilized" within a protein could exhibit conformational transitions at a substantially higher rate than in free solution. Thus, it appears unlikely that conformational transitions of this nature contribute significantly to the motional broadening.

(b) From nitroxide structural data reviewed by Lajzerowicz-Bonneteau (1976), it appears that the N-O bond in most six-member rings is oriented at an angle of 16-20° from the C-N-C plane. Inversion of the N-O bond about this plane would reorient both the principal x and z axes by approximately 35°. However, this inversion would move the oxygen of the N-O group by only about 0.8 Å and thus might well be possible even in a sterically restricted environment. In the slow-motion limit this would again give an inversion rate, $1/\tau \simeq 2\text{-}3 \times 10^6\ s^{-1}$. If this mechanism is of importance, we would also expect to see a constant hyperfine splitting until the onset of motional broadening and then a decrease in the hyperfine splitting at some temperature after the onset of motional broadening (Pople et al., 1959). In fact, however, we find that the rigid-limit hyperfine splitting displays a linear $1/T$ dependence, decreasing from 70.6 G at 4.5 °C to 68.9 G at 25 °C to 67.3 G at 45 °C (M. Johnson, unpublished). Thus, while the inversion mechanism would appear to be physically plausible, the spectra also exhibit other temperature dependencies which may be inconsistent with this being the sole motional broadening mechanism.

(c) Gelin and Karplus (1975) have shown that the steric restrictions which inhibit rotation of a tyrosine ring within the interior of a protein can be represented by a rotational potential barrier with an activation energy E_A. Within the potential well formed by this barrier, classical mechanics indicate that the ring will exhibit torsional oscillations with a frequency, $f \sim \sqrt{E_A/I}$, where I is the moment of inertia of the ring. The oscillation amplitude will depend on the temperature and the shape of the potential barrier. Using appropriate values for E_A (Gelin and Karplus, 1975) and I gives $f \sim 10^{12}$ to 10^{13} per second for the torsional oscillation frequency. Since the rigidly bound nitroxide ring should be quite analogous to the sterically hindered tyrosine ring, it would appear quite plausible for the nitroxide ring to exhibit torsional oscillations about the C-N bond. These torsional oscillations would then produce high-frequency fluctuations in the orientation of the nitroxide principal z axis with an angular deviation of a few degrees from the average orientation. Using a torsional frequency of $10^{12}\ s^{-1}$ and assuming that all of the temperature dependence of the hyperfine separation as listed above is due to motional averaging, eq 4 then indicates that the motionally induced broadening would be less than 10^{-3} G. This value is much smaller than is physically observed, indicating that torsional oscillations within a rigid binding site do not play a significant role in the observed motional broadening. (Note that this does *not* eliminate the *possibility* of torsional oscillation within the

spin-label binding site; it simply states that any reasonable torsional oscillation frequency will be sufficiently rapid that complete motional narrowing will occur.)

(d) Thermally induced fluctuations in protein conformation (Cooper, 1976) might also be expected to affect the conformation of the label binding site. These binding-site conformational fluctuations, in turn, would be expected either to induce transient changes in the orientation of the label or, perhaps more likely, to modulate the intramolecular modes of label motion (i.e., torsional oscillation about the C-N bond and/or inversion of the N-O group about the C-N-C plane). The amplitude of such fluctuations would be expected to increase with temperature, suggesting that motional broadening would probably follow eq 4. If we calculate $(\nu_A - \nu_B)$ from the temperature dependence of the hyperfine separation (Dwek, 1975; M. Johnson, unpublished), we obtain an estimate of the fluctuational frequency as $f \sim 10^8\ s^{-1}$ from eq 4. This estimate is within an order of magnitude of the inverse correlation time for internal motion within tobacco mosaic virus subunits as measured by NMR (deWit et al., 1977). Thus, this librational motion mechanism appears to be quite consistent with the available data.

In summary, it appears that the two most likely mechanisms for librational motion of the spin label are modulation of the nitroxide ring conformation/orientation by protein structural fluctuations and inversion of the N-O group about the C-N-C plane. A combination of these mechanisms with torsional oscillation may also be possible. Further work is in progress to more quantitatively describe the mechanism and effects of librational motion.

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