Rotational Motions in Myoglobin Assessed by Carbon 13 Relaxation Measurements at Two Magnetic Field Strengths*

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SUMMARY

Proton-decoupled Fourier transform nuclear magnetic resonance spectroscopy of natural abundance $^{13}$C was used to obtain spectra of cyanoferrimyoglobin of sperm whale (Physeter catodon) at 14.1 and 23.5 kG. Comparison of the spin-lattice relaxation times at these two field strengths allowed the unambiguous assignment of a rotational correlation time of $22 \pm 5$ ns for the $\alpha$ carbon resonances. The spin lattice relaxation time value for a major band attributable to aromatic carbon atoms also corresponded to a single correlation time, attributable to over-all tumbling of the molecule. Certain narrower resonances reflect other modes of rotational motion in addition to the over-all tumbling. Observations of nuclear Overhauser enhancement and line widths accord with these conclusions.

The dipole-dipole relaxation mechanism has been shown to be dominant for protonated carbon nuclei in molecules of the size of proteins and peptides when observed by $^{13}$C nuclear magnetic resonance spectroscopy (2, 3). The dominance of this relaxation mechanism for $^{13}$C nuclei makes possible the interpretation in terms of rotational correlation times of several measurable parameters such as spin-lattice relaxation time, $T_1$, spin-spin relaxation time, $T_2$, and the nuclear Overhauser enhancement under conditions of proton decoupling (4). The adaptation of the inversion recovery method (5) to proton-decoupled Fourier transform $^{13}$C NMR spectroscopy allows for direct determination of $T_1$ for the particular resonance frequency of the spectrometer (6). For molecules of the size of proteins undergoing simple isotropic rotational motion, a $T_1$ value measured at a given magnetic field strength will yield two possible values for the rotational correlation time (4, 6-8). Measurement of $T_1$ in two spectrometers operating at different field strengths offers a method of choosing unambiguously the correct correlation time values if the protein system may be suitably fit to the theory (6, 8, 9). In the present work the $T_1$ values were obtained for resonances at natural abundance of sperm whale cyanoferrimyoglobin at 14.1 and 23.5 kG.

The present results show that the relaxation of the broad $\alpha$ carbon resonance envelope is described by a single rotational correlation time. The aromatic carbon envelope shows generally similar behavior. Certain narrower resonances clearly reflect additional rotational contributions. These results form a background for the interpretation of the results of experiments in which given loci in the protein are specifically enriched with $^{13}$C.

EXPERIMENTAL PROCEDURE

Myoglobin Preparation—The main chromatographic fraction of sperm whale (Physeter catodon) myoglobin was prepared according to Hapner et al. (10). The cyano derivative of the ferrimyoglobin was prepared by adding 3 eq of NaCN and passing the protein through Sephadex G-25 in 0.01 M Tris buffer, pH 7.50. Samples were concentrated at 4°C by ultrafiltration in a 15-ml Amicon Diaflo cell fitted with a UM-2 filter. Concentrations were checked by measurement of absorbance at 541 nm, employing a millimolar extinction coefficient of 10.8. All NMR measurements were made at a protein concentration of 16.0 mM.

$^{13}$C NMR—Spectra were obtained at 14.1 kG (15.08 MHz) and at 23.5 kG (25.2 MHz) by means of the pulsed Fourier transform technique with complete $^1$H decoupling (2). Coupled spectra for nuclear Overhauser enhancement determinations were obtained at 25.2 MHz using single frequency proton decoupling 75,000 Hz below the resonance frequency of the protons of ethylene glycol (1). The decoupling power was maintained to avoid temperature change. Intensities were obtained by integration of the digitized spectra with a Nicolet 1089 computer.

Spin-lattice relaxation times ($T_1$) were obtained by the inversion-recovery method (5, 6). Where the intensity of a resonance is due to more than a single carbon, the relaxation behavior observed will not conform to a single exponential, except for the case in which all contributing carbon nuclei relax with identical time constants. The determination of $T_1$ represents the best fit of a single time constant to the decay (7). In general, $T_1$ will tend to reflect most heavily the contributions from resonances with large Overhauser effects and narrow line widths.

Chemical shifts are defined in terms of parts per million upfield of external Cs$.^i$ Probe temperatures were measured following each run. At 23.5 kG the value was $28 \pm 1^\circ$, and at 14.1 kG it was $32 \pm 1^\circ$.

Treatment of Results—If $^{13}$C-$^1$H dipolar relaxation is assumed, the $T_1$ values can be related to the rotational correlation time, $\tau_R$, of a sphere undergoing isotropic reorientation (4) as follows:

\[ \frac{1}{T_1} = \frac{1}{\tau_R} \frac{\langle r^2 \rangle}{\langle r^4 \rangle} + \frac{1}{\tau_1} \]

where $\langle r^2 \rangle$ and $\langle r^4 \rangle$ are the mean-squared and mean-fourth power of the carbon-carbon bond lengths, respectively, and $\tau_1$ is the spin-lattice relaxation time.
where

$$\chi_R = \frac{T_R}{1 + (\frac{\mu_{H} - \mu_{C}}{C-H})^2 T_R^2} + \frac{3T_R}{1 + \mu_{C}^2 T_R^2} + \frac{6T_R}{1 + (\frac{\mu_{H} + \mu_{C}}{C-H})^2 T_R^2}$$

(2)

and

$$\chi = \frac{h}{2\pi} \gamma_{H} \gamma_{C}$$

(3)

$$w_{1} = \gamma_{1}^i H_{O}$$

(4)

The symbols $\gamma_{H}$ and $\gamma_{C}$ represent the gyromagnetic ratios for hydrogen and carbon, respectively. The applied field strength is $H_{0}$ and $\hbar$ is Planck's constant. The resonance frequencies of hydrogen and carbon are $\omega_{H}$ and $\omega_{C}$, respectively. The hydrogen-carbon bond length, $r_{CH}$, is assumed to be constant for all the $\delta$ protons directly bonded to the carbon atom in question. This bond length was taken to be 1.09 Å for aliphatic carbons and 1.084 Å for aromatic carbons (11,12). Sperm whale myoglobin can be taken to be a roughly spherical molecule (13).

For a rotating group with one degree of rotational freedom attached to a rigid sphere undergoing isotropic reorientation, $\chi$ is a function of two correlation times. One, $\tau_{\alpha}$, is the correlation time for the internal motion of the attached rotating group, and the other, $\tau_{R}$, is the correlation time for the over-all reorientation of the sphere. The relaxation behavior for a model of this type may be described by the following (14):

$$\frac{1}{NT_{1}} = \frac{A}{NT_{1} r_{R}} + \frac{B}{NT_{1} r_{C}} + \frac{C}{NT_{1} r_{C}}$$

(5)

where

$$\frac{1}{NT_{1,j}} = \frac{1}{10} \ K^2 \gamma_j \gamma_{C-H} \chi_j$$

(6)

$$\Lambda = \frac{1}{4} \ (3 \ cos^2 \theta - 1)^2$$

(7)

$$B = 3 \ \sin^2 \theta \ \cos^2 \theta$$

(8)

$$C = \frac{3}{4} \ \sin^4 \theta$$

(9)

The dependence of $\chi$ on $\tau_{i}$ is as in Equation 2 and

$$\frac{1}{\tau_{\alpha}} = \frac{1}{\tau_{\alpha}} + \frac{1}{6\tau_{\alpha}}$$

$$\frac{1}{\tau_{C}} = \frac{1}{\tau_{R}} + \frac{2}{5\tau_{R}}$$

The angle between the carbon-hydrogen bond vector and the axis of internal reorientation is $\theta$. This angle was taken to be the tetrahedral angle with one exception. For unrestricted phenyl rings a predominant motion has been shown to be that about the C1-C2 axis of the ring (12, 15). The angle between this axis and the carbon-hydrogen vector for four of the ring carbons is 60°, which was the value assigned to $\theta$ for this case. Note that for both models $T_{1}$ is field-dependent, while $\tau_{R}$ and $\tau_{\alpha}$, which are due to thermal motions, are not.

Plots of Equations 1 and 5 were constructed (4) and the experimental $T_{1}$ values were fitted to these plots. For determined values of $\tau_{R}$ and $\tau_{\alpha}$, nuclear Overhauser enhancement values and transverse relaxation times, $T_{2}$, were calculated according to Doddrell et al. (4). Line widths were calculated as $(\tau T_{2})^{-1}$.

RESULTS AND DISCUSSION

General Characteristics of Spectra—Fig. 1A shows the $^{13}$C NMR spectrum of the cyanoferriyoglobin at 23.5 kG and Fig. 1B shows the corresponding spectrum at 14.1 kG. The measurements were made on the same solution. Both spectra show the presence of a small amount of Tris buffer represented by the sharp resonance at 132.2 ppm on the downfield side of the $\alpha$ carbon envelope.

The natural abundance $^{13}$C spectra in Fig. 1 are made up of sums of resonances at each chemical shift position. The spectral overlap results from chemical shift differences arising from local environment that are comparable to the line width (7,8). Since measured intensities are used in computing $T_{1}$ values, variations in nuclear Overhauser enhancement and line width will result in preferential sampling of those resonances which are narrow and intense. In most cases these resonances will originate from mobile carbons, as opposed to those more rigidly held. Since the resolution is finer and line width narrower at the higher magnetic field strength, the detailed characteristics of the two spectra in Fig. 1 are slightly different. It follows that the contributions from the different nuclei in the collection sampled at a given chemical shift position will not be identical in the two spectra.

$\alpha$ Carbon Resonances—$T_{1}$ values for the $\alpha$ carbon resonances (7, 8, 15-19) were determined at 14.1 and 23.5 kG. This region of the spectrum between about 130 and 142 ppm upfield of CS₂ forms a broad envelope. The maximum of this envelope, at 139 ppm upfield of external CS₂, is marked by 2 in Fig. 1. The relaxation times obtained from this maximum of the envelope were 36 and 74 ms, respectively, and corresponded to a $\tau_{R}$ value of 25 ns for 14.1 kG or 20 ns for 23.5 kG. The basis of the choice of the particular root for $\tau_{R}$, Equation 2, is shown in Fig. 2 in which several points on the $\alpha$ carbon envelope were sampled to obtain $T_{1}$ values at the two spectrometer field strengths. The range of $T_{1}$ values was 28 to 40 ms at 14.1 kG and 65 to 82 ms at 23.5 kG. The $\tau_{R}$ range of 20 to 25 ns matches this sample adequately, and the shorter $\tau_{R}$ root does not. The values noted above were taken to indicate a working value of $\tau_{R}$ of 22 ± 5 ns, comparable to that for over-all tumbling obtained by other methods (20-23).

The chosen value of $\tau_{R}$ was employed in the appropriate equations of Doddrell et al. (4) to compute corresponding values of the
nuclear Overhauser enhancement, and of the line width. The computed values of NOE at the two field strengths were 1.18 and 1.17, respectively. These are close to the theoretical minimum of 1.15. The experimental value of NOE for the α carbon envelope as a whole was 1.3. The region of integration for this measurement was between 124 ppm and 147 ppm upfield of external CS$_2$. The basis for the determination is shown in Fig. 3 where A shows the usual decoupled spectrum and the B shows a corresponding spectrum without proton decoupling. The spin-spin splitting in the latter spectrum leads to doublets for singly protonated carbons. These doublets have the effect in the CY carbon region of producing a broadening suggesting an underlying summation of bimodal distributions. An effect that is much more obvious in the aromatic region in Fig. 3B is adjusted between 123 ppm and 148 ppm.

The computed line widths showed a strong field dependence, as expected. That for 14.1 kG was 39 Hz and that for 23.5 kG was 29 Hz. Even at the higher field, the computed line width at half-height was greater than 1 ppm.

**Aromatic Resonances**—The fit of the α carbon envelope to the rigid model described in Equations 1 to 4 was reflected in the results shown in Fig. 2 as well as in the NOE values and broad line widths. Similar observations apply to the region of the aromatic resonances. The maximum for the broad envelope at 64.0 ppm, marked with $T_1$ in Fig. 1A, was characterized by $T_1$ values of 46 ms at 14.1 kG and 89 ms at 23.5 kG. A set of resonance positions on this envelope shows generally similar relaxation behavior, summarized in Fig. 4. Agreement with the simple rigid isotropic tumbling model is good.

The results for the aromatic carbon nuclei fall very close to those for the α carbons, as may be seen by superposition of points on Figs. 2 and 4. By contrast, the fit to the $T_2$ treatment, Equation 5, yielded poor results in both cases in terms of correspondence between values for the matching resonances at the two field strengths.

For the aromatic resonances sampled under the envelope centered at 64 ppm the computed $T_2$ values were 35 ns and 25 ns at the field strengths of 14.1 kG and 23.5 kG, respectively (Fig. 4). The somewhat higher values of $T_2$ compared with those for the α carbon nuclei need not reflect a true difference in rotational behavior, since contributions from more slowly relaxing non-protonated carbon nuclei are to be expected (7, 8, 15, 18).

The computed values of NOE were 1.17 and 1.16 for the two $T_2$ values obtained for the envelope under the 64 ppm maximum (4). The corresponding computed linewidths were 54 Hz and 37 Hz, respectively, at 14.1 kG and 23.5 kG. The observed NOE factor, determined as described above from the experiments in Fig. 3, was 1.2 for the region between 48 and 89 ppm and 52 and 84 ppm for the coupled and decoupled spectra, respectively. This value is in excellent agreement with the computed values, and is close to the theoretical minimum of 1.15. Confidence in the experimental accuracy of the NOE values is supported by an accompanying value of 1.1 for the region of the carbonyl carbon nuclei.

These results indicate that the protonated aromatic carbon nuclei fit the rigid, reorienting sphere model with values of $T_2$ not very much greater than the 22 ns average taken for the α carbon nuclei. In both cases the set of possible values below 1 ns is clearly incorrect.

Since all aromatic residues except certain histidines are more or less buried in the crystalline myoglobin structure (13, 24), it is not surprising that their internal motions appear to be considerably hindered. It should be noted that the histidine residues fall into two classes, one exposed and mobile and the other largely constrained (13, 25), distinguishable in terms of line width and titration properties in proton NMR studies (26). Browne et al. (27) have shown by similar techniques to those employed here that four histidine side chains in the tryptophan synthetase α subunit are highly immobilized.

**Narrower Resonances**—In Fig. 1A four relatively narrow resonances are marked with 3, 4, 5, and 6. In each case a corresponding resonance can be recognized in Fig. 1B. The narrow line width...
can not in each case be assigned to the whole group of resonances contributing at a given chemical shift position. As has been pointed out above, the $T_1$ measurements on resonances such as those will reflect to the greatest extent the behavior of the more mobile nuclei that relax more slowly and confer the narrower line widths. A more rapidly relaxing envelope overlays the base of the narrower resonances, as may be seen by observing the partially relaxed spectra. As shown by Glusker et al. (7) for ribonuclease A and its oxidized derivative the contribution of resonances from less mobile nuclei can be negligible in a protein.

The resonance position marked by $\delta$ in Fig. 1A is at 166.4 ppm of external CS$_2$. From simple peptide studies (15-17, 19), this region of the spectrum probably is weighted with resonances of C$^\beta$ and C$^\gamma$ nuclei. Measurements of NOE on the spectral region between 156 ppm and 186 ppm yield a value of 1.6, implying the presence of a large subset of relatively immobile carbon nuclei. This observation is in keeping with the way the resonance at 166.4 ppm may be seen to rise out of a broader envelope. The $T_1$ values for these resonances were 50 ms and 60 ms at 14.1 kG, respectively. Applying Equation 5, which should be formally applicable, it is found that $\gamma_0$ and $\gamma_8$, assuming a value of $\gamma_0$ of 22 ns, the observed $T_1$ values were found to correspond to rather different values of $\gamma_0$ for the two different spectrometer fields (28). This discrepancy presumably reflects contributions of several modes of motion available to the majority of the lysine side chains in myoglobin which the simple model is inadequate to describe (13).

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The resonance position at 170.3 ppm, marked with $\delta$ in Fig. 1A, corresponds to the chemical shift position of C$^\alpha$ of leucine (10). This tentative assignment is supported by the consideration that the more mobile carbon nuclei that give the sharpest resonance lines are probably least subject to chemical shift nonequivalence stemming from the effects of neighboring structures in the protein. The observed values of $T_1$ were 71 ms and 109 ms at 14.1 kG and 23.5 kG respectively. These values clearly fall beyond the range characteristic of the apparently rigid $\alpha$ carbons, but there is no evidence that the simple treatment embodied in Equation 5 is applicable.

The resonance position at 176.2 ppm, marked with $\delta$ in Fig. 1A, corresponds to that of C$^\beta$ of alanine (16). This assignment invites the direct application of Equation 5. The observed values of $T_1$ were 71 ms and 135 ms at 14.1 kG and 23.5 kG respectively. The computed value of $\gamma_0$ was approximately 20 ps in each case. The corresponding computed line widths were again of the order of 10 Hz. It is worth noting that the computed NOE values from this model fall on a part of the theoretical curve for which the NOE decrease linearly with the decrease in $\tau_R$ (4, 28).

Correlation Times for Over-all Rotation—In the present treatment the average value of $\tau_R$ for $\alpha$ carbon isotropic tumbling of 22 ns has been used as a working estimate for further computations. With $\gamma_0$ and $\gamma_8$ values close to those observed for myoglobin, the calculated $T_1$ values are not sensitive to changes as large as an order of magnitude in $\tau_R$. The model on which the $\tau_R$ computation is based assumes a rigid matrix for the $\alpha$ carbons, without internal motion involving rotation about the $\phi$ and $\psi$ angles or any other motions with rotational components. The quite good fit seen in Fig. 2 supports the model. Some variations in rigidity among the $\alpha$ carbons are not ruled out.

The values for $\tau_R$ obtained for the protonated aromatic carbon nuclei, Fig. 4, indicate somewhat longer values than those computed for the $\alpha$ carbon resonances. Again, it appears that the fit with simple isotropic tumbling is satisfactory. The basis for giving tentatively less weight to the longer $\tau_R$ values obtained in this case has been pointed out, namely, that the values may be biased by contributions of nonprotonated carbon nuclei. However, the possibility cannot be eliminated of secondary motions contributing to the relaxation process with correlation times also of the order of magnitude of $\tau_R$. The value of $\tau_R$ adopted for the $\alpha$ carbon behavior, 22 ± 5 ns, corresponds to a value of 18.0 ns from a similar previous study. The points of correspondence between the $T_1$ values from these separate studies are reasonably satisfactory. In the earlier study the choice between $\tau_R$ values corresponding to the observed $T_1$ values could not be made, and it was necessary to retain as a serious alternative the value of 3 ns (8), which would have implied considerable internal rotational motion of $\alpha$ carbons. The present study establishes unequivocally the correctness of the larger value for $\tau_R$.

The value of 20 to 25 ns for the over-all rotational correlation time is comparable to that estimated by other methods. Anderson et al. (22) with a fluorescent adduct in place of the heme obtained results that would predict for sperm whale myoglobin at 15° a rotational correlation time of 10.3 ns, allowing for a factor of 3 in the approximate conversion to the theoretical basis of the present work (29, 30). Similar results have been obtained by Stryer (20) and by Tao (21) with fluorescent depolarization techniques, and by Marye and Wyman (23) with dielectric dispersion measurements. Considering the various assumptions and approximations involved in these methods, the agreement is good. Undoubtedly it depends very much on the remarkable independence of concentration attested by such a key hydrodynamic property as translational diffusion (31). Nigen et al. (32) have established with myoglobin carboxymethylated with enriched [2-13C] bromoacetate that the spin-lattice relaxation times of the adduct nuclei show no concentration dependence over a considerable range. These adducts do behave more as $\beta$ carbons than as $\alpha$ carbons (32), so that the bearing of the observation on the invariance of $\tau_R$ has limited significance.

A clear departure from $\alpha$ carbon behavior is evident from the relaxation behavior of the narrower resonances discussed above. The correlation times for the inherently less hindered methyl groups are appreciably shorter than the values for the sampled methylene carbons. The ranges of these values are not unreasonable by analogy with smaller molecules (2, 3, 16-19).

Relation to Other Evidences of Motion—There is considerable evidence to support the view that myoglobin is not a rigid, invariant structure (15, 24, 33-44). In fact, the external side chains and the methyl groups have short rotational correlation times. On the other hand, the internal aromatic residues have very little rotational freedom. However, a considerable group of side chain carbon nuclei falls outside the categories listed above and may well contribute to some of the resonances that appear to reflect some degree of internal motion. Two aspects of the myoglobin structure may facilitate the motion of carbons not protruding into the solvent. The first is the presence of cavities within the structure. The second is the case with which surface side chains, especially the nonpolar ones that are less subject to polar constraints, may rotate to protrude into the surrounding solvent.
The present results should serve as a background for future studies in which sites in certain residues of a protein would be specifically enriched in $^{13}$C in order to probe the motional behavior of many parts of the protein (15, 46).

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