solvent, and the effects of the dielectric boundary.[19,34] In addition, an exact treatment of electrokinetic phenomena is very complicated, notwithstanding the Poisson–Boltzmann equation; some radical approximations about the conductivity of the electrolyte in the cavity are usually accepted.[11] In this context, a nonequilibrium molecular dynamics study[37,38] of such a system would be a real step forward toward a better understanding of these important phenomena.

Acknowledgment. The support of the U.S.–Yugoslav Joint Fund for Scientific and Technological Cooperation (Project No. 8717984) is acknowledged gratefully. We thank a reviewer for comments that clarified the meaning of eq 8.

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Solution Deuterium NMR Quadrupolar Relaxation Study of Heme Mobility in Myoglobin

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Abstract: NMR spectroscopy has been used to monitor the quadrupolar relaxation and motional dynamics of ^{2}H selectively incorporated into skeletal and side chain positions of the heme in sperm whale myoglobin. The hyperfine shifts of the heme resonances in paramagnetic states of myoglobin allow resolution of the signals of interest, and paramagnetic contributions to the observed line widths are shown to be insignificant. The ^{2}H line widths for the skeletal positions of deuterohemin-reconstituted myoglobin yield a correlation time identical with that of overall protein tumbling (9 ns at 30 °C) and hence reflect an immobile heme group. The ^{2}H NMR line widths of heme methyl groups exhibit motional narrowing indicative of very rapid internal rotation. Hence the methyl rotation is effectively decoupled from the overall protein tumbling, and the residual quadrupolar line width can be used directly to determine the protein tumbling rate. The ^{2}H NMR lines from heme vinyl groups were found narrower than those from the heme skeleton. However, the range of quadrupolar coupling constants for sp^2 hybridized C–^{2}H bonds does not permit an unequivocal interpretation in terms of mobility.

The importance of dynamics to protein function has been recognized for some time, and it is currently an area of considerable research interest.[2-4] Hemoproteins have received particular attention in this regard, as the X-ray structures of myoglobin[5] and hemoglobin[6] reveal the necessity of structural fluctuations to allow for ligand access to the heme active site. Energy calculations have shown that structure fluctuations in myoglobin make ligand access energetically realistic,[7] and molecular dynamics calculations of amino acid residue fluctuations in cytochrome *c* have shown good agreement with residue mobility results derived from the temperature dependence of X-ray thermal factors.[4,8] The degree of steric interaction with an amino acid side chain, and hence the rotational mobility, of one of the heme vinyl groups in hemoglobin has been implicated in the cooperativity effect in human hemoglobin.[9] Several experimental methods have been employed to probe the great range of motional states in proteins.[2-4,10,11] One of the more versatile methods is NMR spectroscopy, which allows the characterization of the motions of specific atomic sites over a wide range of time scales. Analysis of relaxation rates such as T_1 and T_2 and the nuclear Overhauser effect (NOE) will yield motional information over a large range of rates.[2,3,12,13]

Several NMR relaxation experiments have been carried out on myoglobin. Solution studies include ^{13}C NMR at natural abundance,[14-16] using isotopically enriched ^{13}C methionines,[17] ^{2}H NMR of ^{2}H-labeled modified myoglobin,[18] and proton NOEs.[19,20] Crystalline state NMR has been used with ^{13}C-labeled methionines and ^{2}H-labeled heme methyl and propionic acids.[21-23] While ^{13}C and ^{1}H NMR relaxation experiments have furnished valuable insights into the dynamics of myoglobin, analysis of these experiments is not simple; some problems are the inverse sixth power dependence that the T_1's have on the internuclear distance, whose values are not known with precision, and the possibility of multiple

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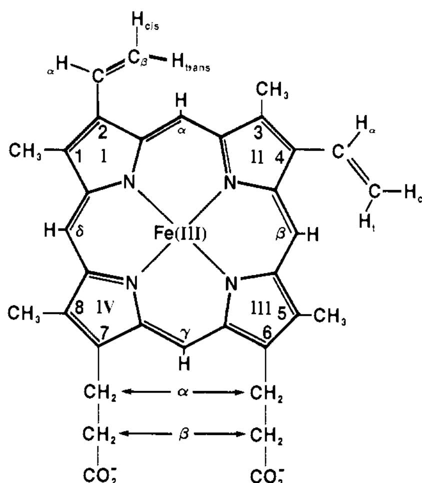

Figure 1. The structure of hemin; deuterohemin has the 2- and 4-vinyl groups replaced with hydrogens.

relaxation mechanisms that contribute significantly to the net relaxation rate.[24,25] Conversely, deuterium NMR[25,26] has the advantage that relaxation rates are dominated by the quadrupolar mechanism, even in paramagnetic systems. While deuterium labeling techniques must be used, the *b*-type hemoproteins such as myoglobin are particularly amenable to such efforts, since the protein may be reconstituted with a ^{2}H-labeled native or modified heme.[18,27–29]

As part of a continuing effort[19,20,30] to probe hemoprotein dynamics by solution NMR techniques, we have examined the ^{2}H NMR solution spectra of myoglobin reconstituted with hemes that have been ^{2}H labeled at various positions[28] (see Figure 1). The labeled myoglobins were observed in the met-aquo,[28] met-cyano,[27,19] and deoxy[31] forms. We have analyzed the ^{2}H relaxation behavior in terms of mobility for a variety of heme labels introduced at functional groups that range in anticipated internal mobility from negligible (porphyrin skeletal probes), to rapid internal rotation (heme methyls), to some degree of restricted rotation (vinyl). The large thermal factors for vinyls,[10] as well as the appearance of disorder in X-ray studies, have been interpreted as evidence for internal mobility.[4,8] Reported intra-vinyl NOEs appear to support this hypothesis.[19] Because of resolution problems, ^{2}H NMR studies are carried out most advantageously on paramagnetic heme protein derivatives that exhibit large chemical shift dispersion for heme substituents.[32,33] As will be shown, the paramagnetic contribution to ^{2}H relaxation rates is negligible for reasonably immobile functional groups, and hence quadrupolar effects dominate. It is noted, however, that unlike ^{13}C relaxation and ^{1}H–^{1}H NOEs, the experimental parameter needed to interpret ^{2}H NMR relaxation quantitatively, $(e^2qQ/\hbar)$, is often not known with precision for the groups of interest.

Principles

Quadrupolar NMR relaxation results from the interaction between the quadrupolar nucleus and an electric field gradient that arises from a chemical bond to the nucleus. The motions of the chemical bond cause this interaction to be time-dependent, and the quadrupolar relaxation rate is reflective of the motions of the field gradient of the chemical bond. For a deuterium nucleus, the quadrupolar relaxation rates in solution are[26,34]

$$1/T_2 = \frac{1}{160}(e^2qQ/\hbar)^2(1 + \eta 2/3)(9J(0) + 15J(\omega_0) + 6J(2\omega_0)) \quad (1)$$

$$1/T_1 = \frac{3}{80}(e^2qQ/\hbar)^2(1 + \eta 2/3)(J(\omega_0) + 4J(2\omega_0)) \quad (2)$$

with $e^2qQ/\hbar$ the quadrupolar coupling constant, η the electric field gradient asymmetry parameter, and $J(\omega_0)$ the spectral density function at the Larmor frequency, ω_0. The asymmetry parameter may be reasonably neglected, as for both aliphatic and aromatic C–^{2}H bonds, $\eta < 0.05$.[25,35] The quadrupolar coupling constant for a deuteron bonded to a carbon atom is dependent on the hybridization and chemical environment of the carbon, with typical values of 165–175 kHz for methyl deuterons, 180–195 kHz for pyrrole deuterons, and 165–195 kHz for aromatic deuterons.[26,36]

The spectral density function, $J(\omega_0)$, is the Fourier transform of the autocorrelation function, $G(t)$, for the reorientation of the C–^{2}H bond in the laboratory frame of reference. Following Wallach,[37,38] the effects of macromolecular tumbling and internal motion can be incorporated into $G(t)$ by rotational transformations from the C–^{2}H bond coordinate system to the laboratory system. In the case of isotropic overall tumbling and no internal motion, we have

$$G(t) = e^{-t/\tau_m} \quad (3a)$$

$$J(\omega) = \frac{2\tau_m}{1 + \omega^2\tau_m^2} \quad (3b)$$

with τ_m the tumbling rate of the protein. For the protein of interest, M_r = 16 kD and $\tau_m \approx 10$ ns; at a spectrometer frequency of 55 MHz, $\omega^2\tau^2 > 1$, leading to a predicted quadrupolar line width, δ_{quad}, in the limit of slow tumbling (and in the absence of internal motion).[25]

$$\delta_{quad}(^2\mathrm{H}) = 1/(\pi T_2) = \frac{9}{80\pi}(e^2qQ/\hbar)^2\tau_m \quad (4)$$

If there is an axis of internal rotation about which the C–^{2}H bond rotates or jumps, a more complex spectral density function results which may depend critically on the motional model. While there are several possible models for methyl rotation, Lipari and Szabo[39] have demonstrated that if the internal motion is fast relative to the overall tumbling, it is possible to calculate the relaxation rates with an approach independent of a particular model of internal motion. This "model free" approach then characterizes the relaxation rate in terms of the two motional rates τ_m, the internal motion correlation time τ_i, and a generalized order parameter S^2, which reflects the geometry of the axis of internal motion to the relaxation vector. The authors note this is exact when the internal motion is fast relative to both molecular tumbling ($\tau_e^{-1} = \tau_i^{-1} + \tau_m^{-1} \gg \tau_m$) and the NMR time scale ($\tau_e\omega_0 \ll 1$). With the assumption of a single internal rotation fast relative to τ_m (e.g. methyl), the relevent line width becomes (in the limit of slow tumbling)[39]

$$\delta(\mathrm{methyl}) = 1/(\pi T_2) = \frac{9}{80\pi}(e^2qQ/\hbar)^2S^2\tau_m \quad (5)$$

with $S^2 = 0.25(1 - 3\cos^2(\beta))^2$, and β the angle between the internal rotation axis and the relaxation vector. For the pertinent

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case of methyl rotation (β = 109°), $S^2 = {}^1/_9$.

Experimental Section

Sperm whale myoglobin, Mb, was purchased as a lyophilized, salt-free powder (Sigma) and used without further purification. Deuterium-depleted water (3×10^{-3} natural abundance) was obtained from Aldrich. Hemin (Figure 1) ^{2}H-labeled at the 2,4-α-vinyl positions (~80%), [2,4-($^2H_\alpha$)$_2$]hemin; ^{2}H-labeled at the 1,5-methyl positions (75% and 90%, respectively), [1,5-(C^2H_3)$_2$]hemin; ^{2}H-labeled at the 1,3-methyl positions (60% and 80%, respectively), [1,3-(C^2H_3)$_2$]hemin; and deuterohemin (2,4 hydrogens rather than vinyls in Figure 1), ^{2}H-labeled at the skeletal 2,4 positions (~90%), [2,4-$^2H_2$]deuterohemin, are the same compounds as reported previously.[28] Sperm whale apomyoglobin was prepared by standard methods to yield the equilibrated hemoproteins.[28,29] The maximum concentration used for data acquisition was 3 mM, and protein solutions were concentrated by ultrafiltration. Protein solutions contained 0.2 M NaCl; the pH (uncorrected for isotope effect) was adjusted by addition of 0.1 M HCl or 0.1 M NaOH, both in deuterium-depleted water. Met-cyano myoglobin was prepared by addition of a 4-fold excess of KCN; deoxy myoglobin was prepared by reduction of myoglobin under nitrogen with a 2-fold excess of sodium dithionite.

Deuterium NMR spectra were recorded at 55.27 MHz on a Nicolet NT-360 NMR spectrometer using a 30 μs 90° pulse over a 7 kHz bandwidth with 4K data points. The number of scans per spectrum varied from 15 000 to 200 000 depending on the sample and the desired signal-to-noise ratio. Spectra simulations were done with use of the Nicolet curve analysis deconvolution routine.

Results and Discussion

Quadrupolar versus Paramagnetic Relaxation Effects. The observed ^{2}H NMR line width, $\delta_{obs}(^2H)$, in a paramagnetic protein will have both quadrupolar and non-quadrupolar contributions, and while it can be expected that the quadrupolar contribution will dominate, an accurate estimate of the non-quadrupolar relaxation is necessary in these paramagnetic systems. The observed ^{2}H NMR line width is given by eq 6

$$\delta_{obs}(^2H) = \delta_{quad}(^2H) + \delta_{dia}(^2H) + \delta_{para}(^2H) \qquad (6)$$

where the first term is given by either eq 4 or 5, and δ_{dia} and δ_{para} are the diamagnetic and paramagnetic contributions, respectively.[40] The corresponding equation for the observed ^{1}H line width for the identical hydrogen is given by

$$\delta_{obs}(^1H) = \delta_{dia}(^1H) + \delta_{para}(^1H) \qquad (7)$$

As both the δ_{dia} and δ_{para} terms are proportional to γ^2, and their frequency dependence is relatively flat at Larmor frequencies in the range 500–100 MHz, this suggests that simply scaling the $\delta_{obs}(^1H)$ by $(\gamma_{^2H}/\gamma_{^1H})^2 = 1/42.6$ will yield a valid estimate of the last two terms in eq 6. Hence, the desired $\delta_{quad}(^2H)$ is obtained from the two available data $\delta_{obs}(^2H)$ and $\delta_{obs}(^1H)$ via eq 8

$$\delta_{quad}(^2H) = \delta_{obs}(^2H) - \delta_{obs}(^1H)/42.6 \qquad (8)$$

Protein Tumbling Time. The ^{2}H NMR line width of an immobilized deuteron in Mb will provide an estimate of τ_m.[25] While no such unambiguously assigned signal is resolved in the ^{1}H NMR spectrum for native Mb in any form, replacement of hemin with deuterohemin (Figure 1) with ^{2}H labels at the 2,4 positions yields a protein with essentially unaltered structure[29] and two convenient peaks as probes of the overall protein motion. Figure 2 shows the ^{2}H NMR spectrum of met-aquo [2,4-$^2H_2$]deuterohemin-Mb at 30 °C; the two 2,4-$^2H_2$ resonances are resolved,[28] as well as the HO^{2}H peak and some residual meso deuteriation.[41] The $\delta_{obs}(^2H)$ for the 2-^{2}H and 4-^{2}H are equivalent and determined to be 525 ± 50 Hz. As the $\delta(^1H)$ has been reported[28] ≈300 Hz, non-quadrupolar contributions to $\delta_{obs}(^2H)$ are then ≤7 Hz, and we note this is well within the experimental error of the deuterium line width. By using 195 kHz ± 10 as the quadrupolar coupling constant for the pyrrole deuterons,[23] application of eq 1 yields τ_m = 8.7 ± 1.7 ns at 30 °C.

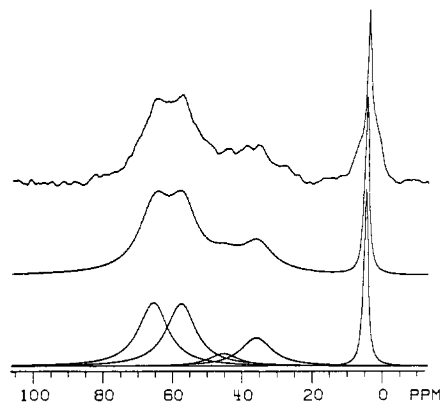

Figure 2. ^{2}H NMR spectrum at 55.27 MHz of [2,4-$^2H_2$]deuterohemin myoglobin in the met-aquo state, with composite and component simulations at pH 6.5, 30 °C. The $\delta_{obs}(^2H)$ for the 2-^{2}H and 4-^{2}H are 525 ± 50 Hz; the intensity in the region 30–45 ppm arises from partial deuteriation of the meso position.[41] Number of scans ~2×10^5.

The tumbling rate of myoglobin at low concentrations has been determined by several methods. Two such studies are γ-γ correlation spectroscopy,[42] which yields a value of $\tau_m \approx 9$ ns (after the usual temperature corrections), and natural abundance ^{13}C α-carbon NMR relaxation,[43] which provides a value of τ_m = 9.2 ns (assuming r_{CH} = 1.11 Å[24]). Both of these experiments show excellent agreement with the ^{2}H NMR result. While the resolution at 25 °C of the [2,4-$^2H_2$]deuterohemin metMb(H_2O) is poor ($\delta(^2H)$ = 640 Hz, data not shown), a value of τ_m = 11 ns at 25 °C is consistent with the ^{2}H, γ–γ, and ^{13}C results.

Methyl Rotation. The quadrupolar ^{2}H NMR relaxation rate of a methyl group will be influenced by two sources of motion: overall protein tumbling, and rapid internal motion of the methyl group. These two rates are expected to be different by several orders of magnitude, as the protein tumbling rate as noted above is 11 ns, and the methyl rotation rate for a compound in solution is expected to be in the range of several picoseconds.[2] Hence eq 5 should be applicable and lead to a reduction of the expected immobile $\delta_{quad}(C^2H_3)$ by a factor of 1/9.[26,39] The ^{2}H NMR spectrum of high-spin [1,5-(C^2H_3)$_2$]Mb(H_2O) at 25 °C is shown in Figure 3A. The computer simulations lead to indistiguishable line widths, and $\delta_{obs}(C^2H_3)$ = 70 ± 7 Hz. The previously reported $\delta_{obs}(C^1H_3) \approx 300$ Hz in this oxidation state[28] leads to a $\delta_{quad}(C^2H_3)$ = 63 ± 7 Hz. The ^{2}H NMR trace for the low-spin derivative, [1,3-(C^2H_3)$_2$]Mb(CN), is shown in Figure 3B. One signal is resolved at ≃19 ppm, the known position for the 1-CH_3;[27] the 3-CH_3 is not resolved in the ^{1}H NMR spectrum. The ^{2}H spectrum suggests that the 3-C^2H_3 peak appears as a shoulder on the upfield side of the $^1HO^2H$ peak (≃4 ppm). This confirms an assignment indicated by NOE studies.[44] The $\delta_{obs}(1\text{-}C^2H_3)$ is 55 ± 6 Hz. Correcting for the $\delta_{obs}(1\text{-}C^1H_3) \approx 35$ Hz[27,29] (≤1 Hz correction for this low-spin oxidation state) gives $\delta_{quad}(1\text{-}C^2H_3)$ = 54 ± 6 Hz. As expected, the $\delta_{quad}(C^2H_3)$ are the same within experimental error for the different methyls, as well as for the same methyls for different oxidation/ligation states of the protein. If $\delta_{quad}(C^2H_3)$ reflects rapid internal rotation, the ratio of eq 5 (with $S^2 = {}^1/_9$) to eq 4 (the pyrrole $\delta(^2H)$) yields, even outside the slow tumbling limit

$$\frac{\delta_{quad}(C^2H_3)}{\delta_{quad}(\text{pyrr}^2H)} = {}^1/_9\left[\frac{(e^2qQ/\hbar)_{C^2H_3}}{(e^2qQ/\hbar)_{\text{pyrr}^2H}}\right]^2 \qquad (9)$$

Inserting the known C^2H_3 quadrupolar coupling constant (170 kHz[23]) and the estimate for the pyrrole (195 ± 10 kHz[23]) yields a ratio of 0.085 ± 0.009 for the right-hand side of eq 9. Inserting the determined δ_{quad} values yield 0.092 ± 0.018. The fact that

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(41) Residual deuteriation of the meso position leads to intensity in the region 30–45 ppm, where the meso-H resonate in the ^{1}H NMR spectrum of deuterohemin-metMbH_2O.[28]

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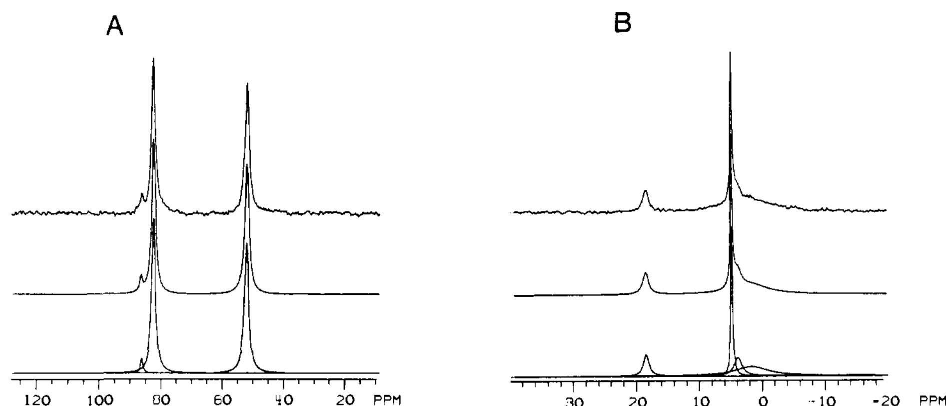

Figure 3. (A) ^{2}H NMR spectra of $[1,5\text{-}(C^2H_3)_2]Mb(H_2O)$ at 55.27 MHz and 25 °C. The 5-CH_3 and 1-CH_3 resonances were previously assigned[28] at 84.9 and 53.2 ppm, respectively. The small peak marked at 87 ppm is from the minor component form of the protein.[29] The $\delta_{obs}(C^2H_3)$ are 70 ± 7 Hz. Number of acquisitions ~15 000, as in spectra B. (B) ^{2}H NMR spectrum of $[1,3\text{-}(C^2H_3)_2]Mb(CN)$. The 1-$CH_3$ resonance has been assigned at 19 ppm;[27] the 3-CH_3 was located at 4 ppm.[44] The $\delta_{obs}(C^2H_3)$ is 55 ± 5 Hz for the 1-C^2H_3.

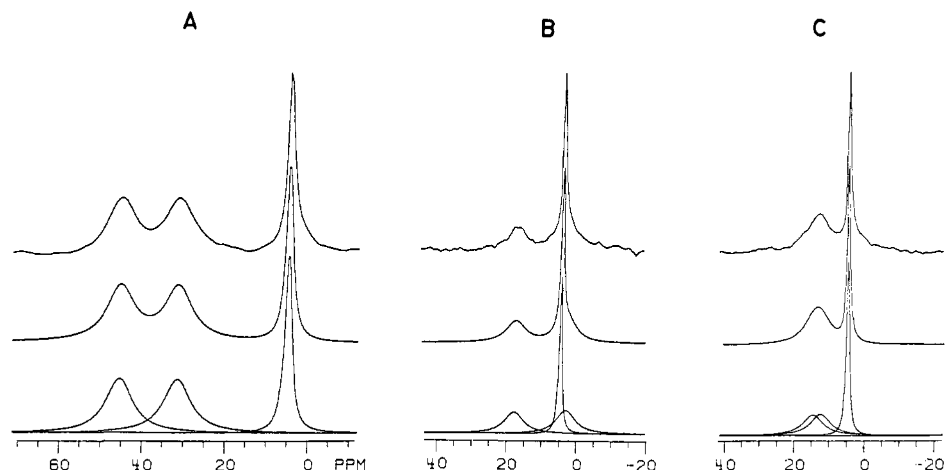

Figure 4. ^{2}H NMR spectrum at 55.27 MHz, 25 °C, of $[2,4\text{-}(^2H_\alpha)_2]Mb$ in H_2O; number of acquisitions ~2 × 10^5. (A) Met-aquo Mb, pH 6.3; simulation line widths are 360 ± 35 Hz. The 4-vinyl and 2-vinyl H_α were assigned at 46.4 and 31.4 ppm, respectively.[28] (B) Met-cyano Mb, pH 7.6; simulation line widths are 350 ± 35 Hz. The 2-vinyl H_α has been assigned at 19 ppm,[19] the 4-vinyl H_α at 4 ppm.[45] (C) Deoxy Mb, pH 7.0; although only one peak is observed (see text), a simulation line width of 360 ± 50 Hz is obtained for the two vinyls of known position (12.6 and 14.6 ppm).[48]

eq 9 holds within experimental error confirms the premise that the methyls are rotating rapidly relative to τ_m.[45] Since narrow lines are easier to detect, characterize, and quantify, we suggest that heme methyl line widths can directly yield protein tumbling times, and hence indirectly the effective M_r. We note that this technique should be particularly advantageous for characterizing the degree of association of oligermic hemoglobins.

Vinyl Mobility. The ^{2}H NMR spectrum of $[2,4\text{-}(^2H_\alpha)_2]Mb(H_2O)$ is shown in Figure 3A. Computer fits yield the same line widths for the two $^2H_\alpha$ signals, 360 ± 30 Hz at 25 °C. The previously reported[28] $\delta_{obs}(^1H) \approx 300$ Hz for the 2-vinyl and 4-vinyl H_α in this complex leads to $\delta_{quad}(^2H_\alpha) = 353 + 30$ Hz. This value reduces to 335 ± 30 Hz at 30 °C (not shown). The ^{2}H NMR trace of $[2,4\text{-}(^2H_\alpha)_2]Mb(CN)$ is shown in Figure 3B. The location of the resolved 2-vinyl H_α (17 ppm) is known from NOE studies.[19] The unresolved 4-vinyl $^2H_\alpha$ peak has been located by indirect detection[48] through ^{13}C labels at the vinyl positions. The shape and position of the residual solvent signal in Figure 3B is consistent with the 4-vinyl $^2H_\alpha$ peak being on the high-field shoulder of the residual solvent peak. The $\delta_{obs}(^2H)$ for this 2-vinyl $^2H_\alpha$ is 350 ± 30 Hz. The $\delta_{obs}(2\text{-}^1H_\alpha) < 30$ Hz,[19] and hence $\delta_{obs}(2\text{-}^2H_\alpha)$ has less than 1 Hz non-quadrupolar contribution. The ^{2}H NMR spectrum of $2,4\text{-}(^2H_\alpha)_2$ deoxy Mb is shown in Figure 3C. A single peak is observed at 13.5 ppm with an apparent line width of ≈500 Hz. The 2-vinyl $^1H_\alpha$ and 4-vinyl $^1H_\alpha$ peak positions have been identified in deoxy Mb by decoupling of ^{13}C labels[48] and were reported at 12.6 and 14.6 ppm. Using this known separation, we simulated the composite line shape using two lorentzians, which yield individual line widths of 360 ± 50 Hz. Deoxy Mb ^{1}H line widths were reported[31] as ≈100 Hz, leading to a correction of ≈2 Hz for non-quadrupolar contributions, and a $\delta_{quad}(^2H) = 358 \pm 50$ Hz. Both the vinyl C-^{2}H and the pyrrole C-^{2}H are sp^2 hybridized bonds, hence the reduced $\delta_{quad}(^2H)$ of the vinyl (350 Hz) vs pyrrole (640 Hz at 25 °C) suggests motional narrowing, and hence internal mobility. However, this presupposes the quadrupolar coupling constants are the same. Brevard reports[36] a range of quadrupolar coupling constants for sp^2 carbons from 160 to 195 kHz. Hence the possible range in the ratio of the quadrupolar line widths for rigid sp^2 deuterons is predicted to vary from 0.7 to 1.5. The observed ratio of the pyrrole to vinyl line widths at 30 °C is 525 ± 50 Hz/335 ± 30 Hz ≈ 1.6 ± 0.3. Hence the range of known quadrupolar coupling constants can account for the narrowing of the $^2H_\alpha$ signals. In the absence of data on vinyl quadrupolar constants,[49] the present line width data cannot be

(45) While long accumulation times prevented T_1 experiments on the pyrrole and vinyl deuterons, an inversion-recovery experiment was performed on the $1,5\text{-}(C^2H_3)_2\text{-}Mb(H_2O)$, which yielded a ^{2}H T_1 rate of ≈17 ± 3 s^{-1}. A ^{1}H T_1 rate of 200 s^{-1} has been reported[46] for the respective C^1H_3 in this ligation state, and as the frequency dependence of this ^{1}H rate is again insensitive,[47] scaling this rate by 1/42.6 yields a residual ^{2}H quadrupolar T_1 rate of 13 s^{-1}. Application of eq 2 with the assumption of infinitely fast methyl rotation yields a calculated T_1 rate of 13.7 s^{-1}. As this is within experimental error of the observed ^{2}H T_1 rate, this supports the assumption of fast methyl internal rotation, and allows an upper limit of ≈50 ps to be placed for the methyl rotational correlation time.

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taken as unequivocal evidence for internal mobility on the nanosecond time scale. We note, however, the ^2H quadrupolar relaxation would yield information on motions whose correlation times $\tau \leq \tau_m$. Hence, ^2H NMR in the liquid state is incapable of characterizing a range of motions slower than τ_m that would be consistent with the intuitive pictures of flexibility implied by other data.[5,6]

Conclusion

We demonstrated that ^2H signals from individual sites in a paramagnetic protein can be detected, resolved, and characterized. The peaks located in the diamagnetic envelope are consistent with assignments obtained by indirect methods.[44,45] The ^2H NMR line widths were shown to be predominantly quadrupolar in origin, even for highly paramagnetic systems, and allow direct interpretation of this data in terms of mobility. Skeletal labels reveal an immobile prosthetic group within the heme cavity. Methyl groups reflected rapid internal rotation. The vinyl ^2H labels did not exhibit sufficient narrowing to be unequivocally interpreted in terms of internal mobility. It is suggested that heme methyl ^2H line widths afford a valuable probe for the determination of protein tumbling times in paramagnetic heme protein derivatives.

(49) Determination of the vinyl ^2H quadrupolar coupling constant, even in the solid state, may be rendered ambiguous if internal motion persists.

Acknowledgment. Useful discussions with J. T. J. Lecomte and Professor R. R. Vold and the experimental assistance of G. B. Matson are gratefully acknowledged. The research was supported by grants from the National Science Foundation (CHE-81-08766, CHE-84-15329) and the National Institutes of Health (HL-22252).

Proton Nuclear Magnetic Resonance Investigation of the Mechanism of the Reconstitution of Myoglobin That Leads to Metastable Heme Orientational Disorder

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Abstract: The nature of the initially formed complexes between sperm whale apomyoglobin and hemin has been investigated by ^1H NMR for the purpose of elucidating the factors that give rise to heme orientational disorder in the reconstitution process. Selective removal of each of the two propionate side chains leads to an initial complex upon reconstitution (in the presence of a non-interacting organic solvent) with strong selectivity for the heme orientation which places the sole propionate into the position occupied by the 6-propionate group in the crystal structure. Hence the propionate contacts with apomyoglobin lead to the heme disorder about the α,γ-meso axis. Equilibration yields a unique heme orientation identical to that found in native Mb single crystals. Reconstitution of apomyoglobin with iron-free protoporphyrin yields only a single heme orientation within the time needed to obtain a NMR spectrum. However, this is concluded to result from rapid equilibration rather than from unique insertion of the porphyrin. The larger ring current for the des-iron myoglobin complex is interpreted in terms of protonation of the His F8 side chain that interacts with the porphyrin core. Pyridine is shown to interact strongly with cyanomet myoglobin, and the induced changes in hyperfine shifts show a spatial selectivity which suggests that pyridine intercalates on the proximal side of the heme.

The reactions between apomyoglobin (apoMb) or apohemoglobin (apoHb) and hemin initially were thought to be complete within seconds to yield the native holoprotein on the basis of optical stopped-flow studies.[1,2] More recently, however, ^1H NMR investigations have demonstrated[3-8] that these reactions instead yield as initial products the 1:1 mixtures of holoproteins with the orientations of the heme in the folded pocket differing by a 180° rotation about the α,γ-meso axis, as shown in A and B of Figure 1 (with $R_6 = R_7$ = propionate). Only one of these orientations corresponds to that found in single crystals[9,10] (Figure 1A). The "reversed" heme orientation (Figure 1B) is kinetically trapped, and this requires many hours to months to equilibrate to the native protein, depending upon the protein origin and the solution conditions.[3,5,6,8] Moreover, this "reversed" heme orientation does not disappear, but it is present to some extent at equilibrium in both Mbs and Hbs, as clearly observed in the ^1H NMR spectra of isolated native proteins.[5-7] ^1H NMR spectroscopy is uniquely and ideally suited for both detecting and quantitatively characterizing this heterogeneity in hemoproteins.[3-8] Qualitative characterization of changes in the ratio of heme orientation can now also be monitored by circular dichroism.[11-14]

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