g, 1.34 mmol) was treated with a pentane solution of *n*-butyllithium (1.1 mL, 1.3 M, 1.45 mmol) (15 min) at -78 °C under an atmosphere of argon. After the reaction mixture was warmed to room temperature over the course of an hour, all volatile components were removed in vacuo. A red-black solid remained, which was dissolved in toluene-d_8 for NMR examination.

(*E*)-1,3,3-Tris(trimethylsilyl)propene (5). Under an argon atmosphere at -78 °C (dry ice-2-propanol) *sec*-butyllithium (66 mL, 1.3 M, 95.8 mmol) in pentane was slowly syringed into a stirred solution of (*E*)-1,3-bis(trimethylsilyl)propene (3; 16 g, 86 mmole) in 50 mL of THF. The mixture immediately turned red. After stirring for 1 h, the resulting solution was allowed to warm to room temperature and then reacted with trimethylsilyl chloride (9.4 g, 11 mL, 86 mmol). Hydrolysis, extraction into diethyl ether, and removal of the ether yielded, after vacuum distillation of the residue, 10.9 g of the title compound: 49% yield; bp 116-120 °C (31 Torr); proton NMR ($CDCl_3$) δ 5.32 (C_1H), 5.87 (C_2H), 1.18 (C_3H), 0.84 and 0.52 (CH_3Si), (1:2); J(X,Y), Hz, C_1H, C_2H 18.2, C_1H, C_3H 0.4, C_2H, C_3H 11.4; ^{13}C NMR δ 127.59 (C_1), 145.22 (C_2), 31.53 (C_3), -0.92 and -0.56 (TMS) (1:2).

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Influence of Molecular Correlation Time on the Homonuclear Overhauser Effect in Paramagnetic Proteins

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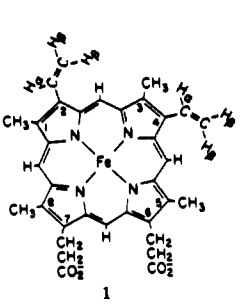
Abstract: The effect of solvent viscosity on the nuclear Overhauser effect, NOE, in paramagnetic myoglobin complexes has been investigated to determine the influence of the overall molecular correlation time on the magnitude of NOEs. We show that steady-state NOEs in these paramagnetic complexes are strongly decreased relative to isostructural diamagnetic systems but that this dramatic diminution characteristic of NOEs in paramagnetic complexes can be moderated by selectively increasing cross-relaxation, provided the electron spin-lattice relaxation time, which dominates intrinsic nuclear relaxation, is independent of the overall molecular motion of the complex. In such cases, the steady-state NOE will increase with the overall correlation time, τ_c, asymptotically approaching that NOE characteristic of an isostructural diamagnetic system. Steady-state NOE experiments on strongly paramagnetically relaxed and hyperfine-shifted resonances in high-spin ferric-aquo and low-spin ferric-cyano complexes of sperm whale myoglobin demonstrate that the NOE doubles upon doubling the viscosity by adding 30% ethylene glycol. The cross-relaxation rates determined via a truncated NOE experiment double, as expected with doubling the viscosity, and the increased steady-state NOE hence indicates that the iron T_{1e} is independent of τ_c. Such an independence of T_{1e} on τ_c in hemes in general is demonstrated for the model compound dicyano[tetrakis(4-sulfophenyl)porphine]iron(III) for which T_{1H} (and hence T_{1e}) is found unchanged when the solvent viscosity is varied 40-fold in mixtures of water and glycerol. The increased NOEs with τ_c provide a rationalization for earlier observations of vastly increased NOEs for model complexes in viscous solvents and improved NOE data in large hemoprotein systems and suggest that not only should NOEs be profitably pursued for a variety of paramagnetic proteins but such studies (in contrast to diamagnetic proteins in the slow-motion limit) may more likely be successful in larger rather than smaller proteins.

The importance of the nuclear Overhauser effect, NOE, in modern structural studies of biopolymers by NMR, either in the one-dimensional variation or in the form of the two-dimensional NOESY map, is underscored not only by its critical role in resonance assignments but also by its ability to provide the unique nonbonded constraints that form the basis of complete structure determination of small diamagnetic biopolymers ($M_r \leq 15 \times 10^3$) in solution.[1-3] The efficacy of the NOE depends on the ^1H-^1H dipolar interaction as the dominant proton relaxation mechanism.[4] Hence, the dominance of electron-nuclear dipolar relaxation of protons in paramagnetic molecules[5,6] was initially thought to render NOEs undetectable. This dominant paramagnetic relaxation mechanism is clearly observed for the hyperfine-shifted resonances of all paramagnetic molecules[5-7] and, in particular, for the class of heme proteins that possess the iron protoporphyrin group **1** capable of existing in no less than seven relatively stable paramagnetic states for the three common oxidation states.[8,9] However, in spite of the strong dominance of paramagnetic relaxation influences in all protein forms studied to date, it has been possible to obtain useful ordinary steady-state or time-dependent



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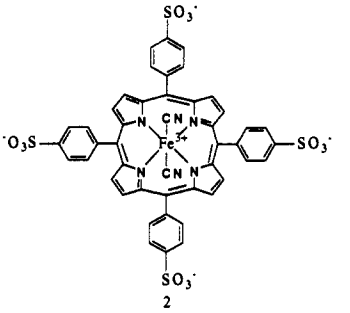

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NOE data for a variety of hemoproteins.[10-13] In two cases, highly informative two-dimensional NOESY maps have been obtained

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that provide a wealth of structural information.[14,15]

A surprising finding was that the quality (or magnitude) of NOEs for similar functional groups appeared superior in some relatively large proteins such as horseradish peroxidase[12,13] ($M_r \sim 42 \times 10^3$) as compared to myoglobin[10,11] ($M_r \sim 16 \times 10^3$) in the same oxidation/spin state. Moreover, the problems with spin diffusion[16] did not appear as serious in large paramagnetic systems as in diamagnetic systems, thus allowing clear detection of fully developed primary NOEs in lactoperoxidase[17] ($M_r \sim 78 \times 10^3$). These preliminary reports suggest that the strategies for NOE studies in paramagnetic systems may not parallel those applied to analogous diamagnetic systems.[1-3] The key difference between the two systems is that, in a paramagnetic system, the intrinsic relaxation is dominated by paramagnetic effects and such paramagnetic relaxation may be independent of molecular motion.[18-20] This is suggested by observing similarly effective nuclear relaxation in isoelectronic heme model compounds[21] and hemoproteins,[7,12] Thus cross-relaxation and intrinsic relaxation, unlike in a diamagnetic system dominated by $^1H-^1H$ dipolar interactions,[4] may be essentially uncoupled and could be extrinsically manipulated.

Large classes of important paramagnetic proteins and enzymes with a variety of metals and oxidation/spin states exhibit reasonably well-resolved ^1H NMR spectra that could potentially provide a wealth of electronic and molecular structural information on the active site.[6,7,22-28] In these systems, the problem of the crucial resonance assignments is complicated by the loss of the usual shift/functional group correlations established in diamagnetic systems. While isotope labeling has been successfully applied to assign heme resonances in *b*-type hemoproteins,[29] the more general class of paramagnetic proteins that do not possess a reversibly extractable prosthetic group has had to rely on necessarily less direct and more ambiguous methods. Metal-centered relaxation provides one recourse to tentative assignments,[7,26,27] but unique assignments are unlikely in view of the demonstrated importance of such relaxation by delocalized spin density.[21]

In view of the potential value of NOE studies on a large variety of paramagnetic proteins for both resonance assignment and structure determination, we investigate here conditions that control the NOEs in paramagnetic systems to provide some guidance for future profitable applications in other paramagnetic molecules. To this end we consider here the influence of overall molecular motion on steady-state NOEs and present such NOE data on both low-spin and high-spin ferric myoglobin complexes that directly support the advantages of carrying out studies on as large a protein as practical. The needed assignments for both spin states of the oxidized derivative of this small hemoprotein ($M_r \sim 16 \times 10^3$), the strongest paramagnetic high-spin,[11,30] $S = {}^5/_2$ and weakest paramagnetic, low-spin, $S = {}^1/_2$ states,[15,31] have been reported.

Principles

The time evolution of an NOE in a two-spin system can be represented by[4]

$$\eta(t)_{j\rightarrow i} = \sigma_{ij}/\rho_i[1 - e^{-\rho_i t}] \tag{1}$$

where

$$\sigma_{ij} = -\gamma^4\hbar^2\tau_c/(10r_{ij}^6) \tag{2}$$

For short saturation time t for spin j, the truncated NOE results with

$$\eta(t)_{j\rightarrow i} = \sigma_{ij}t \tag{3}$$

which is independent of intrinsic relaxation and, hence, independent of paramagnetism. In the limit of long saturation time, the steady-state NOE results[4]

$$\eta_{j\rightarrow i} = \sigma_{ij}/\rho_i \tag{4}$$

For a paramagnetic molecule, $\rho_{total} = \rho_{dia} + \rho_{para}$, allowing us to recast the steady-state NOE of a paramagnetic molecule as

$$\eta(\text{para}) = [\sigma/\rho_{dia}][Q/(Q+1)] = \eta(\text{dia})[Q/(Q+1)] \tag{5}$$

with Q, the ratio of diamagnetic to paramagnetic relaxation rates, given by

$$Q = \rho_{dia}/\rho_{para} \tag{6}$$

where η(dia) is the steady-state NOE had the molecule been diamagnetic. In a case of minimal paramagnetic influences, $Q \gg 1$ and $\eta(\text{para}) \rightarrow \eta(\text{dia})$, as expected. For dominant paramagnetic relaxation, as observed for hyperfine-shifted peaks, $\rho_{para} \gg \rho_{dia}$ and $Q \ll 1$, so that eq 5 simplifies to

$$\eta(\text{para}) = \eta(\text{dia})Q \tag{7}$$

This rationalizes the dramatic decrease of steady-state NOEs in strongly paramagnetic[10-15] as compared to analogous diamagnetic molecules,[32] with the scaling factor $Q \ll 1$. Note, however, that the truncated NOE is unaffected; i.e., eq 3 is still valid, except that the saturation must be made in a shorter time to compensate for large ρ.

Consideration of the components of Q shows that $\rho_{dia} \propto \tau_c$ in the slow-motion limit.[4] However, for a macromolecule with a well-resolved NMR spectrum[5-7]

$$\rho_{para} \propto R_M^{-6} f(T_{1e}) \tag{8}$$

where R_M is the metal–proton distance and T_{1e} is the electron spin–lattice relaxation time. T_{1e} may or may not be determined by some molecular motion.[5,19] For the potential case of interest here, for which nuclear T_1 data suggest very similar T_{1e}s in small models[21] and large proteins,[7,12] a T_{1e} and hence ρ_{para} independent of τ_c would result in $Q \propto \tau_c$. Since, for protons in a diamagnetic protein experiencing primarily $^1H-^1H$ dipolar relaxation in the

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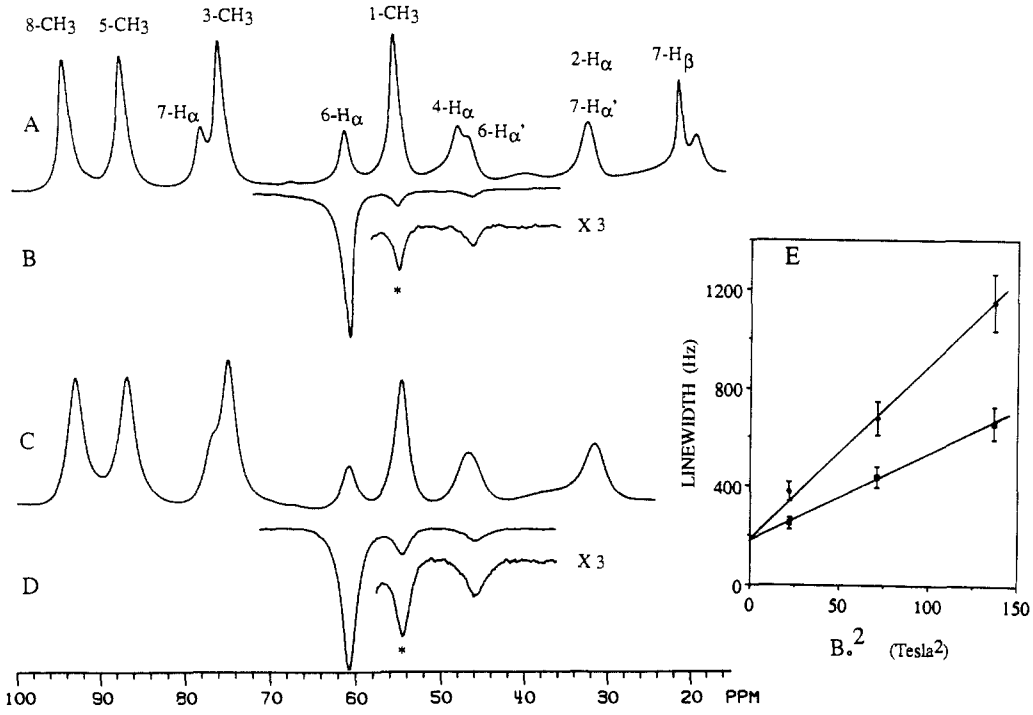

Figure 1. (A) Downfield resolved region of 360-MHz ^1H NMR spectrum of high-spin ferric metMbH_2O in ^2H_2O, pH = 6.0, 20 °C, showing previously assigned[11,30] heme resonances. (B) Difference spectrum with irradiation of a 6-proprionate H_α above. Note −7.0 ± 2% NOE to its geminal partner, 6-H_α' in ^2H_2O. (C) NMR spectrum of metMbH_2O in 30% [$^2H_6$]ethylene glycol/70% ^2H_2O solution, pH 6.0, 20 °C. (D) Difference spectrum upon saturating 6-propionate H_α peak in (C). Note −14 ± 2% NOE to geminal partner 6H_α'. (E) Plot of heme 8-CH_3 line width versus B_0^2 for metMbH_2O in ^2H_2O and 30% [$^2H_6$]ethylene glycol/70% ^2H_2O solution at 20 °C. Note the increased slope (factor 2.0 ± 0.15) in the more viscous solvent. The data points were collected at 4.7, 8.45, and 11.7 T. The asterisk indicates off-resonance saturation effects; the decoupler irradiation for the control experiments in each case was chosen in a way that guarantees no off-resonance effect to the NOE-detected 6-H_α' peak in (B) and (D).

slow-motion limit, both σ and ρ(dia) are proportional to τ_c, η(dia) will be independent of τ_c in the absence of spin diffusion,[16] and we expect at steady state

$$\eta(\text{para}) \propto \tau_c \tag{9}$$

as long as Q remains small. Hence, the steady-state NOE in a paramagnetic protein should increase linearly with molecular reorientation time, as Q becomes much larger than unity, η(para) → η(dia). The overall correlation time for a macromolecule can be estimated by the Stokes–Einstein relationship[4]

$$\tau_c = \frac{4\pi\zeta a^3}{3kT} \tag{10}$$

where a is the molecular radius and ζ the viscosity of the solvent. Thus τ_c is linear in molecular weight or, for a given molecular weight, linear in solvent viscosity.

Experimental Section

Sperm whale myoglobin was purchased from Sigma Chemical Co., and solvents were obtained from Merck, Sharp & Dohme. The tetrakis(4-sulfophenyl)porphine and its iron complex (**2**) were synthesized by standard literature methods.[33,34] The dicyano-ligated complex was prepared by adding 2.5 equiv of KCN. Two stock solutions of identical concentration of this complex in ^2H_2O and [$^2H_8$]glycerol were prepared and mixed in appropriate amounts (mass percent) to obtain solutions of desired viscosity.[35] The final solution concentration of the complex was kept ~1 mM to avoid aggregation.

The NOE experiments on myoglobin samples were performed on aquo-ligated high-spin ferric Mb (metMbH_2O) and cyanide-ligated low-spin ferric Mb (metMbCN) in ^2H_2O and solvent mixtures (mass percent) of [$^2H_6$]ethylene glycol, [$^2H_6$]dimethyl sulfoxide, and sucrose with ^2H_2O. The metMbCN samples were prepared by adding excess KCN to myoglobin in ^2H_2O. The solution pH was adjusted with 0.1 M ^2HCl and/or NaO^2H. The reported pH values are meter readings uncorrected for the isotope effect. Solutions 1.5–3.0 mM in protein were studied, and the magnitudes of the NOEs were found to be invariant: at higher concentrations, the solution viscosity will increase and NOEs could be expected to increase further. The samples in mixed solvent were prepared by adding cooled cosolvent in small fractions to the protein solution in ^2H_2O kept on ice. Solutions were centrifuged and transferred to 5-mm NMR tubes. The samples were stored at 4–5 °C and were found to be stable over several months.

The ^1H NOE experiments on metMbH_2O were performed on a Nicolet NT-360 (360-MHz) spectrometer at 20 °C with Redfield[36] selective excitation pulses by placing a carrier frequency in the downfield region of interest. Typically 3 × 10^4 scans were acquired with 60-ms repetition rate and 30-ms decoupler irradiation time from 8192 data points. The resulting free induction decays, fid, were apodized by 100 Hz to improve signal to noise. The magnitude of a NOE in each case was determined by integrated intensities of peaks of interest. The nonselective spin–lattice relaxation times, T_1, were measured by the standard inversion–recovery method. The data were analyzed by three-parameter nonlinear least-squares fit method with Nicolet software. The T_2 values were estimated from line widths ($T_2^{-1} = \pi \times$ line width). The solution viscosities were estimated from available literature data on nondeuterated solvent mixtures.[37,38] The nominal viscosity was assumed unaffected by deuteration; however, the quantitative measurements of viscosity were also performed independently as discussed in the text. The ^1H NMR experiments on low-spin metMbCN were performed on a Nicolet NM-500 spectrometer operating at 500 MHz. Typically 2500 transients were collected with a 1.25-s^{-1} repetition rate from 8192 data points; exponential line broadening of 20 Hz was applied to fids before Fourier transformation.

Results

High-Spin Ferric Mb. The 360-MHz ^1H NMR trace of metMbH_2O in ^2H_2O at 20 °C is reproduced in A of Figure 1, together with the previous resonance assignments[11,30] on the basis of a combination of isotope labeling and NOEs. The analogous trace in 70% ^2H_2O/30% [$^2H_6$]ethylene glycol is shown in C of Figure 1. The introduction of [$^2H_6$]ethylene glycol caused small but definite perturbations on the resonance positions, as did ad-

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Table I. ^1H NMR Spectral Parameters for Hyperfine-Shifted Heme Resonances of High-Spin metMbH_2O in Various Solvent Mixtures[a]

	neat ^2H_2O		30% [$^2H_6$]ethylene glycol/70% ^2H_2O		25% sucrose in ^2H_2O	25% DMSO/ 75% ^2H_2O
peak	shift (ppm)	T_1 (ms)	shift (ppm)	T_1 (ms)	shift (ppm)	shift (ppm)
8-CH_3	93.6	4 ± 0.2	93.0	4 ± 0.4	93.6	93.4
5-CH_3	86.8	4.1 ± 0.2	86.9	4 ± 0.4	86.8	87.4
7-H_α	77.3	4 ± 0.2	76.3	3.9 ± 0.3	77.0	76.9
3-CH_3	75.0	3.7 ± 0.3	74.7	3.7 ± 0.3	75.0	75.1
6-H_α	60.4	4.5 ± 0.5	60.4	5.5 ± 0.45	60.5	61.1
1-CH_3	54.3	4.3 ± 0.3	54.2	4.0 ± 0.3	54.3	54.6
4-H_α	47.0	*b*	46.4	*b*	47.4	47.0
6-H_α'	45.8	*b*	45.8	*b*	46.5	46.0

[a] Shifts in ppm from DSS at 20.0 °C. [b] Insufficiently resolved to yield T_1.

Table II. ^1H NMR Spectral Parameters for Hyperfine-Shifted Resonances in Low-Spin metMbCN in Various Solvent Mixtures[a]

		neat ^2H_2O		30% [$^2H_6$]ethylene glycol/70% ^2H_2O		25% sucrose in ^2H_2O	25% DMSO/ 75% ^2H_2O
	peak	shift (ppm)	T_1 (ms)	shift (ppm)	T_1 (ms)	shift (ppm)	shift (ppm)
heme	5-CH_3	27.5	127 ± 12	27.75	131 ± 14	27.64	28.0
	1-CH_3	18.90	145 ± 15	19.1	158 ± 17	18.94	19.0
	2-H_α	17.80	95 ± 10	17.90	100 ± 13	17.88	18.1
	8-CH_3	13.0	*b*	12.85	*b*	13.07	12.75
	2-$H_{\beta t}$	−2.70	*b*	−2.80	*b*	−2.76	−2.75
Ile FG5	γ-CH_3	−3.60	200 ± 23	−3.60	205 ± 25	−3.62	−3.60
	δ-CH_3	−4.0	206 ± 24	−4.15	203 ± 28	−4.03	−4.05
	γ-CH	−10.0	45 ± 4	−10.0	50 ± 5	−10.1	−9.7

[a] Shifts in ppm from DSS at 20.0 °C. [b] Insufficiently resolved to yield T_1.

dition of either [$^2H_6$]DMSO or sucrose. The chemical shift data in the different solvents at 20 °C are compared in Table I. More dramatic than the shift changes are the line width increases observed in the more viscous solvents. These result from the well-characterized Curie spin relaxation mechanism[39-42] by which the overall molecular motion modulates the net Curie spin and is typical of high-spin iron systems with narrow ^1H resonances (short T_{1e}s). This Curie spin effect on the line width, δ, is given (in the applicable limit $\omega_1\tau_c \gg 1 \gg \omega_1 T_{1e}$) by the relation[39,40]

$$\delta = bB_0^2\tau_c \qquad (11)$$

with

$$b = \frac{4\gamma H^2 \mu_{eff}^4 r^{-6}}{45k^2T^2}$$

a constant for a given proton at a fixed temperature. A plot of δ versus the magnetic field, B_0, squared leads to a straight line whose slope yields τ_c. Since viscosity calibrations for the solvents utilized here have generally been carried out on the nondeuterated solvent mixtures,[37,38] the relative slopes of δ vs B_0^2 provide a quantitative measure of the ratio of the mixed solvent to ^2H_2O viscosity. Such a plot is illustrated in E of Figure 1 for the optimally resolved 8-CH_3 group, and the ratio of slopes is determined to be 2.0 ± 0.15, in accord with the expectation[37] for a doubling of the viscosity upon adding 30% ethylene glycol to water. The steady-state NOE difference trace in ^2H_2O upon saturating one of the propionate 6-H_αs is shown in B of Figure 1, giving the previously reported[11] ~7 ± 2% NOE to its geminal partner 6-H_α'. When the viscosity is doubled in the 30% ethylene glycol solution, a similar saturation of 6-H_α (D in Figure 1) leads to a 14 ± 2% steady-state NOE.

Low-Spin Ferric Mb. The 500-MHz ^1H NMR traces of metMbCN at 20 °C in ^2H_2O and 30% [$^2H_6$]ethylene glycol are illustrated in A and B, respectively, of Figure 2. The previously reported assignments[10,31] for both heme and amino acid resolved resonances are included. Again, chemical shifts are selectively

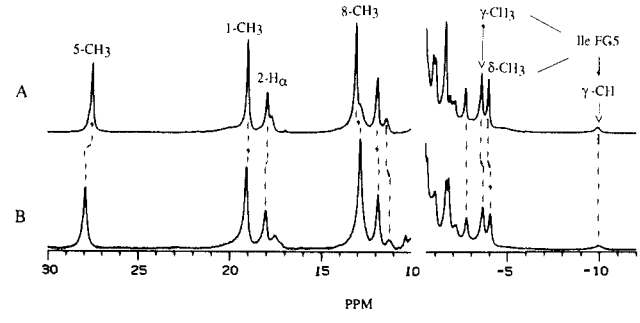

Figure 2. (A) 500-MHz ^1H NMR spectrum of metMbCN in ^2H_2O at 20 °C showing previously reported assignments[10,31] of hyperfine-shifted resonances. (B) Spectrum of the same complex in 30% [$^2H_6$]ethylene glycol/70% ^2H_2O solution. The slight increase in the resonance line widths and selective changes in chemical shifts can be readily noticed and are listed in Table II.

perturbed upon adding organic cosolvent; the shift data in four solvent systems investigated are listed in Table II, where we also include nonselective T_1 values for some of the well-resolved signals of interest in two solvents. The effect of increased viscosity on line width is much less dramatic than for the high-spin ferric derivative, and the ~20-Hz increases observed are consistent with arising essentially from the expected increased diamagnetic T_2 relaxation, as this is the order of line width observed in diamagnetic MbCO in ^2H_2O.[32]

The resulting NOE difference traces upon completely saturating the 5-CH_3 resonance of metMbCN with variable irradiation times (eq 1) and observing the region +10 to −10 ppm are illustrated in Figure 3 in ^2H_2O (A) and 30% [$^2H_6$]ethylene glycol/70% ^2H_2O (B) solvents. The time course of the truncated NOE in ^2H_2O (Figure 3A) reveals the monotonic buildup of NOEs, essentially all of which have been structurally interpreted on the basis of a combination of two-dimensional NOESY and COSY maps.[15] As reported earlier,[10,15] there is no evidence of spin diffusion in the paramagnetic derivative of the relatively small protein ($M_r \sim 16 \times 10^3$). When viscosity is doubled in 30% [$^2H_6$]ethylene glycol (Figure 3B), we observe the buildup of the same peaks (albeit with slightly different chemical shifts, particularly in the region 7–5 ppm), but at a significantly faster rate. Moreover, for the longer irradiation time (300, 500 ms), there is evidence of secondary NOEs (spin diffusion),[16] particularly noticeable in the

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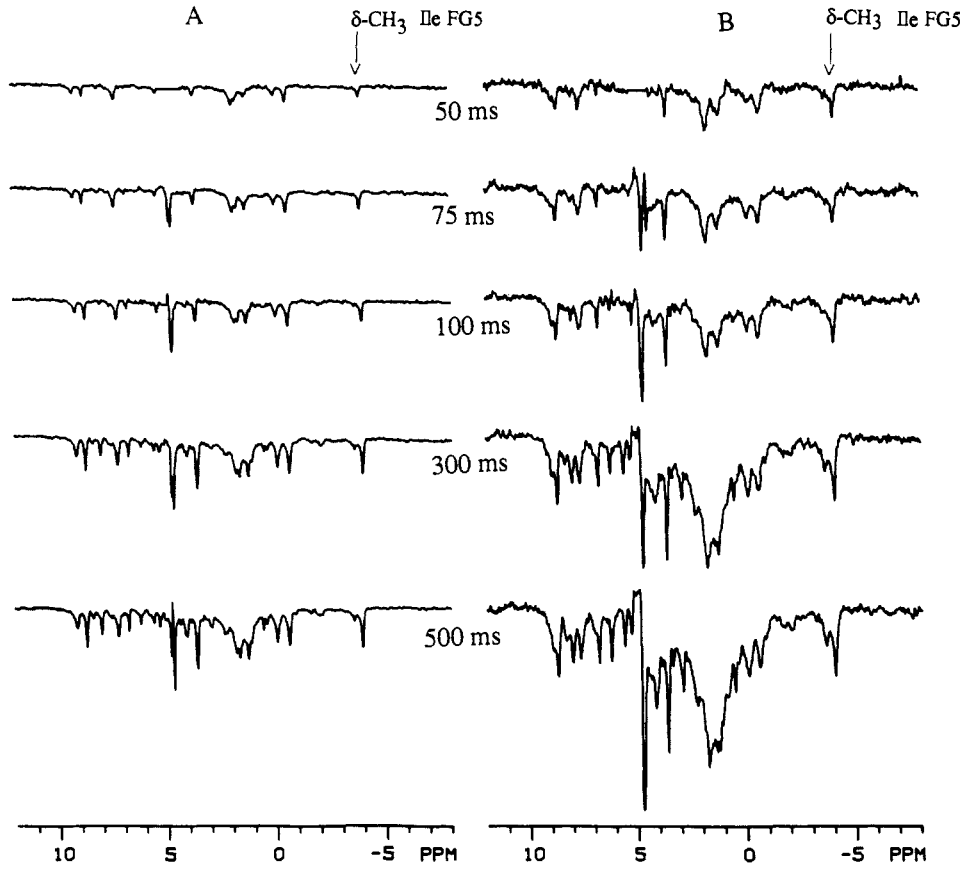

Figure 3. (A) NOE difference spectra in the +12 to −8 ppm region obtained from a truncated NOE experiment in (A) neat ^2H_2O at 20 °C and (B) 30% [$^2H_6$]ethylene glycol/70% ^2H_2O solution at 20 °C upon irradiation of 5-CH_3 resonance of metMbCN for 50-, 75-, 100-, 300-, and 500-ms irradiation time. The difference spectra are presented on a scale where the saturated 5-CH_3 peak (not shown) has the same amplitude for (A) and (B).

window 2–3 ppm. The detected peak of interest here is the δ-CH_3 of Ile FG5,[10,15] which makes dipolar contact with the heme 5-CH_3.

The magnitudes of the NOEs from 5-CH_3 to Ile FG5 δ-CH_3 were determined by integration and are plotted as a function of irradiation time in Figure 4 in ^2H_2O (open markers) and 30% [$^2H_6$]ethylene glycol (closed markers). It is clear that the NOEs are primary in each case and that both the initial slope (σ, i.e., eq 3) and the asymptotic value (σ/ρ, i.e., eq 4) are significantly larger in the latter than the former solvent. Computer fits of the two sets of data point to eq 1 leading to $\sigma = -0.7 \pm 0.2$ s^{-1}, $\rho = 10 \pm 2$ s^{-1}, and $\eta = -7.0 \pm 1.8\%$ in ^2H_2O and $\sigma = -1.4 \pm 0.2$ s^{-1}, $\rho = 10 \pm 2$ s^{-1}, and $\eta = -14.0 \pm 2\%$ in 30% ethylene glycol. Thus, σ doubles with the doubling of viscosity, as is expected (i.e., eq 3), and provides an independent index for the viscosity of the 30% [$^2H_6$]ethylene glycol solvent mixture. The steady-state NOE is also consistent with doubling in the more viscous sample, although the detection of spin diffusion in the ethylene glycol solution (see above) may make quantitative comparison of NOE data at such long irradiation times somewhat questionable. Similar increases in buildup rate and in the magnitude of the apparent steady-state NOE in the more viscous solvent were observed for other peaks (in particular that between[43] 1-CH_3 and 8-CH_3; not shown), but a quantitative analysis was precluded by the large off-resonance saturation[44] on the diamagnetic region that results from the significant decoupler power required to completely saturate other signals for the shorter irradiation times needed to separately obtain σ and ρ for a fit to eq 1.

The increase in the steady-state NOE in 30% ethylene glycol is consistent with an unchanged intrinsic relaxation rate, ρ. While a selective T_1 could not be performed on the resonance of interest,

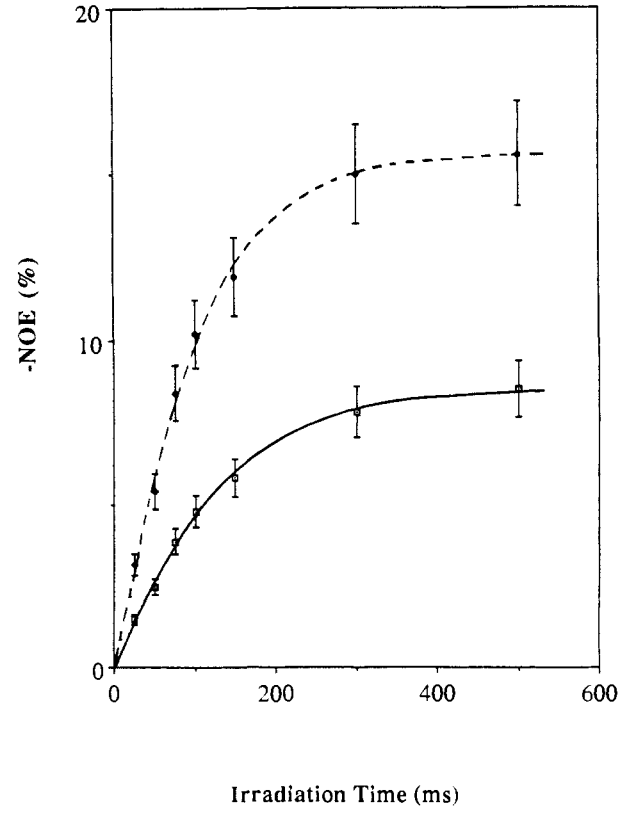

Figure 4. Plot of the magnitude of NOE in the truncated NOE experiment shown in Figure 3 versus irradiation time of 5-CH_3 in ^2H_2O (open markers, solid line) and 30% [$^2H_6$]ethylene glycol/70% ^2H_2O (closed markers, dotted line). The lines represent the least-squares fit of the data points to eq 1.

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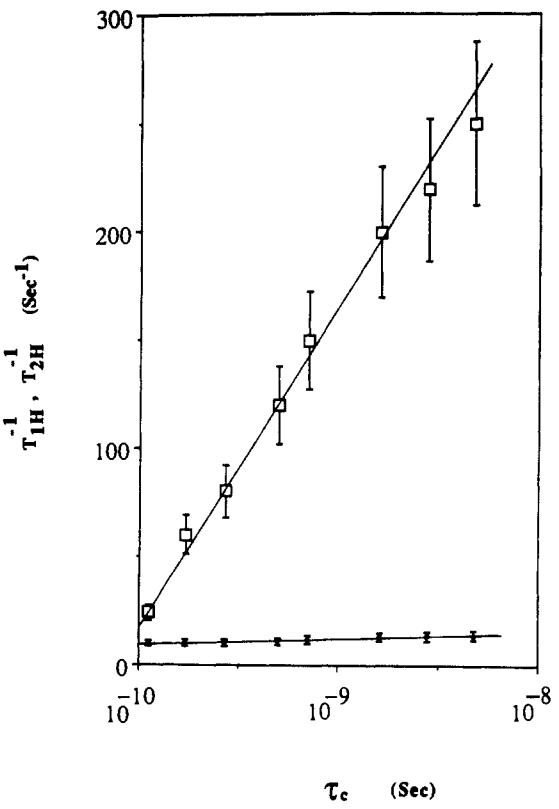

Figure 5. Plot of the pyrrole ^{1}H relaxation rates, T_{1H}^{-1} (closed markers) and T_{2H}^{-1} (open markers) = π line width, of [Fe^{III}(TPP)S(CN)$_2$] as a function of the molecular correlation time obtained through alteration of solution viscosity. The data were obtained at 360 MHz at 25 °C from a variety of mixed solvents ranging from neat ^2H_2O (least viscous) to 80% [$^2H_8$]glycerol (most viscous).[35] The effective viscosity is converted to τ_c for the complex by eq 10.

the nonselective T_1 value for Ile FG5 δ-CH$_3$, as well as for the other resolved resonances that exhibit large hyperfine shifts and large paramagnetic contribution to ρ_{total}, is unchanged within experimental uncertainty (see Table II).

Nuclear Relaxation in Models. The influence of molecular correlation time on nuclear T_1s has been pursued for the model compound dicyano[tetrakis(4-sulfophenyl)porphine]iron(III) (**2**, (TPP)SFe(CN)$_2$) in solvents of variable viscosity[35] between ~1 cp and ~45 cp with the mixed solvent system ^2H_2O and [$^2H_8$]-glycerol. Figure 5 illustrates a viscosity titration of the measured T_{1H}^{-1} and T_{2H}^{-1} (π × line width = T_{2H}^{-1}) at 25 °C and 360 MHz. The viscosity is calibrated to effective τ_c via eq 10 assuming a ~ 5.8 Å. It is clear that T_{1H} is independent of τ_c, while T_{2H} decreases monotonically with τ_c, as expected solely from diamagnetic contributions. A plot of log T_{1H}^{-1} versus reciprocal temperature at 360 MHz yielded a straight line (not shown) with a slope that yields an activation energy ~1.0 kcal/mol ($\tau = \tau_0 \exp(-E_a/RT)$).

Discussion

Hemoprotein NOEs. The observation of increased steady-state NOEs with increasing viscosity provides a quantitative basis for interpreting the earlier qualitative observations of improved NOE data in larger molecular weight isostructural hemoproteins.[10-13,17] The cross-relaxation increases linearly with the correlation time, as expected.[4] The concomitant increase in steady-state NOEs implies that the intrinsic relaxation is independent of the correlation time. The largely unchanged nonselective proton T_1s with the doubling of solvent viscosity for both metMbH$_2$O (Table I) and metMbCN (Table II) are consistent with an $f(T_{1e})$ in eq 8 that is insensitive to τ_c. The large uncertainties in the measured T_1s and the narrow viscosity range, however, provide an insufficiently critical test.

It can then be concluded, largely on the basis of the data available on hemoproteins, that NOE studies may likely be more profitably pursued on large rather than small paramagnetic proteins possessing an electronically similar chromophore. This is in contrast to diamagnetic species, where steady-state NOEs are independent of correlation time in the slow-motion limit,[4] and correlation time longer than ~25 ns leads to spin diffusion[16] that tends to obliterate useful assignments and/or structural information. Spin diffusion, of course, will also increase with τ_c (as witnessed by comparison of the NOE difference traces for long irradiation times in Figure 3). However, as has been noted in previous work with horseradish peroxidase[12,13] (M_r ~ 42 × 10^3) and lactoperoxidase[17] (M_r ~ 78 × 10^3), spin diffusion is considerably less effective in a paramagnetic than an isostructural diamagnetic system.

For systems that exhibit Curie spin relaxation[39-42] (large $S(S+1)$, short T_{1e}), the advantage of increased NOEs with molecular weight can still be realized if the applications are restricted to low magnetic fields (line width $\propto B_0^2$), which minimizes the relaxation mechanism. In the present high-spin ferric metMbH$_2$O case, the steady-state NOE increase with τ_c would translate into a similar increase in signal-to-noise, S/N, in the difference trace if the experiments were carried out at a field <4 T; at 360 MHz, the area in the difference doubles, but the approximately doubled line width leaves the S/N unchanged and, hence, leaves the detectability of the NOE unaltered with change in τ_c.

Electron Spin–Lattice Relaxation. A more quantitative assessment of the influence of overall molecular reorientation on the intrinsic relaxation in a paramagnetic heme system is provided by the study of the viscosity effect on the pyrrole H T_1s of the dicyano[tetrakis(4-sulfophenyl)porphine]iron(III) complex. Here the T_{1H} ~ 100 ms is found unchanged over a factor 45 change in viscosity, with correlation times ranging from 0.1 to 4 ns; the latter corresponds to a 7 × 10^3 molecular weight in ^2H_2O. The T_2 values decrease monotonically with τ_c, as is expected for the diamagnetic contribution to line width.

The heme methyl ~150-ms T_1s of low-spin metMbCN[7,10] (R_{Fe} ~ 6.1 Å) and the 4-ms T_1s of high-spin metMbH$_2$O,[11] as well as the ~100-ms pyrrole H (R_{Fe} ~ 5.2 Å) T_1s in low-spin ferric TPPSFe(CN)$_2$, yield $T_{1e} \lesssim 10^{-12}$ s from the relevant quantitative equation for electron–nuclear dipolar relaxation:[5,6]

$$T_{1H}^{-1} = \frac{2\gamma_H^2 g^2 \beta^2 S(S+1)}{15 R_M^6}\left(\frac{7T_{2e}}{1+\omega_s^2 T_{2e}^2} + 3T_{1e}\right) \quad (12)$$

Thus, T_{1e} and T_{2e} are shorter than any molecular motion, except possibly vibrations at the iron center, and hence it should not be unexpected that they are independent of the overall correlation time.

The mechanism of electron spin–lattice relaxation in low-spin iron(III) is not well understood, and the theoretical difficulties of treating electron spin relaxation times with values close to the motion thought to act as correlation times have been addressed.[45,46] The current data suggest that the overall molecular reorientation motion, not unexpectedly, makes no contribution to T_{1e}. Solvent collision with the chromophore in the ligated metMbCN complex is deemed unlikely. Possible mechanisms that could give rise to such rapid relaxation include dynamic Jahn–Teller effects, Orbach processes, or vibrational modulation of iron **g** tensor anisotropy or zero-field splitting.[18,19,45-49]

The T_{1e}, and hence predominant paramagnetically influenced ρ, being independent of τ_c for the heme model compound also provides the rationalization for our failure to detect steady-state NOEs in related low-spin ferric heme model compounds in normal solvents.[50,51] However, sizable NOEs not only became detectable

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in viscous solvents[50,51] but even allowed determination of a complete NOESY map.[50] Thus, the large NOEs observed[50] earlier for dicyanohemin in ethylene glycol resulted not so much from placing the molecule in the slow-motion limit as from selectively increasing the cross-relaxation rate with viscosity while keeping ρ invariant. *This suggests that the use of viscous solvents may generally allow resonance assignments by NOEs in any paramagnetic model complex whose T_{1e} is independent of the overall molecular tumbling rate.*

Other Metalloproteins. Since well-resolved ^{1}H NMR spectra of any paramagnetic protein imply[5,6] similarly short T_{1e}s $\ll \tau_c$, it is likely that most systems that exhibit useful NMR spectra also have T_{1e}s independent of the protein overall tumbling time. The recent proton relaxation measurements on superoxide dismutase as a function of viscosity also support this conclusion.[20] Some systems for which well-resolved ^{1}H NMR spectra have been reported, in addition to hemoproteins,[10-15,17] are iron–sulfur cluster proteins,[22,23] hemerythrins,[24] uteroferrins,[25] superoxide dismutases,[26] cobalt(II)-substituted Zn proteins,[27] and lanthanide(III)-substituted calcium-binding proteins.[28] It is obvious not only that NOE studies should be profitably pursued for both assignment purposes and structure determination in such systems but that large molecular weight should be considered an advantage rather than a disadvantage in rendering the desired NOEs detectable. For the iron–sulfur cluster proteins, available ^{1}H line width data on small two-iron ferredoxins[22] ($M_r \sim 11 \times 10^3$), as well as the large nitrogenase iron cofactor[23] ($M_r \sim 50 \times 10^3$), support iron T_{1e}s largely independent of molecular size. Indeed, preliminary ^{1}H NOE data confirm cysteine inter-methylene ^{1}H NOEs larger in the larger system.[52] The likely limiting factor on increased size of protein in implementing successful NOE studies may be the substantial decoupler power needed to effect saturation as the lines become necessarily broader solely due to diamagnetic T_2 influences.[11,44] Nevertheless, the currently available data suggest that the ordinary one-dimensional NOE (steady-state[10-14] and truncated[10,12]), as well as the two-dimensional NOESY map,[14,15] may be expected to provide the critically needed assignment and some valuable structural information for a wide range of paramagnetic biopolymers.

Influence of Organic Cosolvents. Organic cosolvents that modulate the medium viscosity are extensively utilized in cryoenzymology[53] and in the assessment of theoretical models for protein dynamics.[54] It is noted here that the addition of these agents to increase viscosity invariably leads to clearly detectable shift changes that depend characteristically on the nature of the cosolvent and are selective for the individual resonances. For example, ethylene glycol significantly affects assigned 7-H_α shifts[11] in metMbH_2O compared to other solvents (Table I). In metMbCN, DMSO perturbs primarily the 5-CH_3, 2-vinyl, and Ile FG5 $C_\gamma H$,[10,31] while ethylene glycol causes both smaller and less spatially discriminant shifts (Table II). The nature of the structural perturbations is not understood at this time, and they are as large (to $\sim$1.0 ppm) because of the nonlinear expansion of the chemical shift scale due to the hyperfine interaction.[6,7,29] Nevertheless, the shift changes clearly demonstrate the role of the cosolvent in modulating the detailed structure in the heme cavity. Considerably more work is needed to determine the extent of such solvent perturbations on the structure. For the moment, the present data should be taken as evidence that the assumption that *only* the solvent viscosity is perturbed in such cosolvent mixtures has definite limitations.

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