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Nuclear Magnetic Resonance Studies of Amino Acids and Proteins. Side-Chain Mobility of Methionine in the Crystalline Amino Acid and in Crystalline Sperm Whale (*Physeter catodon*) Myoglobin†

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ABSTRACT: We have obtained deuterium (^{2}H) nuclear magnetic resonance (NMR) spectra and spin–lattice relaxation times (T_1) of L-[ϵ-$^2H_3$]methionine, L-[ϵ-$^2H_3$]methionine in a D,L lattice, and [*S-methyl*-$^2H_3$]methionine in the crystalline solid state, as a function of temperature, in addition to obtaining ^{2}H T_1 and line-width results as a function of temperature on [ϵ-$^2H_3$]methionine-labeled sperm whale (*Physeter catodon*) myoglobins by using the method of magnetic ordering [Rothgeb, T. M., & Oldfield, E. (1981) *J. Biol. Chem. 256*, 1432–1446]. The results indicate that in the L-amino acid, methyl rotation having an activation energy (ΔE^*) of 8.3 ± 1 kJ dominates T_1 at low temperatures ($\lesssim$−10 °C), while at higher temperatures an additional large-amplitude side-chain motion occurs which causes changes in the ^{2}H NMR line shape and T_1. This motion is inhibited in the D,L lattice, indicating that lattice effects may have a strong effect on the mobility of anhydrous amino acids in the solid state. Further substitution at S^δ to form the sulfonium salt [*S-methyl*-$^2H_3$]-methionine causes a large increase in ΔE^*, to 15.9 ± 2 kJ, a value comparable to the 14–16 kJ found in valine and leucine, which contain the structurally similar isopropyl moiety. These results suggest that the very low barriers to methyl rotation in the methionine side chain are due to long C–S bond lengths and the presence of only two substituents on sulfur, while the anomalous high-temperature behavior is due to a lattice-packing effect. ^{2}H T_1 results with methionine-labeled myoglobin are complex, reflecting the presence of fast large-amplitude side-chain motions, in addition to rapid methyl rotation. Our data indicate that Met-55 and Met-131 are motionally inequivalent in crystalline cyanoferrimyoglobin, in contrast to solution NMR results. We have also recorded ^{13}C cross-polarization "magic-angle" sample-spinning NMR spectra of [ϵ-^{13}C]methionine-labeled crystalline cyanoferrimyoglobin (at 37.7 MHz, corresponding to a magnetic field strength of 3.52 T) and of the same protein in aqueous solution. Cross-polarization transfer rates and proton rotating-frame relaxation time results again indicate that Met-55 and Met-131 are motionally inequivalent in the solid state, and the T_{CH} data indicate that Met-55 is more solidlike. However, we find that ^{13}C chemical shifts in solution and those in the crystalline solid state are in very close agreement, suggesting that the average solution and crystal conformations are the same, in the area of Met-55 and Met-131.

There is currently considerable interest in investigating the dynamic structures of proteins in solution, in membranes, and in the crystalline solid state (Williams, 1978; Frauenfelder et al., 1979; Frauenfelder & Petsko, 1980; Artymiuk et al., 1979; Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Keniry et al., 1982; Schramm et al., 1981; Schramm & Oldfield, 1982). Nuclear magnetic resonance (NMR)[1] spectroscopy, because of its sensitivity to the wide range of time scales over which the motions occur, is one of the best techniques capable of yielding such information (Oldfield et al., 1981; Schramm et al., 1981).

In this paper, we discuss motions of the amino acid methionine, using ^{2}H and ^{13}C NMR spectroscopy. In another paper (Keniry et al., 1982), we have discussed the motions of methyl groups in alanine, valine, threonine, and leucine, in a variety of systems. We showed that, in general, the motions observed were rather simple, consisting solely of fast methyl group rotation. However, with the larger leucine side chain, additional motions occurred in some instances since the observed ^{2}H NMR spectra had nonzero asymmetry parameters (η). Similar effects have been observed by Batchelder et al.

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[1] Abbreviations: NMR, nuclear magnetic resonance; CP/MAS, cross-polarization "magic-angle" spinning; Tempamine, 4-amino-2,2,6,6-tetramethylpiperidinyloxy; TSP, sodium 3-(trimethylsilyl)[2,3-$^2H_4$]propionate; Me_4Si, tetramethylsilane; Mb, myoglobin; CoMb, cobalt derivative of myoglobin, "coboglobin"; MbH_2O, aquoferrimyoglobin; MbCN, cyanoferrimyoglobin; MbF, fluoroferrimyoglobin; MbCO, carboxyferromyoglobin; $\Delta\nu_Q$, quadrupole splitting; T_1, spin–lattice relaxation time.

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(1982) for leucine in collagen and have been attributed to fast (microsecond) interconversions between the two conformations of the leucine side chain, observed previously in crystals of leucine-containing peptides (Benedetti, 1977; Janin et al., 1978). Since the C^{γ}–C^{δ} bond vector is transported over an approximately tetrahedral angle in the transition, an asymmetry parameter of ~1 is observed for the case of equal site populations, as expected theoretically (Soda & Chiba, 1969) and as observed previously for gauche–trans isomerization in polymethylene chains of a glycolipid (Huang et al., 1980).

Based on these observations of increased motional freedom in the solid state for residues distal to the polypeptide backbone, we have investigated the dynamics of the methionine side chain, both in the polycrystalline amino acid and when incorporated into myoglobin from sperm whale (*Physeter catodon*). Previous workers have observed "unusual" NMR behavior for the methionine side chain in the solid state (Zaripov, 1974; Andrew et al., 1976; D. A. Torchia, private communication; C. M. Gall, private communication), principally that the ^{2}H NMR line shape of [ϵ-$^2H_3$]methionine is not always axially symmetric. We have thus investigated the ^{2}H NMR spectral line shapes, and spin–lattice relaxation times, of L-[ϵ-$^2H_3$]methionine in the crystalline solid state as a function of temperature, in addition to obtaining similar measurements on [ϵ-^{13}C]- and [ϵ-$^2H_3$]methionine-labeled crystalline sperm whale myoglobins.

Sperm whale myoglobin is a suitable protein for such studies since it is relatively small, having a molecular weight of about 17 000 (Rothgeb & Gurd, 1978), and it has the amino acid methionine at positions 55 and 131 of its primary sequence. The terminal methyl (C^{ϵ}) groups of these residues may be readily isotopically enriched while maintaining the native structure of the rest of the molecule by means of a simple alkylation/dealkylation reaction (Gurd & Rothgeb, 1979; Jones et al., 1976a,b). [ϵ-^{13}C]Methionine-labeled sperm whale myoglobin has been studied previously in solution by ^{13}C NMR (Jones et al., 1976b; Gurd & Rothgeb, 1979), and more recently, the corresponding [^{2}H]methyl-labeled protein has been studied by ^{2}H NMR in the crystalline solid state by the method of magnetic ordering, which permits resolution of *both* methionine sites (Oldfield & Rothgeb, 1980; Rothgeb & Oldfield, 1981). In this paper, we report a continuation of these studies of the isotopically enriched crystalline protein by carrying out ^{2}H NMR spin–lattice (T_1) relaxation time determinations on the deuterated protein, and chemical-shift and cross-relaxation time studies of the ^{13}C-labeled protein by using the technique of cross-polarization magic-angle sample spinning (CP/MAS) NMR (Schaefer et al.,1977; Stejskal et al., 1977). Our results with the labeled amino acid indicate that methyl rotation dominates the deuterium T_1 and ^{2}H NMR line shape at temperatures below about −40 °C for L-[ϵ-$^2H_3$]methionine, but at higher temperatures (≳10 °C), new large-amplitude motions of the side chain occur, and these then dominate T_1 and the line shape. L-[ϵ-$^2H_3$]Methionine incorporated into a D,L lattice does not show this unusual behavior. Similar evidence for complex side-chain mobility is obtained with the labeled protein, and differences between the two residues, not observed in the solution studies, are obtained in both ^{2}H and ^{13}C relaxation results. These effects likely originate both in lattice packing and in the very low rotational barriers about the two C–S bonds in the methionine side chain.

Experimental Procedures

Nuclear Magnetic Resonance Spectroscopy. "Home-built" Fourier-transform NMR spectrometers were used to record all spectra. Deuterium measurements were made both at 8.45 T, by using a 3.5-in. bore superconducting solenoid (Oxford Instruments, Osney Mead, Oxford, U.K.) together with a variety of digital and ratio-frequency (rf) electronics and a Nicolet 1180 computer interfaced to a Nicolet NIC-2090 50-ns transient recorder system (Nicolet Instrument Corp., Madison, WI; Rothgeb & Oldfield, 1981; Kinsey et al., 1981), and at 5.0 T, by using the system described previously (Oldfield et al., 1978). Spectra were recorded by using an 800-μL sample volume and a quadrupole-echo pulse sequence (Davis et al., 1976), using a 90° pulse width of about 3 μs and $\tau_1 = \tau_2 =$ 50 μs, unless otherwise noted. Henry Radio (Los Angeles, CA) Model 2006 transmitters were employed on both systems at a 1000–1200-W output level to achieve these pulse widths. The spectrometer zero frequencies were established by using a 1% D_2O reference, the zero frequency for the protein samples being set at about 2 ppm upfield from this position. The deuterium resonance frequency at 8.45 T was 55.3 MHz and at 5.0 T was 32.9 MHz. 90° pulse widths and phase quadrature between the two radio-frequency pulses were established by viewing quadrature free induction decay signals of [*S-methyl*-$^2H_3$]methionine. The same settings were used for data acquisition on the ^{2}H-labeled protein. In essentially all cases, no phase corrections were necessary after Fourier transformation. All spectra were obtained by using single-phase detection and were reflected about the carrier frequency in all spectra shown, for the convenience of the reader. Sample temperature was regulated either by use of a liquid nitrogen boil-off system or by use of a heated air flow. The temperatures reported were measured by using a calibrated Doric Trendicator (San Diego, CA) with a copper–constantan thermocouple, and are the gas flow temperatures measured next to the sample. Separate experiments indicate that this temperature is accurate to ±1–2 °C over the entire sample volume.

Deuterium spin–lattice relaxation time measurements on magnetically ordered protein microcrystals (Oldfield & Rothgeb, 1980; Rothgeb & Oldfield, 1981) were made by using either a three-pulse inversion-recovery–echo sequence ($180°_x$–τ_3–$90°_x$–τ_1–$90°_y$–τ_2–echo–T), where τ_i values are the time intervals between pulses and T is the repetition time of the experiment, or a standard two-pulse inversion recovery sequence. In test cases, it was found that the T_1 values obtained were independent of the pulse sequence used. Therefore, in order to maximize the signal to noise ratio and minimize distortion, we routinely used the three-pulse sequence. T_1 values were determined by measuring the resonance intensity as a function of τ_3 and fitting these results to a three-parameter exponential curve of the form $I(\tau_3) = A + B \exp(-\tau_3/T_1)$ (Levy & Peat, 1975). In all cases, the estimated uncertainty in the T_1 values is about ±10%.

High-resolution ^{13}C NMR measurements on aqueous myoglobin were made at a field strength of 5.9 T by using an ambient temperature 20-mm high sensitivity probe (Oldfield & Meadows, 1978; D. Wright, unpublished results). Spectra were obtained (at 62.9 MHz) under conditions of full proton decoupling by using conventional Fourier-transform methods. The multinuclear instrument used was basically the same as that described previously (Oldfield & Meadows, 1978) except that it operates at a higher frequency. The 90° pulse width was generally ~14 μs for a 6.8-mL sample. Solution chemical shifts were referenced relative to internal dioxane, which was taken to be 67.86 ppm downfield of tetramethylsilane (Me_4Si).

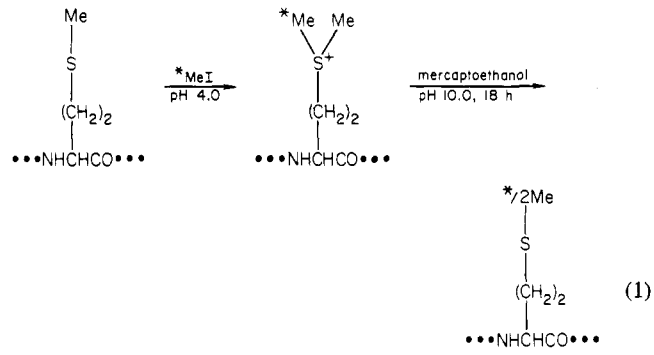
Carbon-13 cross-polarization magic-angle spinning (CP/MAS) NMR experiments at 37.7 MHz were performed on a 3.52-T system (Oldfield & Meadows, 1978; Rothgeb &

Oldfield, 1981). The instrument employed vacuum tube linear amplifiers (Henry Radio, Los Angeles, CA; Models 2006 and 2002), which were capable of delivering up to 1000 W of continuous output power. The probe used was a double-tuned single-turn solenoid design using one-quarter wave elements (Stoll et al., 1977). We used a high-power tubular low-pass filter (Telonic Berkeley Inc., Irvine, CA) having about 100-dB attenuation at 150 MHz, on the input to the ^{13}C preamp, to prevent leakage of 150-MHz radiation into the receiver system. The rotors were made of Delrin and had an Andrew-type design (Andrew, 1971); the sample volume was ~0.60 cm^3. Spinning speeds were in the range 2–3 kHz, and the protein samples occupied approximately two-thirds of the coil volume.

All CP/MAS spectra were obtained under Hartmann–Hahn matched spin-lock, single-contact cross-polarization conditions (Pines et al., 1973) as follows. First, the proton spins were polarized in H_0 and then placed in their rotating frame by a 90° pulse followed immediately by a long 90°-phase-shifted pulse. Next, ^{13}C–^{1}H contact was established for some time (t_m) by continuous ^{13}C spin irradiation such that $\gamma(C)H_1(C) = \gamma(H)H_1(H)$. This leads to a build up of ^{13}C magnetization. Finally, the ^{13}C magnetization was sampled after turning off the $H_1(C)$ field, while maintaining dipolar decoupling of the ^{1}H spins. In all experiments, alternation of spin temperature was performed to minimize artifacts (Stejskal & Schaefer, 1975). Routinely, an $H_1(H)$ field of between 0.87 and 1.50 mT was maintained, the Hartmann–Hahn match being optimized by using a spinning sample of adamantane. The amplitudes of the two rf fields were adjusted to the match condition with a precision of about 1 dB. Experiments were all performed with the spinning axis at the "magic angle" (54.7°) relative to the external field, H_0. Adjustments of this angle were made for each rotor by using a spinning sample of hexamethylbenzene.

CP/MAS spectra of solid ^{13}C-labeled protein were obtained on ~200-mg crystalline samples. Crystals, equilibrated as previously described (Rothgeb & Oldfield, 1981), were filtered free of excess medium and then transferred into the sample rotor, care being taken to ensure the crystals remained hydrated but that a large excess of medium was not cotransferred. Solid TSP was then added and the rotor sealed. UV–visible spectra of dissolved crystals after CP/MAS experiments were identical with those obtained before, and visible microscopic examination of samples after CP/MAS experiments indicated that the protein remained crystalline, although in general the mother liquor was somewhat depleted. All spectra were obtained by using quadrature detection with 10-kHz spectral widths, the carrier frequency being set at 90 ppm downfield from Me_4Si (i.e., on the Delrin resonance). Due to heating of the protein samples because of the presence of large amounts of saturated (or even some crystalline) ammonium sulfate, we applied ^{1}H irradiation for ~100 ms every 5.1 s (the recycle time of the experiment), an *average* power dissipation of only about 3 W. The fast spinner air stream provided further cooling. Under these conditions, the protein remained native, as described above, for periods of up to 12 h, and we estimate that sample temperatures did not rise above ~35–40 °C. Solution ^{13}C NMR spectra of myoglobin samples dissolved *after* CP/MAS appeared quite normal. Chemical shifts in the CP/MAS spectra were measured relative to the ^{13}C methyl resonance of solid TSP, which was taken to be 1.24 ppm upfield of Me_4Si; 150 W of proton decoupling typically provided a 1.2-mT $H_1(H)$ field.

Sample Preparation. Samples of sperm whale myoglobin isotopically enriched in either ^{2}H or ^{13}C at the methionyl methyl groups were synthesized and prepared for NMR spectroscopy as described previously (Oldfield & Rothgeb, 1980; Rothgeb & Oldfield, 1981). The method, briefly, involves formation of the sulfonium salt of apomyoglobin, which is then dealkylated (loss of 50% label) with, e.g., mercaptoethanol:



(1)

with the resultant formation of a statistically 50% labeled protein, which may then be rehemed by addition of hemin chloride, then purified, and crystallized from ~70% saturated ammonium sulfate. [*S-methyl*-$^{2}H_3$]Methionine was prepared as described previously (Rothgeb & Oldfield, 1981). DL-[L-ϵ-$^{2}H_3$]Methionine was prepared by mixing equimolar quantities of D-methionine (Sigma) and L-[ϵ-$^{2}H_3$]methionine (Merck Sharp & Dohme, Montreal, Canada) in warm water, followed by crystallization.

L-[α-^{2}H]Methionine was synthesized by first forming the *N*-acetyl derivative by warming DL-methionine and acetic anhydride at 37 °C in 2 N $NaO^{2}H$ for 5 h (Vigneaud & Meyer, 1932). The solvent was removed under reduced pressure, and the *N*-acetyl-DL-[α-^{2}H]methionine was recrystallized from acetone–water. The acetyl group was enzymatically cleaved from the L isomer by using acylase I (*N*-acylamide amidohydrolase, EC 3.5.1.4; Sigma Chemical Co., St. Louis, MO). The L-[α-^{2}H]methionine was recrystallized from ethanol–water mixtures 3 times (Fodor et al., 1949). The identity of the amino acid was confirmed by microanalysis and by optical rotation at the sodium line.

Sperm whale (*P. catodon*) myoglobin was obtained from Sigma Chemical Co. (St. Louis, MO). Iodo[$^{2}H_3$]methane was from Merck Sharp & Dohme (St. Louis, MO) and had 0.95 mol fraction of ^{2}H. Iodo[^{13}C]methane, 0.90 mol fraction of ^{13}C, was from the Los Alamos Stable Isotope Research Resource and was kindly provided by Dr. B. Whaley and Professor N. Matwiyoff.

All other chemicals were reagent grade and were used without further purification. All amino acid samples were dried over P_4O_{10} at 60 °C in vacuo prior to NMR spectroscopy. The purity of all amino acid samples was verified by microchemical analysis and by thin-layer chromatography.

Spectral Simulations. Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corp. Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory. We assumed that efficient quadrupole relaxation gives a Lorentzian contribution to the ^{2}H NMR line widths, leading to the powder pattern line shape shown in eq 2, where δ is the half-width at

$$g(\omega,\Delta\nu_Q) = \int_0^{\pi/2} (d\theta \sin\theta) \times (\delta/\pi)/\{\delta^2 + [[\omega \pm (\Delta\nu_Q/2)](3\cos^2\theta - 1)]^2\} \quad (2)$$

half-height (HWHH) of the Lorentzian broadening function and $\Delta\nu_Q$ is the quadrupole splitting. For ordered systems, there

Table I: Experimental Deuterium NMR Spin-Lattice Relaxation Times (T_1), Quadrupole Splittings (QS), Asymmetry Parameters (η), and Computed Correlation Times (τ_c) for L-[ϵ-$^2H_3$]Methionine and [*S-methyl*-$^2H_3$]Methionine as a Function of Temperature at 5.0 T

	L-[ϵ-$^2H_3$]methionine					[*S-methyl*-$^2H_3$]methionine			
temp (°C)	QS[a] (kHz)	η[b]	T_1[c] (ms)	τ_c[d] (ps)	temp (°C)	QS[a] (kHz)	η[b]	T_1[c] (ms)	τ_c[d] (ps)
33	23	0.27	92	*e*	37	41	0	50.5	52
27	29	0.22	114	*e*	21	41	0	35.4	74
−10	40	0	227	*f*	−8	41	0	16.8	160
−40	40	0	256	10	−23	41	0	12.6	210
−75	40	0	158	17	−40	41	0	6.4	420

[a] Obtained from a spectral simulation, as described in the text. Error is ±1 kHz. [b] Obtained from a spectral simulation, as described in the text. Error is ±0.02. [c] T_1 determinations were made by using an inversion-recovery quadrupole-echo sequence ($180°_x$-τ_3-$90°_x$-τ_1-$90°_y$-τ_2-echo-T). Signal intensities were measured at the major singularity and have an accuracy of about ±10–15%. See Figures 2 and 3 for representative results and Arrhenius behavior. [d] Correlation times were obtained from the T_1 data as described elsewhere (Keniry et al., 1983). Errors for the τ_c values are about ±20%. [e] The decrease in T_1 and the line-shape and line-breadth changes indicate large amplitude side-chain motion. The correlation time for the latter is ≈50 ns for a two-site hop model giving best agreement with experimental QS, η. [f] Not estimated.

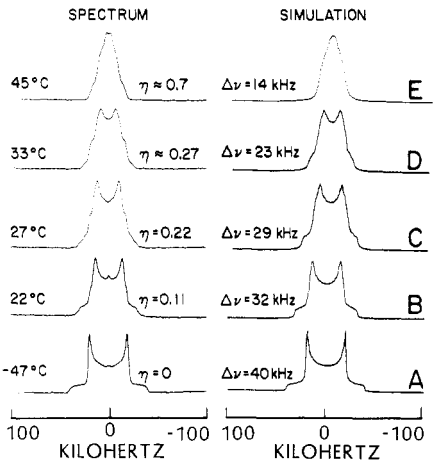


FIGURE 1: Deuterium NMR spectra obtained by using the quadrupole echo Fourier-transform method at 55.3 MHz (corresponding to a magnetic field strength of 8.45 T) of L-[ϵ-$^2H_3$]methionine, in the crystalline solid state as a function of temperature (left) together with some computer simulations (right) of the experimental spectra. The 2δ values used in the simulations are 600, 1000, 1200, 1600, and 2200 Hz for spectra A–E, respectively.

is simply a Lorentzian line at a frequency ω corresponding to the appropriate θ. An asymmetry parameter η [$(V_{xx} - V_{yy})/V_{zz}$] may be introduced into eq 2 to calculate $\eta \neq 0$ ^2H line shapes; however, the resulting line-shape equations are even more complex, and the interested reader is referred to the literature (Bloembergen & Rowland, 1953; Cohen & Reif, 1957).

Results and Discussion

Amino Acids. We show in Figure 1 typical spectra of L-[ϵ-$^2H_3$]methionine as a function of temperature, obtained by the quadrupole-echo Fourier-transform technique, at an operating field strength of 8.45 T (corresponding to a ^2H resonance frequency of 55.3 MHz). Shown in Figure 2 are typical partially relaxed Fourier-transform (PRFT) NMR spectra of L-[ϵ-$^2H_3$]methionine and [*S-methyl*-$^2H_3$]methionine, and in Figure 3, we show the results of Figure 2, together with additional points and data on DL-methionine in the form of Arrhenius plots.

The results of Figures 1 and 3 indicate that motions in addition to methyl rotation affect both the ^2H NMR line shape of methionine (Figure 1) and the ^2H NMR spin–lattice relaxation behavior of methionine (Figure 3 and Table I). At low temperatures, below about 0 °C, the deuterium NMR powder pattern has both the breadth ($\Delta\nu_Q$ ~40 kHz, 2δ = 1.2 kHz) and asymmetry parameter (η = 0) expected for a deu-

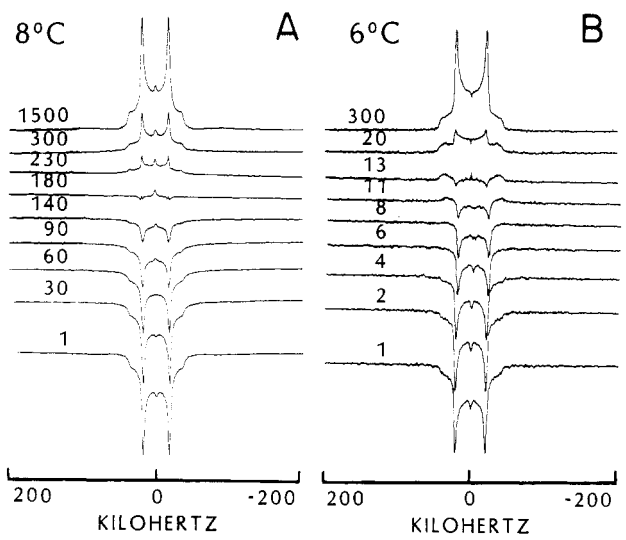


FIGURE 2: Partially relaxed Fourier-transform deuterium NMR spectra of L-[ϵ-$^2H_3$]methionine and [*S-methyl*-$^2H_3$]methionine in the crystalline solid state at 6–8 °C. Spectra were recorded by using a $180°_x$-τ_3-$90°_x$-τ_1-$90°_y$-τ_2-echo pulse sequence at 55.3 MHz. (A) Methionine; (B) *S*-methylmethionine. The τ values (τ_3), in milliseconds, are shown to the left of each spectrum. τ_1 and τ_2 were as in Figure 1.

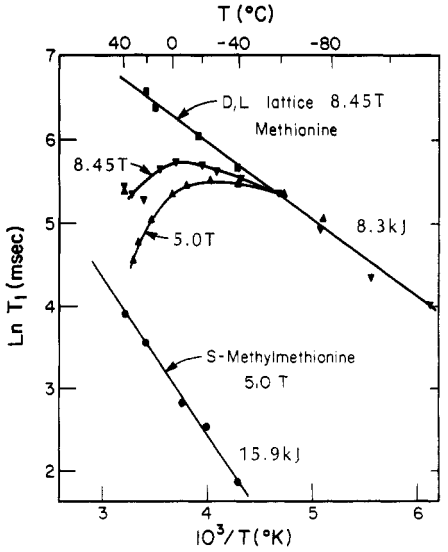


FIGURE 3: Arrhenius plot showing the temperature dependence of the deuterium spin–lattice relaxation time (in milliseconds) for L-[ϵ-$^2H_3$]methionine at 8.45 (▼) and 5.0 T (▲), for L-[ϵ-$^2H_3$]methionine in a D,L lattice at 8.45 T (■), and for [*S-methyl*-$^2H_3$]methionine at 5.0 T (●).

teriomethyl group rotating rapidly ($\geq 10^7$ s^{-1}) about its C_3 axis. This is in accord with our previous studies of this system (Rothgeb & Oldfield, 1981), in addition to studies of L-[β-$^2H_3$]alanine, DL-[γ-$^2H_6$]valine, DL-[β,γ-$^2H_4$]threonine, and L-[δ-$^2H_3$]leucine (Keniry et al., 1983). However, as the sample temperature is increased, there are large changes in the ^2H NMR line shape (Figure 1B–E and Table I), indicating the presence of a new type(s) of side-chain motion. These line-shape changes are accompanied by considerable changes in the ^2H spin–lattice relaxation rate, as shown in Figure 3 and Table I.

At temperatures below about −35 °C, and down to at least −110 °C, the results of Figures 1–3 are consistent with fast methyl rotation being the only source of ^2H line narrowing and spin–lattice relaxation. The activation energy ($\Delta E^{\ddagger}$) for methyl rotation obtained from Figure 3 and Table I is 8.3 ± 1 kJ, in good agreement with the value of 6.7 kJ determined from ^1H NMR measurements by Zaripov (1974) and Andrew et al. (1976). As noted by these previous workers using ^1H NMR spectroscopy, the methyl group in methionine has the lowest activation energy and the lowest temperature T_1 minimum (∼−180 °C at 30 MHz) of any methyl group in an amino acid. This we believe is due primarily to the lack of any significant steric hindrance to methyl rotation in the methionine side chain, due to the presence of only two substituents on sulfur (compared with four for carbon), and the relatively long C–S bond lengths.

Using the mathematical model described elsewhere (Torchia & Szabo, 1982), we have analyzed the ^2H PRFT data shown in Figures 2 and 3 to determine the rotational correlation times of the methyl group in L-[ϵ-$^2H_3$]methionine. Results are given in Table I. Clearly, the methionine methyl group undergoes extremely fast rotation at all temperatures investigated, due to the presence of the unhindered C^{γ}–S^{δ}–C^{ϵ} moiety. That this is the origin of the fast methyl rotation is substantiated by comparison with the relaxation behavior of the sulfonium species, [*S-methyl*-$^2H_3$]methionine (Figures 2 and 3 and Table I).

For [*S-methyl*-$^2H_3$]methionine in the crystalline solid state, relaxation times are at least an order of magnitude shorter than those obtained for L-[ϵ-$^2H_3$]methionine at comparable temperatures (below ∼−10 °C), and the activation energy for methyl rotation is 15.9 kJ, considerably in excess of that found for methionine. These results are all consistent with our suggestion that the increased steric crowding found in the sulfonium derivative, which contains the $-S(CH_3)_2^+$ fragment, plays a major role in causing an increase in $\Delta E^{\ddagger}$, as shown by comparison of the following $\Delta E^{\ddagger}$ and τ_c(−40 °C) values:

	$\Delta E^{\ddagger}$ (kJ)	τ_c(−40 °C) (ps)
$>CHCH_2CH_2SCH_3$	8.3	∼10
$>CHCH_2CH_2\overset{+}{S}<^{CH_3}_{CH_3}$	15.9	∼150
$>CHCH<^{CH_3}_{CH_3}$	13.8	∼400
$>CHCH_2CH<^{CH_3}_{CH_3}$	15.5	∼300

Results with [*S-methyl*-$^2H_3$]methionine are remarkably similar to those obtained with the isopropyl-containing amino acids DL-[γ-$^2H_6$]valine and L-[δ-$^2H_3$]leucine (Keniry et al., 1982), in which $\Delta E^{\ddagger}$ values of 14–16 kJ are obtained.

At temperatures above about −40 °C, for L-[ϵ-$^2H_3$]-methionine there are observable deviations from the linear Arrhenius plot behavior (Figure 3) together with changes in

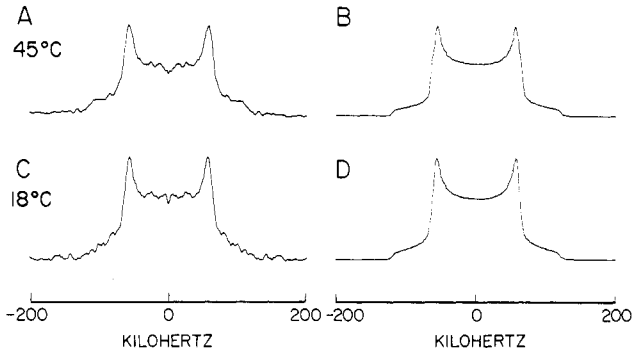


FIGURE 4: Deuterium quadrupole echo spectra of L-[α-^2H]methionine in the crystalline solid state at 32.9 MHz, showing the lack of molecular rotation from 18 to 45 °C. (A) 45 °C spectrum, 800 scans, 100-s recycle time, $\tau_1 = \tau_2 = 90$ μs, 2.7-μs 90° pulse widths. (B) Simulation of spectrum A using $\Delta\nu_Q = 120$ kHz, $\eta = 0.05$, and $2\delta = 3.0$ kHz. (C) 18 °C spectrum, 600 scans, other conditions as in spectrum A. (D) Simulation of spectrum C using $\Delta\nu_Q = 121$ kHz, $\eta = 0.08$, and $2\delta = 2.0$ kHz.

the ^2H NMR line shape (Figure 1), indicating the onset of new, rapid motions in the amino acid side chain, as observed, for example, for leucine in collagen (Batchelder et al., 1982). As expected, the influence of the new motion is first observed (upon increasing temperature) on our lower frequency instrument. However, without additional ^2H (and perhaps ^{13}C and ^{33}S) labeling experiments, it is not possible to be sure of the exact nature of these new motions, but in order to greatly affect deuterium spin–lattice relaxation, they must be fast, of the order of the Larmor frequency. In this case, a two- (or more) site hop model may be applicable (Soda & Chiba, 1969; Huang et al., 1980). We show in Figure 1 spectral simulations of the simplest case of a rapid 2-fold hop, using as the single adjustable parameter the angle (β) over which the hop occurs. The asymmetry parameters and spectral breadth reductions calculated for a given β are in quite good agreement with experimentally determined values up to about 27 °C (Figure 1C) and can be interpreted in terms of the Soda–Chiba theory in terms of hops over angles of up to 45° (at 27 °C). At higher temperatures, the observed spectra are narrower than those calculated for the 2-fold hop, with equally populated sites. Thus, it seems clear that a more complex, multiple-site unequally populated exchange model must be used in order to fit the high-temperature results, but the lack of crystallographic data discourages this approach. We have ruled out the possibility that the high-temperature line shapes are the result of overall molecular tumbling or rigid body motions by obtaining spectra of L-[α-^2H]methionine at 45 and 18 °C (Figure 4A–D). These spectra show only rigid powder patterns, indicating that C^{α} is not undergoing fast motion. Thus, these high-temperature line shapes are due to the onset of additional fast motion about bonds along the methionine side chain. Further understanding of the high-temperature methionine side-chain motions may be gained from two-dimensional ^{13}C NMR spectroscopy in the solid state (work in progress).

Interestingly, the rapid, large-amplitude, high-temperature motion of the methionine side chain is observed only in the optically active L-methionine crystals. When we crystallized a D,L racemate containing L-[ϵ-$^2H_3$]methionine, the ln T_1 vs. $1/T$ plot for it was linear up to temperatures of at least 21 °C, well beyond −40 °C, where the L-methionine T_1 values begin to deviate from the low-temperature methyl relaxation curve (Figure 3). In addition, the line shapes in the racemate were typical of methyl rotation, having a quadrupole splitting of 40 ± 1 kHz and an asymmetry parameter of $\eta = 0$. Thus, there appears to be a strong crystal lattice contribution to the

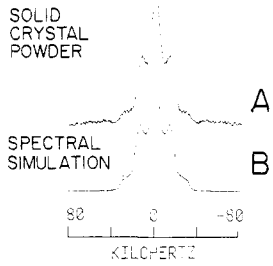


FIGURE 5: Deuterium quadrupole echo Fourier-transform NMR spectrum at 55.3 MHz and 20 ± 2 °C of [ϵ-$^2H_3$]methionine-labeled sperm whale aquoferrimyoglobin as a solid hydrated crystal powder together with its spectral simulation. (A) Solid crystal powder hydrated with ~90% saturated $(NH_4)_2SO_4$: 176 685 scans, 65-ms recycle time, $\tau_1 = \tau_2 = 65$ μs, 7-μs 90° pulse widths, 167-kHz spectral width, 4K real data points, line broadening = 400 Hz. (B) Spectral simulation of (A) using $\Delta\nu_Q = 31.0$ kHz, $\eta = 0.15$, and $2\delta = 2.8$ kHz together with the central, isotropic background HO^2H contribution. The latter is absent in dehydrated samples (Rothgeb & Oldfield, 1981).

barrier to rotation of the side chain in the racemate that is not present in L-methionine.

In summary, the results of Figures 1–4 show that at or below −40 °C, the only significant motion of the amino acid methyl group is fast rotation about its C_3 axis. The activation energy for this motion is about 8.3 ± 1 kJ. At higher temperatures, new side-chain motions are important in determining both the ^2H NMR line shape and the ^2H spin–lattice relaxation, at least in the optically pure L enantiomer. The activation energy for this (these) new motion(s) is ~14 kJ, as determined from the high-temperature relaxation data of Figure 3. In the next section, these results for the ^2H NMR spectra of methionine are compared with those for methionine-labeled myoglobin crystals.

Deuterium NMR of Methionine-Labeled Myoglobin. In the case of methionine-labeled myoglobin microcrystals, only a limited range of temperatures is accessible because of (i) solvent freezing at low temperatures, resulting in some static disordering, and (ii) protein denaturation at temperatures above ~40 °C. These limitations on the ^2H NMR data make the results more difficult to interpret.

We show in Figure 5 the ^2H NMR spectra of polycrystalline [^2H]methionine-labeled sperm whale (*Physeter catodon*) aquoferrimyoglobin microcrystals in excess, 70%-saturated ammonium sulfate solution, at 20 °C. The observed line shape (A) is not the characteristic spin $I = 1$, $\eta = 0$ powder pattern of $\Delta\nu_Q$ ~40-kHz width expected for purely methyl rotation. Instead, it has $\Delta\nu_Q$ ~32 kHz and $\eta = 0.15$. Only at lower temperatures does $\Delta\nu_Q \rightarrow 40$ kHz and $\eta \rightarrow 0$ (Rothgeb & Oldfield, 1981). Moreover, the observed line shape cannot be simulated by using two different component line shapes. Therefore, both Met-55 and Met-131 at 20 °C must undergo motions in addition to fast C_3-axis methyl rotation. The main question we wish to answer is the following: Are the methionine methyl group motions in crystalline myoglobin the same as those in crystalline methionine, or do they differ? For example, the ΔE^* and τ_c values for valine, threonine, and leucine were found to be basically the same as those obtained for the corresponding isotopically enriched bacteriorhodopsins from the purple membrane of *Halobacterium halobium* R_1 (Keniry et al., 1983). Also, we would like to know whether there are differences in dynamical behavior *between* the two methionine residues in the crystal, even though NMR studies have indicated their motions to be equivalent in solution (Jones et al., 1976b). Qualitative answers to these questions have been obtained by the method of magnetic ordering, which permits T_1 determinations on resolved, assigned resonances in the solid

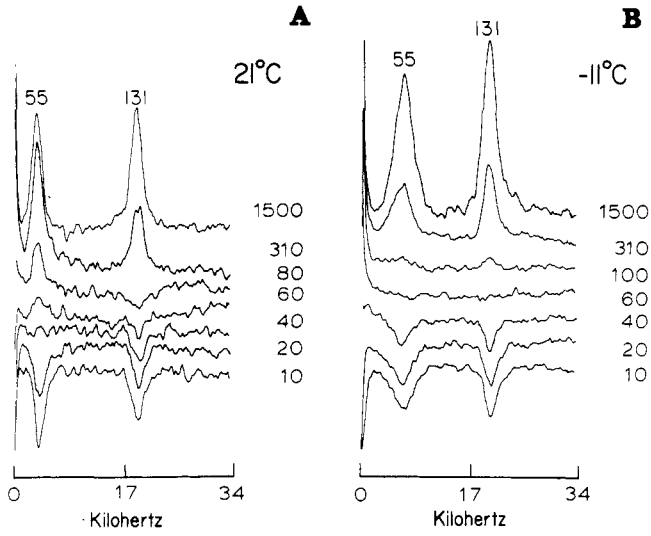


FIGURE 6: Typical partially relaxed deuterium Fourier-transform NMR spectra at 34.1 MHz of magnetically ordered [ϵ-$^2H_3$]-methionine-labeled cyanoferrimyoglobin crystals from *P. catodon* at pH 7.4 and temperatures of (A) 21 and (B) −11 °C. Each spectrum was obtained by using 16 384 accumulations with 4096 time domain data points, sampled at a rate of 15 μs per point. A three-pulse inversion-recovery sequence was used ($180°_x$–τ_3–$90°_x$–τ_1–$90°_y$–τ_2–echo–T), with values for $\tau_1 = \tau_2 = 90$ μs held constant throughout. The values of τ_3 (in milliseconds) are shown at the right of each spectrum. Other details are given the text. The resonance giving rise to a quadrupole splitting ($\Delta\nu_Q$) of 9.4 kHz has been assigned to Met-55, while the resonance having $\Delta\nu_Q = 39$ kHz is assigned to Met-131. Spectra were obtained by using single-phase detection on-resonance and are thus mirrored about the carrier frequency.

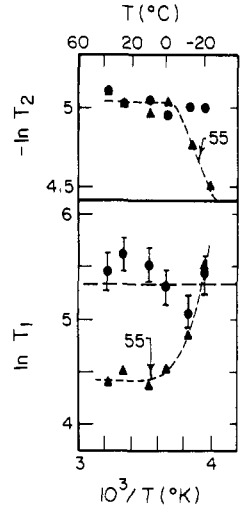


FIGURE 7: Temperature dependence of deuterium spin–lattice relaxation times and inverse line widths [$T_2 = 1/(\pi W)$] for methionines in cyanoferrimyoglobin, obtained as described in the legend of Figure 6. Met-55 and Met-131 are indicated by (▲) and (●), respectively. The line widths used to determine "T_2" were the full widths at half-height. T_1 is in milliseconds and T_2 in microseconds. The dashed line serves only to draw attention to the behavior of the Met-55 resonance at low temperature.

state (Rothgeb & Oldfield, 1981).

Figures 6 and 7 present typical deuterium inversion-recovery spin–lattice relaxation time measurements on magnetically ordered [ϵ-$^2H_3$]methionine-labeled *P. catodon* cyanoferrimyoglobin microcrystals at 21 and −11 °C (Figure 6), and as a more complete function of temperature (Figure 7). Similar ambient temperature (21 °C) measurements were made on Fe(III) protein crystals having other ligands attached to the heme iron, as well as for the deoxycobalt(II) form, coboglobin (data not shown). In the following discussion, we neglect any

paramagnetic contribution to the ^2H T_1 values because of the large distance of the Met-55 and Met-131 C^ϵ groups from the heme iron, the absence of any measurable hyperfine shifts in the solid-state ^2H NMR spectra, and the lack of any major T_1 differences between proteins containing different paramagnetic centers.

One might expect spin–lattice relaxation of the methionine C^ϵ deuterons in myoglobin to be dominated by methyl rotation, and to be essentially identical for each residue, as deduced from previous solution NMR results (Jones et al., 1976b). However, several factors indicate that this is not the case. First, we see from Figure 7 that the Met-55 T_1 decreases with increasing temperature while that of Met-131 is essentially constant, indicating a clear difference in motional behavior between the two side chains. Neither residue shows the characteristic $\Delta E^\ddagger \sim 8.3$ kJ activation energy behavior observed with L-[ϵ-$^2H_3$]methionine at low temperature, or with the D,L material over a wider temperature range, given in Figure 3, attributed to methyl rotation. Thus, it appears that side-chain motions in addition to methyl rotation must account for the T_1 relaxation behavior of both residues. The apparent ~0-kJ activation energy for both residues at $T \gtrsim 0$ °C is not due to being near a methyl T_1 minimum, since the magnitudes of T_1 observed (~100 ms) are about 2 orders of magnitude higher than those predicted and found experimentally with [β-$^2H_3$]alanine (Keniry et al., 1982). These results taken together strongly suggest that new fast motions other than methyl rotation are responsible for the high-temperature myoglobin deuterium T_1 results and that these motions are "quenched out" for Met-55 below ~0 °C. Additional labeling and relaxation experiments will be required in order to permit determination of this motion.

As seen from Figure 7, the line-width parameters for both methionine sites are also essentially temperature independent in the range 0–40 °C, while for Met-55, there is a dramatic increase in line width below ~0 °C. We believe the line-width increase does not have a motional origin since a freezing-in of conformational substates having a total angular spread of only ~±3° is enough to explain the observed broadening. This type of temperature-dependent behavior has already been noted by Frauenfelder et al. (1979) and Frauenfelder & Petsko (1980) in their study of the temperature dependence of the X-ray structure of aquoferrimyoglobin from *P. catodon*, although the structure of aquoferrimyoglobin differs somewhat from that of the low-spin cyanoferri form (Watson & Chance, 1966; Takano, 1977). Interestingly, below ~−40 °C, the resonance of Met-55 becomes too broad to detect (in a time that gives a well-resolved Met-131 signal), consistent with an interpretation of the X-ray results in terms of a static disordering for this residue.

Even though this description of Met-55 and Met-131 dynamics is extremely rudimentary, it is important to note that the T_1 and line-width results clearly show very large differences between Met-55 and Met-131 behavior in the crystal, differences not detected in solution.

Carbon-13 NMR of Methionine-Labeled Myoglobin. In solids without fast molecular tumbling, it is possible to use NMR relaxation methods to study motions which occur in the $\approx 10^{-3}$–10^{-5}-s time scale, corresponding to the strengths of local (H_1 or dipolar) magnetic fields.

In Figure 8A, we compare the high-resolution ^{13}C NMR spectrum of [*methyl*-^{13}C]methionine-labeled *P. catodon* cyanoferrimyoglobin in the crystalline solid state (upper spectrum) with that in solution (lower spectrum). The solution spectrum was obtained by using the *same* protein sample, after CP/MAS data acquisition. It serves not only for solid/solution comparative purposes but also as a control to assure that the protein was not denatured during the high-power CP/MAS experiment. Examination of Figure 8A reveals three main points: First, the protein is not denatured by the CP/MAS experiment, since the solution spectrum obtained on the protein after CP/MAS is identical with one obtained from the same batch of crystals before CP/MAS. Second, the two ^{13}C-enriched methionine sites give rise to resonances in the crystalline protein easily distinguishable from the natural-abundance ^{13}C envelope, although each methionine resonance displays a significantly different integrated intensity. This difference between the two resonances is a result of different cross-polarization mixing behavior and is discussed in more detail below. Finally, we note that the chemical shifts and line widths of the resonances in the crystalline protein are very similar to those in solution (Jones et al., 1976b), after account is taken of the ~20-Hz instrumental broadening associated with the CP/MAS experiment due to exponential multiplication, digital resolution, and inhomogeneity broadening.

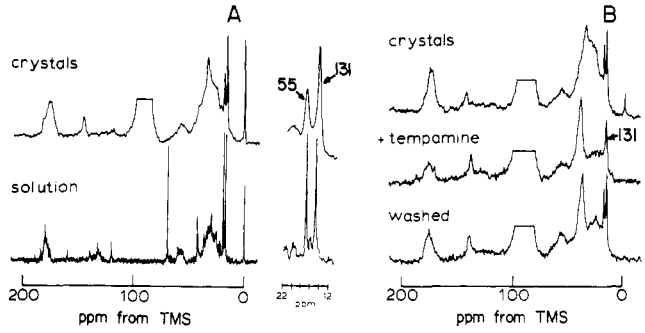


FIGURE 8: Carbon-13 NMR spectra of [ϵ-^{13}C]methionine-labeled *P. catodon* cyanoferrimyoglobin in the crystalline solid state and in solution, pH 7.4. Solid-state spectra were obtained by using CP/MAS conditions as described in the text, with an H_1(H) field of 0.87 mT. In (A), the upper spectrum was obtained by using 4000 accumulations on ~200 mg of labeled protein at a 5.1-s recycle time with a 3-ms cross-polarization mix time and a 100-ms proton decoupling pulse. The truncated resonance at ~90 ppm arises from natural-abundance ^{13}C in the Delrin rotor, while the resonance at about 0 ppm is due to the "internal" standard, TSP. The lower spectrum resulted from the *same* protein crystals, dissolved in H_2O after the CP/MAS spectrum was recorded. It was obtained by using 20000 accumulations on ~75 mg of labeled protein at a 2.5-s recycle time. The additional resonance at about 68 ppm is due to dioxane added as an internal reference. The inset in (A) is an expanded region from 12 to 22 ppm and shows the methionine sites resolved. In (B), one sees the effect of addition of the free-radical Tempamine to the protein crystals. Spectra were recorded as in (A), except that only 1000 accumulations and a 1-ms cross-polarization mix time were used. Tempamine selectively, and reversibly, broadens the downfield methionine resonance, which is assigned to Met-55 (see the text).

The following question arises: Even though the chemical shifts are similar in the crystal and solution spectra (Table II), does the downfield resonance in the crystal correspond to the downfield resonance in solution? In solution, resonance assignments have been made by using the nonbinding spin-label Tempamine, which selectively broadens the downfield methionine resonance, previously assigned to Met-55 (Jones et al., 1976b; Gurd & Rothgeb, 1979). Tempamine has also been successfully used on the crystalline protein in deuterium NMR experiments (Rothgeb & Oldfield, 1981), and as may be seen from Figure 8B, it has similar broadening effects in the CP/MAS experiment. The downfield resonance in Figure 8B is selectively broadened by Tempamine, and this broadening effect is readily reversed by washing out the spin-label with $(NH_4)_2SO_4$ solution. On this basis, therefore, the downfield methionine resonance in the CP/MAS spectrum is assigned

Table II: Crystal vs. Solution Chemical Shifts for [*methyl*-^{13}C]Methionine-Labeled *Physeter catodon* Myoglobin[a]

protein form[b]	Met-55 shift[c] (ppm)		Met-131 shift[c] (ppm)	
	crystal	solution	crystal	solution
fluoroferri, pH 7.2	16.4_2	17.1_4	16.4_2	16.2_2
aquoferri, pH 6.5	16.5_3	17.1_4	16.5_3	16.2_2
hydroxyferri, pH 9.2	17.0_0	17.1_6	14.9_7	16.1_2
cyanoferri, pH 7.3	17.4_1	17.8_8	14.7_9	15.9_2
carboxyferro, pH 7.8	16.9_6	17.3_9	15.0_0	15.9_8

[a] Results were obtained from the data of Figure 8 and additional unpublished results. Chemical shifts are expressed relative to external Me_4Si by using in solution an internal dioxane reference taken as 67.86 ppm downfield and in the solid state crystalline TSP taken as 1.24 ppm upfield from Me_4Si. [b] In solution, the protein was equilibrated with the appropriate ligand at the pH indicated. In the crystalline form, the protein was equilibrated in a mother liquor of ammonium sulfate with the appropriate ligand, buffered at the pH indicated, and then filtered free of excess medium as described under Experimental Procedures. [c] Resonance assignments in the crystalline solid state and in solution are based upon the spin-label titration shown in Figure 8 and discussed in the text (Jones et al., 1976b).

to Met-55, and by elimination, the upfield one is Met-131.

We summarize in Table II the ^{13}C chemical shifts for Met-55 and Met-131 in the crystalline and solution forms of myoglobin in a variety of ligand and redox states. In all cases where both residues were resolved, we found the same line width and intensity pattern as given in Figure 8A. There is also a strong correspondence between the crystalline and solution chemical shifts. The deviations shown in Table II are much smaller than those observed in amino acids and smaller peptides (Opella et al., 1979; Opella & Frey, 1979; Frey & Opella, 1980; Hexem et al., 1981a,b) and are close to experimental error. It is apparent that the factors determining the ^{13}C chemical shifts of the protein in solution also govern the shifts in the crystalline solid state. Such differences that do exist between the crystalline and solution forms of the protein are visible primarily as differences in the rates of motion between the solid and the liquid state.

These observations are the first detailed comparison between the chemical shifts of amino acid residues in protein crystals with those in solution. Previous reports have compared ^{13}CO ligand chemical shifts in solution with those in the solid state (Maciel et al., 1980) and ^{13}C Trp C$^{\gamma}$ chemical shifts between a lyophilized bacteriophage coat protein powder and the protein dissolved in sodium dodecyl sulfate (Opella et al., 1979). The similarity between the solution and crystal ^{13}C chemical shifts of myoglobin, compared with the rather large differences between solution and crystal shifts in the crystalline amino acids (Frey & Opella, 1980; Hexem et al., 1981a,b), reinforces the idea of a significant "lattice" effect in the amino acids. Such lattice effects may well be much smaller in proteins because of their large solvent content.

Another point of interest is the large difference in the integrated intensities of the two methionine resonances in the CP/MAS spectra of Figure 8. These differences originate from the factors influencing signal intensity in this type of solid-state NMR experiment. The intensity of a given resonance in a proton-enhanced spectrum is dependent both on the time required to exchange magnetization from the proton to the carbon nuclei (T_{CH}) and on the rate at which the

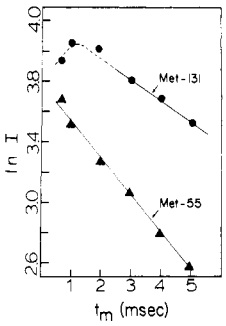


FIGURE 9: [^{13}C]Methionine resonance intensity as a function of the cross-polarization mix time in CP/MAS spectra of cyanoferrimyoglobin. The intensities for Met-55 (▲) and Met-131 (●) were measured from spectra by using 1000 accumulations, each at an H_1(H) field of 0.87 mT.

magnetization of the proton population is lost due to spin–lattice relaxation effects ($T_{1\rho}$) (Mehring, 1976; Schaefer et al., 1977). Assuming that both the cross-polarization and spin–lattice relaxation processes are characterized by exponential time constants, we may describe the overall signal intensity, $I(t)$, by

$$I(t) \propto \exp(-t_m/T_{1\rho})[1 - \exp(-t_m/T_{CH})] \quad (3)$$

where t_m is the cross-polarization or mix time, $T_{1\rho}$ is the proton rotating frame spin–lattice relaxation time, and T_{CH} is the time constant for the cross-polarization transfer process (Mehring, 1976). $T_{1\rho}$ is proportional to the spectral density function at $\omega_1 = \gamma H_1$ and thus provides a measure of low-frequency motions undergone by the group in question. T_{CH} is related to the strength of the carbon–hydrogen dipolar interaction, and, in general, the greater the reorientational freedom permitted a particular residue, the smaller will be the residual dipolar interaction and the longer will be T_{CH} (Mehring, 1976; Stejskal et al., 1977). The time scales for correlation times affecting $T_{1\rho}$ and T_{CH} are on the order of $\sim 10^{-3}$–10^{-5} s. In order to characterize the intensity differences between the methionine resonances in the crystalline protein, we have measured signal intensities as a function of mix time (t_m), and these intensities are plotted vs. t_m in Figure 9. So that the analysis of these data can be facilitated, eq 3 can be rearranged to

$$\ln I(t) = \text{const} - \frac{t_m}{T_{1\rho}} + \ln\left[1 - \exp\left(-\frac{t_m}{T_{CH}}\right)\right] \quad (4)$$

For $t_m >> T_{CH}$, the ln $\{1 - \exp[-(t_m/T_{CH})]\}$ term becomes negligible, and the curve, for longer t_m values, is a straight line with slope $-(1/T_{1\rho})$. Such an analysis yields a proton $T_{1\rho}$ of 4.2 ms for Met-55 and 7.4 ms for Met-131. For $t_m \sim T_{CH}$, the ln $\{1 - \exp[-(t_m/T_{CH})]\}$ term is no longer negligible, and the curve becomes nonlinear. This nonlinearity is evident for Met-131 at mixing times shorter than 1.0 ms. However, the Met-55 curve remains linear down to mixing times of 0.7 ms. On the basis of the T_{CH} behavior, this implies a weaker carbon–hydrogen dipolar interaction for Met-131, and therefore more reorientational freedom (Mehring, 1976; Stejskal et al., 1977; Schaefer et al., 1977).

Interpretation of the long-time behavior, reflecting the proton $T_{1\rho}$ values, is unfortunately not as straightforward, for a number of reasons. First, the ^{1}H $T_{1\rho}$ is itself not a unique function of the reorientational correlation time (τ) but rather has a minimum value at $\omega_1\tau = 1$ and increases for either $\omega_1\tau << 1$ or $\omega_1\tau \gg 1$ (McCall, 1971). Therefore, to interpret any $T_{1\rho}$ difference in terms of correlation times, one must first know on which side of the $T_{1\rho}$ minimum one is, i.e., whether

$\omega_1\tau$ is greater or less than 1. Second, the extent of a spin-diffusion contribution to the observed decay is unclear, especially since there could be a nonmotional contribution due to the presence of the paramagnetic center. However, we have obtained similar mix-time behavior with the diamagnetic carboxymyoglobin (at t_m = 1 and 6 ms, pH 7.8), suggesting the lack of a paramagnetic contribution, but clearly, additional detailed temperature and H_1 field dependence studies would be required for detailed interpretation of the mix-time behavior shown in Figure 9. The results of Figure 9 do, however, explain the signal intensity anomaly shown in Figure 8A and also suggest that Met-131 is more mobile than Met-55, at least in the crystalline solid state and on the $\approx 10^{-4}$-s time scale.

Concluding Remarks. In solution, relaxation of the methionine methyl group in myoglobin is dominated by rotation of the methyl group about its C_3 axis, together with protein rotation (Jones et al., 1976b; Gurd & Rothgeb, 1979), and the two residues (Met-55 and Met-131) appear indistinguishable by means of ^{13}C T_1 relaxation results. By contrast, in the crystalline solid state, Met-55 and Met-131 show differences in S^δ–C^ϵ vector motion in each of three different relaxation (or cross-relaxation) experiments: ^2H T_1 ($\tau \approx$ 100-ps time scale) and ^1H $T_{1\rho}$ and ^{13}C T_{CH} (approximately millisecond time scales). Nonetheless, in all forms of myoglobin investigated, the solid-state ^{13}C chemical shifts obtained by using CP/MAS techniques are within experimental error the same as the shifts in solution, suggesting (but not proving) that the local average solid and solution structures are the same.

A possible explanation for this apparent anomaly is that the solution NMR measurements may simply not be sensitive to the differences in dynamical behavior between the two residues noted on longer time scales, since in solution overall tumbling ($\sim 10^{-8}$ s) and methyl rotation ($\sim 10^{-10}$ s) dominate the ^{13}C T_1 relaxation process. Differential mobility in the 10^{-3}–10^{-7}-s time-scale range, for example, affecting ^2H T_1, ^1H $T_{1\rho}$, and ^{13}C T_{CH} values, may simply be too slow to be reflected in the ^{13}C solution results. The correlation times of both methyl residues in myoglobin in the solid state may be the same (as suggested by solution results), but in this case, their correlation times will be just too short ($\sim 10^{-10}$ s) to have any influence on the ^2H T_1 relaxation process, which is dominated by some other type of side-chain motion. Clear evidence for a side-chain motion other than methyl rotation is seen in the ^2H NMR spectra of the crystalline amino acid L-[ϵ-$^2H_3$]-methionine itself, a result consistent with the observation of large thermal amplitudes for the methionine side chains observed in X-ray crystallographic studies of the amino acid (McL. Mathieson, 1952; Torii & Iitaka, 1973), and of both methionine residues in sperm whale myoglobin (Frauenfelder et al., 1979).

Our results with methionine suggest that it will be one of the most flexible amino acids found in proteins. This is due to the presence of two long ($\sim$1.8 Å) C–S bonds, the lack of substituents on sulfur (cf. the two hydrogens in a methylene group), and the lack of any side-chain group capable of undergoing strong hydrogen bonding. Lysine and arginine, although longer, may frequently be involved in strong hydrogen-bond interactions, reducing overall mobility. This is especially likely to be true when these residues are involved in salt bridges, or when buried withn a membrane protein such as bacteriorhodopsin (Engelman & Zaccai, 1980), although surface lysines and arginines may, of course, be quite flexible, as observed, for example, in ^{13}C solution NMR studies by Allerhand et al. (1971).

Finally, we have demonstrated the feasibility of making extensive studies of individual ^2H- and ^{13}C-labeled sites in solid proteins of up to $\sim$30 000 daltons by using a combination of magnetic ordering, CP/MAS experiments, and state of the art spectrometer sensitivity. This opens up the possibility of obtaining detailed information on individual amino acid side-chain dynamics in a variety of proteins in condensed phases.

Acknowledgments

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Registry No. Methionine, 63-68-3.

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