Carbon-13 Nuclear Magnetic Resonance Study of the Motional Behavior of Ethyl Isocyanide Bound to Myoglobin and Hemoglobin†

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ABSTRACT: The interaction of ethyl isocyanide with myoglobin and hemoglobin has been studied by means of ^{13}C nuclear magnetic resonance spectroscopy by using ethyl isocyanide labeled in the ethyl side chain. Chemical shifts, longitudinal relaxation times, and approximate line widths are reported for the resonances of bound [1-^{13}C]ethyl isocyanide ($CH_3{}^{13}CH_2NC$) and bound [2-^{13}C]ethyl isocyanide ($^{13}CH_3CH_2NC$), as well as nuclear Overhauser enhancement values for the latter compound. Bound [1-^{13}C]ethyl isocyanide shows a chemical shift which is practically the same for sperm whale myoglobin, harbor seal myoglobin, and human hemoglobin. The same is true for bound [2-^{13}C]ethyl isocyanide. This suggests that the heme environment experienced by the ethyl side chain of ethyl isocyanide is similar in harbor seal myoglobin, sperm whale myoglobin, and the liganded (R) form of human hemoglobin. A theoretical analysis of the longitudinal relaxation time and nuclear Overhauser enhancement data, by using the restricted diffusion model [Wittebort, R. J., & Szabo, A. (1978) *J. Chem. Phys. 69*, 1722–1736; London, R. E., & Avitabile, J. (1978) *J. Am. Chem. Soc. 100*, 7159–7165], reinforces this conclusion. It suggests that the motion of the ethyl group of bound ethyl isocyanide is somewhat restricted and that the motional behavior and degree of restriction of motion may be similar for myoglobin and the R state of hemoglobin.

Studies of alkyl isocyanide binding to hemoglobin have contributed to an understanding of the role of steric effects in hemoglobin functioning (Szabo, 1978). The alkyl isocyanides, with general formula R—N=C, were first introduced as oxygen analogues by Pauling and colleagues (St. George & Pauling, 1951; Lein & Pauling, 1956). Their results showed that steric hindrance at the heme pocket has a strong influence on the binding of the alkyl isocyanides to hemoglobin and myoglobin.

Mansuy et al. (1976, 1978) and Dill et al. (1978) used ^{13}C NMR[1] spectroscopy to study the interaction of ethyl [^{13}C]-isocyanide ($CH_3CH_2N{}^{13}C$) with hemoglobin and myoglobin. They showed that structurally different myoglobins and hemoglobins differed in the chemical shifts of their bound ethyl [^{13}C]isocyanide. This suggested that the chemical shift of bound ethyl [^{13}C]isocyanide is highly sensitive to the environment at the heme pocket.

^{13}C NMR spectroscopy may also be used to study the motional properties of carbon atoms, such as those of the ethyl side chain of ethyl isocyanide. Motion of ^{13}C nuclei affects the longitudinal and transverse relaxation times (T_1 and T_2) and NOE values of their NMR lines. Theories have been developed which relate the motion of a protonated carbon atom to those observable parameters (Wallach, 1967; Doddrell et al., 1972; London & Avitabile, 1976, 1978; Wittebort & Szabo, 1978).

This report presents NMR spectra and longitudinal relaxation times for the ethyl side chain carbons of ethyl isocyanide bound to sperm whale myoglobin, harbor seal myoglobin, and human hemoglobin. The results are interpreted by using the "restricted diffusion" theory of Wittebort & Szabo (1978) and London & Avitabile (1978), with the assumption that the primary relaxation mechanism is ^{13}C–^{1}H dipolar interaction. This analysis suggests that the motion of the ethyl side chain of bound ethyl isocyanide is somewhat restricted and is practically the same for myoglobin and the R state of hemoglobin.

Materials and Methods

Preparation of Myoglobin for NMR. The major component of harbor seal metmyoglobin was prepared by using the procedure of Hapner et al. (1968), with Sephadex CM-50 ion-exchange chromatography at pH 6.4 (0.1 μ phosphate buffer); it was stored as lyophilized powder until use. Sperm whale metmyoglobin was obtained from Sigma Chemical Co. and used without further purification.

Just prior to an NMR experiment, 4 mM metmyoglobin solution in 0.1 μ phosphate buffer (pH 7.4, containing 3 mg/L of EDTA) was gently bubbled with nitrogen for 30 min at room temperature. Deoxygenated ascorbic acid solution was added to a concentration of 6 mM, and, after 30 min of additional nitrogen bubbling, the solution was 60% reduced to the ferrous form. About 4.7 mL of myoglobin solution was transferred by syringe to a nitrogen-flushed NMR tube, sealed with a rubber stopper. The NMR tube was then flushed with nitrogen (without bubbling) for 30 min more.

Up to 100 μL of ethyl isocyanide solution (0.5–1 M, in water) was introduced into the sealed NMR tube with a 50-μL Hamilton syringe. The level of metmyoglobin decreased shortly after ethyl isocyanide addition, from about 40% to less than 5%. The amount of metmyoglobin in solution after a run was generally negligible and always less than 5%. The final pH of the myoglobin solutions was 7.1 ± 0.1, measured in the presence of oxygen after a run.

Preparation of Hemoglobin for NMR. Human hemoglobin was prepared from the author's blood just prior to use. Red cells were washed three times with saline solution and hemolyzed by freezing and thawing. The hemolysate was dialyzed against several changes of 0.1 μ phosphate buffer (pH 7.4, containing 3 mg/L of EDTA) for 2 days at 4 °C. The hemoglobin solution was then concentrated to 8–9 mM (in heme) and used for NMR experiments within 1 week.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane; NOE, nuclear Overhauser enhancement; LW, line width; EtNC, ethyl isocyanide; EDTA, ethylenediaminetetraacetic acid; SWM, sperm whale myoglobin; HSM, harbor seal myoglobin; HHB, human hemoglobin.

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The procedure for adding hemoglobin and ethyl isocyanide to NMR tubes was as described above for myoglobin, except that at every stage bubbling or flushing with nitrogen proceeded 60 min, to ensure thorough deoxygenation. The amount of metmyoglobin before and after a run was negligible. pH was measured as 7.10 ± 0.05 after an NMR run, in the presence of oxygen.

Ethyl Isocyanide Preparation. Ethyl isocyanide was prepared by a miniaturized version of the method of Jackson & McKusick (1955); a full characterization of the procedure is given by Gautier (1869). For preparation of [2-^{13}C]ethyl isocyanide, 400 mg of AgCN was added to a small vial containing a magnetic stirrer. To this was added about 240 μL of 90% enriched [2-^{13}C]ethyl iodide (Stohler Isotope Chemicals, Rutherford, NJ). The two reagents were stirred together very briefly with a small rod, and the vial was clamped shut. The sealed vial was then placed in a boiling water bath, and the reaction proceeded with continual stirring for 1 h, at which time the reaction mixture was a dark brown, viscous, mass. The vial was cooled, and 270 μL of water was added, followed by 540 mg of KCN and another 230 μL of water. The mixture was stirred manually until the dark, oily mass rose to the surface. A small distillation side arm was then attached to the vial in place of the sealable cover. About 150 μL of ethyl isocyanide (a colorless oil) was collected during distillation. The ethyl isocyanide was not further purified; it was diluted 1:17 with water to a concentration of 0.7–0.8 M, placed in smaller rubber-sealed vials, and stored until use at −20 °C.

^{13}C Fourier Transform NMR Measurements. NMR spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer equipped with a Nicolet TT100 Fourier transform modification. It had an internal fluorine field-frequency lock, quadrature phase detection or optional single-channel detection, a 90° pulse width of 20 μs, and a sample tube diameter of 12 mm. Sample temperature was measured as 31 ± 1 °C.

For the experiments of this paper, the radio-frequency pulse was set at 0.1 ppm upfield from the ^{13}C resonance of Me_4Si. A spectral width of 3610 Hz was used in quadrature mode, and 1805 Hz in single-channel mode. The free induction decay was accumulated in 2048 addresses per channel of the Nicolet 1080 computer, giving 1.76-Hz digital resolution. Except for some determinations of approximate line width, data were processed by using 2.5 Hz of digital line broadening, to reduce background noise. Chemical-shift values were obtained digitally, by reference to an internal standard of dioxane (67.40 ppm downfield from Me_4Si, according to Sillerud et al., 1978).

Fully proton-decoupled spectra were obtained using 10 W of ^{1}H irradiation centered 2.9 ppm downfield from the ^{1}H resonance of Me_4Si at 100 MHz, with a random noise modulation bandwidth of 600 Hz. For normal spectra, 8192 scans were accumulated, and a 90° pulse was used, with a recycle time of 0.6 s. For partially relaxed spectra used for T_1 measurements, the 180°–τ–90° pulse sequence was employed, with an interval of 0.6 s between pulse sequences. The fast inversion recovery method (Canet et al., 1975) was used, and the longest value of τ was four to five times the T_1 value of the resonance line under study. Data were accumulated using single-channel detection, except for the longest τ value, where quadrature detection was used. Single-channel data for all τ values were processed identically, and T_1 values were calculated by fitting the data to the expression $I = A + B \exp(-\tau/T_1)$ (Kowalewski et al., 1977) by using the nonlinear least-squares fitting program BMDX85 (Dixon, 1965); the error limits are calculated by the program as ± standard deviation on the T_1 estimate.

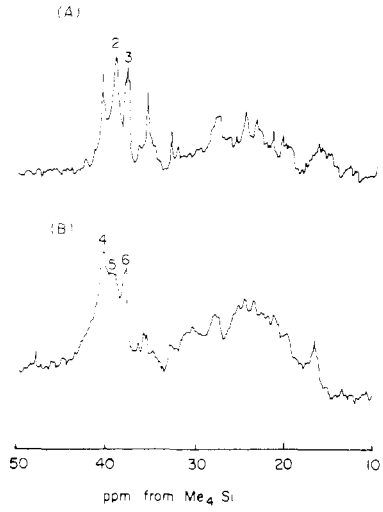

FIGURE 1: ^{13}C NMR spectra at 25.2 MHz for [1-^{13}C]ethyl isocyanide bound to (A) sperm whale myoglobin and (B) human hemoglobin. Peaks 1 and 4 are due to ε carbons of lysine (Visscher & Gurd, 1975), peaks 2 and 5 are due to bound ethyl isocyanide, and peaks 3 and 6 are due to free ethyl isocyanide. For both spectra, 8192 transients were accumulated in quadrature detection mode. Other conditions are as described in the text.

For measurements of the nuclear Overhauser enhancement (NOE), the gated decoupler technique was used (Kuhlman & Grant, 1971). ^{1}H irradiation was turned on at the same time as the 90° pulse, and it was maintained for 0.6 s, during which time the data were collected. The decoupler was then turned off for a time interval T. As a general rule, $T/T_1 \geq 8$ should allow sufficient time for the nuclei to relax to an equilibrium Boltzmann distribution, according to Opella et al. (1976). In the experiments of this paper, T/T_1 is always greater than eight, except for the case of hemoglobin ($T/T_1 = 6.5$). Calculations which use eq 3 of Opella et al. (1976) show that, in this case also, relaxation should have been virtually complete before a new pulse was initiated. NOE values were calculated as the ratio of the integrated intensity of the fully decoupled peak (by using the largest τ-value spectrum of the T_1 series) to the integrated intensity of the peak obtained with gated decoupling. The relative values of the integrated intensities were obtained by cutting and weighing the resonance peaks by using photocopies of the NMR spectra (Matthew et al., 1977); in obtaining these spectra, data for fully decoupled experiments and gated decoupling were processed identically.

To determine approximate line widths, spectra were generally processed without the use of digital line broadening. The line width in Hz of a peak was measured at one-half the peak height. When digital line broadening of 2.5 Hz was introduced in processing a spectrum used in line-width determination, 2.5 Hz were subtracted from the measured line width. The line-width values given in Tables I and II, below, were not corrected for the instrumental contribution to line broadening, which was typically about 1–2 Hz.

Results

^{13}C NMR of [1-^{13}C]Ethyl Isocyanide Bound to Myoglobin and Hemoglobin. Figure 1A shows the 25.2-MHz spectrum for [1-^{13}C]ethyl isocyanide ($CH_3{}^{13}CH_2NC$) bound to 4 mM sperm whale myoglobin. Free [1-^{13}C]ethyl isocyanide is seen at 37.6 ppm downfield from Me_4Si (peak 3), while the resonance due to [1-^{13}C]ethyl isocyanide bound to myoglobin occurs as a relatively broad peak at 38.9 ppm (peak 2). At

Table 1: Chemical Shifts, Longitudinal Relaxation Times (T_1), and Approximate Line Widths for [1-^{13}C]Ethyl Isocyanide Bound to Myoglobin and Hemoglobin[a]

protein	chemical shift (ppm from Me_4Si)	T_1 (s)	line width[b] (Hz)
SWM	39.0	0.055 ± 0.004	26
	38.9	0.046 ± 0.007	23
	38.9	0.052 ± 0.006	22
HSM	38.7	0.047 ± 0.006	23
	38.7	0.054 ± 0.009	24
HHb	38.9	0.124 ± 0.015	48
	39.1	0.124 ± 0.013	52

[a] Each entry represents an experiment on an independently prepared sample. Concentration of myoglobin was 4 mM, and of hemoglobin was 8.2 mM (in heme) for one experiment, and 8.7 mM for the other. [b] Line-width values shown are not corrected for the instrumental contribution to the line width, which is estimated to be 1–2 Hz.

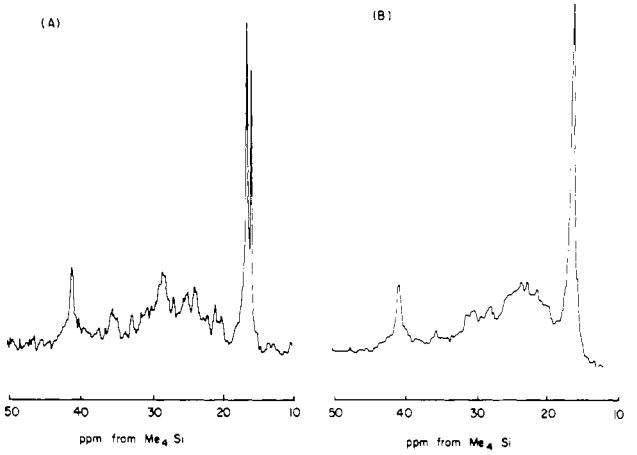

FIGURE 2: ^{13}C NMR spectra at 25.2 MHz for [2-^{13}C]ethyl isocyanide bound to (A) sperm whale myoglobin and (B) human hemoglobin. For both spectra, 8192 transients were accumulated in quadrature detection mode. Other conditions are as described in the text.

35.2 ppm is an unidentified peak, which may represent a breakdown product of ethyl isocyanide; longitudinal relaxation time data suggest that this peak has a relatively long T_1 value characteristic of small organic compounds not bound to protein.

Figure 1B shows the equivalent NMR spectrum for [1-^{13}C]ethyl isocyanide bound to human hemoglobin 8.2 mM in heme. The peak of bound ethyl isocyanide (peak 5), at 39.1 ppm, is considerably broader than in the case of myoglobin. The sharper peaks 4 and 6, at 40.2 and 37.6 ppm (ϵ carbon of lysines and free [1-^{13}]ethyl isocyanide, respectively), appear to rise off of it.

Table I summarizes the data on chemical shift, longitudinal relaxation time (T_1), and approximate line-width measurements for [1-^{13}C]ethyl isocyanide bound to sperm whale and harbor seal myoglobins and human hemoglobin.

^{13}C NMR of [2-^{13}C]Ethyl Isocyanide Bound to Myoglobin and Hemoglobin. Figure 2A shows the 25.2-MHz NMR spectrum of 4 mM sperm whale myoglobin saturated with [2-^{13}C]ethyl isocyanide ($^{13}CH_3CH_2NC$). Bound [2-^{13}C]ethyl isocyanide is seen at 15.9 ppm downfield from Me_4Si, while the peak of free [2-^{13}C]ethyl isocyanide is at 15.4 ppm.

Figure 2B shows the equivalent spectrum for human hemoglobin at 8.7 mM (in heme) concentration in the presence of about 10 mM [2-^{13}C]ethyl isocyanide. In this case, the bound [2-^{13}C]ethyl isocyanide appears at 16.1 ppm. The shoulder seen on the upfield side of the peak is due to free [2-^{13}C]ethyl isocyanide.

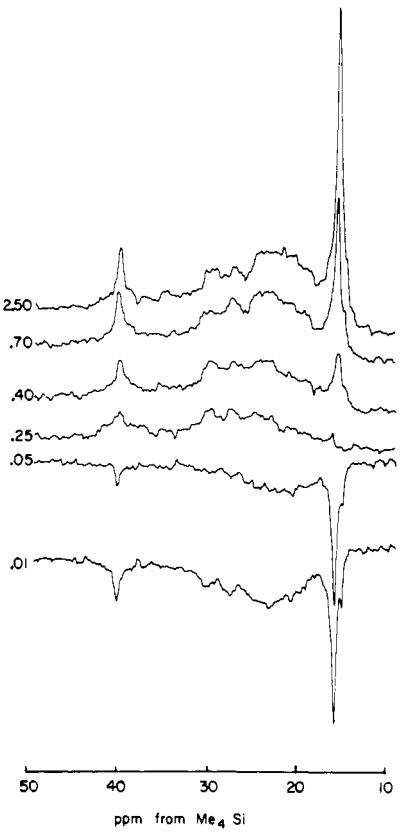

FIGURE 3: Partially relaxed ^{13}C NMR spectra at 25.2 MHz for [2-^{13}C]ethyl isocyanide bound to human hemoglobin. The number to the left of each spectrum is τ in seconds (τ is the time interval between 180° and 90° pulses in the pulse sequence 180–τ–90°). For all spectra, 8192 transients were accumulated in single channel mode. Other conditions are as described in the text.

Table II: Chemical Shifts, Longitudinal Relaxation Times (T_1), Nuclear Overhauser Enhancement (NOE) Values, and Approximate Line Widths for [2-^{13}C]Ethyl Isocyanide Bound to Myoglobin and Hemoglobin[a]

protein	chemical shift (ppm from Me_4Si)	T_1 (s)	NOE	line width[b] (Hz)
SWM	15.9	0.306 ± 0.009	1.7	5
	15.9	0.325 ± 0.018	1.5	5
HSM	15.7	0.300 ± 0.029	1.3	7
	15.9	0.332 ± 0.042		5
HHb	16.1	0.613 ± 0.038	1.5	11

[a] Each entry represents an experiment on an independently prepared sample. Concentration of myoglobin was 4 mM, and of hemoglobin was 8.7 mM (in heme). [b] Line-width values shown are not corrected for the instrumental contribution to the line width, which is estimated to be 1–2 Hz.

Figure 3 shows partially relaxed spectra for [2-^{13}C]ethyl isocyanide bound to human hemoglobin. The T_1 value obtained from the data of this figure is presented in Table II, along with T_1 values calculated for sperm whale and harbor seal myoglobins. Table II also gives values for chemical shifts, NOE, and approximate line widths for [2-^{13}C]ethyl isocyanide bound to all three proteins.

T_1 Data for α Carbons and Estimation of the Rotational Correlation Time τ_r. The ^{13}C NMR resonances of the α carbons form a broad envelope about 50–70 ppm downfield of the ^{13}C resonance of Me_4Si. This region is not shown in

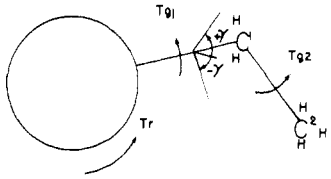

FIGURE 4: An illustration of the theoretical models used to interpret the data of Tables I and II. The ethyl isocyanide is assumed rigidly bound to the heme, such that only the ethyl side chain carbons are free to move within the heme pocket. The protein molecule is assumed spherical, with rotational correlation time τ_r; τ_r is defined as $(6D)^{-1}$, where D is the diffusion constant for the rotational motion of the macromolecule. For the free diffusion model of Wallach (1967), rotation of carbon-1 (C^1) is assumed to occur as a free diffusional process, with correlation time τ_{g1}, about an axis along the bond between carbon-1 and the sphere. Carbon-2 (C^2) is assumed to rotate freely about an axis along its bond with carbon-1, with correlation time τ_{g2}. τ_{gi} is defined as $(6D_i)^{-1}$, where D_i is the diffusion constant for the internal rotation about the ith carbon–carbon bond. For the restricted diffusion model of Wittebort & Szabo (1978) and London & Avitabile (1978), carbon-1 rotates only within the restricted angular region of $\pm\gamma$, and its rotational motion is reflected back at the boundary of this region.

Table III: Longitudinal Relaxation Times (T_1) for the α-Carbon Envelope of Myoglobin and Hemoglobin, and Calculation of the Rotational Correlation Times (τ_r)[a]

protein	α-carbon T_1 (s)	calcd τ_r[b] (s)
SWM	0.051 ± 0.003	$1.2 \pm 0.1 \times 10^{-8}$
HSM	0.051 ± 0.004	$1.2 \pm 0.1 \times 10^{-8}$
HHb	0.145 ± 0.022	$4.1 \pm 0.6 \times 10^{-8}$
HHb	0.131 ± 0.022	$3.7 \pm 0.7 \times 10^{-8}$

[a] Each entry represents an experiment on an independently prepared sample. Concentration of myoglobin was 4 mM, and of hemoglobin was 8.7 mM (in heme) for the experiment giving the larger T_1 value, and 8.2 mM for the other experiment. [b] τ_r values were calculated from the α-carbon T_1 values as described by Oldfield et al. (1975).

Figures 1 to 3, but it was included in all of the spectra obtained for this paper. These experiments, therefore, provide information on the α-carbon T_1 values, which are not very different from those of bound [1-^{13}C]ethyl isocyanide. The α-carbon T_1 values permit a calculation of the rotational correlation times τ_r for myoglobin and hemoglobin, under the experimental conditions of this paper. These τ_r values will be used in theoretical analysis of the relaxation data of Tables I and II for bound ethyl isocyanide (see Figure 4 and Discussion, below).

Table III gives the T_1 values for the α-carbon envelope of myoglobin (4 mM) and hemoglobin (8–9 mM in heme). The τ_r values calculated from the corresponding T_1 values are also shown; they were computed as described by Oldfield et al. (1975).

Discussion

Ethyl isocyanide and other alkyl isocyanides have been used extensively in studying the heme pocket environment of hemoglobin and myoglobin. The alkyl isocyanides offer a series of similar ligands which differ only in the size of the alkyl side chain, and hemoglobin shows the same general characteristics in binding the isocyanides as in binding oxygen: cooperative effects (Hill's constant n is 2.4 for ethyl isocyanide), a Bohr effect, and lowered ligand affinity in the presence of organic phosphates (Brunori et al., 1972; Lo & Schimmel, 1972).

Studies on a series of alkyl isocyanides of varying sizes showed that steric hindrance at the heme pocket limits their binding to hemoglobin and myoglobin (St. George & Pauling, 1951; Lein & Pauling, 1956). However, isocyanide binding may be favored by hydrophobic interactions at the heme pocket involving the alkyl side chain (Brunori et al., 1972; Olson & Binger, 1976).

Ethyl isocyanide has the formula CH_3–CH_2–N=C. In this report, ^{13}C NMR data were presented on the binding of ^{13}C-labeled ethyl isocyanide to sperm whale and harbor seal myoglobin and human hemoglobin. Two labeled compounds were used to obtain data separately on each of the carbons of the ethyl side chain: [1-^{13}C]ethyl isocyanide ($CH_3{}^{13}CH_2NC$) and [2-^{13}C]ethyl isocyanide ($^{13}CH_3CH_2NC$).

Data of Table I show that [1-^{13}C]ethyl isocyanide has almost the same chemical shift when bound to harbor seal myoglobin and sperm whale myoglobin. Table II shows that there is no difference in chemical shift for [2-^{13}C]ethyl isocyanide bound to sperm whale vs. harbor seal myoglobin. This similarity in chemical shift values for the ethyl side chain in ethyl isocyanide bound to the two myoglobins is seen in spite of the fact that the two myoglobins crystallize into different crystal types (Scouloudi, 1960), differ at 26 positions in the amino acid sequence (Bradshaw & Gurd, 1969), and show significantly different chemical shifts for ethyl [^{13}C]isocyanide binding (Gilman, unpublished observation).

The NMR spectra for human hemoglobin (Figures 1 and 2) show chemical shifts for bound [1-^{13}C]- and [2-^{13}C]ethyl isocyanide almost identical with those of myoglobin. No separate α and β chain peaks are visible, although they are seen for ethyl [^{13}C]isocyanide (Mansuy et al., 1976, 1978; Dill et al., 1978). One concludes from these observations that the heme environment experienced by the ethyl side chain of ethyl isocyanide is similar in harbor seal and sperm whale myoglobin and fully liganded human hemoglobin.

A comparison of T_1 values obtained for ethyl isocyanide bound to sperm whale and harbor seal myoglobin reinforces the conclusion that the heme environment of the ethyl group is similar in the two myoglobins. For both [1-^{13}C]ethyl isocyanide (Table I) and [2-^{13}C]ethyl isocyanide (Table II), T_1 values are the same, within experimental error, for sperm whale and harbor seal myoglobin.

Theoretical Considerations. By analyzing the T_1 data of Tables I and II, using the "free diffusion" theory of Wallach (1967) or its extension to the "restricted diffusion" case (Wittebort & Szabo, 1978; London & Avitabile, 1978), one can make quantitative deductions concerning the motion of the ethyl side chain carbons. There are two principal assumptions: (1) That relaxation is occurring solely by dipole–dipole interactions between a ^{13}C nucleus and its directly bonded ^{1}H nuclei. This assumption is apparently justified for protonated carbons (Norton et al., 1977). (2) That the ethyl side chain of ethyl isocyanide is bonded to a rigid sphere with rotational correlation time τ_r, as shown in Figure 4. This is the simplest assumption one can make in order to deduce the motional behavior of both ethyl group carbons using these theories.

One may first consider the free diffusion model (Wallach, 1967), which assumes free rotation of carbon-1 about an axis along the bond which connects it to the sphere; correlation time for the rotation is τ_{g1}. Similarly, unrestricted rotation of carbon-2 about an axis connecting it with carbon-1 is assumed; correlation time for carbon-2 rotation is τ_{g2}. This model and the restricted diffusion model are illustrated in Figure 4.

By using the free diffusion model, it is possible to fit the T_1 data for carbons 1 and 2 ([1-^{13}C]- and [2-^{13}C]ethyl isocyanide, respectively). Calling these T_1 values $T_1(1)$ and $T_1(2)$, respectively, Table IV shows the fit that can be obtained by adjusting the values of τ_{g1} and τ_{g2}. These calculations were

Table IV: Theoretical Fit to the Free Diffusion Model of Wallach (1967) of T_1 Data for [1-^{13}C]- and [2-^{13}C]Ethyl Isocyanide Bound to Myoglobin and Hemoglobin

τ_r (s)	τ_{g1} (s)	τ_{g2} (s)	$T_1(1)^a$ (s)	LW(1) (Hz)	$T_1(2)^b$ (s)	LW(2) (Hz)	NOE(2)
			Myoglobin				
1.2×10^{-8}	1.6×10^{-10}	8×10^{-13}	0.052	9.2	0.304	1.6	2.42
			Hemoglobin				
3.7×10^{-8}	6×10^{-11}	1.8×10^{-12}	0.120	13.6	0.598	2.4	2.65
4.1×10^{-8}	6×10^{-11}	1.8×10^{-12}	0.123	14.8	0.607	2.6	2.67

[a] The number 1 in parentheses refers to carbon-1 of Figure 4; the correct value for $T_1(1)$ is assumed to be between 0.047 and 0.058 s for myoglobin and between 0.11 and 0.14 s for hemoglobin, as suggested by the data of Table I for [1-^{13}C]ethyl isocyanide. LW(1) is the line width for carbon-1. [b] The number 2 in parentheses refers to carbon-2 of Figure 4; the correct value for $T_1(2)$ is assumed to be between 0.29 and 0.34 s for myoglobin and between 0.57 and 0.65 s for hemoglobin, as suggested by the data of Table II for [2-^{13}C]ethyl isocyanide. LW(2) is the line width, and NOE(2) is the NOE value, for carbon-2.

performed by using the experimentally determined values of τ_r given in Table III: 1.2×10^{-8} s for 4 mM myoglobin, and 3.7×10^{-8} or 4.1×10^{-8} s for 8–9 mM (in heme) hemoglobin. For myoglobin, the published values for τ_r are $1.8 \pm 0.5 \times 10^{-8}$ s at 8.6 mM concentration (Oldfield et al., 1975; Wilbur et al., 1976) and $2.2 \pm 0.5 \times 10^{-8}$ s at 16 mM concentration (Visscher and Gurd, 1975). For hemoglobin (14.8 mM heme concentration), the published value is $4.7 \pm 0.7 \times 10^{-8}$ s (Oldfield et al., 1975). The τ_r values given in Table III are at the lower limit of the published observations. This is probably due to the lower protein concentrations used to obtain the data of this paper.

One sees, by comparing the calculations of Table IV with the data of Tables I and II, that the free diffusion model, with two adjustable parameters (τ_{g1} and τ_{g2}), can be made to fit the T_1 data for carbons 1 and 2. However, it cannot then account for the line width or NOE data.

The restricted diffusion theory (Wittebort & Szabo, 1978; London & Avitabile, 1978) was developed to be able to account for the effect of excluded volumes on the rotational motion of side chains. In the case of the ethyl side chain of ethyl isocyanide bound to myoglobin or hemoglobin, one imagines that rotation of carbon-1 may be restricted due to collision of the side chain with groups present in the heme pocket. Rotation of carbon-2 may not be restricted, however, since rotation of carbon-2 around the carbon-1–carbon-2 bond axis would not change the orientation of the ethyl side chain.

The restricted diffusion model applied to the bound ethyl isocyanide situation thus has a third adjustable parameter γ, in addition to τ_{g1} and τ_{g2}: As illustrated in Figure 4, rotation of carbon-1 occurs as a diffusional process within an angular region of $\pm\gamma$ about its bond with the sphere.

In fitting the data of Tables I and II to the restricted diffusion model, a wide range of the values of the parameters τ_{g1}, τ_{g2}, and γ was tested, in order to set approximate limits on the parameters. τ_r was fixed at the experimentally determined values given in Table III: 1.2×10^{-8} s for myoglobin, and 3.7×10^{-8} or 4.1×10^{-8} s for hemoglobin. Three values of the ratio τ_{g1}/τ_{g2} were examined: 2, 100, and 1000 (values of less than 1 were not considered, as they would indicate faster motion for carbon-1 than for carbon-2, which appears physically unlikely). For each value of τ_{g1}/τ_{g2}, a range of γ values was considered, between 30° and 80° in increments of 5°. Finally, for each value of τ_{g1}/τ_{g2} and γ, τ_{g1} was varied systematically ($\tau_{g1} = 10^{-12}, 2 \times 10^{-12}, 4 \times 10^{-12}, \ldots, 8 \times 10^{-10}, 10^{-9}$).

Table V shows values of γ, τ_{g1}, and τ_{g1}/τ_{g2}, which allow a fit to observed T_1 values for carbon-1 and carbon-2, and to the NOE of carbon-2. From this table, one sees that γ = 50–65° fits the myoglobin data. Restricting the angular displacement γ to 50–60° will also permit a reasonably good fit to the myoglobin line-width data (after adding 2 Hz to the calculated value to allow for instrumental contributions to the line width). The acceptable ranges of τ_{g1} and τ_{g2} for myoglobin are then $2 \times 10^{-12} \leq \tau_{g1} \leq 8 \times 10^{-11}$ and $2 \times 10^{-14} \leq \tau_{g2} \leq 3 \times 10^{-12}$. For hemoglobin, $\tau_r = 3.7 \times 10^{-8}$ and γ = 50–55° comes close to fitting the line-width data, in addition to the T_1 and NOE data; the allowed ranges for τ_{g1} and τ_{g2} are then $4 \times 10^{-12} \leq \tau_{g1} \leq 4 \times 10^{-11}$ and $4 \times 10^{-14} \leq \tau_{g2} \leq 3 \times 10^{-12}$.

Examination of Table V suggests that the motion of bound ethyl isocyanide may be quantitatively very similar in myoglobin and the fully liganded (R) state of hemoglobin. In particular, there is one set of values for τ_{g1}/τ_{g2} and γ which is able to fit the data for both myoglobin and hemoglobin: $\tau_{g1} = 6 \times 10^{-12}$ s, $\tau_{g2} = 3 \times 10^{-12}$ s, and γ = 55°. Setting τ_r equal to 1.2×10^{-8} s gives values for T_1, line width, and NOE which agree with the data for myoglobin of Tables I and II. With $\tau_r = 3.7 \times 10^{-8}$ s, the values are consistent with the data for hemoglobin.

The analysis of Table V shows that a fit can be obtained between the restricted diffusion model and the data of Tables I and II, and that approximate limits can be set on the values of the parameters τ_{g1}, τ_{g2}, and γ. Another way to determine acceptable limits for the parameters of the restricted diffusion model is to use the nonlinear least-squares fitting procedure, which minimizes the sum of squared error of the fit to the data.

Table VI gives the results of the least-squares analysis applied to the data of Tables I and II, for sperm whale myoglobin and human hemoglobin. For this analysis, τ_r was treated as a parameter to be fitted, along with τ_{g1}, τ_{g2}, and γ. The estimated value of τ_r by using this procedure is $1.30 \pm 0.10 \times 10^{-8}$ s for myoglobin and is largely determined by the line-width data. For hemoglobin, τ_r is estimated as $3.50 \pm 0.17 \times 10^{-8}$ s. The τ_r values estimated in this manner agree with the values determined by the T_1 values of the α carbons (Table III). The ranges of values for τ_{g1}, τ_{g2}, and γ are, on the whole, consistent with the ranges determined by the analysis of Table V. Table VI suggests that τ_{g1} may be larger for ethyl isocyanide bound to sperm whale myoglobin than for ethyl isocyanide bound to hemoglobin. Otherwise, no significant differences are seen between the motional parameters for ethyl isocyanide bound to hemoglobin compared with those for ethyl isocyanide bound to myoglobin.

Table VI also analyzes the motion of the methionine side chains of myoglobin by using the data of Jones et al. (1976) on ^{13}C-labeled methionines. These data, obtained on 5 mM myoglobin, are also compatible with a τ_r value of about 1.3×10^{-8} s, and τ_{g1} and τ_{g2} values are not very different from those for bound ethyl isocyanide. The value of γ, however, is smaller for methionine than for ethyl isocyanide, suggesting a greater restriction on the movement of the methionines. For the enzyme dihydrofolate reductase, the analysis of Blakley et al. (1978) suggested γ values as small as 45°, for the broadest methionine resonances observed, although some

Table V: Theoretical Fit to the Restricted Diffusion Model (Wittebort & Szabo, 1978; London & Avitabile, 1978) of T_1 Data on [1-^{13}C]- and [2-^{13}C]Ethyl Isocyanide Bound to Hemoglobin and Myoglobin[a]

τ_r (s)	τ_{g_1}/τ_{g_2}	γ (deg)	τ_{g_2} (s)	$T_1(1)$ (s)	LW(1) (Hz)	$T_1(2)$ (s)	LW(2) (Hz)	NOE(2)
				Myoglobin				
1.2×10^{-8}	2	50	10^{-12}	0.049	21.0	0.283	3.5	1.27
1.2×10^{-8}	2	55	10^{-12}	0.055	18.7	0.318	3.1	1.28
1.2×10^{-8}	2	55	2×10^{-12}	0.055	18.7	0.303	3.2	1.35
1.2×10^{-8}	2	55	3×10^{-12}	0.055	18.7	0.290	3.2	1.42
1.2×10^{-8}	1000	50	2×10^{-14}	0.048	21.1	0.286	3.5	1.25
1.2×10^{-8}	1000	55	4×10^{-14}	0.051	19.2	0.304	3.2	1.35
1.2×10^{-8}	1000	55	6×10^{-14}	0.049	19.5	0.291	3.2	1.42
1.2×10^{-8}	1000	60	4×10^{-14}	0.055	17.2	0.331	2.9	1.41
1.2×10^{-8}	1000	60	6×10^{-14}	0.052	17.5	0.313	2.9	1.49
1.2×10^{-8}	1000	60	8×10^{-14}	0.050	17.9	0.297	3.0	1.57
1.2×10^{-8}	1000	65	6×10^{-14}	0.055	15.9	0.333	2.6	1.58
1.2×10^{-8}	1000	65	8×10^{-14}	0.052	16.3	0.311	2.7	1.66
1.2×10^{-8}	1000	65	10^{-13}	0.049	16.6	0.293	2.8	1.73
				Hemoglobin				
3.7×10^{-8}	2	45	10^{-12}	0.111	61.5	0.612	10.3	1.32
3.7×10^{-8}	2	50	2×10^{-12}	0.123	54.9	0.617	9.2	1.49
3.7×10^{-8}	2	50	3×10^{-12}	0.122	54.9	0.566	9.3	1.61
3.7×10^{-8}	2	55	3×10^{-12}	0.136	48.8	0.617	8.2	1.66
3.7×10^{-8}	1000	45	2×10^{-14}	0.106	61.6	0.637	10.2	1.25
3.7×10^{-8}	1000	50	4×10^{-14}	0.108	55.3	0.645	9.2	1.42
4.1×10^{-8}	2	40	10^{-12}	0.110	75.3	0.607	12.6	1.32
4.1×10^{-8}	2	45	2×10^{-12}	0.122	67.8	0.609	11.4	1.48
4.1×10^{-8}	2	50	3×10^{-12}	0.134	60.6	0.609	10.2	1.65
4.1×10^{-8}	1000	45	6×10^{-14}	0.105	68.2	0.628	11.3	1.44
4.1×10^{-8}	1000	50	6×10^{-14}	0.109	61.1	0.651	10.2	1.55

[a] For myoglobin, τ_r was assumed to be 1.2×10^{-8} s, and for hemoglobin it was assumed to be 3.7×10^{-8} or 4.1×10^{-8} s, as discussed in the text. The correct T_1 values for [1-^{13}C]- and [2-^{13}C]ethyl isocyanide, $T_1(1)$ and $T_1(2)$, respectively, were assumed to lie in the range described in the footnotes to Table IV. In addition, the NOE value for [2-^{13}C]ethyl isocyanide bound to myoglobin [NOE(2)] was assumed to be in the range of 1.3–1.7, as suggested by the data of Table II. For hemoglobin, NOE(2) was assumed to lie within essentially the same range. The ranges of τ_{g_1}/τ_{g_2}, γ, and τ_{g_1} were chosen as described in the text. Values of τ_{g_1}/τ_{g_2}, of 100 were tested, but the results are not shown as they do not alter the conclusions that one might draw from the table.

Table VI: Nonlinear Least-Squares Estimation of Restricted Diffusion Parameters τ_r, τ_{g_1}, τ_{g_2}, and γ for Data of Tables I and II, and Other Published Observations[a]

protein	carbon-13 label	spectrometer frequency (MHz)	$\tau_r \times 10^8$ (s)	$\tau_{g_1} \times 10^{11}$ (s)	$\tau_{g_2} \times 10^{12}$ (s)	γ (deg)	T_1 (s)	LW (Hz)	NOE
SWM[b]	[1-^{13}C]EtNC	25.2	1.30 ± 0.10	7.4 ± 3.8			0.051	19.7	
	[2-^{13}C]EtNC	25.2	1.30 ± 0.10	7.4 ± 3.8	0.028 ± 1.70	57.6 ± 3.1	0.306	3.3	1.51
HHb[b]	[1-^{13}C]EtNC	25.2	3.50 ± 0.17	0.17 ± 2.0			0.124	49.5	
	[2-^{13}C]EtNC	25.2	3.50 ± 0.17	0.17 ± 2.0	2.4 ± 3.8	52.2 ± 2.1	0.613	8.3	1.50
SWM[c]	[^{13}C]Me of Met's	25.2	1.40	6.4	8.3	39.9	0.195	5.2	1.70
	[^{13}C]Me of Met's	25.2	1.22 ± 0.12	2.9	2.7	33.8 ± 5.2	0.199	5.0	1.36
	[^{13}C]Me of Met's	67.9	1.22 ± 0.12	2.9	2.7	33.8 ± 5.2	0.815	4.3	
	[^{13}C]Me of Met's	67.9	1.40	1.1	4.3	41.9	0.833	4.2	

[a] The nonlinear least-squares program used for these calculations was devised by Dr. L. Homer (Naval Medical Research Institute, Bethesda, MD). Applied to the estimation of the parameters of the restricted diffusion model (Wittebort & Szabo, 1978; London & Avitabile, 1978), it minimized the sum of separated values of $1 - y_c/y_0$ where y_0 are the observed data points, and y_c are their calculated theoretical values. The standard error of the estimate for each parameter is given for cases in which a meaningful error estimate was obtained. For calculations on the ethyl isocyanide data of this paper, the angles β between successive internal rotation axes, and between the final internal rotation axis and the carbon–hydrogen vector, are taken as the complement of the tetrahedral angle (Wittebort & Szabo, 1978). For calculations on the data for the [^{13}C]methyls of methionines of sperm whale myoglobin, the assumptions, and the values of the angles β, are as described by London & Avitabile (1978). [b] The values of the parameters τ_r, τ_{g_1}, τ_{g_2}, and γ were estimated by using the combined data of Tables I and II on bound [1-^{13}C]- and [2-^{13}C]ethyl isocyanide. Separate computations were made for sperm whale myoglobin (12 data points) and human hemoglobin (7 data points). The instrumental contribution to the line width was assumed to be 2 Hz, and this was subtracted from the observed line width prior to the computation. [c] The data upon which the parameter estimation is based are those of Jones et al. (1976), on ^{13}C-labeled labeled methyl groups of methionines, at positions 55 and 131 of sperm whale myoglobin (5 mM concentration). For computation purposes, data for both methionines were pooled, giving two T_1 values and two line-width values at each of the spectrometer frequencies 25.2 and 67.9 MHz, and one NOE value at 25.2 MHz. Line-width values were corrected for the instrumental contribution by using the values given by Jones et al. (1976): 1 Hz at 25.2 MHz, and 5 Hz at 67.9 MHz. Values of τ_r, τ_{g_1}, τ_{g_2}, and γ were estimated on the five data points at 25.2 MHz, and this gave a τ_r estimate of 1.40×10^{-8} s (with other values as shown in the table). All four parameters were also estimated on the nine data points for 25.2 and 67.9 MHz, giving the τ_r estimate of 1.22×10^{-8} s (with other values as shown in the table). For the four data points at 67.9 MHz alone, the value of τ_r of 1.4×10^{-8} s was assumed, and only the value of τ_{g_1}, τ_{g_2}, and γ were estimated, as shown in the table.

methionine residues appeared to be much less restricted.

In conclusion, the restricted diffusion theory of Wittebort & Szabo (1978) and London & Avitabile (1978) is able to account for the T_1, NOE, and approximate line-width data that were presented for [1-^{13}C]- and [2-^{13}C]ethyl isocyanide bound to sperm whale myoglobin, harbor seal myoglobin, and human hemoglobin. The theory suggests that motion of the ethyl group of ethyl isocyanide is somewhat restricted, but nowhere near to the point of complete immobilization. The motional behavior and degree of restriction of motion of the ethyl group may be similar for myoglobin and the R state of hemoglobin.

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