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Iron-57 Nuclear Magnetic Resonance Spectroscopic Study of Alkyl Isocyanide Myoglobins and a Comparison of the ^{57}Fe Chemical-Shift Anisotropies of Alkyl Isocyanide Myoglobins, Carbonmonoxymyoglobin, Ferrocytochrome *c*, and $[^{57}Fe(bipy)_3]X_2 \cdot 5H_2O$ (X = Cl, Br, I)*

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The ^{57}Fe nuclear magnetic resonance spectra and spin–lattice relaxation times (T_1) of ethyl isocyanide (EtNC), isopropyl isocyanide (iPrNC), and *n*-butyl isocyanide (*n*-BuNC) ligated ferrous myoglobins (~ 12 m*M*, pH 7.1, 22°C) at 8.45 T (corresponding to a ^{57}Fe Larmor frequency of 11.7 MHz) have been obtained. The isotropic chemical shifts are 9223, 9257, and 9238 ppm downfield from $Fe(CO)_5$, which yields chemical-shift anisotropies, $|\delta_\perp - \delta_\parallel|$, of 1288, 1260, and 1205 ppm, for the EtNC, and *n*-BuNC species, respectively. The T_1 values (of about 140 ms) are very much longer than those found previously for carbonmonoxymyoglobin (~17 ms) and are consistent with a change in sign of the chemical-shift tensor upon moving from carbonmonoxymyoglobin to ferrocytochrome *c*, as previously postulated by L. Baltzer, (*J. Am. Chem. Soc.* **109**, 3479 (1987)). The solution- and solid-state ^{57}Fe NMR chemical shifts of $[^{57}Fe(bipy)_3]X_2 \cdot 5H_2O$ (X = Cl, Br, I) complexes are also reported, and for the solids it is found that $\delta_\perp = \delta_\parallel = \delta_i$ = 11,854, 11,721, and 11,635 ppm, for X = Cl, Br, and I, respectively. T_1 values for the solids are in the range ~1–5 s, and are probably dominated by electron exchange with paramagnetic impurities. © 1990 Academic Press, Inc.

There has been increasing interest in the use of ^{57}Fe as a probe of heme protein structure over the past 10 years, since Fe is a central feature of all heme proteins, and ^{57}Fe NMR offers, in principle, a direct probe of the functionally interesting diamagnetic, low-spin ferrous state. Earlier workers synthesized a variety of labeled species, such as $^{57}Fe^{13}C^{15}NR$ myoglobin (*1*) and $^{57}Fe^{13}CO$ myoglobin (*2*), and determined ^1J coupling constants, but did not directly observe ^{57}Fe NMR, due presumably to technical limitations. LaMar determined, using double resonance, the iron-57 chemical shift of $[^{57}Fe]Mb^{13}CO$, and we (*3*) and others (*4*) determined by direct detection the isotropic chemical shift due in MbCO, as well as by inference the anisotropy ($|\delta_\perp - \delta_\parallel|$) of the chemical-shift tensor. More recently, Baltzer (*5*) investigated the isotropic shift (δ_i)

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of ^{57}Fe in ^{57}Fe-labeled ferrocytochrome *c*. He found δ_i = 11,197 ppm (IUPAC δ scale), considerably deshielded from the δ_i = 8227 value found in MbCO, and he put forward the interesting idea that $\delta_\perp$, the chemical-*shift* (as opposed to the shielding) tensor element in the plane of the porphyrin ring, is at $\approx$9000 ppm, for both MbCO and cytochrome *c* and for high symmetry porphyrins. Baltzer's results imply that it is primarily $\delta_{||}$, the shift tensor element perpendicular to the heme plane, that dominates the observed changes in δ_i with axial ligation, and his work specifically implied a *change in sign* for $\delta_\perp - \delta_{||}$ on transition from MbCO to ferrocytochrome *c*.

Baltzer's ideas thus imply that there may be a series of heme proteins that have $\delta_\perp \approx \delta_{||}$, in which case spin-lattice relaxation times (T_1) will be extremely long. We show in this publication that the alkyl isocyanide derivatives of ferrous myoglobin appear to fall into this category, supporting the notion that $\delta_{||}$ dominates the observed δ_i.

EXPERIMENTAL SECTION

Sample preparation. Protoporphyrin IX dimethyl ester (Sigma Chemical Co., St. Louis, Missouri) was metallated using $^{57}Fe(II)(OAc)_2$ prepared from ^{57}Fe metal (94.5% ^{57}Fe, Oak Ridge National Laboratory, Oak Ridge, Tennessee). Sperm whale (*Physeter catodon*) myoglobin was also obtained from Sigma, and was purified chromatographically (*6*). Reconstitution of apomyoglobin with [^{57}Fe] hemin chloride was carried out basically as described previously (*6, 7*). iPrNC and *n*-BuNC were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin), while EtNC was synthesized in this laboratory from AgCN and EtI, basically as described by Jackson and McKusick (*8*). $[^{57}Fe(bipy)_3]Cl_2 \cdot 5H_2O$ was prepared according to standard procedures (*9*), starting from 94.5% ^{57}Fe powder, using concentrated hydrochloric acid and 2,2′-bipyridyl (Aldrich). After ether extraction of the aqueous phase to remove unreacted starting material, the sample was recrystallized from Me_2CO/H_2O. The resulting $[^{57}Fe(bipy)_3]Cl_2 \cdot 5H_2O$ had a satisfactory ^1H NMR spectrum (200 MHz) and microchemical analysis. $[^{57}Fe(bipy)_3]Br_2 \cdot 5H_2O$ was made by ion exchange of the chloride salt on Amberlite IRA-400 (Mallinkrodt Chemical Works, St. Louis, Missouri) and the bromide salt was subsequently exchanged with I^- (to make $[^{57}Fe(bipy)_3]I_2 \cdot 5H_2O$), after ^{57}Fe NMR spectroscopy, in the same way. Myoglobin samples for NMR spectroscopy were prepared by equilibrating the ferric protein in 50 m*M* phosphate buffer (pH 7.1), purging the sample with N_2, adding a slight excess of sodium dithionite (Fisher Scientific, Fairlawn, New Jersey) in buffer to achieve full reduction, followed by adding a five-fold excess of the respective alkyl isocyanide. Visible absorption spectra showed the expected absorption maxima at 532 and 563 nm (*10*).

NMR spectroscopy. ^{57}Fe NMR spectra were obtained on "homebuilt" NMR spectrometers, which consist of either an 8.45 T, 3.5 inch bore superconductive solenoid magnet (Oxford Instruments, Osney Mead, UK) or an 11.7 T, 2.0 inch bore solenoid (Oxford), together with Nicolet (Madison, Wisconsin) Model 1280 computer systems, and a Henry Radio (Los Angeles, California) Model 2KD radiofrequency amplifier, together with assorted ancillary digital and radiofrequency electronics. For solution ^{57}Fe NMR spectra, we used a homebuilt static 20 mm solenoidal-coil probe; the sample volume was ~7 ml. The 90° pulse widths used varied between 35 and 55 μs, and were determined on a saturated solution of ferrocene in toluene. Chemical shifts are

all referenced with respect to $Fe(CO)_5$ at 0 ppm, using as a secondary standard a saturated solution of ferrocene in toluene (δ = 1531 ppm). Low-field, high-frequency, paramagnetic or deshielded shifts are taken to be positive (IUPAC δ scale). For consistency, we report chemical-*shift* tensor elements using the same convention, which is opposite to the *shielding* convention used by Baltzer (*5*).

RESULTS AND DISCUSSION

We show in Figs. 1A–C the 8.45 T (11.7 MHz) ^{57}Fe NMR spectra of [^{57}Fe] ethyl, isopropyl, and *n*-butyl isocyanide ligated ferrous myoglobins (all 12 ± 1 m*M* in pH 7.1 phosphate buffer, at 22 ± 2°C). The isotropic chemical shifts (Table 1) are at 9223 ± 2, 9257 ± 2, and 9238 ± 2 ppm downfield from $Fe(CO)_5$. Figure 1D shows a typical inversion-recovery determination of T_1 for *n*-BuNC Mb, and we find T_1 = 133 ± 37, 139 ± 23, and 152 ± 9 ms for the EtNC, iPrNC, and *n*-BuNC species (Table 1).

As can be seen from Table 1, these T_1 values (of ≈140 ms) are almost an order of magnitude larger than those found previously for MbCO (~17 ms, at essentially the

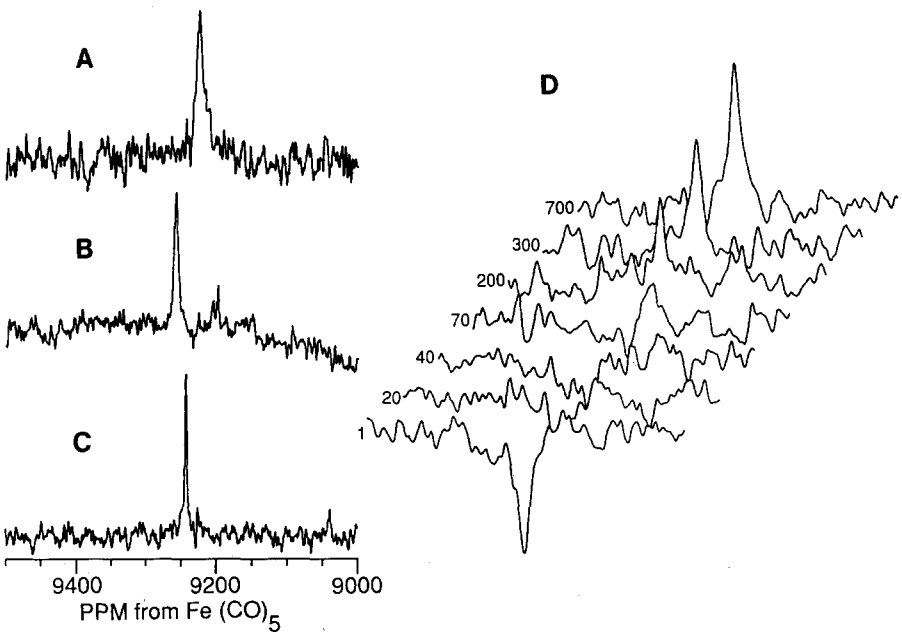

FIG. 1. The 8.45 T (11.7 MHz) ^{57}Fe NMR spectra and spin–lattice relaxation time behavior of alkyl isocyanide myoglobins. (A) EtNC Mb, 12 m*M*, pH 7.1, 22° ± 2°, 100,000 scans, 55 μs pulse excitation, and 50 Hz line broadening due to exponential multiplication. (B) iPrNC Mb, 12 m*M*, pH 7.1, 22° ± 2°, 184,000 scans, 35 μs pulse excitation, and 20 Hz line broadening due to exponential multiplication. (C) *n*-BuNC Mb, 12 m*M*, pH 7.1, 22° ± 2°, 54,000 scans, 45 μs pulse excitation, and 10 Hz line broadening due to exponential multiplication. (D) Partially relaxed inversion-recovery series for *n*BuNC Mb; same sample conditions as (C) but with 100, 50 μs pulse excitation, 14,000 scans per spectrum, and 10 Hz line broadening due to exponential multiplication. The "τ" values between the two (180°, 90°) pulses used are given on the figure, in ms. The recycle time was 500 ms in each case.

TABLE 1

Iron-57 NMR Spin–Lattice Relaxation Times and Isotropic Chemical Shifts for Heme Proteins and Derived Chemical-Shift Tensor Elements and Anisotropies

System	T_1 (ms)	τ_R (ns)	δ_i (ppm)[a]	$\lvert\Delta\delta\rvert$ (ppm)	$\delta_\perp$ (ppm)[b]	$\delta_\parallel$ (ppm)[b]
Myoglobin						
EtNC	133 ± 37	20[c]	9,223	1288	8794 (9,652)	10,082 (8,364)
iPrNC	139 ± 23	20[c]	9,257	1260	8837 (9,677)	10,097 (8,417)
n-BuNC	152 ± 9	20[c]	9,238	1205	8836 (9,640)	10,041 (8,435)
CO	17 ± 3[d]	20[c]	8,227	3600	9427 (7,027)	5,827 (10,627)
Cytochrome *c*[e]	(4)[f]	6[g]	11,197	7630	8655 (13,739)	16,282 (6,112)

[a] Isotropic chemical shift, in ppm, downfield from $Fe(CO)_5$. Error is ±2–3 ppm.
[b] Shift tensor element. Alternative sets of solutions are shown in parentheses.
[c] Rotational correlation time, τ_R, taken as 20 ns (Ref. (*3*)).
[d] From Ref. (*3*).
[e] 3 m*M* [^{57}Fe] ferrocytochrome *c*, pH 7, 25°C, from Ref. (*5*).
[f] Computed T_1, from Ref. (*5*).
[g] Value of τ_R used by Baltzer in Ref. (*5*).

same concentration, pH, and temperature). Since the alkyl isocyanides are known to bind tightly to Mb, at least on the time scale we are interested in, and since any ligand exchange with ferrous Mb would almost assuredly give a very rapid T_1, we must find an explanation for the very long T_1 values in the chemical-shift anisotropy of the ^{57}Fe in the alkyl isocyanide myoglobins, since we previously ruled out all other possible contributions to T_1 values on this time scale (*3*). For spin–lattice relaxation via the chemical-shift anisotropy mechanism, we have (*11*)

$$1/T_1 = (1/15)\gamma^2 H_0^2(\delta_\perp - \delta_\parallel)^2 \frac{2\tau_R}{1 + \omega^2\tau_R^2} \qquad [1]$$

and for the spin–spin relaxation (T_2) we similarly find (*11*)

$$1/T_2 = (1/90)\gamma^2 H_0^2(\delta_\perp - \delta_\parallel)^2 \left[\frac{6\tau_R}{1 + \omega^2\tau_R^2} + 8\tau_R\right], \qquad [2]$$

where γ is the gyromagnetic ratio of the ^{57}Fe nucleus, H_0 is the magnetic field strength, $(\delta_\perp - \delta_\parallel)$ is the anisotropy of the chemical-shift tensor (assumed to be axially symmetric with $\delta_\perp = \delta_{xx}$, δ_{yy}, the in-plane element, and $\delta_\parallel = \delta_{zz}$, the element perpendicular to the heme plane), ω is the Larmor frequency ($=\gamma H_0$), and τ_R is the rotational correlation time of the protein (or more specifically, the correlation time of the ^{57}Fe nucleus, assumed to be equal to that of the protein).

We can immediately estimate $\Delta\delta = |\delta_\perp - \delta_\parallel|$ by using the observed T_1 values and a $\tau_R = 20$ ns, as we did previously for MbCO (*3*), in which case we find the $\Delta\delta$ values listed in Table 1. For each of the alkyl isocyanide myoglobins, $\Delta\delta \sim 1250$ ppm. Typical graphical solutions to Eq. [1] are shown in Fig. 2A.

Unfortunately, we were not able to perform an independent check on τ_R by using the observed linewidths, since clearly there seem to be rather substantial differences

in observed widths, even though pH, temperature, concentration, and T_1 values are all about the same. Use of the observed widths gives unphysical τ_R values, and so these widths arise, we believe, primarily from a large distribution of conformational substates in the alkyl isocyanide myoglobins. This notion has some support: for example, (1) Johnson *et al.* (*12*) suggest a "dynamic disorder" for the proximal His E7 in EtNC Mb, (2) Gilman has reported restricted motion (albeit fast) in EtNC Mb (*13*), and (3) Mims *et al.* (*14*) have suggested the possibility of multiple, slowly interconverting conformations of the bound ligand, in *n*-BuNC Mb.

If we use the observed T_1 and the linewidth for the narrowest ^{57}Fe resonance (*n*-BuNC Mb), we can place an upper bound on $\Delta\delta$ of $\sim$1700 ppm, but this yields the physically unreasonable τ_R $\sim$53 ns. We thus conclude that the intrinsic linewidths are $\approx$8 Hz and thus $\Delta\delta$ $\sim$1250 ppm, for each of the alkyl isocyanides, as estimated from the observed T_1 values and a τ_R of 20 ns. The linewidth difference for *n*-BuNC Mb can, however, readily be accounted for by a 2.5 ppm rather than a 1 ppm broadening, due to magnetic field inhomogeneity and temperature gradients in our large volume static probe.

In any case, our results clearly indicate that T_1 values increase by about a factor of $\sim$8.3 on replacement of CO by RNC, so $\Delta\delta$ changes by $\sim\sqrt{8.3}$; that is, it decreases from 3600 ppm to $\sim 3600 \times 8.3^{1/2} = 1250$ ppm (Eq. [1]), and in addition δ_i becomes deshielded by $\sim$1000 ppm. We now consider the possible reasons for such changes.

In a recent communication, Baltzer (*5*) reported a ^{57}Fe NMR study of ferrocytochrome *c*, enriched with ^{57}Fe. He found that the ^{57}Fe isotropic chemical shift was 11,197 ppm downfield from $Fe(CO)_5$, some 3000 ppm downfield from that of MbCO. Using corrected linewidth measurements and published τ_R values for a dilute cytochrome *c* solution, he determined that $\Delta\delta = 7630$ ppm, a much larger CSA value than those found for any other iron compound so far. He also calculated $T_1 = 4$ ms (Table 1), and although he did not actually determine T_1, he noted that pulse repetition times of 5 ms could be used, implying that T_1 must indeed be very short.

For such a $|\Delta\delta|$, there are two possible $\delta_\perp$ and $\delta_\parallel$ values: either $\delta_\perp = 13{,}739$ and $\delta_\parallel = 6112$ ppm or $\delta_\perp = 8655$ and $\delta_\parallel = 16{,}282$ ppm. On the basis of δ_i values for high symmetry porphyrin complexes in the vicinity of 7300 ppm (*15*) Baltzer argued that $\delta_\perp = 8655$ and $\sigma_\parallel = 16{,}282$ is the correct solution for ferrocytochrome *c* and then applied similar arguments to MbCO ($\delta_i = 8227$ ppm, $\Delta\delta = 3600$ ppm) to suggest that the solution $\delta_\perp = 9427$, $\delta_\parallel = 5827$ ppm was correct, rather than the alternative, $\delta_\perp = 7027$, $\delta_\parallel = 10{,}627$ ppm. While the counterargument that $\delta_\perp = 7027$ is actually closer to the "symmetric" case of $\delta \cong 7300$ ppm could be made, Baltzer put forward the hypothesis that "between the several experimental errors involved, an estimate of $\sigma_\perp$ ($\delta_\perp$) for the porphyrins is roughly −9000 ppm (9000 ppm)," where we have added in parentheses the equivalent chemical-shift tensor values using our convention.

We believe that our observation of very long T_1 values for each of the alkyl isocyanide myoglobins fits very well into the picture sketched above and we show in Table 1 the possible shift tensor elements that can be deduced from our δ_i and $\Delta\delta$ values. Consistent with Baltzer's "prediction," we find that one possible set of solutions has $\delta_\perp = 8794$, 8837, and 8836 ppm, close to the "roughly (−)9000 ppm" value suggested (*5*), the mean value of all systems investigated so far, using the solutions given in Table 1, being $\sim$8900 ppm. While we do not wish to definitely assign the tensor elements for

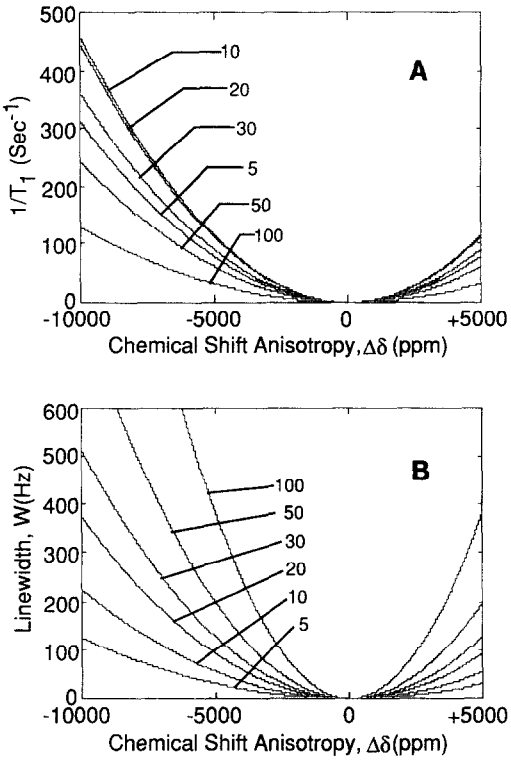

FIG. 2. Computed $1/T_1$ (A) and linewidth (B) values for ^{57}Fe NMR at 8.45 T, as a function of chemical-shift anisotropy ($\Delta\delta$) and correlation time (τ_R), for relaxation via the chemical-shift anisotropy mechanism. The τ_R values used are shown on the graphs, in nanoseconds.

the isocyanides at this time (since the alternative solutions do not alter our basic conclusions), we do believe that it seems very probable, on the basis of our ^{57}NMR observations, that, as Baltzer suggested, there is indeed a sign reversal of ^{57}Fe chemical-shift anisotropy on going from MbCO to ferrocytochrome *c*.

This is shown in a graphical fashion in Figs. 2 and 3, where we show the T_1 and W values calculated for a range of τ_R and $\Delta\delta$ (Fig. 2), together with proposed ^{57}Fe chemical-shift anisotropy powder patterns for ferrocytochrome *c*, RNC myoglobins, and MbCO. The MbCO and cytochrome *c* results are based on Baltzer's work (*5*) (and our $\Delta\delta$ results for MbCO (*3*)), while the RNC result is based on this work (and the alternative sign for $\Delta\delta$ does not affect our conclusions appreciably, since $\Delta\delta$ is always very small given that $\delta_\perp \approx \delta_\parallel$).

The significance of these results on the alkyl isocyanides is that, in our view, they can *only* be explained by a very small chemical-shift anisotropy. This in turn argues for the sign change in $\Delta\delta$ upon going from MbCO to cytochrome *c* put forward by Baltzer, using the argument that $\delta_\perp$ is primarily governed by shielding in the heme plane, which would not be expected to change significantly due to minor vinyl cross-linkage in cytochrome *c*. There is thus not only a large deshielding of the isotropic chemical shift upon going from CO and RNC to RSR′ (methionine) axial ligation

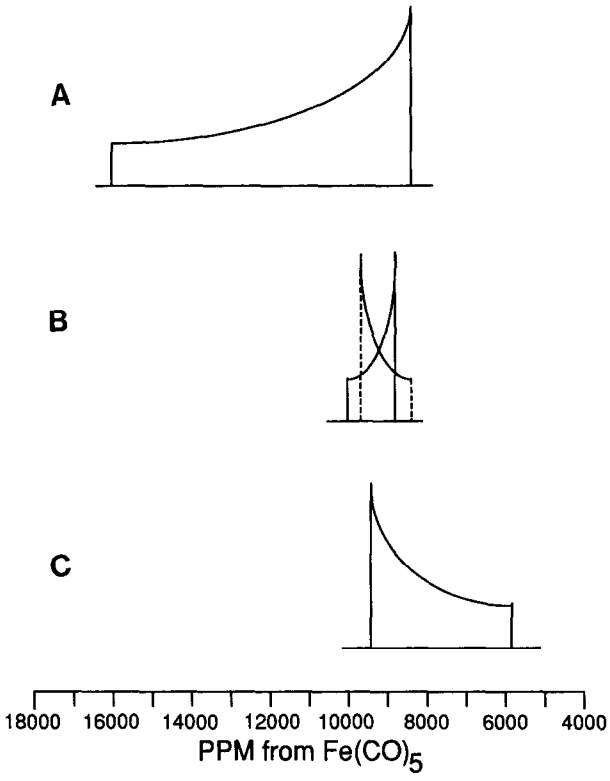

FIG. 3. Suggested chemical-shift tensors expected for (A) ferrocytochrome *c*, (B) RNC Mb, and (C) MbCO, on the basis of this work and Ref. (*5*). The sign of the CSA for RNC Mb could be reversed (dotted line). The large deshielding of $\delta_{\parallel}$ on moving from CO → RNC → RSR′ is thought to be responsible for the long T_1 values for the alkyl isocyanide myoglobin, where $\delta_{\perp} \approx \delta_{\parallel}$.

(the second axial ligand in each system is histidine) but also an even larger change in $\delta_{\parallel}$, from ~5827 to ≈10,073 (8405) to ~16,282 ppm, upon transition from CO to RNC to RSR′. These observed shift changes may not be unreasonable based on the π-acceptor behavior of the sixth ligands, which by virtue of increasing the ligand field splitting Δ for the $^1T_{1g} \leftarrow {}^1A_{1g}$ metal *d–d* transition (from RSR′, to RNC and CO) will decrease the paramagnetic shift, as observed experimentally (*15, 16*).

A direct determination of the magnitude and sign of the chemical-shift anisotropy is, in principle, possible by use of solid-state "magic-angle" sample-spinning techniques. We have begun to explore this possibility by investigating the symmetric complexes $[^{57}Fe(bipy)_3]X_2$, X = Cl, Br, I. To date, there have been no MASS NMR studies of ^{57}Fe in diamagnetic solids, so studies of the feasibility of such experiments on proteins are required. We synthesized symmetric complexes which we thought would have very small chemical-shift anisotropies, and typical solid- and solution-state results are shown in Fig. 4. Isotropic chemical shifts and spin–lattice relaxation times are given in Table 2.

Figures 4A–4C show the 16.4 MHz (11.7 T) ^{57}Fe MASS NMR spectra of Cl, Br, and I salts, respectively, which are some 300–600 ppm deshielded from the aqueous

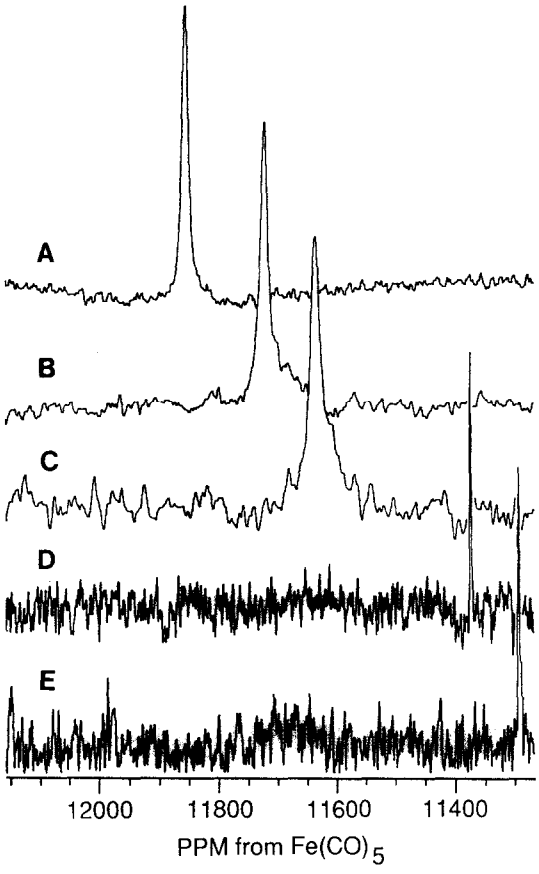

FIG. 4. Solution- and solid-state ^{57}Fe NMR of $[^{57}Fe(bipy)_3]X_2 \cdot \sim 5H_2O$, X = Cl, Br, I. (A) X = Cl, 2.4 kHz MASS at 11.7 T, 3600 scans, 2.5 s recycle time, 36 μs pulse excitation, and 50 Hz line broadening due to exponential multiplication. (B) X = Br, 2.5 kHz MASS at 11.7 T, 12,000 scans, 5.4 s recycle time, 41 μs pulse excitation, and 75 Hz line broadening due to exponential multiplication. (C) X = I, 2.5 kHz MASS at 11.7 T, 2000 scans, 2.6 s recycle time, 25 μs pulse excitation, and 100 Hz line broadening due to exponential multiplication. (D) X = Cl, in methanol, 8.45 T, 9200 scans, 3.6 s recycle time, 30 μs pulse excitation, and 10 Hz line broadening due to exponential multiplication. (E) X = Cl, in water, 8.45 T, 8560 scans, 3.6 s recycle time, 30 μs pulse excitation, and 10 Hz line broadening due to exponential multiplication.

(or methanolic) solution shift values, (Figs. 4D and 4E and Table 2). As expected, our results indicate that $\Delta\delta \approx 0$ in all these complexes, in the solid state, i.e., $\delta_\perp = \delta_{||}$, as expected on symmetry grounds. T_1 values determined on the solids vary from $\approx$1–5 s, (Table 2). This is surprisingly rapid and arises, we believe, from electron exchange with small amounts of d^5 iron impurity centers—as suggested previously for solution NMR of this system (*5*). A primary piece of evidence in support of this idea is that T_1 increased significantly from the Cl → Br → I complexes, and we attribute this to the sequential elimination of ferric salts during each ion-exchange chromatographic step, i.e., the final product $[^{57}Fe(bipy)_3]I_2$ had the least amount of paramagnetic impurities of any of the materials investigated. In each case, the linewidths observed are

TABLE 2

Iron-57 Isotropic Chemical Shifts and Spin–Lattice Relaxation Times for $[^{57}Fe(bipy)_3]X_2 \cdot nH_2O$, X = Cl, Br, I; $n \sim 5.0$

System	δ_i (ppm)[a]	T_1 (s)[b]	T_1 (s)[c]
$[^{57}Fe(bipy)_3]Cl_2$[d] $\cdot nH_2O$	11,854[e]	0.8 ± 0.1	1.23 ± 0.06
$[^{57}Fe(bipy)_3]Br_2$[d] $\cdot nH_2O$	11,721[e]	[f]	2.7 ± 0.2
$[^{57}Fe(bipy)_3]I_2$[d] $\cdot nH_2O$	11,635[e]	5.2 ± 0.2	4 ± 1
$[^{57}Fe(bipy)_3]X_2$[g]	11,291[h]	[f]	5 ± 2[i]
$[^{57}Fe(bipy)_3]X_2$[j]	11,380[h]	[f]	[f]

[a] Isotropic chemical shift, in ppm, downfield from $Fe(CO)_5$.
[b] Spin–lattice relaxation time, T_1, at 8.45 T.
[c] Spin–lattice relaxation time, T_1, at 11.7 T.
[d] Solid-state sample, $n \sim 5.0$.
[e] Error is ±10 ppm.
[f] Not measured.
[g] Aqueous solution of X = Cl, Br, or I species.
[h] Error is ±2 ppm.
[i] From Ref. (5)
[j] Methanolic solution of X = Cl, Br, or I species.

quite broad ($\approx$10 ppm), and presumably arise from a combination of ^{57}Fe–^{14}N and ^{57}Fe–^{1}H dipolar interactions (which are insignificant in solution). These linewidths, T_1 values, and signal-to-noise ratio results imply that ^{57}MASS NMR spectra of heme model systems may be feasible, but investigation of even the smallest proteins will be quite challenging.

In conclusion, our result of rather long spin-lattice relaxation times in the alkyl isocyanide myoglobins can only, we believe, be due to a very small chemical-shift anisotropy ($\delta_\perp \approx \delta_\parallel$), a result consistent with a change in sign of the ^{57}Fe chemical-shift anisotropy between MbCO, containing the strongly π-bonding CO ligand, and ferrocytochrome *c*, with the much weaker π-bonding mercaptide (methionine) axial ligand (*5*). Proof of such ideas must await independent solid-state NMR determination of $\Delta\delta$, which, on the basis of observation of $[^{57}Fe(bipy)_3]X_2$ (X = Cl, Br, I), may be feasible at least for heme model systems, so long as T_1 values are relatively short. In the absence of electron exchange, this may not be the case, although it is possible that cross-polarization with abundant ^{1}H spins, perhaps in paramagnetically doped systems, may be helpful. The recent observation of ^{113}Cd NMR in a Cd myoglobin by Kennedy and Ellis (*17*) suggests the feasibility of this approach. These authors also observed a sign reversal among a series of Cd-substituted hemes with axial ligation, although their results are not directly comparable with ours because of the differences in coordination number and the nature of the axial ligands involved.

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REFERENCES

1. I. MORISHIMA, T. HAYASHI, T. INUBUSHI, T. YONEZAWA, AND S. UEMURA, *J. Chem. Soc. Chem. Commun.*, 483 (1979).
2. G. N. LAMAR, C. M. DELLINGER, AND S. S. SANKAR, *Biochem. Biophys. Res. Commun.* **128,** 628 (1985).
3. H. C. LEE, J. K. GARD, T. L. BROWN, AND E. OLDFIELD, *J. Am. Chem. Soc.* **107,** 4087 (1985).
4. L. BALTZER, E. D. BECKER, R. G. TSCHUDIN, AND O. A. GANSOW, *J. Chem. Soc. Chem. Commun.*, 1040 (1985).
5. L. BALTZER, *J. Am. Chem. Soc.* **109,** 3479 (1987).
6. K. D. HAPNER, R. A. BRADSHAW, C. R. HARTZELL, AND F. R. N. GURD, *J. Biol. Chem.* **243,** 683 (1968).
7. T. M. ROTHGEB AND F. R. N. GURD, "Methods in Enzymology," (S. Fleisher and L. Packer, Eds.), Vol. 52, p. 473, Academic Press, New York, 1978.
8. H. L. JACKSON AND B. C. MCKUSICK, *Org. Synth.* **35,** 62 (1955).
9. F. BLAU, *Monatsh. Chem.* **19,** 647 (1898).
10. A. LEIN AND L. PAULING, *Biochemistry* **42,** 51 (1956).
11. T. C. FARRAR AND E. D. BECKER, "Pulse and Fourier Transform NMR," Academic Press, New York, 1971.
12. K. A. JOHNSON, J. S. OLSON, AND G. N. PHILLIPS, JR., *J. Mol. Biol.* **207,** 459 (1989).
13. J. G. GILMAN, *Biochemistry* **18,** 2273 (1979).
14. M. P. MIMS, J. S. OLSON, I. M. RUSSU, S. MIURA, T. E. CEDAEL, AND C. HO, *J. Biol. Chem.* **258,** 6125 (1983).
15. T. NOZAWA, M. SATO, N. KOBAYASHI, AND T. OSA, *Chem. Lett.*, 1289 (1983).
16. K. I. HAGEN, C. M. SCHWAB, J. O. EDWARDS, AND D. A. SWEIGART, *Inorg. Chem.* **25,** 978 (1986).
17. M. A. KENNEDY AND P. D. ELLIS, *J. Am. Chem. Soc.* **111,** 3195 (1989).