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# CARBON-13-IRON-57 SPIN COUPLING AS A NEW STRUCTURAL PROBE ON HEMOPROTEINS. CARBON-13 NMR SPECTRUM OF IRON-57-ENRICHED CARBONYL MYOGLOBIN*

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SUMMARY

Carbon-13 Fourier transform nmr has been used to make the first observation of a carbon-13-iron-57 spin-spin coupling constant in a protein, sperm whale carbonyl myoglobin enriched to 90% in both iron-57 and carbon-13. The coupling constant, 27.1±0.2 Hz, is found to be essentially identical to that of a model compound, supporting the view that the carbonyl is not tilted with respect to the heme plane in solution. Such carbon-13-iron-57 couplings, and the resultant iron-57 chemical shifts obtained from decoupling experiments, should provide valuable new tools for studying the different affinity states of tetrameric hemoglobins.

Elucidation of structure-function relationships in hemoproteins have relied heavily on solution nmr methods (1). These studies have focused on the protein structural changes accompanying variations in ligand affinity in hemoglobins (2) using proton hyperfine and ring current shifts, as well as some $^{13}C$, $^{15}N$, $^{19}F$ and $^{31}P$ spectra in native and modified proteins (1). However, current nmr probes provide little direct information on the iron-ligand bond or the iron itself, in spite of the central position of the heme iron in the control of $O_2$ and CO affinity.

The most direct probe of the metal in the physiologically important diamagnetic ligated states of hemoglobin, (2) Hb, and myoglobin, Mb, would be the iron nmr signal. Although the majority of the iron isotopes (97.8%) possess no magnetic moments, the rare $^{57}Fe$ isotope (2.2% abundant, $\mu_N$ = 0.09204) can serve as an ideal nmr probe since I = 1/2 (3). However, even though $^{57}Fe$-

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enriched hemoproteins have been used extensively in Mossbauer studies (4), we are unaware of any attempts to exploit their nmr potential. A possibly severe experimental obstacle to the direct FTNMR detection of $^{57}Fe$ is its very low frequency (3) (3.23 MHz in a 2.35 T field). However, indirect detection *via* spin coupling to more easily detectible nuclei may circumvent this problem. As a result of our initial efforts to assess the potential of $^{57}Fe$ nmr as a structural probe in hemoproteins, we report here on the observation *via* $^{13}C$ nmr of $^{13}C$-$^{57}Fe$ spin coupling in a model compound and in sperm whale carbonyl-myoglobin.

## METHODS

The model complex, mono-carbonyl,mono-N-methylimidazole protoporphinato-iron(II), was prepared according to the method of Barlow (5) using both normal hemin and hemin enriched 90% in $^{57}Fe$, and 90%-enriched $^{13}CO$. The complex was prepared ~2 mM in dimethylformamide.

Sperm whale apomyoglobin (Sigma) was prepared in the standard manner (6), and then reconstituted with hemin enriched to 90% with $^{57}Fe$. Samples ~5 mM in normal sperm whale myoglobin and that enriched in $^{57}Fe$ were prepared in 0.2 M NaCl $H_2O$, the iron reduced to the ferrous state with excess $Na_2S_2O_4$, and $^{13}CO$ (enriched to 90%) bubbled through the solution until ligation was complete. The solution pH was adjusted to 7.6.

Carbon-13 Fourier transform nmr spectra were obtained in 10 mm tubes on a JEOL PFT-100 spectrometer operating at 25.0 MHz. 10-40 K transients were collected using a ~30° pulse, 8 K points, a 5 KHz bandwidth and a pulse repetion rate of 0.8 $sec^{-1}$. The probe temperature was maintained at 25°C. Chemical shifts are given in parts per million, ppm, downfield from tetramethylsilane, TMS.

## RESULTS AND DISCUSSION

The $^{13}C$ FTNMR trace (5) of mono-carbonyl[$^{13}C$],mono-N-methylimidazole pro-protophinato-iron(II), (6) (enriched to 90% in $^{13}CO$) in dimethylformamide is illustrated in A of Figure 1. The strong downfield singlet at 205.5 ppm arises from the coordinated $^{13}CO$. When the iron is simultaneously enriched to 90% $^{57}Fe$, the trace in B of Figure 1 results, where the downfield singlet is replaced by a doublet centered at 205.5 ppm with $J(^{13}C-^{57}Fe) = 27.0 \pm 0.2$ Hz. There are only a few previous reports (7-10) on the detection of $^{13}C$-$^{57}Fe$ coupling constants, all in small inorganic complexes at natural abundance $^{57}Fe$, and all but one (9) involving the Fe-CO moeity; values of $J(^{13}C-^{57}Fe)$ for bound CO ranged from 23 to 28 Hz.

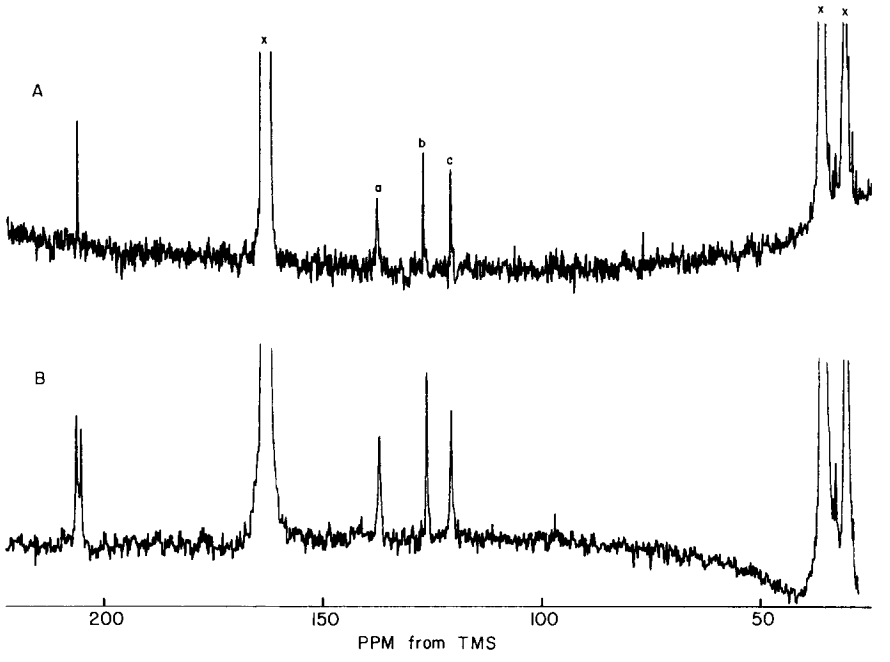


Figure 1: Carbon-13 FTNMR Spectra of Model Compounds.

Proton-decoupled $^{13}C$ FTNMR traces of A, 2 mM mono-carbonyl($^{13}C$),mono-N-methyl-imidazole protoporphinato-iron(III) in dimethylformamide, and B, 2 mM of the same complex but 90% enriched in iron-57, in dimethylformamide. Peaks a, b, c are from excess uncoordinated imidazole; x = solvent resonances. Probe temperature was maintained at 25°C and tetramethyl silane, TMS, was used as reference.

The $^{13}C$ FTNMR trace of sperm whale carbonyl-myoglobin, Mb($^{13}CO$) in $H_2O$ is illustrated in A of Figure 2. The sharp singlet at 207.2 ppm is due to the coordinated $^{13}CO$ (11). Reconstitution (6) of the protein with 90% $^{57}Fe$-enriched heme, i.e. Mb[$^{57}Fe$]($^{13}CO$), yields trace B in Figure 2. The $^{13}C$ signal at 207.2 ppm is a clear doublet with J($^{13}C$-$^{57}Fe$) = 27.1±0.2 Hz. The narrow $^{13}C$ signals for bound $^{13}CO$ in carbonyl-Hb (12) indicate that $^{13}C$-$^{57}Fe$ couplings should also be readily obtainable from the $^{57}Fe$-enriched tetrameric proteins.

The major potential of the method would be to monitor changes in Fe-CO

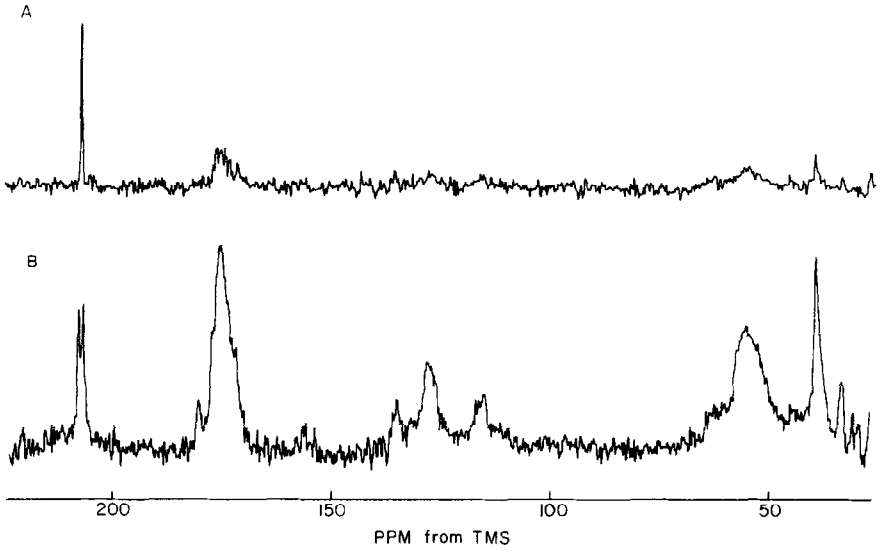


Figure 2: Carbon-13 FTNMR Spectra of Sperm Whale Carbonyl-Myoglobin.

The proton-decoupled $^{13}C$ FTNMR trace, A, of 10 mM sperm whale carbonyl[$^{13}C$]-myoglobin in 0.2 M NaCl $H_2O$ solution, and, B, of 5 mM sperm whale carbonyl[$^{13}C$]-myoglobin[$^{57}Fe$] in 0.2 M NaCl $H_2O$ solution. Chemical shifts are referenced to internal dioxane which is in turn referenced against TMS. Both solutions were at pH 7.6 and 25°C.

bonding (i.e., changes in $J(^{13}C\text{-}^{57}Fe)$) upon conversion between the T and R states (2) in a tetrameric hemoglobin such as Hb Kansas, which exhibits this allosteric transition solely within the ligated state (13). Similar efforts can also be directed towards characterizing the change in Fe-CO bonding in the monomeric insect hemoglobins which exhibit sizable Bohr effects (14). Decoupling experiments should be able to yield the $^{57}Fe$ chemical shift change, which may provide the most direct information on the correlation between electronic structure of the iron and its ligand affinity.

Another potential area of interest relates to the question of the "tilting" of the CO in relation to the heme plane, which has been suggested based on both x-ray (15) and neutron (16) diffraction data. This tilting was attributed to steric effects of the distal histidine. However, recent optical

studies of sperm whale MbCO single crystals have indicated (17) that, while the CO may be bent in the solid, it is "normal" in solution. Although there exists no simple model on which to base estimates of changes in $J(^{13}C-^{57}Fe)$ with degree of tilting (18), a significant change could certainly be expected for a tilt of ~130°. Our observation of essentially identical $J(^{13}C-^{57}Fe)$ ~27 Hz in model and protein tentatively lend support for the hypothesis that the CO is not tilted in solution.

Thus our preliminary results presented here demonstrate that the determination of $^{13}C-^{57}Fe$ spin coupling in carbonyl-ligated ferrous hemoproteins is practical, and that these couplings will provide not only a new probe for iron-carbonyl bonding, but may also lead to the indirect determination of $^{57}Fe$ chemical shifts in these hemoproteins.


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