# Nmr and Esr of Myoglobin Crystals Using Magnetic Ordering

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The structures of a variety of heme proteins have been deduced in the crystalline state by x-ray and neutron diffraction methods (Kendrew and Parrish, 1956; Kendrew et al., 1960; Perutz et al., 1960, 1968; Takano, 1977; Norrell et al., 1975) and in solution by nmr methods (Wuthrich, 1976; Wuthrich et al., 1970; Oldfield et al., 1975; Fung and Ho, 1975, 1977; Ho and Russu, 1978; Moore and Williams, 1980) during the past few years. However, it has until now been impossible to obtain structural information about protein crystals using nmr techniques, due to low spectral resolution in the solid state, or about solution conformations using diffraction methods. In this publication, however, we report on our progress with a new method for obtaining nmr spectra of individual sites in protein *crystals*, which permits direct extraction of static, and in principle dynamic, molecular structural information.

The difficulty of using nmr methods to obtain structural information about protein crystals is due to a variety of circumstances, including the following:

1. Lack of long-range coherence effects—no diffraction.
2. Tensor interactions in the solid contain structural information, but generally "obliterate" signals.
3. Long relaxation times in solids.

Examples of (2) include the chemical shift anisotropy, dipole-dipole and quadrupole interactions. These interactions give rise to individual resonances spread over many kilohertz making detection and resolution of nmr signals from different sites impossible. We know of no remedy for (1). However, (2) and (3) can be alleviated by carrying out *single-crystal* studies on a quadrupolar (in general rapidly relaxing) nucleus such as $^2H$. The problem is then reduced to that of growing ~ 1-g crystals!

Fortunately, we have found, however, that microcrystals of some paramagnetic heme proteins may be oriented or aligned along an external magnetic field, due to the large anisotropy in the magnetic susceptibility of the protein.

Our technique to obtain static structural information therefore involves determination of the electric quadrupole splitting ($\Delta\nu_Q$) or chemical shift ($\sigma$) of a specifically $^2H$ or $^{13}C$-labeled group in *magnetically ordered* paramagnetic protein microcrystals. From $\Delta\nu_Q$ (and in principle $\sigma$) it is possible to determine the orientation of the appropriate labeled group with respect to the applied dc magnetic field, $H_0$.

This work was supported in part by the U.S. National Institutes of Health (Grants HL-19461, CA-00595), and in part by the Alfred P. Sloan Foundation.

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 Chien Ho (ed.), Hemoglobin and Oxygen Binding

## Materials and Methods

Specifically $^2H$ and $^{13}C$ methionine methyl-group labeled sperm whale (*Physeter catodon*) myoglobin was prepared as described elsewhere by Jones et al. (1975, 1976) and nmr spectra at 3.5 and 8.5 Tesla recorded using two "home-built" nmr spectrometers.

## Results and Discussion

We show in Figure 1 a highly ordered array of crystals of the paramagnetic protein metmyoglobin. The crystals orient with the crystallographic $c^*$ axis parallel to the field directions, $H_0$. Such an ordered array permits a "single-crystal" nmr (or esr) spectrum to be recorded, albeit for only one crystal orientation.

In Figure 2 we illustrate the general resolution-enhancement obtained for $^2H$ nmr using the magnetic ordering method. In Figure 2A we show the $^2H$ nmr spectrum, obtained by the quadrupole-echo Fourier transform nmr method (Davis et al., 1976) at 8.5 Tesla (corresponding to a deuterium resonance frequency of 55 MHz), of cyanometmyoglobin labeled as $CD_3$ (50%) at Met-55 and Met-131. For a random powder distribution of methyl groups (or $C^{\delta}$-$S^{\epsilon}$ vectors), we obtain a broad "powder pattern" having, as expected (Oldfield et al., 1978) a breadth $\Delta\nu_Q$ of ~ 33 kHz. Such a width is typical of a rotating methyl group in a solid. Note that the two inequivalent sites are not resolved. The spectrum of Figure 2A may be attributed to two overlapping spin $I = 1$ $\eta \sim 0.15$ powder patterns having a quadrupole splitting ($\Delta\nu_Q$) of 33 kHz. We have not been able to obtain good spectral simulations of Figure 2A when using $\eta = 0$ asymmetry parameters, even when two overlapping absorptions having different $\Delta\nu_Q$ values are used. It thus appears that in the crystal the motions of the two methionine groups are similar and involve, in addition to fast methyl $C_3$ rotation, restricted torsional oscillations of the main $C^{\alpha}$-$C^{\gamma}$ chain resulting in nonzero asymmetry parameters. The narrow central component in Figure 2A arises from residual $HO^2H$.

By contrast to the "powder" spectrum of Figure 2A, two very narrow-line resonances having $\Delta\nu_Q = 13.6$, 38.1 kHz are obtained when the micro-

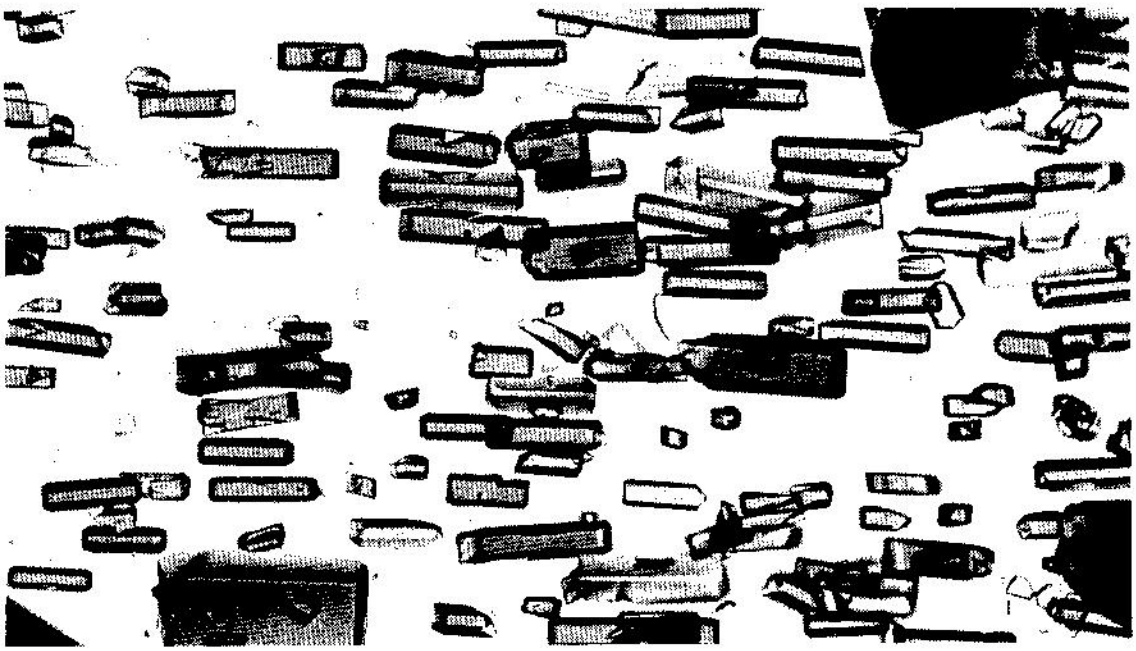


**Figure 1.** Photomicrograph [100× on a 5" x 4" Polaroid] of sperm whale (*Physeter catodon*) metmyoglobin suspended in ~90% saturated $(NH_4)_2SO_4$ at 23° after ordering 10 min in a dc magnetic field of ~3 kG. The long crystallographic axis is perpendicular to the field direction.

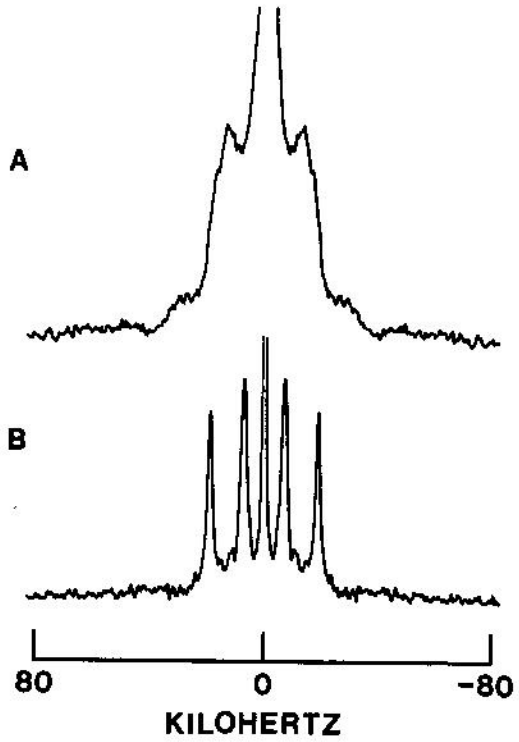
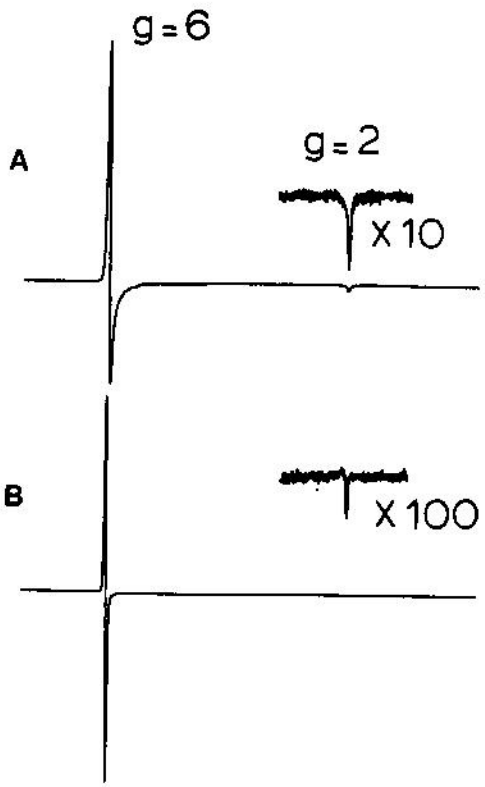



**Figure 2.** Deuterium Fourier transform nmr spectra obtained using the quadrupole echo pulse method at 55.3 MHz (corresponding to a magnetic field strength of about 8.5 Tesla) of sperm whale (*Physeter catodon*) cyanometferrimyoglobin microcrystals labeled as $CD_3$ at Met-55 and Met-131 at 21 ±2°, pH 6.4 (**A**) pelleted microcrystals (**B**) microcrystals suspended in saturated ammonium sulfate solution and magnetically ordered at 8.5T.

crystals of Figure 2A are resuspended in $^2H$-depleted saturated ammonium sulfate (pH 6.4) (Figure 2B). This spectrum originates from a *magnetically ordered* sample of protein microcrystals similar to that of Figure 1, and from the splittings ($\Delta\nu_Q$) we may deduce that one Met $S^\delta$-$C^\varepsilon$ vector is oriented at 32° to the field direction, and that the other Met $S^\delta$-$C^\varepsilon$ vector is oriented at 47 or 64°. The results of calculations (based on the x-ray structure—(Takano, 1977; Watson and Chance, 1966) and spin-label titration experiments (unpublished results) suggest that Met-131 is oriented at 32° and that the correct solution for Met-55 is $\theta = 64°$. Similar results obtained on $Mb \cdot H_2O$ directly give excellent agreement with x-ray data (Takano, 1977). These results represent the first nmr spectra of protein crystals in which individual sites may be resolved, and structural information obtained, and will open the way for numerous studies of protein crystal dynamics, using nmr spectroscopy.

We have also obtained well-resolved esr spectra using the magnetic-ordering method. Shown in Figure 3A is the 9.3 GHz continuous-wave esr spectrum (at 12°K) of a powder sample of specifically deuterated metmyoglobin, and in Figure 3B the same sample after being magnetically ordered at 0.9 Tesla for 15 min at 20°C, then cooled to 6°K. The spectrum of Figure 3A obviously corresponds to a normal high-spin

**Figure 3.** Electron spin resonance spectra of samples similar to those used in Figure 1. Spectra were recorded on a Varian E-4 instrument at 9.29 GHz. (**A**) powder sample of aquoferrimyoglobin microcrystals, 12°K, 1.25 G modulation amplitude, 1 mW power level (**B**) sample as in Figure 1B, except magnetically ordered at 0.9 Tesla at 20° then frozen. Sample temperature 6°K, 0.32 G modulation amplitude, 0.2 mW power level. The results of additional experiments (data not shown) indicate little saturation of these spectra.



ferric-iron powder pattern signal having $g_\perp = 5.95 \pm 0.05$ and $g_\parallel = 2.00 \pm 0.01$. It is assumed that the lowest doublet is $S_z = \pm 1/2$ with a zero-field splitting to the other spin doublets much greater than that of the microwave quanta (Bennett et al., 1957; Griffith, 1956). The spectrum of Figure 3A is essentially identical to that of Hori (1971). In contrast, the spectrum of Figure 3B shows a very symmetric derivative absorption centered at a $g$ value of 5.95, with only a very small component at a $g$ value of 2.00. The spectrum of Figure 3B corresponds therefore to ferrimyoglobin molecules oriented with their maximum $g$ value (~6) along the field direction, together with a very small contribution from nonoriented material (at a $g = 2$). This $g = 2.00$ component is even smaller in a sample oriented at 8.5 Tesla, then frozen for esr (data not shown).

The results presented in this communication open a new area for nmr of protein crystals, since the difficulties of preparing large single crystals are eliminated. Observation of "sharp" resonances in magnetically ordered samples (Figure 2B) permits rapid data acquisition due to increased signal-to-noise ratios, and naturally permits resolution of signals from numerous sites that would normally all overlap. Dynamic studies of individual resolved sites may now be carried out, and results compared with those obtained from crystallography. (Frauenfelder et al., 1979; Artymiuk et al., 1979). Magnetic resonance of ligands bound to protein crystals, e.g., $^{31}P$ nmr of 1,3-diphosphoglycerate bound to methemoglobin, may be investigated as may paramagnetic ion binding by esr. The structures of diamagnetic species may be determined using suitable redox state conversions in mixed crystals, e.g., by using mixed Fe(II)-Fe(III) systems. Nmr of "other metals" may perhaps be investigated by using one paramagnetic ion to cause protein ordering as in, e.g., the Cu(II)-$^{67}$Zn(II) protein, superoxide dismutase. In addition paramagnetic ions may be bound to otherwise diamagnetic proteins to enable their static and dynamic structures to be investigated. perfectly ordered by an intense (8.5 Tesla) magnetic field due to the large anisotropy in the magnetic susceptibility of the protein. The method permits determination of a "single-crystal" nmr spectrum without the difficulties associated with growing large protein single-crystals. Measurement of $^2H$ nmr quadrupole splittings in these systems permits accurate determination of protein structural parameters.

## Summary

Selectively deuterated microcrystals of the paramagnetic protein metmyoglobin suspended in ammonium sulfate mother liquor are essentially

This work was supported in part by the U.S. National Institutes of Health (Grants HL-19481 and CA-00595) and in part by the Alfred P. Sloan Foundation. One of us (E.O.) was an Alfred P. Sloan Research Fellow (1978–1980) and is a U.S. Public Health Service Research Career Development Awardee (1979–1984).

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