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USE OF THE NUCLEAR OVERHAUSER EFFECT TO ASSIGN ^{1}H NMR RESONANCES IN A LOW-SPIN PARAMAGNETIC HEMIN

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The nuclear Overhauser effect has been used to assign the ^{1}H resonances of the paramagnetic low-spin biscyano complex of Fe(III) protoporphyrin. When the meso protons are irradiated, changes in integrated signal intensity are seen at neighboring methyl or methylene groups and vice versa. Although the changes are small (< 1% negative NOEs for Fe(III)protoporphyrin$(CN)_2$ in Me_2SO-d_6 at 30°C and 360 MHz), they can be seen clearly. This technique has been used to assign the 6-α-CH_2 (6.21), 7-α-CH_2 (5.82), β-meso (0.50) and δ-meso (0.03 ppm) resonances of this species. The nuclear Overhauser effect will allow rapid assignment of ^{1}H NMR resonances in a wide variety of low-spin paramagnetic hemins.

Iron porphyrins are the active site of a variety of proteins including cytochromes, globins, peroxidases and catalases. This has led to substantial interest in the structure and reactivity of these iron porphryins. NMR has proved the most useful tool for heme characterization (1,2). Historically, heme resonances in the ^{1}H NMR spectra have been assigned by selective deuteration of specific resonances (3). This technique is unambiguous, but synthesis of specifically deuterated porphyrins is very time consuming. More recently, paramagnetic line broadening reagents, notably Mn^{2+} and Gd^{3+}, have been used to assign resonances (4). The protons nearest the paramagnetic center relax most quickly: the effect is proportional to r^{-6}. This allows rapid assignment of some of the resonances. However, in general not all of the resonances can be assigned by this method. In addition, there must be a site at which the Mn^{2+} or Gd^{3+} can

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Abbreviations: Fe(III)PPIX, ferric protoporphyrin IX; NOE, nuclear Overhauser enhancement.

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bind. To date this has involved either one (Gd^{3+}) (4d) or two (Gd^{3+} or Mn^{2+}) (4a-c) propionic acid moieties on the heme.

Although nuclear Overhauser enhancements (5-7) have been used very successfully in the assignment of resonances in diamagnetic porphyrins and metalloporphyrins (7,8), this technique has not been extended to the paramagnetic Fe(III) porphyrins. It generally appears to have been considered that a nuclear Overhauser enhancement will not be observed because the relaxation due to the paramagnetic iron is so efficient (9). However, there have been a few reports of successful NOE studies in low-spin paramagnetic heme proteins. These include studies of metcyanoleghemoglobin (10), metcyanomyoglobin (11), cytochrome b_5 (12) and cytochrome c (13).

Successful observation of the nuclear Overhauser effect in paramagnetic heme proteins does not imply success in the iron porphyrins because tumbing times and the paramagnetic contribution to the spin-lattice relaxation may be very different in the two species. We have, however, found that the nuclear Overhauser effect can be seen in low-spin paramagnetic iron porphyrins under certain conditions. In this communication we report that NOEs can be used to assign resonances in low-spin paramagnetic hemins.

MATERIALS AND METHODS

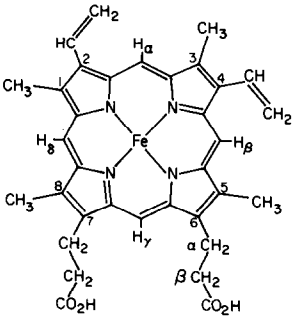
Ferriprotoporphyrin IX chloride (Fe(III)PPIXCl) was obtained from Midcentury Chemicals and used as received. Fe(III)PPIXCl (3.5 mg, 5.4 x 10^{-3} mmol) was dissolved in 0.500 mL Me_2SO-d_6 (99.5 atom %, MSD Isotope). Potassium cyanide (4 eq., 10 µL of a 2.2 M solution in D_2O) was added in the presence of oxygen (to prevent autoreduction).

Proton NMR spectra were recorded at 30°C (methanol thermometer) (14) using a 5 mm probe on a Bruker WH-360 spectrometer operating at 360.13 MHz. Since chemical shifts of paramagnetic hemins are very temperature dependent (9), the sample was equilibrated to probe temperature prior to spectral accumulation. Spectra had 16 K data points with a spectral width of 20000 Hz. Chemical shifts in parts per million (ppm) are referenced to the residual Me_2SO-d_5 signal assigned as 2.50 ppm. NOE difference spectra were obtained using the pulse sequence: [relaxation delay (1 sec) - irradiation (variable 0.195-0.995 sec) - ringdown delay (0.005 sec) - 90° pulse (5.0 µsec) - acquisition (0.413 sec)]. Continuous wave irradiation (Bruker HG mode, decoupling power $\gamma H/2\pi$ = 8.6 Hz) was applied alternately to the resonances of interest and a resonance-free area of the spectrum. Spectra were obtained by taking 12 scans (4 scans receiver off, 8 scans receiver on) at a series of desired irradiation frequencies followed by 12 scans (again 4 receiver off, 8 receiver on) at a control position in a blank area of the spectrum. This acquisition cycle was repeated to give a total of 200-1000 scans at each irradiation frequency. Data workup consisted of subtraction of the NOE and control free induction decays followed by multiplication of the resulting FID by a 20 Hz exponential and Fourier transformation.

RESULTS AND DISCUSSION

Figure 1 shows a spectrum of the low-spin biscyano complex of ferric protoporphyrin IX (4b). Figure 2 shows a series of experiments in which the meso protons were irradiated in turn. Irradiation of the α-meso proton shows an enhanced signal for the 3-Me group. Irradiation of the β-meso proton gives enhanced signals for the 5-Me and 4-α-CH=CH_2 protons. Irradiation of the γ-meso proton shows no enhancements in the methyl region, but does produce enhancements in the α-CH_2 groups of the propionic acid moieties (not shown). Finally, in this run irradiation of the δ-meso proton shows enhancements for all four methyl groups, with the 8-Me and 1-Me signals largest. In other runs only the 8-Me and 1-Me enhancements were seen. The out-of-phase appearance of the 5- and 3-Me groups in this run indicate that these signals are largely subtraction artifacts (7).

When the methyl protons are irradiated, signal enhancements are generally seen in the meso positions. The effect is not as large as that seen when the meso protons are irradiated. This is in part due simply to the relative intensities of the methyl and meso signals (3:1) but may be related to differences in relaxation as well. Pulse sequences have not been optimized, but it should be noted that NOEs from the meso to the methyl protons were most easily seen with shorter irradiation times (0.195 s) while those from the methyl to the meso protons were most easily seen with longer irradiation times (0.995 s). Irradiation of the 5-Me group also resulted in an NOE enhancement of the resonance at 6.2



Fe(III) protoporphyrin IX

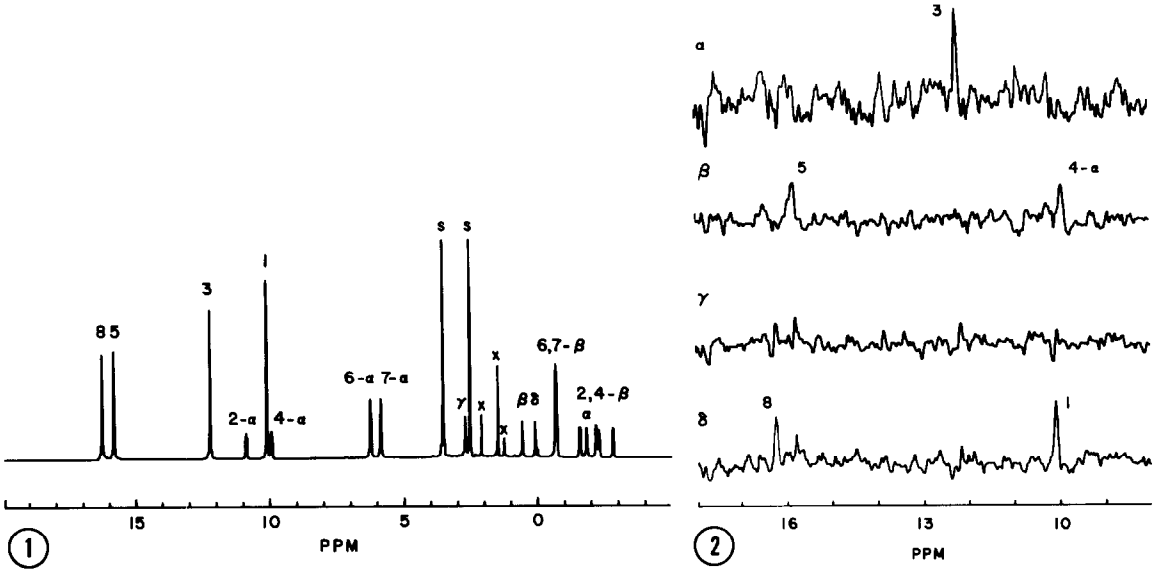


Figure 1. ^1H NMR of Fe(III)PPIX$(CN)_2$ in Me_2SO-d_6 at 30 °C. Peak assignments are indicated on the spectrum. s indicates solvent resonances; x indicates impurities.

Figure 2. NOE difference spectra of Fe(III)PPIX$(CN)_2$ in Me_2SO-d_6 at 30 °C after preirradiation of the α, β, γ, and δ meso proton resonances. Assignments of the enhanced resonances are indicated on the spectra. The irradiation time was 0.195 sec; 200 scans each.

ppm, indicating that this is the α-CH_2 of the propionic acid side chain at position 6 on the ring.

Assignment of the α-CH_2 groups of the propionic acid side chains was confirmed by irradiation of the α-CH_2 resonances and observation of the methyl resonances. As seen in Figure 3, irradiation of the downfield α-CH_2 resonance resulted in enhancement of the 5-Me resonance, again establishing the former as the α-CH_2 of the propionic acid at position 6 on the ring. Irradiation of the upfield α-CH_2 resonance resulted in an enhancement of the 8-Me resonance, as expected. To our knowledge, this is the first time that the β- and δ-meso protons and 6- and 7-α-CH_2 protons of the propionic acid side chains have been assigned.

The nuclear Overhauser effect depends upon proton-proton dipolar relaxation (5-7). Although the dominant relaxation pathway in these low-spin paramagnetic complexes is via interaction with the metal center, there is still a proton dipolar component. Spin-lattice (T_1) relaxation times for the methyl groups of

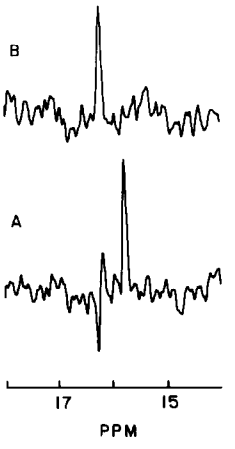


Figure 3. NOE difference spectra of Fe(III)PPIX$(CN)_2$ in Me_2SO-d_6 at 30 °C after preirradiation of 6-α-CH_2 (A) and 7-α-CH_2 (B) resonances. The irradiation time was 0.195 sec; 1000 scans each. Spectrum A shows an enhancement of the 5-Me peak and a subtraction artifact (out-of-phase) at the position of the 8-Me peak. Spectrum B shows an enhancement of the 8-Me peak.

the low-spin paramagnetic Fe(III)PPIX$(CN)_2$ in methanol are 0.15-0.2 s (15); those for diamagnetic metalloporphyrins in low viscosity solvents are 0.5-0.6 s (8a,c). Thus, both diamagnetic and paramagnetic relaxation pathways contribute to relaxation in this sytem and NOEs can be seen. To the extent that the relaxation is metal-related however, the NOEs are smaller than they would be in the corresponding diamagnetic system. The NOEs observed in these experiments were < 1%. The NOEs may also be small because the molecule has a rate of reorientation of the internuclear proton-proton vector ($1/\tau_c$) similar to that of the Larmor frequency (ω) of the protons. NOEs in this study were negative, indicating that $\omega\tau_c > 1$ (6).

Finally, the NOE experiment allows overdetermination of the resonance assignments. Enhancements can be seen both from single proton resonances (meso) to two- and three-proton resonances (CH_2 and CH_3 groups) and vice versa. This allows assignment of most of the resonances from two experiments rather than only one. Nuclear Overhauser effect studies allow rapid and unambiguous assignment of ^1H NMR resonances in low-spin paramagnetic hemes. This technique should find widespread application in the characterization of these species.

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