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Supplementary Material Available: Physical properties, NMR (^1H and ^{13}C) and IR data, and analyses of all new compounds described (5 pages). Ordering information is given on any current masthead page.

A Method of Assigning Functionally Relevant Amino Acid Residue Resonances in Paramagnetic Hemoproteins Using Proton NOE Measurements

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Solution ^1H NMR spectroscopy has contributed significantly to our understanding of structure–function relationships in hemoproteins.[1-3] A major emphasis in this research area has been placed on paramagnetic forms because of the improved spectral resolution for the hyperfine-shifted resonances in the heme cavity.[4,5] The interpretation of spectral parameters, however, depends critically on unambiguous assignment of relevant resonances.[6] For b-type hemoproteins (Mb, Hb) the assignment of heme (A in Figure 1) resonances has been largely accomplished on the basis of systematic deuteration studies.[6-8] Such methods are not applicable to amino acid residues in the heme pocket, and consequently resonance of few of the functionally relevant residues have been assigned. Two such unassigned residues are the distal His E7 and FG5 (Ile-99 in Mb, Val-98 in Hb). The former sterically interacts with the ligand binding site,[9-12] while the latter has been implicated in the intrasubunit interaction in Hb.[13] While nuclear Overhauser effect, NOE, measurements provide one of the most powerful tools for assignments in diamagnetic proteins,[14-16] such applications to paramagnetic proteins have been rare,[17-20] probably because it is considered likely that paramagnetic leakage will render NOEs too small to observe. Another important reason for such assignments is that the observed dipolar shifts, together with the protein structure as determined from both X-ray crystallography and the NOE's themselves, will yield the elusive magnetic coordinate system for the magnetic anisotropic heme iron.[1,21]

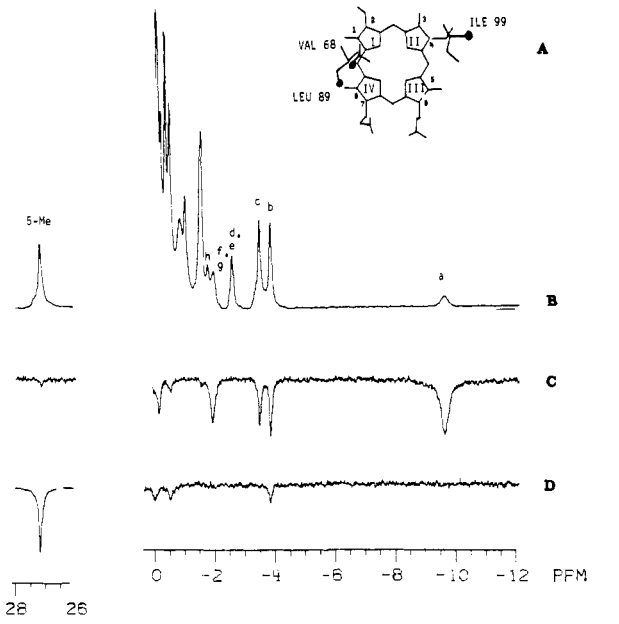

Figure 1. (A) The protohemin structure, with the non-hydrogen atoms of the Ile-99, Leu-89, and Val-68 amino acid side chains in sperm whale myoglobin. The dark circles are the α-carbon positions. Leu-86 and Ile-99 residues are on the proximal side of the heme, the Val-68 residue on the distal side. (B) The upfield and 5-methyl regions of the 360-MHz proton NMR spectrum of 3 mM sperm whale metcyanomyoglobin in D_2O, with 0.2 M NaCl, at 25 °C, pH 8.5 (uncorrected for isotope effect). All spectra shown were taken on a Nicolet 360-MHz spectrometer, using 8K data points over a ±10000-Hz bandwidth, with delays between scans of 0.5 s. (C) NOE difference spectrum resulting from saturation of peak a, with sample as in B, spectrum intensity ×5 that of B. For a given difference spectrum, two spectra were obatined in an interleaved fashion: the first spectrum with the decoupler on the peak of interest, the second with the decoupler offset by 1500 Hz to provide a reference spectrum. (D) NOE difference spectrum resulting from saturation of the 5-methyl with sample as in B. Spectrum intensity of the region 28–26 ppm is ×1, the region 0 to −12 ppm is ×5.

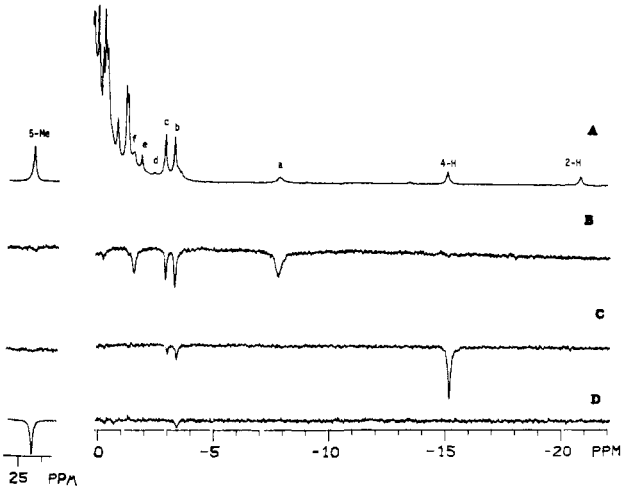

Figure 2. (A) Upfield of 5-methyl region of the 360-MHz spectrum of 3 mM sperm whale metcyanodeuteromyoglobin, 0.2 M NaCl, 25 °C, pH 8.5. (B) NOE difference spectrum resulting from saturation of peak a, spectrum intensity ×5. (C) NOE difference spectrum resulting from saturation of the heme 4-H resonance, spectrum intensity ×5. (D) NOE difference spectrum resulting from saturation of the 5-methyl resonance; spectrum intensity of the region 26–24 ppm ×1, for 0 to −12 ppm region, ×5.

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We report here on ^1H NMR experiments on low-spin ferric metMbCN, which demonstrate not only the presence of numerous large NOEs but permit the assignment for the first time of the Ile-99 (-FG5) side chain. metMbCN is a suitable model for MbCO and has the advantage of large magnetic anisotropy whose resulting dipolar field influences both coordinated and noncoordinated residues in the heme pocket.[22,23] The highly resolved upfield portion of the 360-MHz ^1H NMR spectrum of sperm whale metMbCN is illustrated in B of Figure 1. The NOE difference spectrum resulting from saturating peak a is shown in C. Clearly, proton peak f[24] and methyl resonances b and c, as well as some unresolved peak(s) near 0 ppm, exhibit substantial NOEs, indicative of a covalently localized network involving at least two methyls and two single protons.[24] Both Val-E11 and Ile-FG5, as well as Leu-F4, possess such functional groups; the significant line broadening of a,f and b,c eliminates other residues more distant from the iron. The positions of the possible residues are indicated on the projection onto the heme plane in Figure 1A. Saturating individual assigned heme resonances yields zero difference spectra for peaks a, b, c, and f, except for 5-CH_3, which yields a small NOE for peak b (D in Figure 2). Only Ile-99 (-FG5) lies in the proximity of 5-CH_3 but is more directly over pyrrole II with 3-CH_3 and 4-vinyl groups, whose resonances, unfortunately, are unresolved in the crowded diamagnetic region.

Clear identification, however, can be effected by taking advantage of the substitution of deuterohemin (R = H) for hemin (R = vinyl), which yields essentially the same ^1H NMR spectrum whose methyl assignments have established the same heme orientation as the native protein.[5,25] While 3-CH_3 is still obscured in the diamagnetic envelope, the 4-H signal now appears at -15 ppm (A in Figure 2).[25] Saturation of deuterohemin-metMbCN peaks a (B in Figure 2) and 5-CH_3 (D in Figure 2) show the same NOE connectivity as in native metMbCN. However, upon saturation of the deuterohemin-metMbCN peak 4-H (C in Figure 2), significant NOE's are observed for both methyl peaks b and c. Thus the resonances a, b, c, and f in native metMbCN can be definitely assigned to Ile-99 (-FG5).

Our results demonstrate that substantial NOEs are observable even among hyperfine-shifted resonance in paramagnetic hemoproteins and open up the possibility of assigning numerous other important residues in such systems. The unambiguous assignment of Ile-99 (-FG5) in metMbCN now permits investigation of the iron-induced dipolar relaxation, the magnetic coordinate system responsible for the Ile-99 dipolar shifts,[21,22] and the time dependence of the NOEs from which solution spatial orientations and internal mobility of the side chain may be determined.[17,26] Extension of these methods to other side chains may provide insight into the side-chain dynamics considered crucial for the function of oxygen-binding hemoprotein.[27] Variable steric interaction between 4-vinyl and the analogous Val-FG5 have been proposed for the molecular mechanism of cooperativity in hemoglobin.[13] The similarity of the ^1H NMR spectrum of metHbCN[28] to that of metMbCN[4] suggests the present method could be extended to Hb once the relevant heme methyl assignments have been made. Such studies are in progress in this laboratory.

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Surface Titration of Silica-Alumina Monitored by Nitrogen-15 NMR with Cross Polarization and Magic-Angle Spinning[1]

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The characterization of acid sites on solid surfaces is a central problem in catalysis. The total concentration of all acid sites on silica-alumina can be determined by various nonspectroscopic techniques (e.g., standard titration), which are flawed in that they do not distinguish between Brønsted acid sites and Lewis acid sites.[2] Infrared spectroscopy of adsorbed bases such as pyridine is able to distinguish between adsorption at Brønsted sites and adsorption at Lewis sites, but the molar absorbtivities must be estimated.[2-5] In this communication we report an ^{15}N CP/MAS technique that yields independent concentration values for both Brønsted and Lewis acid sites. In addition, this technique is potentially capable of revealing information on the dynamics of molecules adsorbed at the various surface sites. ^{15}N NMR has recently emerged as a promising technique in surface studies.[6-9]

Samples were prepared from silica-alumina[10] that had been activated for 18 h at 160 °C. Following activation, each sample was cooled to room temperature and exposed to a known quantity of 99%-^{15}N-enriched pyridine vapor to give a loading level of 0.19 mmol pyridine/g silica-alumina. On the basis of a reasonable model,[6] this loading is equivalent to a surface coverage of 0.082 monolayer. Each sample was then exposed to a variable but known quantity of *n*-butylamine vapor. Quantitative adsorption was confirmed in all cases by manometer readings. All samples were handled in an inert atmosphere. ^{15}N CP/MAS spectra were obtained at 20.3 MHz on a modified Nicolet NT-200 spectrometer, using a home-built probe. Samples were spun at room temperature at ~2.2 kHz, using dry nitrogen (the boil-off gas from a liquid nitrogen Dewar).

Several samples with *n*-butylamine loading levels ranging from 0 to 11 equiv (relative to pyridine) were examined. Each equivalent of *n*-butylamine is equal to 0.19 mmol/g silica-alumina. The detailed character of the ^{15}N spectra obtained depends markedly on sample history and handling, and reproducibility is difficult. Furthermore, the presence of water in a sample dramatically alters the kinetics of pyridine exchange between surface sites and the relative proportions of sites occupied by pyridine. The ^{15}N CP/MAS spectra of a representative series of samples are shown in Figure 1, which also lists the concentrations of species that we believe to be present in these samples on the basis of our interpretations of the spectra. These interpretations are based on the assumption that *n*-butylamine displaces pyridine from the strongest surface acid sites, with pyridine then occupying the strongest sites not occupied by *n*-butylamine.

The ^{15}N chemical shift of pyridine is a sensitive indicator of chemical environment,[6,7] Natural-abundance *n*-butylamine was

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(10) 75% SiO_2, 25% Al_2O_3, surface area 485 m^2/g by BET.

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