*PROTON MAGNETIC RESONANCE STUDIES OF HUMAN CYANOMETHEMOGLOBIN**

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Human hemoglobin has a molecular weight of 64,500 and is composed of four subunits, two each of two types, α and β. Each subunit consists of an α- or β-polypeptide chain and one protoheme IX group. In the biologically active state, each heme group contains one iron (II) ion which binds molecular oxygen in its first coordination sphere. The entire hemoglobin molecule, $\alpha_2\beta_2$, thus contains four heme groups and binds four oxygen molecules in the fully oxygenated state. It has been found that the oxygen affinity is closely related to the structure of hemoglobins.[1] In the present paper we give a preliminary account of proton nuclear magnetic resonance (NMR) studies of human cyanomethemoglobin. From the NMR data, electron spin densities at various positions in the heme group are derived. We indicate how this kind of NMR experiment can give new information about some aspects of the relations between structure and function in hemoglobin.

In the NMR spectra of proteins, essentially all the proton resonances are observed in a narrow range which extends from the internal standard DSS (2,2-dimethyl-2-silapentane-5-sulfonate) downfield to *ca.* -10 ppm (parts per million relative to DSS). The larger the protein molecule, the more the resonances of the protons of the individual amino acid side chains overlap. Therefore most NMR studies are done with relatively small proteins with molecular weights less than 20,000. In paramagnetic heme proteins, local magnetic fields arising both from aromatic ring currents[2, 3] and from the unpaired electron spins have been shown to shift certain proton resonances out of the range between DSS and -10 ppm.[4–6] For these reasons the NMR spectrum of cyanomethemoglobin contains a considerable number of resolved resonances despite its high molecular weight.

Experimental.—Human hemoglobin solutions were prepared from the freshly drawn blood of one of us (K. W.) by the following method. The erythrocytes were separated from the plasma by centrifugation within 15 min after the addition of the anticoagulant (sodium oxalate), then washed four times with 1 vol of 1% NaCl. After hemolysis, the solution was subjected to 105,000 *g* centrifugation for $2^1/_2$ hr. The upper two thirds of the supernatant, free of any flocculus precipitate, was separated off and then further centrifuged. This procedure was repeated twice. The hemoglobin solution was dialyzed overnight at 4°C against 0.1 *M* phosphate buffer. Some hemoglobin solutions were also prepared by the widely used toluene method.[7] The NMR spectra of cyanomethemoglobin solutions prepared by these two different methods were found not to differ noticeably.

Methemoglobin was prepared by addition of a sixfold excess of $K_3Fe(CN)_6$ to the hemoglobin solution that was then dialyzed extensively against 0.1 *M* phosphate buffer. Methemoglobin was purified on the cation exchange resin Bio-Rex 70 (Bio-Rad) and concentrated by vacuum dialysis. Sodium phosphate buffer, pH 6.42, total ionic strength of 0.304, was used for the elution of methemoglobin.[8] The homogeneity of isolated hemoglobin fractions was checked by starch gel electrophoresis[9] with the discontinuous buffer system.[10] Methemoglobin A_1 was then converted to cyanomethemoglobin by the addition of a freshly prepared solution of KCN in phosphate buffer.

"Mixed state" hemoglobin, $\alpha_2(Fe^{2+}O_2)\beta_2(Fe^{3+})$, in which only the β-chains were oxidized to the ferric state, was isolated from the aged hemolysate by Bio-Rex 70 chromatography.[11] Phosphate buffer, pH 6.42, ionic strength of 0.259, was used for the elution. A concentrated solution of $\alpha_2(Fe^{2+}O_2)\beta_2$ $(Fe^{3+}CN^-)$ was then obtained by vacuum dialysis and addition of KCN.

To replace water with D_2O, the solutions were dialyzed at 4° against 0.1 M deuterated phosphate buffer pD 7.0 in a collodion-bag suction apparatus purchased from Schleicher and Schuell Company. The hemoglobin concentration was determined spectrophotometrically.

The proton NMR spectra were obtained on a Varian 220-Mc high-resolution spectrometer. A standard Varian variable-temperature control unit was used. The temperature in the sample zone was determined from the chemical shifts of the resonances of an ethylene glycol sample. In the temperature range 5–40°C, the regulation was better than $\pm 1°$ over the period of time required for any of the experiments described in this paper. Signal-to-noise ratios were improved in some experiments by employing a Varian C-1024 computer of average transients.

The hemoglobin concentration was varied from 3.5×10^{-4} M to 3.5×10^{-3} M, which corresponds to heme concentrations from 1.4×10^{-3} M to 1.4×10^{-2} M, respectively. No noticeable dependence of the NMR spectra on concentration has been observed. All the changes with temperature of the NMR spectra were found to be reversible. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal NMR standard. Chemical shifts are expressed in parts per million from DSS, where shifts to low field are assigned negative values.

Results and Discussion.—The proton NMR spectrum at 220 Mc of cyanomethemoglobin is shown in Figure 1. It contains the HDO resonance at −4.63 ppm with its first and second spinning sidebands, and the strongly overlapping resonances of the 3500 protons of the protein part of the hemoglobin molecule between 0 and −10 ppm. In addition a number of rather well-resolved lines are observed upfield from DSS between +2 and +5 ppm and at low fields between

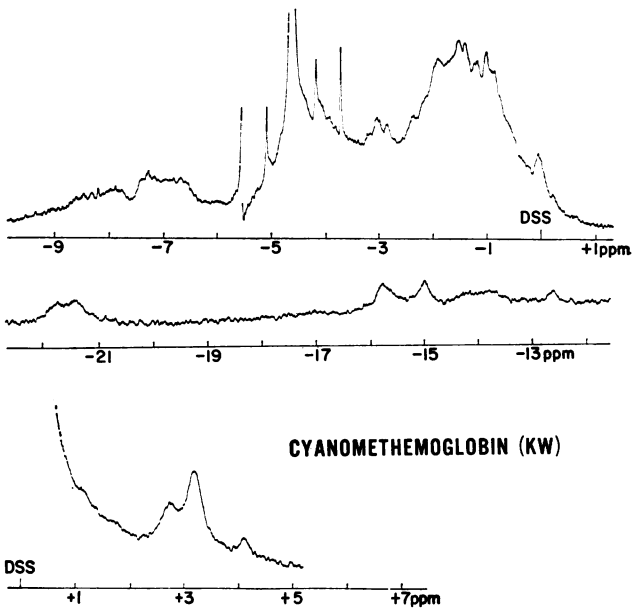

FIG. 1.—Proton NMR spectrum at 220 Mc of cyanomethemoglobin 1.5 × 10^{-3} M in deuterated phosphate buffer, pD 7.0, at 36°C. The five sharp lines between −3.5 and −6.0 ppm correspond to the resonance of HDO and its first and second spinning side bands. Different instrument settings were used to record the three portions of the spectrum.

−12 and −23 ppm. No other resonances were observed in the sweep range of our instrument, i.e., −40 ppm to +15 ppm.

The proton NMR spectra of the common amino acids have no resonances outside of the region 0 to −10 ppm.[12] However, interactions of aliphatic amino acid side chains with the ring current fields induced by the external magnetic field in aromatic residues and in the porphyrin ring may give rise to resonances upfield from DSS in the spectra of heme proteins.[2, 3] Furthermore, hyperfine interactions with the delocalized unpaired electrons of the paramagnetic heme iron may give rise to large upfield or downfield shifts of the resonances of groups of protons on the ligands bound directly to Fe^{3+}, i.e., protoporphyrin IX and histidine. Ring current shifts and shifts arising from hyperfine interactions with Fe^{3+} can be distinguished by their different temperature dependences.[6] Ring current shifts are independent of temperature unless there are conformational changes in the protein. On the other hand, hyperfine interactions are proportional to the reciprocal of temperature, as given for shifts $\Delta\nu_c$ arising from contact interactions by[13]

$$\Delta\nu_c = -A\frac{|\gamma_e|}{|\gamma_H|}S(S+1)\frac{\nu}{3kT}, \qquad (1)$$

where positive values of $\Delta\nu_c$ describe shifts to higher fields at fixed frequency, A is the contact interaction constant of the proton, γ_e and γ_H are the gyromagnetic ratios of the electron and of the proton, S is the total electronic spin, ν is the resonance frequency of the proton, k the Boltzmann constant, and T the absolute temperature. The temperature dependence of the NMR spectrum of cyanomethemoglobin (Fig. 2) then implies that the three resonances between +2 and +6 ppm and all the resonances between −10 and −25 ppm are shifted by interactions with the paramagnetic iron (III), while the resonance at 0 ppm appears to be shifted to its high-field position by ring current effects. The number of protons corresponding to the intensities of the individual resonances (Fig. 2) were obtained from comparisons of the relative intensities of the various resonances in the spectrum. As a standard it was assumed that the resonances observed between 0 and −10 ppm (Fig. 1) correspond to 3500 protons.

To understand the NMR spectra of hemoglobins, one must know if the four hemes give rise to one, two, or even three or four different sets of proton resonances. For this we compared the NMR spectra of cyanomethemoglobin, $\alpha_2(Fe^{3+}CN^-)\beta_2(Fe^{3+}CN^-)$, and the "mixed state" hemoglobin $\alpha_2(Fe^{2+}O_2)$-$\beta_2(Fe^{3+}CN^-)$. The latter contains the α-subunits in the diamagnetic low-spin ferrous state so that only proton resonances of the β-subunits can be shifted by hyperfine interactions with the iron. The two spectra contain the same number of resonances in the same high- and low-field positions, but for the mixed-state hemoglobin the intensities of these largely shifted lines relative to the resonances between 0 and −10 ppm are only half of those observed for cyanomethemoglobin. This experiment shows that in cyanomethemoglobin the NMR spectrum of the hemes in the two α-subunits is essentially identical to that of the two β-subunits. From the observation that the widths of the resonances in the two spectra are the same, we can set an upper limit of *ca.* 10 cps for possible small differ-

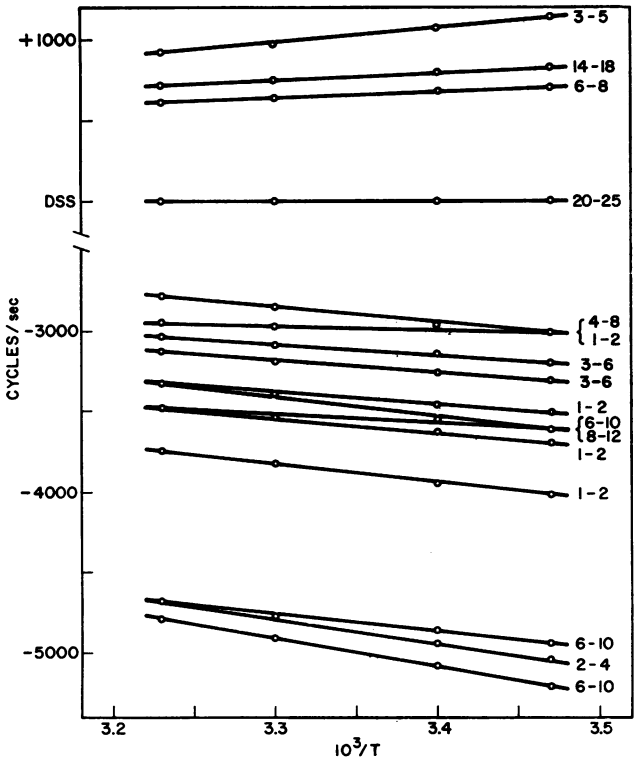

FIG. 2.—Dependence on the reciprocal of temperature of the positions relative to DSS of the proton resonances of cyanomethemoglobin in the regions DSS to +6 ppm, and −10 to −25 ppm. The number of protons estimated to correspond to the intensities of the individual resonances is indicated on the right-hand side.

ences in the positions of the resonances corresponding to the two α and β subunits, respectively. Since it then appears rather unlikely that differences should exist between the heme spectra of the two α- or β-subunits, we assume for the present interpretation that the NMR spectra of the hemes in the four subunits of cyanomethemoglobin are identical.

From the NMR spectra of cyanoprotoporphyrin IX iron (III)[14] and cyanometmyoglobin,[6, 15] we expect the resonances of 12 protons per heme to be shifted upfield by hyperfine interactions. These are the four meso protons, the two groups of two protons of the vinyl side chains, and the protons of two methylene groups of the two propionic acid side chains. Not all of these resonances are observed in the cyanomethemoglobin spectrum (Fig. 1.) From their intensities (Fig. 2), the lines at +2.8 and +4.1 ppm correspond most likely to two and one meso proton per heme, while the resonance at +3.2 ppm arises from four protons per heme of either the vinyl or propionic acid side chains. We hope to be able to distinguish between these two possible assignments by studying reconstituted hemoglobin, with protoheme IX replaced by deuteroheme IX as we have done for myoglobin.[16] The resonances of the five other protons per heme expected to be shifted upfield are probably not shifted far enough to be resolved. In the low-field range of the spectrum (Figs. 1 and 3), we expect from each of the four heme groups the resonances of the four ring methyls and the two single protons of the vinyl groups. In addition, two resonances corresponding to the 2,4-protons of

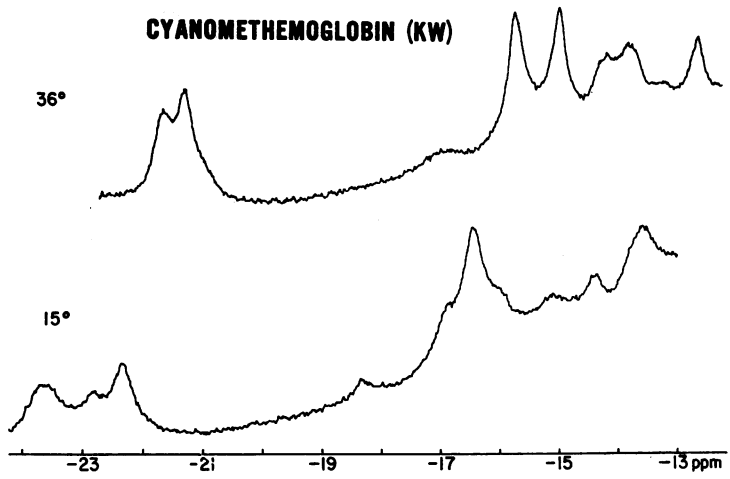

FIG. 3.—The range −12 to −24 ppm of the spectrum in Fig. 1 at 15° and 36° after signal averaging for 12 hr.

the histidine coordinated to Fe^{3+} of each heme might be resolved. In the cyanomethemoglobin spectrum we observe four resonances which could be attributed to one methyl group per heme, although their intensities appear rather small. The large ranges for the intensities given in Figure 2 correspond to uncertainties in the base line. At 36° these resonances are observed at −15.0, −15.6, −21.3, and −21.6 ppm (Fig. 3). In addition, at least four resonances of intensity *ca.* 1 proton per heme are observed (Figs. 2 and 3). Hence the low-field region of the cyanomethemoglobin spectrum agrees quite well with the spectrum predicted in analogy to those of cyanoprotoporphyrin IX iron (III) and cyanometmyoglobin, if we assume that the resonances of the four heme groups per molecule are identical. However, there appear to be some additional weak resonances corresponding in intensity to less than one proton per heme (Figs. 2 and 3) which we cannot assign at this time.

The temperature-independent high-field resonance at 0 ppm (Figs. 1 and 2) which comes from ring current shifts may prove to be useful as an indicator of conformational changes. The intensity of the resonance corresponds to six protons per subunit, which could be two methyl groups of a valine or a leucine side chain, or two different amino acid side chains. With the availability in the future of accurate coordinates of the structure of hemoglobin,[17] one might be able to assign the resonance at 0 ppm to specific amino acid side chains, and to decide if its upfield shift comes from interactions with the porphyrin ring or with aromatic amino acid side chains.[18] Since ring current shifts are very sensitive to changes in the relative positions of the aromatic ring and the protons which experience its local ring current field,[6] variations of the position of this resonance could then be related to conformational changes in specific parts of the hemoglobin molecule.

From the studies of heme proteins we can draw some qualitative conclusions about the line widths of the strongly overlapping resonances which occur in the NMR spectra of proteins in the range 0 to −10 ppm (Fig. 1). The width $\delta\nu$ of

the resolved lines observed in the NMR spectra of cyanometmyoglobin and cyanomethemoglobin arises from three types of interactions[19]

$$\delta\nu = \delta\nu_{H-H} + \delta\nu_d + \delta\nu_{sc} \quad (2)$$

where $\delta\nu_{H-H}$ is the line-broadening from dipolar coupling between protons modulated by random tumbling of the entire protein molecule or parts thereof, $\delta\nu_d$ arises from dipolar coupling between the unpaired electron of the iron and the protons, and $\delta\nu_{sc}$ from scalar coupling between electronic and nuclear spins. Because of the very short electronic relaxation time, $\delta\nu_d$ and $\delta\nu_{sc}$ are small compared to $\delta\nu_{H-H}$ in low-spin ferric heme proteins.[18] From the above comparison with "mixed state" hemoglobin, it appears unlikely that the lines in the cyanomethemoglobin spectrum are broadened by small differences in the positions of the resonances corresponding to the four individual heme groups in the molecule. Hence the observed half widths of the resonances at half height of *ca.* 15 cps in cyanometmyoglobin[6] and *ca.* 40 cps in cyanomethemoglobin are essentially entirely determined by $\delta\nu_{H-H}$. From the structures of hemoglobin[17] and myoglobin,[20] it appears that very few protons in the entire molecules could be more restricted in their intramolecular tumbling than the protons attached to the large rigid porphyrin rings. Hence 15 cps for myoglobin and 40 cps for hemoglobin appear to be good estimates for the upper limits of the half widths at half height of essentially all the strongly overlapping resonances between 0 and -10 ppm. It is interesting to note at this point that the increase of $\delta\nu_{H-H}$ by a factor of 3 when going from myoglobin to hemoglobin corresponds closely to the increase of the rotational correlation times from 2.9×10^{-8} sec to 8.5×10^{-8} sec, which were obtained from dielectric dispersion experiments with horse myoglobin and horse hemoglobin,[21] and fluorescence polarization with a modified myoglobin.[22] This implies that the effective correlation time for the transverse nuclear relaxation by proton-proton dipolar coupling is largely determined by the rotational tumbling of the entire molecules rather than by intramolecular motions of individual sections of the protein molecules.

At present it appears that the most interesting information from NMR experiments on the relations between structure and function in hemoglobins can be obtained from studies of the hyperfine interactions between protons and the paramagnetic heme iron. In the low-spin ferric hemes, pseudo-contact interactions[23] between the unpaired electron and the protons appear to be small compared to contact interactions.[14] We therefore assume for the present discussion that the hyperfine shifts, which are obtained as the differences between the positions of corresponding resonances for the paramagnetic compounds and for diamagnetic protoporphyrin iron (II),[24] come entirely from contact coupling.[23] The contact coupling constants A corresponding to the hyperfine shifts of the various protons of the heme group are then obtained from Equation (1). It has been shown that the contact coupling constants of aromatic protons[25] and of the protons of methyl groups attached to the aromatic ring[26] can be related to the unpaired electron density on the neighboring ring carbon atom by

$$A = Q\rho_C^{\pi} \quad (3)$$

where ρ_C^π is the integrated spin density centered on the π-orbital of the ring carbon next to the observed protons, and Q is a proportionality constant. Q has been found to be approximately constant for all aromatic protons, i.e., $Q \approx -6.3 \times 10^7$ cps. For methyl groups attached to aromatic rings, a value of $Q \approx 7.5 \times 10^7$ cps has been proposed,[26, 27] but rather large variations have been observed in different molecules.[28] However, as long as we consider the same molecule, cyanoprotoporphyrin IX iron (III), in different environments, it seems a good approximation to assume that Q is constant also for methyl protons. Spin densities at various positions of the heme group can thus be derived from NMR studies of hyperfine interactions. Table 1 gives a comparison of the spin densities on the ring carbons next to the four methyl groups in cyanoprotoporphyrin IX iron (III), cyanometmyoglobin,[6] and cyanomethemoglobin. It

TABLE 1. *Experimental NMR hyperfine shifts and contact coupling constants of the four ring methyls, and spin densities on the ring carbons next to the methyl groups in cyanoprotoporphyrin IX iron (III) in a mixed solvent of 80 per cent d_5-pyridine and 20 per cent D_2O,[14] and in the heme groups of sperm whale cyanometmyoglobin[6] and human cyanomethemoglobin.*

Cyanomethemoglobin			Cyanometmyoglobin			Cyanoprotoporphyrin IX Iron (III)		
$\Delta\nu^a$	$A(10^5$ cps$)^b$	$\rho_C^{\pi c}$	$\Delta\nu^a$	$A(10^5$ cps$)^b$	$\rho_C^{\pi c}$	$\Delta\nu^a$	$A(10^5$ cps$)^b$	$\rho_C^{\pi c}$
−4090	6.8	0.90	−5090	8.5	1.12	−2850	4.7	0.63
−3860	6.4	0.85	−3170	5.3	0.70	−2740	4.5	0.60
−2660	4.4	0.58	−1970	3.3	0.44	−1940	3.2	0.43
−2610	4.3	0.57	−1870	3.1	0.41	−1530	2.5	0.33

[a] $\Delta\nu$ is the difference in cps of the positions of corresponding resonances in the paramagnetic compounds and in diamagnetic protoporphyrin IX dimethylester iron (II)[24] at 25° C.

[b] Derived from a through Eq. (1), assuming that $\Delta\nu$ comes entirely from contact coupling.

[c] Derived from b through Eq. (3), with $Q = 7.5 \times 10^7$ cps. ρ_C^π in percentage of one unpaired electron.

is seen that the spin densities vary quite markedly. Further experiments are needed to decide if the observed changes come from variations of the ligands in the axial positions of Fe^{3+} or from other interactions of the heme groups with the polypeptide chains. Another interesting aspect for future work is to correlate the observed spin densities with the function of the heme proteins. It is well known that in the biologically active iron (II) state the three compounds in the table react quite differently with O_2. Since a large number of modified hemoglobins with different oxygen affinities have been reported,[1] it appears worth while to extend the NMR studies to some of these compounds. From this it might be possible to derive relations between oxygen affinities and spin distribution in the heme groups. NMR studies might also differentiate between possible mechanisms proposed to explain variations of the oxygen affinities of hemoglobins.

Summary.—The proton nuclear magnetic resonance (NMR) spectrum of human cyanomethemoglobin, $\alpha_2(Fe^{3+}CN^-)\beta_2(Fe^{3+}CN^-)$, has been measured at 220 Mc and at several temperatures between 15°C and 36°C. Despite the high molecular weight a considerable number of largely shifted, resolved resonances are observed. From their temperature dependence, these resonances are assigned to protons of the heme groups, and the shifts explained by hyperfine interactions with the paramagnetic iron (III). The mixed-state hemoglobin α_2-

$(Fe^{2+}O_2)\beta_2(Fe^{3+}CN^-)$ has the same shifted resonances; this shows that there is no difference between the resonances of the α- and β-subunits in cyanomethemoglobin. Electron densities at various positions of the heme group have been derived from the NMR data. Possible applications of NMR experiments to the study of relationships between structure and function in hemoglobins are discussed.

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