NUCLEAR MAGNETIC RESONANCES OF RECONSTITUTED MYOGLOBINS

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Abstract.—In proton nuclear magnetic resonance spectra of paramagnetic heme proteins, the resonances of the heme groups are shifted away from those of the many hundred protons of the polypeptide chains by interactions with the unpaired electrons and are therefore well resolved at 220 Mc. This paper describes experiments from which these resolved resonances are assigned to specific protons of the heme group in cyanoferrimyoglobin.

From a comparison with the proton nuclear magnetic resonance spectra of reconstituted cyanoferrimyoglobins and the corresponding cyanoporphyrin iron (III) complexes, the following groups of heme-protons have been assigned to specific resonances in the NMR spectrum of native cyanoferrimyoglobin: the four ring methyls, the four meso protons, the two —CH groups of the vinyls, and the four methylenes of the propionate side chains. Two resonances of intensity one proton have been assigned to the proximal histidyl residue. The only heme protons whose resonances were not observed are the $=CH_2$ groups of the vinyl groups which are probably buried in the bulk spectrum of the polypeptide chain. The present data indicate that the protein environment is more important in determining the distribution of unpaired electron density in the heme group than are the heme substituents.

Recently we have reported well-resolved proton nuclear magnetic resonances (NMR) at 220 MHz in cyanoferrimyoglobin[1] (MbCN) and cyanoferrihemoglobin[2] (HbCN). From their intensities and from a comparison with the NMR spectrum of cyanoprotoporphyrin IX iron (III)[3] (ProtoCN), it has been possible to show that these resonances come from the protons of the heme group. The temperature dependences of the shifts indicate that the resonances are shifted by hyperfine interactions with the unpaired electron. Spin densities in the carbon π orbitals of the porphyrin ring are obtained from the observed shifts, giving a point-by-point measure of the distribution of the unpaired electron in the porphyrin ring.

In this paper, we present a more detailed identification of these shifted resonances, which is obtained from a comparison of the NMR spectra of native MbCN and myoglobin reconstituted[4, 5] with cyanodeuteroporphyrin IX iron (III) (DeutMbCN) and cyanomesoporphyrin IX iron (III) (MesoMbCN) (Fig. 1). In addition to allowing an identification of some of the resonances, these NMR studies support previous investigations of myoglobin reconstituted from globin and protoheme in which it was indistinguishable from the native material.[4–6] Furthermore, the present experiments show that the protein environ-

ment is more important in determining the distribution of unpaired electron density than are the heme substituents.

Experimental.—Sperm-whale myoglobin was a commercial preparation obtained from Seravac Chemical Company (England) and repurified by fractional precipitation with ammonium sulfate before use. Globin was prepared from repurified myoglobin by using the acid procedure.[4] Coupling of native globin with proto, meso, or deutero hemes was performed in accordance with the procedure reported before.[5] The O_2 dissociation curve of the reconstituted myoglobins used in these experiments was measured and found to be indistinguishable from that reported in previous investigations.[6, 7] Approximately 5×10^{-3} *M* solutions of the reconstituted myoglobins in 0.1 *M* deuterated phosphate buffer pD 7.0 were used for the NMR experiments.

High-resolution NMR spectra were obtained on a Varian 220 Mc spectrometer. With a standard Varian variable temperature control unit, the temperature was varied between 5 and 40°C. The temperature in the sample zone was determined from the chemical shifts of ethylene glycol. The regulation is better than ±1°C over the interval required for the experiments described in this paper. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard. Chemical shifts are expressed in parts per million (ppm) from DDS, where shifts to lower fields are assigned negative values.

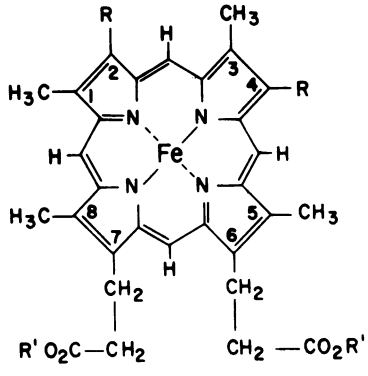

FIG. 1.—Heme groups of the compounds studied. Cyanoprotoporphyrin IX iron (III) (ProtoCN), R = —CH=CH_2; cyanodeuteroporphyrin IX iron (III) (DeutCN), R = —H; cyanomesoporphyrin IX iron (III) (MesoCN), R = —CH_2–CH_3.

Results and Discussion.—Previously, myoglobin reconstituted from protoheme IX and globin was shown to have identical physicochemical and functional properties as the native myoglobin.[6] The NMR spectra of native and reconstituted MbCN are compared in Table 1. The positions and intensities of all the observed resolved resonances are essentially identical. Since even minor changes of the conformation of the polypeptide chain near the heme were found to have marked effects on the NMR spectra,[8–10] this implies that the interactions between the heme group and the polypeptide chain are very similar in native and reconstituted MbCN.

When deuteroporphyrin IX is substituted for the naturally occurring protoporphyrin IX, the general features of the NMR spectrum remain the same, even though definite changes are observed. A comparison of the spectra of DeutMbCN and native MbCN (Fig. 2) allows certain definite assignments of proton resonances to be made. First consider the low-field region. The ring methyl resonances (*a*) are easily identified from their intensities and from a comparison[3] with the cyanoporphyrin iron (III) complexes (ProtoCN and DeutCN). Qualitatively they are very similar in MbCN and DeutMbCN. Two methyl resonances are closely grouped at −12 to −13 ppm, while the other methyl

TABLE 1. *Positions of the heme proton resonances in sperm whale cyanoferrimyoglobin in cycles per second from DSS at 25°C before and after reconstitution.*

Intensity	Native	Reconstituted
1 *b*	+2130	+2130
2 *c*	+ 845	+ 840
2 *c*	+ 760	+ 750
1 *b*	+ 560	+ 550
1 *b*	+ 420	+ 410
2 *d*	+ 350	+ 340
2 *d*	+ 330	+ 340
1 *f*	−2350	−2390
1 *x*	−2510	−2540
1 } *a*	−2570	−2540
2 } *a*	−2760	−2750
3 *a*	−2860	−2850
1 *x*	−3770	−3790
1 *f*	−3920	−3950
3 *a*	−4060	−4030
3 *a*	−5980	−5960

The letters refer to the assignment of the resonances (Fig. 2). The number of protons corresponding to the intensity of the resonances is listed in the first column.

groups are observed at much lower fields. The two weak lines labeled *f* with the intensities of one proton at −17.8 ppm and −10.7 ppm in the MbCN spectrum are not observed for DeutMbCN. From their intensities and disappearance, we have identified these resonances as the —CH protons of the vinyl groups in MbCN (see Fig. 1). Further support for this assignment is obtained from a comparison with the NMR spectra of ProtoCN and DeutCN.[3] Two lines of intensity one proton are found in the low-field region of the former but not in that of the latter. Finally, the low-field regions of the spectra of MbCN and DeutMbCN contain two additional resonances with intensities of one proton (Fig. 2, *x*). From the known features of the ProtoCN and DeutCN spectra,[3] it

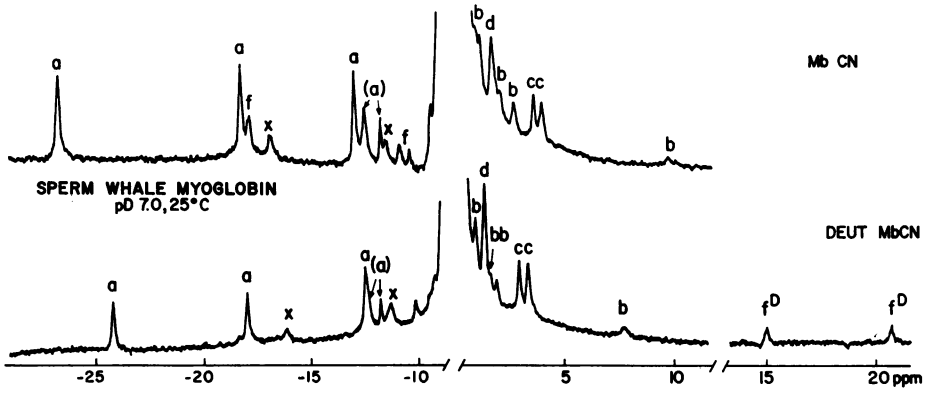

FIG. 2.—Comparison of the NMR spectra at 25°C and 220 MHz of native sperm whale cyanoferrimyoglobin (MbCN) and reconstituted cyanodeuteroferrimyoglobin (DeutMbCN). The assignments of the resonances to heme protons are: (*a*) ring methyls, (*b*) mesoprotons, (*c, d*) methylene protons of propionic acid groups, (*f*) —CH protons of the vinyl groups, (f^D) 2, 4-protons in DeutMbCN, (*x*) tentatively assigned to the imidazole protons of the proximal histidyl.

appears unlikely that these resonances correspond to protons of the heme group. On the other hand, unpaired electron density from Fe(III) should be delocalized not only into the prophyrin ring but also into the imidazole ring of the proximal histidine. Since the porphyrin and the proximal histidine are the only groups of protons coordinated to the iron in MbCN, it appears most likely that the resonances x correspond to the protons of the proximal imidazole.

Of the high-field shifted resonances of MbCN and DeutMbCN (Fig. 2), four lines (b) of intensity one proton, which correspond to the four meso protons, cover about the same range in both spectra. In particular, both have one of these resonances well separated to higher field. The four resonances (c) and (d), with intensities corresponding to two protons each, are observed in both MbCN and DeutMbCN and are assigned to the propionic acid side chains (Fig. 1). As in the spectra of ProtoCN and DeutCN, it has not been possible to decide which of the resonances c and d correspond to the methylene groups next to the porphyrin ring. In the DeutMbCN spectrum at 25°C, two resonances (f^D) of intensity one proton are observed at +15.0 and +20.9 ppm. From a comparison with the spectra of MbCN, ProtoCN, and DeutCN, these resonances are assigned to the 2,4-protons. Finally, it should be noticed that we have not been able to resolve the two $=CH_2$ groups of the vinyls which are presumably buried under the bulk spectrum of the globin.

The temperature dependence of the DeutMbCN resonances (Fig. 3) confirms that they are shifted by hyperfine interactions.

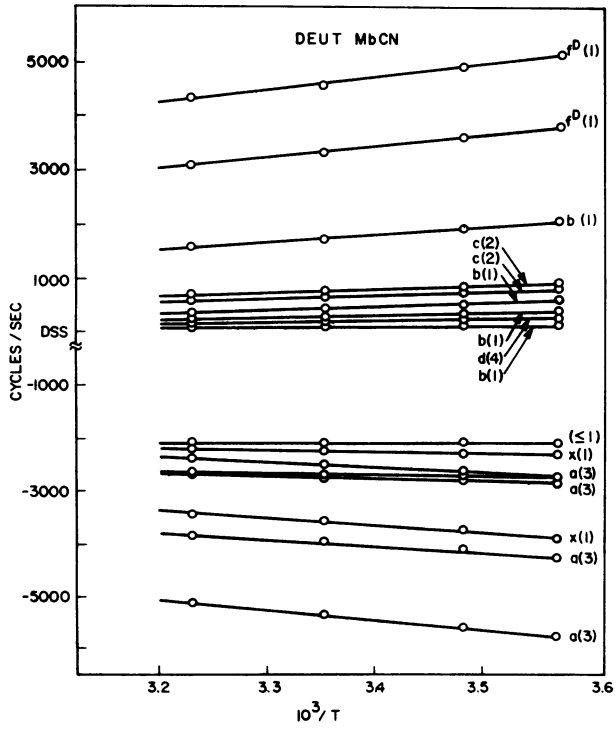

FIG. 3.—Dependence on the reciprocal of temperature of the positions relative to DSS of the proton resonances in reconstituted DeutMbCN. The letters and numbers on the right-hand side indicate the assignments (Fig. 2) and the relative intensities of the resonances.

The NMR spectrum of reconstituted MesoMbCN has the same general features as those of MbCN and DeutMbCN. In particular, four lines at low fields can be assigned to the four ring methyl groups.

Figure 4 shows the positions of the ring methyl resonances for the different reconstituted cyanoferrimyoglobins and for the corresponding cyanoporphyrin iron (III) complexes. From a comparison of ProtoCN, DeutCN, and MesoCN, one finds that the different substitutents in the 2,4 positions of the porphyrin ring do not have a great influence on the unpaired electron distribution in the heme group.[3] It is not surprising that replacing the 2,4-protons of DeutCN with ethylene groups in MesoCN causes no large changes. On the other hand, one might have anticipated larger effects to arise from the introduction of vinyl groups into positions 2 and 4 in ProtoCN, since the vinyl groups become part of the conjugated system.

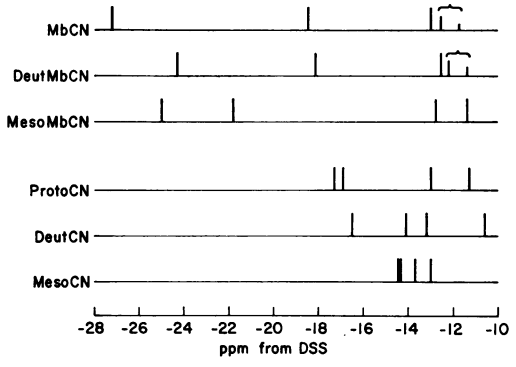

FIG. 4.—Comparison of the positions relative to DSS at 25°C of the ring methyl resonances of the heme groups of MbCN, DeutMbCN, and MesoMbCN with those of ProtoCN, DeutCN, and MesoCN in DMSO solution.

In contrast to the moderate effects of the porphyrin substituents, the spin delocalization from Fe^{3+} into the porphyrin ring is strongly affected by interactions with the polypeptide environment. For example, it can be seen in Figure 4 that the methyl resonances in MbCN, DeutMbCN, and MesoMbCN are at similar positions, while on the other hand the myoglobin spectra are quite different from those of the corresponding cyanoporphyrin iron (III) complexes. The effects of the globin environment upon the positions of the methyl resonances can be explained in terms of nonequivalence of the x and y directions of the heme group in the protein. A detailed molecular orbital model will be presented elsewhere.[11] Comparison of the positions of the 2,4-proton resonances in DeutMbCN and DeutCN provides another indication of the differences between the spin density distribution in the cyanoporphyrin iron (III) complexes in solution and in the protein. In DeutCN at 25°C the 2 and 4 protons are observed at 16.9 and 17.8 ppm upfield from DSS, while in DeutMbCN they are at 15.0 and 20.9 ppm. Here again, quite clearly, the near equivalence of the x and y directions observed in DeutCN is lost through the interaction with the globin. However, just as for the ring methyls, these resonances are found in the same region of the spectra of both DeutCN and DeutMbCN.

One might have predicted that interactions of the polypeptide chain with the vinyl side chains of the heme group in MbCN could greatly affect the electronic

structure, since the vinyl groups are part of the conjugated system. However, the comparison of MbCN, DeutMbCN, and MesoMbCN shows that the vinyl groups in MbCN do not function as unique "handles" for globin-heme interactions. This nonuniqueness of the vinyl substituents is also reflected in the oxygen affinities of reconstituted myoglobins and hemoglobins which are changed, but not destroyed, when protoporphyrin IX is replaced by deuteroporphyrin IX.[6]

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