MAGNETIC RESONANCE IN BIOLOGY

High-resolution proton nuclear magnetic-resonance spectroscopy leads to new insights in structure–function relations in heme proteins.

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DURING THE LAST FEW YEARS high-resolution nuclear magnetic-resonance (NMR) spectroscopy has become a powerful technique for investigating the molecular and electronic structure of biological compounds.[1–5] The high sensitivity and resolution of recently developed spectrometers with superconducting solenoids has made possible many new applications and has yielded data not obtainable by other methods. NMR experiments can be done under conditions similar to the physiological environment of the molecules: in aqueous solution and at body temperature. Thus with NMR it is often possible to establish relations between data on the structure of biological compounds in the solid state, obtained by techniques like x-ray crystallography, and the corresponding molecular properties in solution.

In the present survey some biological applications of NMR spectroscopy are illustrated with examples from recent investigations of heme proteins. NMR studies of this class of iron-containing proteins have given detailed insights into the electronic wave functions of the active sites and have helped towards clarifying the reaction mechanisms involved in the biological roles of these molecules.

PRINCIPLES

In NMR spectroscopy the sample under study is placed in a strong magnetic field. Transitions between the nuclear Zeeman levels are then observed in the radiofrequency range. The proton is at present the most extensively studied nucleus. Since high-resolution proton NMR experiments are usually done with liquid samples, the resonance positions yield information on the isotropic parts of proton–nucleus and proton–electron interactions. Sometimes one can even obtain information on the anisotropic interactions from the line widths and the saturation behavior of the resonances. As all these interactions are closely related to the molecular structures, and since essentially all organic and biological compounds contain protons, high-resolution proton NMR spectroscopy is widely used for structural studies.

Instrumentation

One observes nuclear resonances either by sweeping the polarizing magnetic field at constant radiofrequency, or by sweeping the frequency at constant field. The common spectrometers operate at field strengths corresponding to proton resonance frequencies of 60, 100 and 220 megahertz. Since the differences between the resonance positions of different protons are proportional to the applied field (see box), the resolution is greatly enhanced at higher field strength, and because of the greater effective magnetization the sensitivity is also improved.

At present a commercially available instrument (Varian HR-220), which uses a superconducting solenoid for the polarizing magnetic field and operates at 220 MHz, is widely used for biological applications. Field homogeneity and time stability over the sample area of close to 1 part in

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Nuclear Zeeman splitting in a static magnetic field

$$E = -\gamma h H_0 I_z / 2\pi$$

proton gyromagnetic ratio: $\gamma/2\pi = 4.260 \times 10^3$ Hz gauss^{-1}

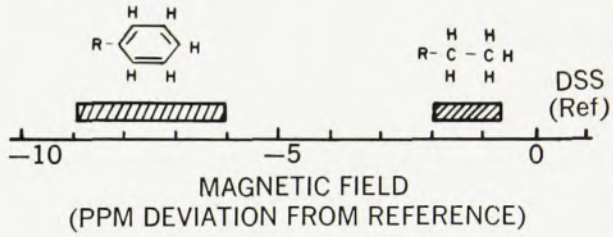
proton spin quantum number: $I = 1/2$

$$\Delta E = h\nu = \gamma h H_0 \quad \begin{cases} \nu = & 60 \text{ MHz if } H_0 = 14\,100 \text{ gauss} \\ & 100 \qquad\quad 23\,500 \\ & 220 \qquad\quad 51\,700 \end{cases}$$

Isotropic proton NMR spectrum of a diamagnetic molecule

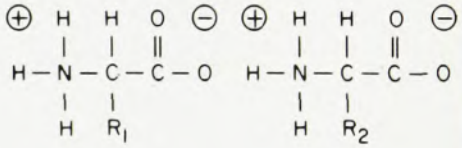
$$E = -\frac{\gamma h H_0}{2\pi} \sum_i (1 - \sigma_i) I_{zi}$$

σ_i, the isotropic screening constant of the ith proton, is a small number of order 10^{-6}. The summation is over all the protons in the molecule. The resonance positions are referred to resonance in a standard internal reference compound, in biological work usually "DSS" (2,2-dimethyl-2-silapentane-5-sulfonate).



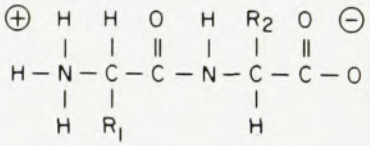
Amino acids and proteins

amino acids



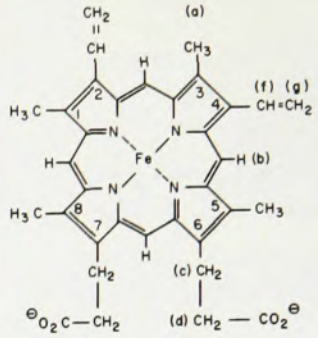
In nature 20 different amino acids are common, where the groups "R_i" are either aliphatic or aromatic substituents.

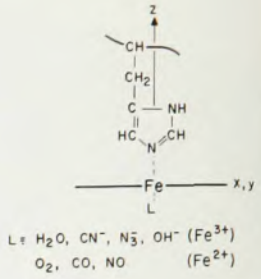
peptide bond



Through peptide bonds the amino acids combine into polypeptides, which then consist of the "back-bone" with variable side chains R_i.

The heme group of myoglobin and hemoglobin

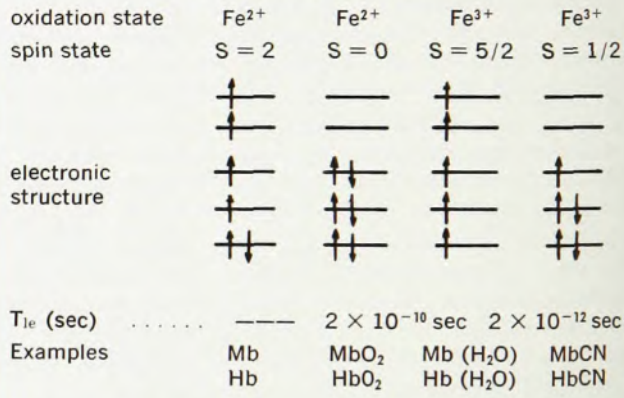




In this heme group, which is protoporphyrin iron (III) complex (sometimes called "protoheme IX"), the iron is bound to four nitrogen atoms in the plane of the porphyrin. A fifth nitrogen is provided by a histidine amino acid of the polypeptide. The sixth coördination site of the iron is part of the active site of the molecule, where oxygen and other small molecules such as H_2O or CN^- are bound as ligands of the iron. The different heme-substituents are referred to as (a) ring methyls, (b) meso protons, (c, d) propionates, (f, g) 2,4 vinyls.

The heme groups of cytochromes and other heme proteins have very similar molecular structures.

Oxidation and spin states of the heme iron



T_{1e} longitudinal electron spin relaxation time

Mb—deoxymyoglobin, MbO_2—oxymyoglobin, (H_2O)—ferrimyoglobin, MbCN—cyanoferrimyoglobin

Hb—deoxyhemoglobin, etc.

Myoglobin and hemoglobin

The myoglobin molecule consists of a polypeptide chain of 153 amino acid residues and one protoheme IX group. The molecular weight is 18 000. There are about 1000 protons per molecule, of which 30 are bound to protoheme IX, and two to the ring of the axial histidyl residue. Myoglobin can bind one oxygen molecule.

Hemoglobin consists of four subunits, two each of two types, called α and β. Each subunit contains one polypeptide chain of about 150 amino acid residues and one protoheme IX group. The molecular weight is 65 000. There are 3500 protons per molecule of which 128 are bound to the molecules attached directly to the iron atoms. One molcule of hemoglobin binds four oxygen molecules to the four iron atoms.

10^9 is achieved with electric shim coils and fast mechanical spinning of the sample tube. The HR-220 spectrometer operates at constant frequency and the magnetic field is swept to observe the spectrum. The samples can be studied at temperatures between −60 and 150°C. For many biological experiments it is necessary to improve the signal-to-noise ratio by employing a computer of average transients, with observation times of several hours.

Diamagnetic molecules

In organic molecules each proton is shielded differently from the external magnetic field by its environment, that is by the other atoms in the molecule, by the surrounding chemical bonds and by the solvent. The resulting differences in resonance conditions for individual protons are called chemical shifts, which are expressed relative to the resonance conditions of standard reference compounds (see diagram in box). In a field-sweep experiment at constant radiofrequency all proton resonances of diamagnetic organic molecules occur at field values that are lower than for resonances in the standard references within about 10 parts per million (ppm). For example, the resonances of aliphatic hydrocarbons are observed between −0.5 and −2.0 ppm and those of aromatic molecules between −6.0 and −8.5 ppm. For small organic molecules the nuclear relaxation times T_1 and T_2 are rather long, in the range 1–20 sec, so that the observed linewidths of the resonances are determined by the resolution of the apparatus rather than by the nuclear relaxation. However, in large molecules, which rotate more slowly, the nuclear dipole interaction creates considerably broader NMR lines.

Paramagnetic molecules

Interactions with unpaired electrons greatly affect the proton NMR spectra of paramagnetic molecules. These interactions, which include isotropic and anisotropic hyperfine interactions as well as dipolar contributions, can both shift and broaden individual proton resonances. We refer to NMR lines shifted by these interactions with the unpaired electrons as hyperfine-shifted lines. It is easy to show that these NMR shifts depend upon the polarization of the electron spins, and for this reason the hyperfine shifts in paramagnetic molecules are inversely proportional to the absolute temperature.

The NMR hyperfine shifts can usually be interpreted in terms of electron spin densities, and in that way can give detailed information about the electronic structure of the molecule. The NMR line widths also depend upon the strength of the interaction, but in addition depend upon the correlation time of the interaction. The correlation time can be determined from the electron spin–lattice relaxation time or the molecular tumbling time, and each case requires a separate analysis. In general, when this correlation time is decreased, the magnetic electrons make a smaller contribution to the NMR line width and the resolution is improved.

HEME PROTEINS

Heme proteins are involved in many vital processes in living organisms. For example, hemoglobin takes oxygen from air in the lungs and transports it in the blood to muscles and the brain. Myoglobin binds and stores oxygen in muscles. A variety of different cytochromes, also heme proteins, function as electron-transferring oxidation-reduction carriers in the "respiratory chain" where oxygen is reduced and the resulting energy gradually released to the body. Still other heme proteins function as enzymes that control diverse biochemical reactions. Thus a large variety of biological roles have been observed for these structurally quite closely related compounds.

Structural units

Amino acids, which combine in long strings or "polypeptide chains" (see box), are the basic structural units in proteins. The polypeptide chains in typical proteins contain between one hundred and several thousand amino-acid residues, corresponding to molecular weights between ten thousand and several hundred thousand. In the molecular structures determined by x-ray crystallography of protein single crystals, the polypeptide chains are uniquely arranged in space. The molecules are fixed in three-dimensional structures by a multitude of weak bonds, namely hydrogen bonds, ionic bonds and van der Waals interactions.

In heme proteins one or several heme groups (see box) are located in crevices formed by the three-dimensional arrangement of the polypeptide chains. They are bound to the latter by a few covalent or coördinative bonds, and by a multitude of weaker interactions. The heme groups consist of a porphyrin ring, which is a conjugated aromatic ring system, and an iron ion. The heme iron can be in different oxidation and spin states (see box). Correspondingly, the heme proteins are either diamagnetic or paramagnetic.

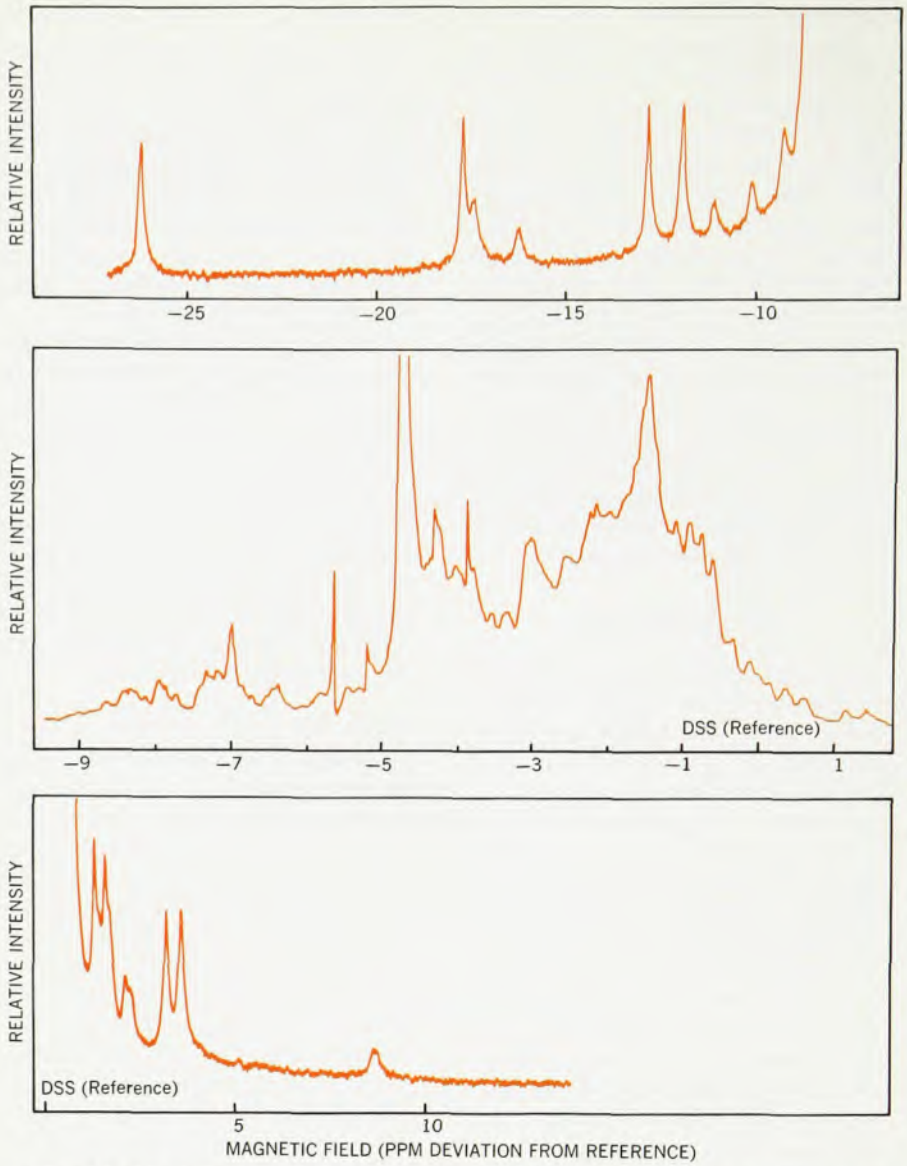
For example, in physiologically normal myoglobin and hemoglobin the heme iron is in the diamagnetic, low-spin ferrous state when oxygen is bound and in the high-spin ferrous state otherwise. Interconversion between the ferric and ferrous oxidation states is an important factor in the biological roles of cytochromes. For structural studies the state of the heme iron can easily be modified with chemical reagents.

Active sites

In all the heme proteins the heme group is part of the active site and thus directly involved in the biological role of the molecule. Even though the molecular structures of heme groups in different heme proteins are very similar, greatly different chemical reactivities of heme iron, corresponding to different biological roles of heme proteins, result from extremely specific interactions between polypeptide chains and heme groups. High-resolution proton NMR spectroscopy yields information on these heme–polypeptide interactions. In addition, structural changes during biochemical reactions, for example upon oxygen binding to myoglobin or hemoglobin, can be studied by NMR and then compared with the structures examined by x-ray crystallography in single crystals.

MYOGLOBINS

Three regions of the NMR spectrum of cyanoferrimyoglobin, MbCN, which contain all the observed resonances, are reproduced in figure 1 with different vertical and horizontal scales. Many of the spectral features are easily understood from the introductory statements. The resonances of nearly all the 950 protons of the polypeptide chain fall between −0.5 and −9.0 ppm. Because of the slow rotation time of the molecule, the widths of the individual protein resonances are broadened by nuclear dipole interactions to about 20–40



PROTON NMR SPECTRUM at 220 MHz of a solution of porpoise cyanoferrimyoglobin (MbCN) in D_2O. Different horizontal and vertical scales were used for the spectral regions 1 to −9 ppm, +1 to +10, and −10 to −30 ppm. The sharp lines between −3.8 and −6 ppm, correspond to the resonance of HDO and its side bands introduced by spinning the NMR sample tube. —FIG. 1

Hz, and therefore this spectral region is not well resolved, even at 220 MHz.

From comparison with the NMR spectra of the individual amino acids, the resonances between −0.5 and −3.0 ppm come mostly from aliphatic amino-acid residues. Resonance between −3.0 and −4.0 ppm come from the protons of the backbone of the polypeptide chain, and those between −6.0 and −9.0 ppm from the aromatic amino-acid residues and from nondeuterated amide protons. The sharp lines between −4.0 and −6.0 are the HDO resonance and spinning side bands introduced by the spectrometer.

The relative intensities in the different spectral regions agree well with those expected from the known amino-acid composition of porpoise myoglobin. The spectral region from −0.5 to −9.0 ppm for different proteins with molecular weights between 10 000 and 25 000 is very similar to that of figure 1. A more detailed assignment of specific polypeptide resonances is possible in certain favorable instances, as will be discussed below.

Since the region from the DSS reference position to −9 ppm of a protein spectrum is not well resolved, most of the structural information is obtained from resonances in other spectral regions. Because MbCN is paramagnetic and has a very short electron spin–lattice relaxation time, the proton resonances of the molecules bound to the iron are shifted but not appreciably broadened by the interactions with the unpaired electron. Therefore, well resolved resonances, with intensities corresponding to one, two or three protons, are observed outside the range DSS to −10 ppm. From their intensities and from comparison with isolated heme groups and with myoglobins in which protoheme IX (see box) was replaced with different heme groups, the hyperfine-shifted resonances of MbCN were assigned to specific protons of the heme group and the axial histidyl residue (figures 1 and 2).

Ring currents

In addition to the hyperfine-shifted lines, which are dependent on temperature, the spectrum of figure 1 contains resonances at fields higher than −0.5 ppm that are shifted by a temperature independent mechanism. This is a general feature of the NMR spectra of most proteins, because even in the absence of paramagnetic centers the proton resonances of the amino-acid residues are shifted by local magnetic fields of the neighboring groups.

The strongest of these local fields come from so-called ring currents in aromatic rings, namely the aromatic amino-acid residues and the heme groups. These ring currents arise because the π electrons of the aromatic molecules are subject to a Larmor precession in the closed conjugated ring, if an external magnetic field is applied perpendicular to the plane of the molecule. The resulting magnetic field opposes the external field above and below the molecule's plane, and reinforces it otherwise. Thus if aliphatic amino-acid residues are located near the planes of one or several aromatic rings in the three-dimensional structure of the protein molecule, their proton resonances can be shifted to higher fields than DSS and may be well resolved (figures 1 and 3). These ring-current shifts are very sensitive to the relative positions of observed protons and aromatic rings, and therefore to structural changes in the proteins.

Compared to the spectrum of cyanoferrimyoglobin (figure 1) the hyperfine shifts are much larger and the lines much broader for the heme resonances in high-spin ferrimyoglobin, in which the sixth coördination site of

the iron is occupied by a water molecules. This was to be expected from the larger total electronic spin and from the longer electronic relaxation time. For deoxymyoglobin, where the sixth coördination site of the ferrous heme iron is not occupied, hyperfine-shifted resonances were observed at high and low fields. The hyperfine shifts are comparable to those of cyanoferrimyoglobin (figure 1), but the lines are somewhat broader.

Oxygen binding

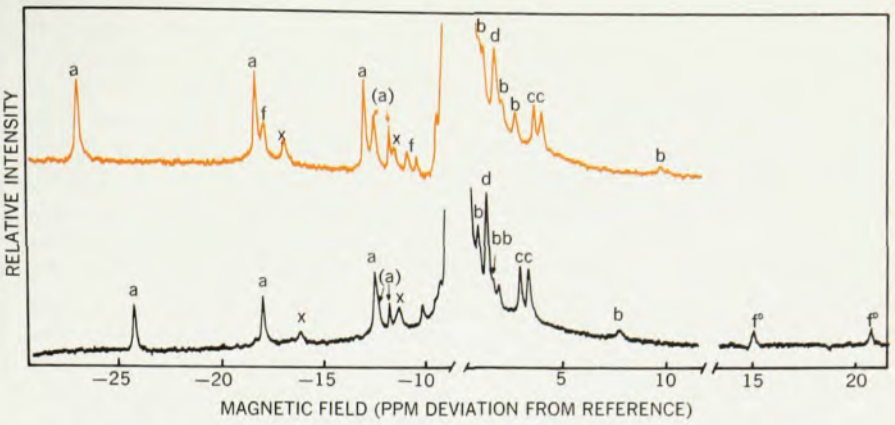
Both hyperfine-shifted resonances and ring-current shifts have been observed for paramagnetic deoxymyoglobin, while all the resolved resonances of diamagnetic oxymyoglobin are shifted by ring-current fields. For an understanding of the biological function of myoglobin it was of particular interest to determine if structural changes occur upon oxygen binding.

Using the x-ray coördinates of myoglobin determined by John Kendrew and coworkers (unpublished), and empirical expressions to simulate the local ring-current fields, we calculated the approximate NMR positions of the different amino-acid residues. From comparisons of the calculated and experimental spectra it appears to us that the protein structures are quite similar in single crystals and in solution.

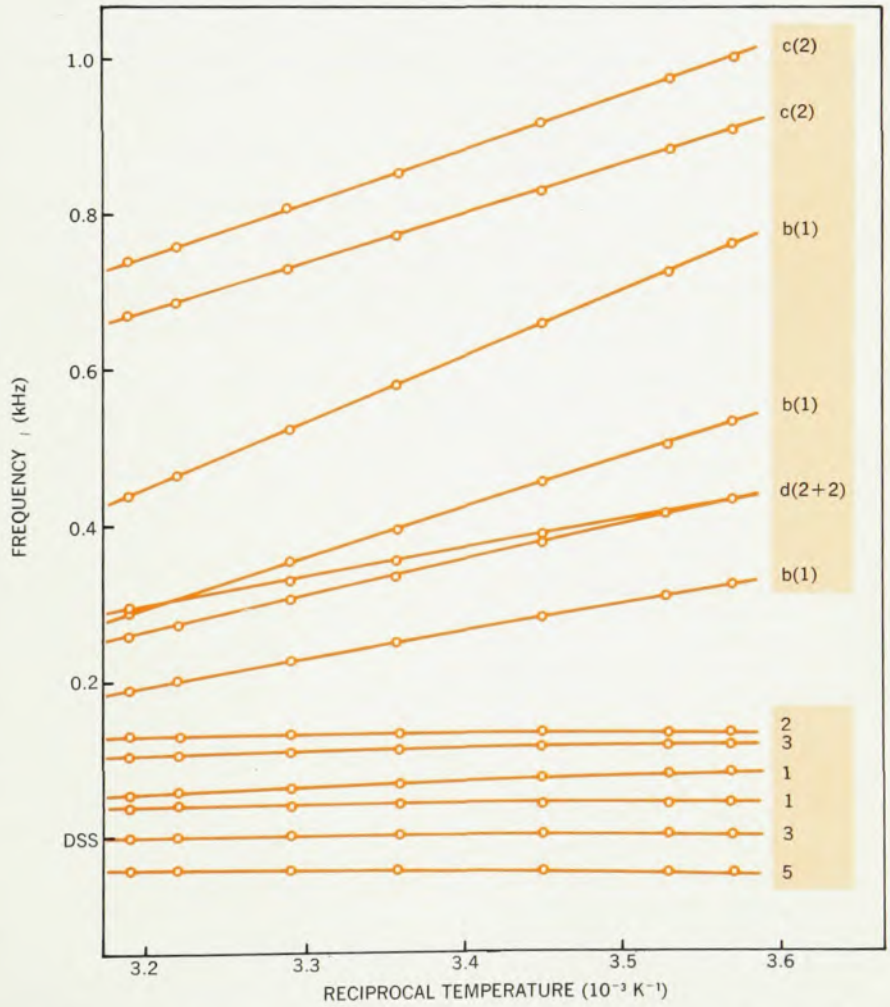
Furthermore, some of the resolved ring-current shifted resonances could be assigned to specific amino-acid residues located near the heme group. From a comparison of the positions of these resonances in the spectra of oxy- and deoxymyoglobin it was apparent that oxygenation does change the structure of the myoglobin molecule. Even though these changes may be too small to be resolved in x-ray studies (they are estimated from the NMR data to be of the order of 0.2 Å), they are observed in the NMR spectra as shifts of more than 100 Hz.

HEME GROUPS

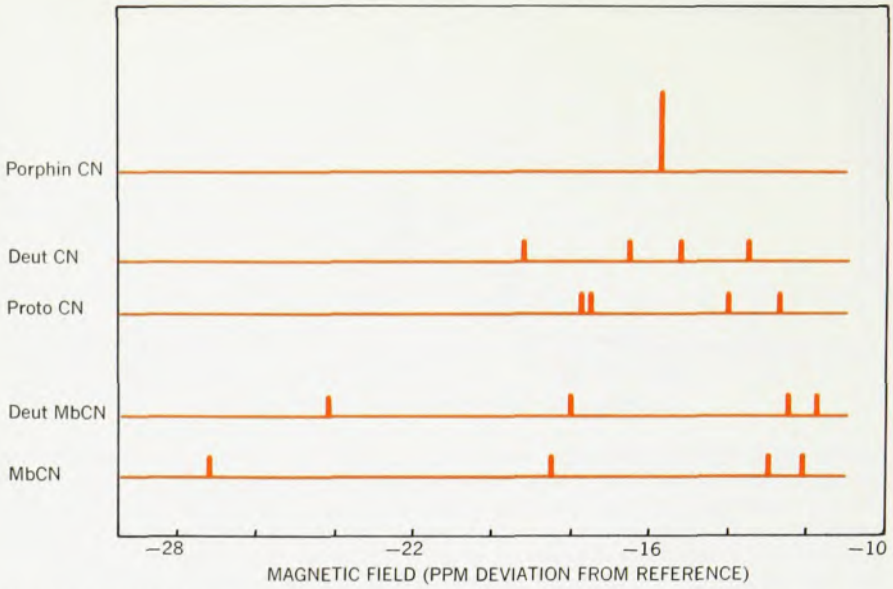
The electronic structure of a heme group can be described by molecular-orbital wave functions that extend over all atoms of the conjugated ring system, including the iron. Hence in paramagnetic heme groups the unpaired electrons are delocalized from the iron to the porphyrin ring. Unpaired electron density is then further



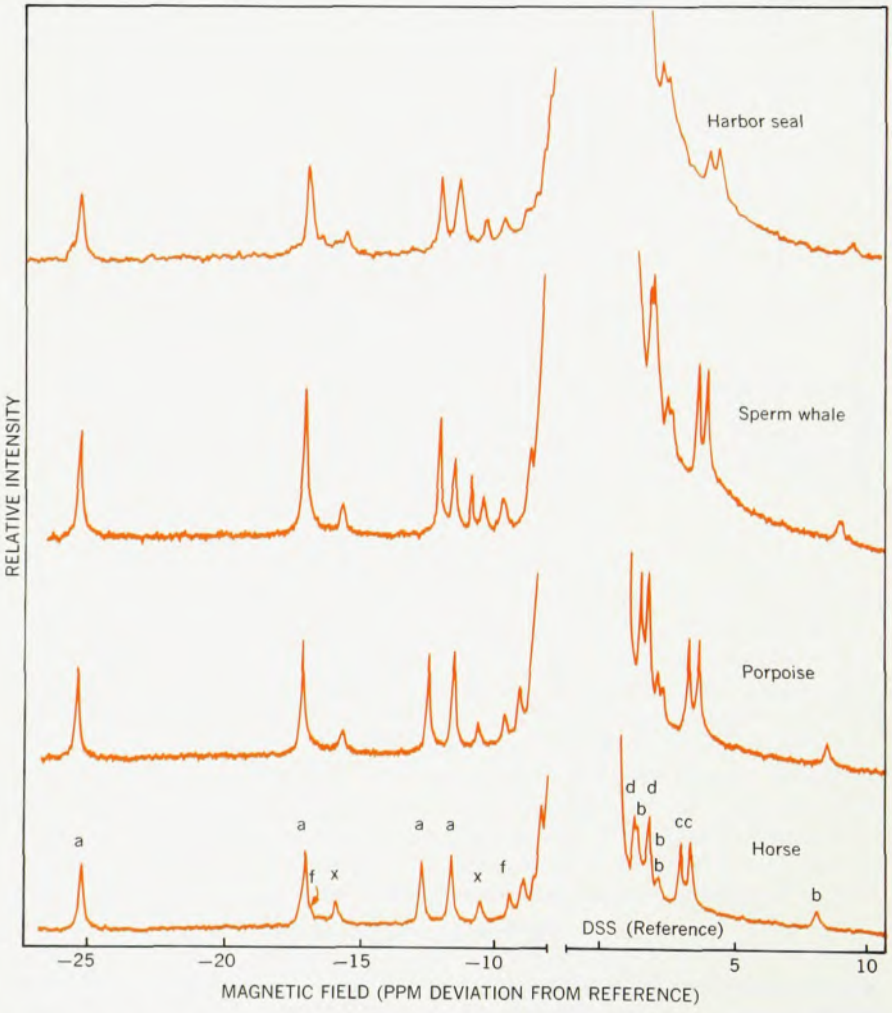
COMPARISON of the 220 MHz spectra taken at 25°C of native sperm whale cyanoferrimyoglobin (color), and reconstituted cyanodeuteroferrimyoglobin (black), where the 2,4 substituents of the heme group are —H instead of vinyls (see box). The assignments of the resonances to heme protons are: (a) ring methyls, (b) meso protons, (c,d) methylene protons of propionic acid groups, (f) —CH protons of the vinyl groups, (f^D) 2,4-protons in Deut MbCN, (x) protons of the axial histidine. The f resonances were assigned from their absence in Deut MbCN, the other resonances from their intensities and presence in both spectra. —FIG. 2



RESONANCE POSITION dependence on reciprocal temperature of sperm whale MbCN between −1 and +4 ppm (1 ppm = 220 Hz). From their temperature dependence the hyperfine-shifted resonances can be distinguished from the ring current shifted lines. The number of protons corresponding to the intensities and the assignments of the contact-shifted resonances as labelled in figure 2 are indicated on the right-hand side. —FIG. 3



RING-METHYL-RESONANCE positions of the dicyano iron (III) complexes of porphin, deuteroporphyrin, protoporphyrin, and of reconstituted Deut MbCN and native cyanoferrimyoglobin, MbCN. NMR shifts for fictional methyl groups in porphin were calculated from those observed for the protons attached to the ring carbon atoms. —FIG. 4



HYPERFINE-SHIFTED RESONANCES of cyanoferrimyoglobins from different animals. The heme group of all the different myoglobins is protoheme IX, but a number of amino-acid residues of the polypeptide chains are different. —FIG. 5

transferred from the ring carbon atoms to the directly attached protons, or to CH_3 and CH_2 groups (see box) by spin polarization or by additional spin delocalization. The resulting hyperfine shifts of the proton resonances are proportional to the unpaired electron density at the nearest ring carbon atom. Therefore, if the proton resonances have been assigned to specific heme protons, they provide a point by point measurement of the unpaired electron distribution around the heme group.

Low-spin ferric hemes

The unpaired electron of low-spin ferric hemes is localized in molecular orbitals of π symmetry. The isotropic part of the hyperfine coupling tensor, A_i, which is the quantity measured in the NMR experiments, has been shown to be related to the spin density ρ_c^π at the ring carbon atoms by the equation:

$$A_i = Q\rho_c^\pi$$

where $Q \approx -6.3\times10^7$ Hz for directly attached protons, and $Q \approx +3.0\times10^7$ Hz for protons of the methyl groups. The following discussion will be based on the four ring methyl resonances, which yield spin densities at the four adjacent carbon atoms of the heme (see box). Studies of the other proton resonances lead to the same conclusions.

If all the substituents of the porphyrin ring are identical, the molecule has four-fold symmetry. Correspondingly, NMR studies of solutions in various solvents of dicyanoporphin iron (III), in which all the heme substituents are protons, show that the electron densities at the ring carbon atoms 1–8 are identical. Small perturbations of the four-fold symmetry arise in the different heme groups in solution from the different substituents, as shown in figure 4. In the heme proteins, however, the interactions of the heme group with the polypeptide chains change both the total extent of the spin delocalization from the iron to the porphyrin ring, and the symmetry of the spin distribution (figure 4).

These interactions appear to be very specific and have a larger effect upon the spin densities than does changing the heme substituents. The modifications of the electronic structure of the heme groups in heme proteins seem to come from interactions with amino-acid residues that are either coördina-

tively bound to the iron, or interact directly with the porphyrin ring.

Structure and role

The biological role of a protein molecule is largely determined by its chemical reactivity towards other molecules. Even though the reactivities of heme proteins are closely related to those of the heme groups, they are greatly different from those of the isolated hemes, which by themselves cannot perform the biological roles of any known heme proteins. To clarify the action mechanisms of heme proteins it is then of interest to try to relate the NMR data on the electronic structures of hemes and heme proteins to their chemical reactivities and biological roles.

The following observations imply that specific modifications of the electronic structure of the heme group are important functions of the polypeptide chains in heme proteins. Characteristic differences were observed between the electronic structures of isolated hemes and heme groups in heme proteins (figure 4). Furthermore, the electronic heme structure was found to be different in heme proteins with different biological functions, as for example in myoglobin, hemoglobin and cytochrome c.

On the other hand, essentially identical spin distributions were found for myoglobins from different mammals (figure 5), which all contain protoheme IX but not identical polypeptide chains. Similar results were obtained in comparative studies of other heme proteins from different species, indicating that both biological activity and electronic heme structure were not appreciably affected by the evolutionary mutations in the polypeptide chains. Since only limited regions of the polypeptide chains of given heme proteins from different species are identical, one also learns from the near constancy of the electronic heme structures which amino-acid residues are most important in the heme-polypeptide interactions.

Although the electronic structure of the heme groups may be only one of many factors that determine the biological role of heme proteins, it is of great interest to investigate its impact. Because both biochemical functioning and electronic heme structure can now easily be studied for a variety of heme proteins, detailed insights into the relations between electronic structure and biochemical functioning should be obtainable.

HEMOGLOBIN

With the background of the myoglobin studies, it has been possible to apply these techniques to the far more complex hemoglobin molecule. Because of its larger size one observes a greater number of proton resonances than for myoglobin and the individual resonances are broader and overlap more strongly. Yet it is still possible to observe the hyperfine-shifted heme resonances of cyanoferrihemoglobin and to assign them at least in part to specific heme protons.

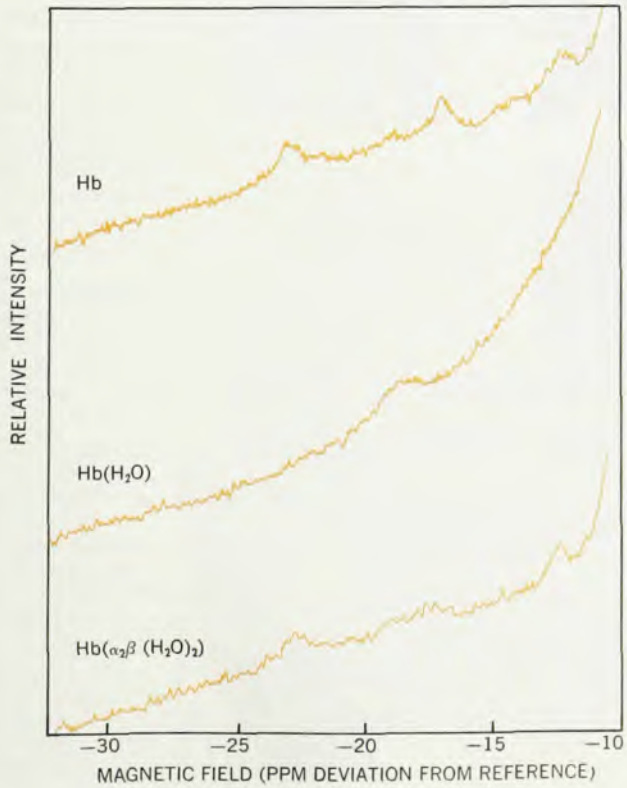
Coöperative oxygen binding

In its biological role, hemoglobin binds one oxygen molecule to each of its four heme groups. It has been known for a long time that binding of oxygen or other ligands to hemoglobin is "coöperative." This means that the affinity for oxygen or other ligands of the partially ligated hemoglobins formed through binding of one, two or three ligand molecules is greater than that of deoxyhemoglobin. The question arises as to the mechanism responsible for the free energy of interaction between the subunits that cause the coöperativity.

We have described above the NMR experiments that show the structural changes that occur upon oxygenation of myoglobin. Since the structures of myoglobin and of individual hemoglobin subunits are very similar, the implication is that ligand binding should induce corresponding structural changes within the subunits. For an understanding of the coöperativity of ligand binding to hemoglobin it is then essential to know how far these ligand-induced structural changes in the subunits are propagated through the structure of the entire molecule. Do they affect the neighboring heme groups and thereby change the oxygen affinity or do they operate only on the protein part? An answer to these questions was obtained from recent NMR experiments.

If an appreciable fraction of the increased free energy of ligand binding attributed to subunit interactions was related to changes in the electronic structure of the heme groups, the hyperfine-shifted heme proton resonances of partially ligated hemoglobin would have to be different from those in the completely ligated and unligated forms. NMR studies of a series of partially ligated forms of hemoglobin showed that ligand binding does not affect the heme group of the neighboring subunits.

In a typical experiment the low-field resonances of completely unligated deoxyhemoglobin, Hb, and completely ligated ferrihemoglobin, $Hb(H_2O)$, are compared to those of



COMPARISON of the hyperfine shifted resonances at low fields of deoxyhemoglobin Hb (no ligands bound), ferrihemoglobin Hb-(H_2O) (completely ligated), and partially ligated Hb($\alpha_2\beta(H_2O)_2$). It is seen that the spectrum of the partially ligated hemoglobin is a superposition of the spectra of Hb and Hb-(H_2O). These spectra were recorded over a period of several hours, employing a computer of average transients.
—FIG. 6

the partially ligated form Hb $(\alpha_2,\beta\text{-}(H_2O)_2)$. Figure 6 shows that the spectrum of the partially ligated hemoglobin is a superposition of the spectra of Hb and $Hb(H_2O)$, and thus no effects of ligand binding on the electronic structure of the hemes in neighboring subunits were observed even though the partially ligated form has a higher oxygen affinity than does Hb. On the other hand there is evidence from NMR spectroscopy that structural changes occur upon ligand binding to both hemoglobin and myoglobin. It appears therefore that subunit interactions in hemoglobin are linked with rearrangements of the polypeptide chains, and not caused by direct heme–heme interactions.

The study of heme proteins is one of many examples that might have been chosen to illustrate the applications of high-resolution NMR spectroscopy in biology. Through combination with different chemical and physical techniques it has been possible to identify the resonances of specific protons in the NMR spectra of various biological molecules, and hence to study the structures of these molecules under variable conditions. Many of these experiments are reviewed in references 3 and 4.

The amount of new information on the solution structures of a variety of biological molecules obtained from NMR experiments during the past few years is very encouraging. The availability of high-resolution spectrometers and future improvements in instrumentation, in particular faster data accumulation through use of Fourier transformation and perhaps the availability of even higher polarizing fields, promise to establish NMR spectroscopy as one of the basic techniques for structural studies in biological systems.

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5. For more detailed accounts of our investigations of heme proteins see Proc. Nat. Acad. Sci. U. S., **60**, 373 (1968); **61**, 1199 (1968); **62**, 637 (1969); Science **165**, 251 (1969). □