Characterization of Proteins by Nuclear Magnetic Resonance

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The availability of 100 MHz and particularly 200 MHz pmr spectrometers has increased the potentiality of the direct application of nmr to proteins. The present article makes no attempt to survey the current literature, but rather demonstrates the various types of approach to study the structure of protein molecules. Emphasis is placed on effects produced by interaction of the chains in the native and denatured forms, binding of small molecules, substrates, and inhibitors to protein, and types of interactions likely to cause shifts in resonance positions, namely ring-current fields and paramagnetic center.

The physical methods used for the characterization of biological macromolecules can be roughly classified into two groups: the methods which yield information about gross molecular features such as the shape, electrical charge, viscosity, and molecular weight of a macromolecule; and the methods which can provide details at the atomic level as X-ray methods.

The usual methods, like light scattering, osmosis, electrophoresis, and diffusion, measurements of viscosities, and ultracentrifugation belong to the first group, and spectroscopic techniques belong to the second.

The most recent applied spectroscopy technique is nuclear magnetic resonance (nmr) which has been used for studying the structures and structural changes of macromolecules.

Until recently, the direct examination of the resonances of the H atoms (protons) of the protein itself was severely limited by insufficient sensitivity and resolution, but developments in nmr technology have alleviated, though certainly not eliminated, these problems.

Valuable developments which have led to a sudden upsurge of biologically-inclined studies are: field-frequency locked spectrometers which provide increased sensitivity and resolution by eliminating drift and consequent broadening of spectral lines; time-averaging computers or computers of average transients (CAT) which make use of this stability to accumulate many traces of weak spectra, enhancing the signal to noise ratio in proportion to the square root of the number of scans accumulated; and most recently a spectrometer operating at 220 MHz became available, which has improved sensitivity and increased peak separations. The Varian 220 MHz spectrometer, which has a helium-cooled super-conducting magnet, has sufficient magnetic field stability to obviate the need for a lock system.

The present paper tries to demonstrate the various types of approaches and degrees of success of the nmr method at this stage in its development. The theory and experimental details of the nmr method are adequately covered in reference works (Pople *et al.*, 1959; Roberts, 1959, 1962; Slichter, 1963; Emsley *et al.*, 1965; Carrington and McLachlin, 1967; Ehrenberg *et al.*, 1967; Jardetzky and Jardetzky, 1962). Several review articles serve to cover early applications (Johnson and Bovey, 1958; Kowalsky and Cohn, 1964; Dwek and Richards, 1967; Cohen, 1969) and more recent reviews are listed (Sheard and Bradbury, 1970; Rowe *et al.*, 1970; Mildvan and Cohn, 1970; McDonald and Phillips, 1970; Roberts and Jardetzky, 1970).

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BASIC CONCEPTS

The theory of nmr can be described qualitatively in the following manner. The position of a peak in the spectrum, its chemical shift, is determined by the electronic or chemical environment of the nucleus. Nuclei having identical resonance frequencies are said to be magnetically equivalent. The area under a resonance signal gives a quantitative measure of the number of resonating nuclei. For a molecule containing magnetically nonequivalent nuclei, the ratio of areas in a spectrum will thus reflect the ratio of the number of nuclei in each chemical group. The coupling constant, J_{AB}, is a measure of the strength of the coupling between two nuclei A and B; it depends on the nature of the bond between them, and, if they are separated by more than two bonds, it depends on the relative orientations of the chemical bonds. It is independent of the external magnetic field. In macromolecules these splittings are usually smaller than the intrinsic line widths. Nuclear spins exchange between their energy levels at a rate determined by the rapidity of movement of the molecule or molecular group containing them. The rate is characterized by so-called "spin relaxation times" T_1 and T_2. T_1 (longitudinal or spin-lattice relaxation time) and T_2 (transverse or spin–spin relaxation time) decrease as molecular motion becomes slower. The time scale for molecular tumbling is the so-called correlation time, τ_c. In liquids, generally, $T_1 = T_2$, but at high viscosity or in macromolecules it is often observed that $T_1 > T_2$. When $T_1 \sim T_2$, one can often write $1/T_1 \propto \tau_c \propto \eta$, where η is the viscosity. T_2 is inversely related to the peak width, *i.e.*, $T_2 = 1/\pi\Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$ is the width at half height.

EXPERIMENTAL CONSIDERATIONS

Nmr measurements are carried out under biologically reasonable conditions and are nondestructive.

The volume of sample required for nmr measurements is generally 0.5 ml. A lower concentration limit to observe a proton resonance on a single sweep is about 1×10^{-3} in any given proton, depending on the spectrometer sensitivity. However, this concentration limit may be reduced to less than 1×10^{-4} *M* by the application of Fourier transform spectroscopy (Ernst and Anderson, 1966; Ernst, 1966). Microquantities of solution may be used in specially designed microcells (Shoolery, 1962; Hall, 1968) with 0.03 to 0.05 ml capacity, although loss of resolution usually results.

The sample is usually in D_2O solution in order to suppress the strong H_2O resonance that can overwhelm the central and aromatic regions of a polypeptide. The exchangeable NH, NH_2, and OH protons of polypeptides are usually

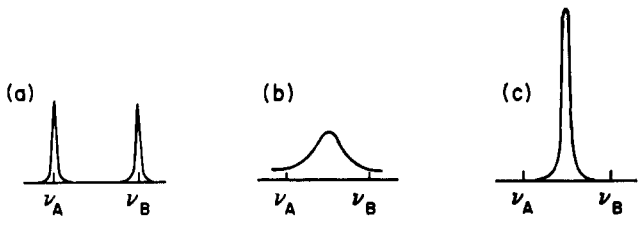

Figure 1. Change of line shape as a result of chemical exchange between two sites A and B at successively increasing exchange rates. (a) Slow exchange, $\tau \gg {}^1/_2\pi\Delta\delta$; (b) intermediate exchange, $\tau = {}^1/_2\pi\Delta\delta$; (c) fast exchange, $\tau \ll {}^1/_2\pi\Delta\delta$

replaced by exchange preliminary to running in D_2O, to decrease further the intensity of the residual HDO resonance and its side bands. While the replacement of NH protons by deuterium results in great simplifications of the pmr spectra of polypeptide, much potentially valuable information is lost because NH protons are intimately involved in the secondary and tertiary structures (McDonald and Phillips, 1970). For work in D_2O solution, a pD is defined such that (Glascol and Long, 1960)

$$pD = pH + 0.4 \quad (1)$$

where the symbol pH refers to the reading obtained with a conventional pH meter.

REFERENCE STANDARDS

The difficulties inherent in obtaining accurate measurements of field strength make an absolute scale for chemical shift impractical. Therefore, line positions are measured relative to a given standard. Thus in frequency units, $\Delta\nu = \nu_s - \nu_r$ (in Hz; *i.e.*, cycles per second) where $\Delta\nu$ is the peak separation, and ν_s and ν_r are the resonance frequencies of the sample and of the reference. Since $\Delta\nu$ is proportional to the strength of the applied field, it is often expressed as a dimensionless parameter δ, where

$$\delta = \frac{\Delta\nu}{\nu_{osc}} \times 10^6 \text{ (in parts per million)} \quad (2)$$

The most commonly used reference standard is tetramethyl silane (TMS), $(CH_3)_4Si$, owing to its chemical inertness and the fact that its resonance position is at one extreme of the usual range of absorption. Most other resonances occur in a lower field, usually within a range of 10 ppm. The less volatile hexamethyldisiloxane (HMS) or water soluble 2,2-dimethylsilapentane-5-sulfonate (DSS), $(CH_3)_3Si-(CH_2)_3-SO_3^-\ Na^+$, are also used. Water is not a useful standard since its chemical shift is sensitive to temperature and to the nature of the solution. The reference compound may be within the sample as an internal reference or contained within a capillary as an external reference. These will usually give slightly different results depending on the difference of bulk diamagnetic susceptibility between the pure reference compound (external) (Tiers, 1958). Internal referencing is probably avoided since biological macromolecules have a tendency to bind small molecules. Micelle formation has been described for DSS in D_2O (Donaldson and Schwarz, 1968).

The difficulties inherent in external referencing, the instability and large spinning side-bands associated with spinning capillaries may be minimized by the use of precision coaxial inserts (Wilmad Glass Co., Buena, N. J., Cat. No. 520). Quite accurate values for chemical shifts may be obtained using the signal of the desired reference compound as the lock signal and reading directly the difference between the fixed (lock) and variable (sweep) frequencies.

The high-resolution nmr can, in principle, provide the following information: the mode of binding of small molecules, and protein conformation in solution.

BINDING OF SMALL MOLECULES

The study of small molecules in the presence and absence of macromolecules with which they are supposed to have specific interactions has had considerable success. The underlying theory of this method is covered in reviews by Jardetzky (1964), Mildvan and Cohn (1970), and a paper by Shulman *et al.* (1966).

When a small molecule binds to a macromolecule, two types of change are commonly observed in its nmr spectrum: a change in chemical shift of one or more resonances, reflecting a change in the magnetic environment of one or more of the groups on binding; and a broadening of one or more peaks due to decreased motional freedom of a particular part of the small molecule, or of the molecule as a whole, with a corresponding increase in correlation time and hence of relaxation rate. A working assumption has often been that those groups which interact directly with groups on the macromolecule will show both the largest change in magnetic environment and the largest decrease in motional freedom.

Depending on the lifetime of the complex, the appearance of the spectrum falls into one of three categories, referred to as the fast, intermediate, and slow exchange cases. The mathematical treatment has been thoroughly reviewed (Johnson, 1965).

Consider the case that involves exchange of magnetic nuclei between two equally populated sites A and B. If

$$\frac{\tau_A + \tau_B}{2} = \tau \quad (3)$$

where τ is the mean lifetime, the shape of the resonance signals observed will depend upon the ratio of τ to $1/\Delta\nu$, where $\Delta\nu = (\nu_A - \nu_B)$ measured in Hz. Three conditions may be delineated. (a) Slow exchange, $\tau \gg {}^1/_2\pi\Delta\nu$. In this case there are separate resonance signals for the nuclei at each site (Figure 1). (b) Intermediate exchange, $\tau = {}^1/_2\pi\Delta\nu$. If the exchange rate becomes faster (and the lifetime, τ, shorter), the two signals broaden and coalesce, giving finally a single broad resonance centered between ν_A and ν_B. (c) Fast exchange $\tau \ll {}^1/_2\pi\Delta\nu$. A single sharp peak is observed whose chemical shift is the weighted average ($\nu_{mean} = P_A\nu_A + P_B\nu_B$ where P_A and P_B are the fractional populations of A and B sites) of the shifts in the two states. Rates of the order of 5 sec^{-1} up to 10^7 sec^{-1} are measurable by this method (Saunders, 1967).

When metal ion binds to a molecule, two quite distinct effects are observed depending on the nature of the metal ion. Diamagnetic metal ions, such as Mg^{2+}, Zn^{2+}, Ca^{2+}, cause small chemical shifts which can be explained on the basis of charge effects. Paramagnetic ions, such as Mn^{2+}, Co^{2+}, Cu^{2+}, cause a much greater chemical shift (paramagnetic shift) and line broadening of nmr spectra due to the interaction of the nucleus with unpaired electrons. If the broadening effect is observed on more than one nucleus, it is possible to define the position of the metal ion with respect to the ligand because the degree of broadening is proportional to $1/r^6$, r being the distance between the nucleus and the unpaired electron.

The relaxation enhancement technique (Eisinger *et al.*,

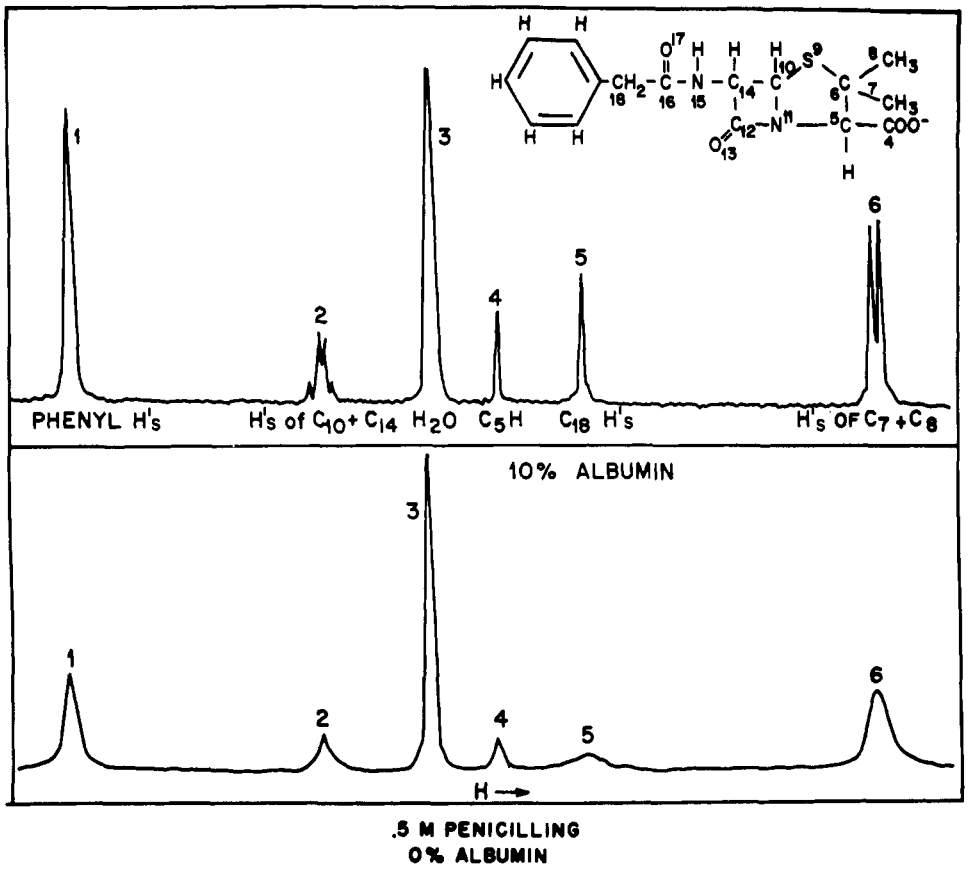

Figure 2. 60 MHz spectra of a 0.5 *M* D_2O solution of penicillin G alone, and in the presence of 10% albumin (Fischer and Jardetzky, 1965). Reprinted with permission of: *J. Amer. Chem. Soc.* 87, 3237 (1965)

1961, 1962; Cohn and Leigh, 1962) is very useful for the study of a wide range of chemical and biological problems involving magnetic ions.

In solutions of paramagnetic ions such as Mn^{2+}, spin relaxation of the water protons is dominated by the metal ions. The effective correlation time τ_c is thought to be determined by Brownian rotation of the hydrated Mn^{2+} complex. In the presence of a macromolecule, τ_c may be increased by hindered rotation in the bound state, in which case spin relaxation of the solvent water protons becomes even more efficient. A proton relaxation enhancement parameter, ϵ, may be defined as $\epsilon = R^*/R$, where R^* and R are the proton relaxation rates for a certain ion concentration in the presence of the macromolecules under study and for the aqueous solution, respectively. R is obtained from the experimentally observed relaxation rate $1/T_1$ by subtracting the contribution to the relaxation rate which has nothing to do with the paramagnetic ions (generally $1/T_1$ for pure water). R^* is obtained in a corresponding manner. The three limiting cases of no ion binding, ion binding in accessible sites, and exterior ion binding may be characterized by $\epsilon = 1$, $\epsilon \ll 1$, and $\epsilon \gg 1$, respectively. By suitable titrations it is possible to determine the characteristic value of ϵ for each complex at the tight binding sites, as well as calculate the number of binding sites and association constants. A review indicating the type of information relevant to enzyme function that can be obtained from measurements of nuclear spin relaxation rates due to paramagnetic probes has appeared (Mildvan and Cohn, 1970).

An early example of the mode of binding of small molecules to proteins is a study by Fischer and Jardetzky (1965) on the binding of penicillin G to bovine plasma albumin. In Figure 2 their 60 MHz spectra of penicillin G in the absence and presence of albumin are presented. Measurements of the widths of the various nmr lines indicated that the aromatic end of penicillin (peak 1 of Figure 1) was bound more tightly in the complex than the rest of the molecule. The dependence of the line broadening on ionic strength implied that the binding was hydrophobic, *i.e.*, stronger binding was observed at higher ionic strengths. Although the accuracy of the measurements was rather low, they were able to demonstrate that only one or two binding sites on albumin were involved and thereby to conclude that the binding was specific. This approach has been applied by Hollis (1967) to the interactions between alcohol dehydrogenases YADH and LADH and the coenzymes NADH and NAD, by Gerig (1968) to binding of the inhibitor tryptophan to α-chymotrypsin, and by Raftery *et al.* (1968, 1969) to associations between lysozyme and monosaccharide inhibitors (Dahlquist and Raftery, 1968, 1969).

Navon *et al.* (1968, 1970) have used the method of relaxation enhancement to study the binding of inhibitors to carboxypeptidase A. The quantitative aspects of the nmr experiment were improved by employing the metalloenzyme with manganese substituted for zinc. Because divalent manganese is paramagnetic, any proton coming near the metal ion site will experience a very large dipolar field due to the unpaired electrons. This will produce a substantial decrease in the characteristic relaxation times T_1 and T_2 which determine nmr linewidths, and thereby broaden the nmr lines. Weak inhibitors, whose exchange rates between bound and free states are relatively high, had as the principal source of broadening the dipolar interactions in the bound

state, *i.e.*, the relaxation times were in the fast exchange limit. Thus, from the relative widths of the nmr lines of the inhibitor, it was possible to estimate the distances of the individual inhibitor protons from the metal ion at the active site of the enzyme. For stronger inhibitors, which exchanged much more slowly, the line broadening was dominated by the lifetime of the bound state (the slow exchange limit). In this case all protons are affected equally, but one has a measure of the kinetics of binding. For a substrate such as glycyltyrosine the measured kinetic constant corresponds to k_{-1} of the Michaelis–Menten scheme, which is difficult to measure by more conventional methods.

Proton relaxation rate enhancement by divalent manganese has also been used by Cohn, Mildvan, and coworkers to elucidate the role of the metal ion in manganese-containing biological systems. Measuring the relaxation enhancement of the water proton resonance caused by enzyme-bound Mn^{2+}, Mildvan and Cohn found that pyruvate kinase binds two manganous ions per mole (Mildvan and Cohn, 1965). The measured binding constant was shown to be equal to the activator constant for Mn^{2+} in the pyruvate kinase reaction. Further detailed studies suggested that an intermediate in the reaction was a complex containing an enzyme–Mn^{2+}–phosphoenolpyruvate bridge, and that formation of this complex involved a change in the conformation of the protein at the binding site (Mildvan and Cohn, 1966). Pyruvate carboxylase is a manganese metallobiotin enzyme. Nmr studies have demonstrated the formation of enzyme-Mn-substrate bridge complexes and have implicated the bound metal in carboxyl transfer from the E–biotin–CO_2 intermediate to pyruvate. The bound Mn has no apparent role in the formation of E–biotin–CO_2 from ATP + HCO_3^-. The measurements of $1/T_1$ of water protons have shown that L-malate, an inhibitor of the enzyme, binds to pyruvate carboxylase 30-fold more tightly than D-malate due to a more favorable entropy of interaction, and also that L-malate is a bidentate and D-malate a monodentate ligand for the bound manganese ion (Mildvan and Scrutton, 1967; Mildvan, 1970).

The magnetic resonance of nuclei other than protons has also been of some utility in biological systems. Navon *et al.* (1970) found that an Mn^{2+} ion at the active site of carboxypeptidase A caused broadening of the F^{19} resonance of NaF in aqueous solution, just as it did for the proton resonance of H_2O (Shulman *et al.*, 1966). The presence of enzyme inhibitors in the solution prevented this effect, indicating that they inhibited the enzyme by impeding access to the metal ion. Measurements of relaxation enhancement of the F^{19} resonance due to $FPO_3^=$ have suggested that an enzyme–Mn^{2+}–phosphoryl bridge complex is an intermediate in the fluorokinase reaction catalyzed by pyruvate kinase (Mildvan *et al.*, 1967).

A novel use of ^{35}Cl nmr in studying the conformation of biological macromolecules was proposed by Stengle and Baldeschwieler (1966). The quadrupole moment of ^{35}Cl provides a very efficient source of nuclear relaxation if there exists an appreciable electric field gradient at the nucleus, and if the correlation time for rotational motion is sufficiently long. Both these requirements are satisfied if a chloride ion is firmly bound in the first coordination sphere of a metal ion attached to a large molecule—such as mercuric ions bound to the sulfhydryl groups of a protein. Line broadening can be observed for binding site concentrations as low as 5×10^{-6} *M*. Marshall (1968) has applied this method to study the active site environment of α-chymotrypsin, employing a *p*-mercuribenzenesulfonyl group attached to serine as a chloride binding site. The data indicated that the degree of line broadening was sensitive to the conformation of the enzyme, *e.g.*, alkylation of the methionine residue in the active site caused a decrease in mobility of the chloride ion probe.

PROTEIN CONFORMATION

The amount of information that can be obtained from a spectrum is directly proportional to the number of lines observed. Resonances of aliphatic amino acids are observed between −0.5 and −3 ppm, protons of the backbone of the polypeptide chain between −3 and −4 ppm, and those of aromatic amino acids and nondeuterated amide protons between −6 and −9 ppm.

For single amino acids the nuclear relaxation times T_1 and T_2 are rather long, in the range 1 to 20 sec, so that the observed linewidths of the resonances are determined by the resolution of the spectrometer rather than by the nuclear relaxation. However, in proteins, which rotate more slowly, the nuclear dipole interaction creates considerably broader nmr lines. Therefore the problem of resolving individual proton resonance in the pmr spectrum of protein has been and remains a severe one. Usually certain amino acids in special chemical environment in protein can be identified, *i.e.*, peaks shifted outside the usual 0 to −9 ppm region, although under favorable circumstances imidazole residues in proteins can be identified.

Some of the types of interactions likely to cause shifts in resonance positions are effects of ring-current fields and paramagnetic center.

Ring-Current Shift. When aromatic rings are placed in a magnetic field, circulating currents of delocalized π-electrons are set up which generate a subsidiary magnetic field opposing the main field (Figure 3). This is analogous with the flow of current in a closed loop of wire. Protons above or below the plane of the ring are partially shielded from the applied magnetic field and higher fields H_0 have to be applied to achieve the resonance condition. Protons on the periphery of the ring are deshielded and their resonance peaks appear at lower fields than usual. Such ring-current fields in proteins arise not only from the aromatic rings of phe, tyr, try, and his residues, but also from prosthetic groups such as flavin and porphyrin.

McDonald and Phillips (1967a,b) presented 220 MHz

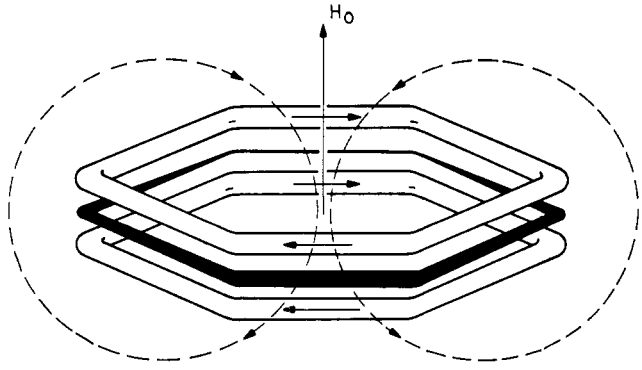

Figure 3. If the external magnetic field H_0 has a component perpendicular to the plane of an aromatic ring, ring-currents are induced in the π-orbitals and subsidiary magnetic fields are generated. Nuclei in groups lying above or below in aromatic ring experience upfield resonance shifts, and nuclei in groups lying to one side of an aromatic ring experience downfield shifts

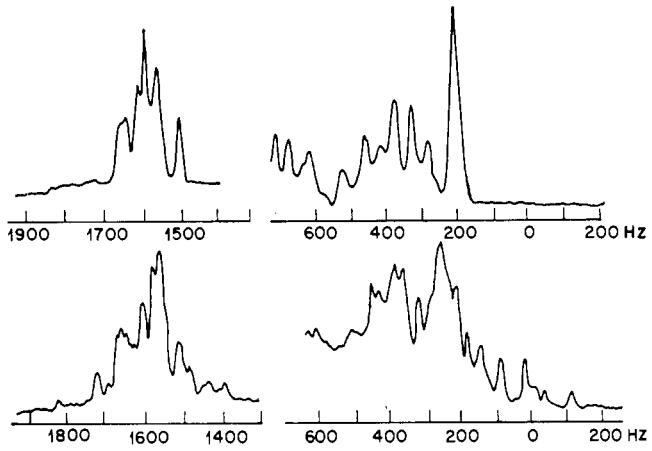

Figure 4. Changes in the 220 MHz spectrum of egg white lysozyme on cooling through its refolding temperature. The sharp peak at −0.85 ppm, which is attributed to methyl groups of apolar residues, gives way to many different peaks in the range −0.7 to +0.7 ppm when the protein adopts a native conformation (McDonald and Phillips, 1970). Reprinted with permission of: "Biological Macromolecules," Vol. 2, Marcel Dekker, New York, N. Y., 1970

spectra of native and denatured forms of RNase, lysozyme, and cytochrome c. In the denatured state, *i.e.*, random-coil, lysozyme shows a sharp peak at −0.85 ppm corresponding to methyl groups of apolar residues (40 methyl groups as components of 8 leu, 6 ileu, and 6 val) (Figure 4). A striking feature of the refolding of lysozyme was the loss of area of sharp peak at −0.85 ppm and the simultaneous appearance of many new high-field peaks in the range +0.7 ppm to −0.7 ppm. It was presumed that the folding process placed some of the methyl groups in position near the ring of aromatic residues and listed from inspection of an X-ray lysozyme model, which was already known in detail at that time (Blake *et al.*, 1967), the residues which may be subjected to ring-current shifts. An explanation of the nmr spectra of lysozyme, made by reference to the amino acid chemical shifts and the known structure, has been attempted with some success (Sternlicht and Wilson, 1967). It was postulated that a considerable amount of the absorption in the high-field arises from resonances of methyl groups of leu, ileu, and val that are shifted into this region because these residues are in an internal hydrophobic environment in the protein.

The 220 MHz spectrum of native RNase does not exhibit resonances substantially shifted to a high field from the random-coil positions of methyl resonances, indicating that for the protein there are no close interactions of methyl groups with aromatic residues. This is in accord with inspection of an X-ray model of the protein (Kartha *et al.*, 1967).

The aromatic region of resonance absorption of proteins is of particular interest because only four amino acids contribute to this region (histidine, phenylalanine, tyrosine, and tryptophan) and these residues are often implicated in the active site of an enzyme or in structural interactions. Since NH protons are replaced by deuterons in D_2O solution, the C-2 protons of histidine residues are usually the lowest field resonances observed.

The succession of nmr observations on RNase exemplifies how the technological developments have concomitantly improved the ability to resolve pmr spectra. Early 40 and 60 MHz spectra of RNase in D_2O contained six principal broad peaks whose assignment in terms of amino acids was somewhat tenuous (Saunders *et al.*, 1957; Jardetzky and

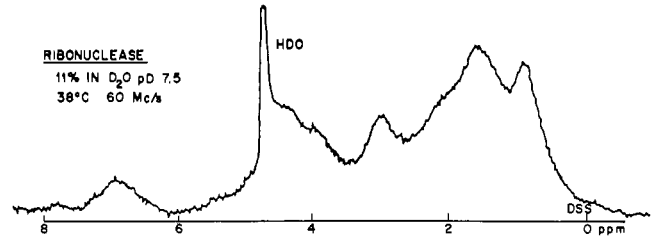

Figure 5. 60 MHz spectrum of ribonuclease A in D_2O (McDonald and Phillips, 1967a). Reprinted with permission of: *J. Amer. Chem. Soc.* 89, 6332 (1967)

Jardetzky, 1957; Kowalsky and Boyer, 1960; Boyer, 1960; Kowalsky, 1962, 1964). A typical spectrum under these conditions is shown in Figure 5. The spectra of oxidized RNase, or RNase in 8 *M* urea, showed better resolution of peaks, and a more certain assignment of the aromatic proton resonances was possible (Kowalsky, 1962). More detailed investigation at 60 MHz resulted in identification of a peak due to a C-2 proton of histidine (RNase A contains four such protons per mole) (Kowalsky, 1964; Mandel, 1965). Since histidine residues are involved in the active site "this," as Mandel put it, "provides us with a window to observe the active site under various conditions." Bradbury and Scheraga (1966), working at 60 MHz, found that the chemical shift of this resonance was very sensitive to pH, and that between pH 4.9 and 8.2 the single line became three distinct peaks corresponding to one, one, and two protons per mole. The approximate p*K* values derived from the nmr measurements led them to assign the resonances corresponding to one proton each to the active site histidines at positions 12 and 119 in the sequence, and the resonance corresponding to two protons to the histidines at positions 48 and 105. Meadows *et al.* (1967) extended this work using a 100 MHz instrument, and identified similar histidine resonances in the spectra of RNase, staphylococcal nuclease, and human and hen egg lysozyme. The increased resolution at 100 MHz showed that in fact four individual histidine C-2 proton

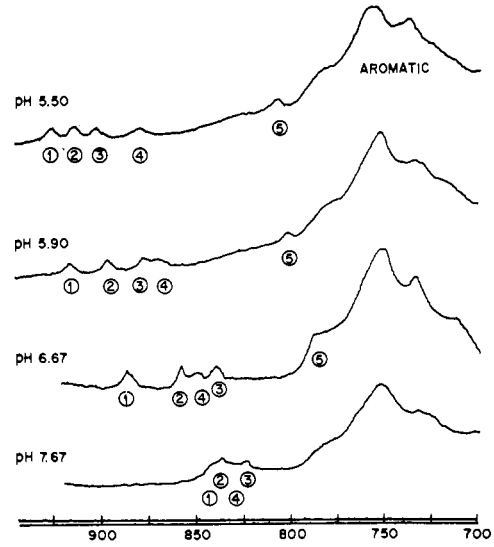

Figure 6. Time-averaged 100 MHz spectra of the aromatic region of RNase A in deuteroacetate buffer at various pH values. Peaks 1–4 are C-2 imidazole peaks due to the four histidine residues, peak 5 is a C-4 imidazole resonance, and the envelope designated aromatic includes three other C-4 imidazole peaks as well as those due to six tyrosine and three phenylalanine residues (Meadows *et al.*, 1967). Reprinted with permission of: *Proc. Nat. Acad. Sci.* 58, 1307 (1967)

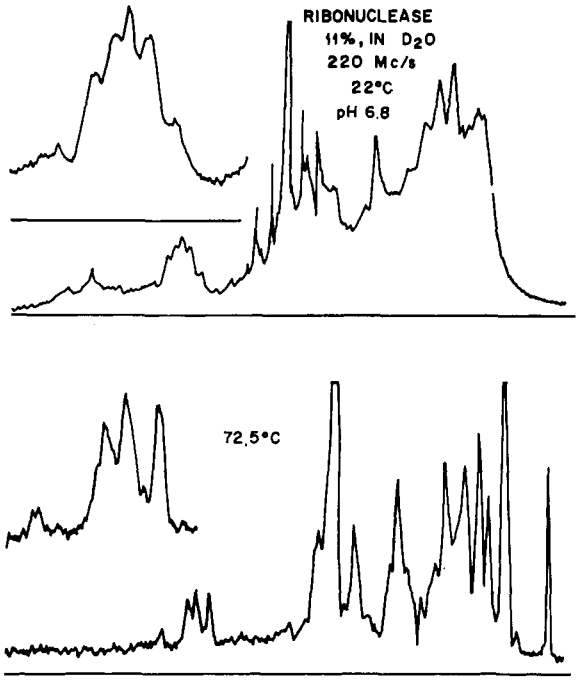

Figure 7. 220 MHz spectra of ribonuclease A in the native (upper spectrum) and denatured (lower spectrum) states. The inserts show enlarged versions of the aromatic region of the spectra (6 to 8 ppm downfield from the internal reference sodium 2,2-dimethyl-2-silapentane-5-sulfonate). The strong narrow line between 4 and 5 ppm is due to a residual amount of HDO (McDonald and Phillips, 1967a). Reprinted with permission of: *J. Amer. Chem. Soc.* 89, 6332 (1967)

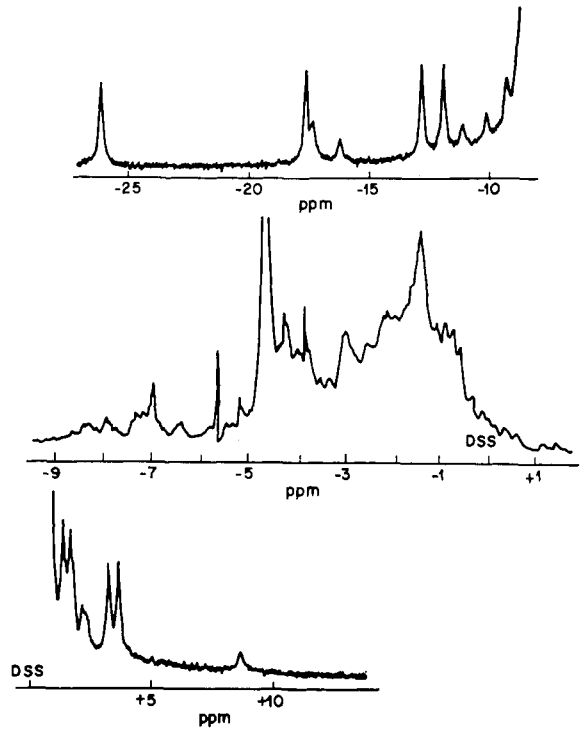

Figure 8. Proton nmr spectrum at 220 MHz of a solution in D_2O (pD 7.0) of cyanoferrimyoglobin (porpoise) at 35° C. Different horizontal and vertical scales were used for the spectral regions 1 to −9 ppm, and +1 to +10 and −10 to −30 ppm. The sharp lines between −3.8 and −6 ppm correspond to the resonance of HDO and its first and second spinning side-bands (Wüthrich *et al.*, 1968). Reprinted with permission of: *Proc. Nat. Acad. Sci.* 60, 373 (1968)

resonances could be distinguished in RNase, as well as a resonance due to one C-4 histidine proton (Figure 6). At low ratios of inhibitor to enzyme they showed that peaks 2 and 3 were shifted downfield appreciably by cytidine-5′-phosphate, whereas the other two were unaffected. This was taken as further evidence that these two peaks are attributable to histidines 12 and 119 at the active site, although on this information alone specific assignment was not possible. At a high concentration of inhibitor all four histidine C-2 peaks were shifted, suggesting a conformational change in the enzyme. The corresponding nucleoside, cytidine, had no effect on the nmr spectrum under the same conditions. Peak 4 was assigned to his-48, the buried histidine of RNase A, because of its anomalous chemical shift in the acid pH region and its greater line width, reflecting a lower mobility than the three other relatively exterior histidine residues. Peak 1 therefore should correspond to his-105. The assignment of peaks 2 and 3 to his-12 and his-119 residues was made in the following way. RNase A can be cleaved at the 20-21 peptide bond with the enzyme subtilisin. The C-2 proton of his-12 was replaced by a deuteron by exposing the *S*-peptide (residues 1–20) to D_2O at 40° C for 5 days. Then RNase was reconstituted from the *S*-peptide with nondeuterated *S*-protein (residues 21–124). From this, it was relatively straightforward to associate peak 2 with his-12 and peak 3 with his-119.

A detailed analysis of similar effects in the 220 MHz spectrum of RNase A has also been completed recently (Patel and Woodward, 1971). McDonald and Phillips (1970) have studied the thermal denaturation of RNase by 220 MHz proton nmr. A comparison of the spectra of Figure 7 with those of Figure 5 shows the vastly improved resolution obtained in the nmr spectrum of the native enzyme. The lower spectrum of Figure 7 demonstrates the dramatic spectral changes produced by thermal denaturation of the tertiary structure. Assignment of the peaks in the lower spectrum of Figure 7 is relatively unambiguous, and it should be possible to follow the gradual disintegration of the tertiary structure in detail by analysis of changes in the 220 MHz spectra with small increments in temperature.

PARAMAGNETIC SHIFT

The largest shifts of protein proton resonance from their normal random-coil positions occur when the protons are in the vicinity of a paramagnetic center in the protein-contact shifts. Such shifts, of magnitude ranging up to 90 ppm, have been detected in pmr spectra of several proteins having heme–iron prosthetic groups.

Contact shifts were first observed in the paramagnetic forms of cytochrome c and myoglobin by Kowalsky in 1965. Kurland (1968) and Wüthrich *et al.* (1968) reexamined these recently by employing improved instrumentation.

Working with cyanometmyoglobin, in which the Fe(III) is low-spin, Wüthrich *et al.* (1968) observed about nine peaks in the high-field region of the spectrum from 0 to +5 ppm relative to DSS, though none were found at higher fields and only about nine peaks at low fields in the range −10 to −27 ppm. It was clear from the magnitudes of the low-field shifts that ring-current effects could not be responsible and that the shifts were probably due to contact interactions, but in the high-field region the shifts could be attributed either to ring-current effects or to contact interactions.

Three regions of the nmr spectrum of cyanoferrimyoglobin are shown in Figure 8 (Wüthrich *et al.*, 1968). The reso-

nances of nearly all the 950 protons of the polypeptide chain fall between −0.5 and −9 ppm. Because of the large size of the molecule and its slow tumbling, the widths of the individual protein resonances are broadened to *ca.* 20–40 cps, and therefore this spectral region is not well resolved even at 220 Mc. It can be easily calculated from the chemical shifts of the amino acids that even at 1000 MHz the resolution of a protein spectrum would be far from complete.

The resonances between −0.5 and −3.0 ppm come mostly from aliphatic amino acid residues, those between −3.0 and −4.0 ppm 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 its spinning side bands. 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 7.

Since the region from DSS 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 cyanoferrimyoglobin is paramagnetic and has a very short electronic 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 was replaced with different heme groups, the contact-shifted resonances of MbCN were assigned to specific protons of the heme group and the axial histidyl residue (Shulman *et al.*, 1969a; Wüthrich *et al.*, 1970). In addition to the hyperfine shifted lines, which are dependent on temperature, the spectrum of Figure 8 contains resonances at fields higher than −0.5 ppm which are shifted by a temperature independent mechanism, *i.e.*, ring currents caused by the aromatic amino acid residues and the heme groups. These ring current shifts are very sensitive to the relative positions of the observed protons and the aromatic rings and therefore to structural changes in the proteins. The hyperfine shifts of the heme resonances in high spin ferrimyoglobin, where the sixth coordination site of the iron is occupied by a water molecule, are much larger and the lines are much broader than in cyanoferrimyoglobin. This was to be expected from the larger total electronic spin and from the longer electronic relaxation time. For deoxymyoglobin, where the sixth coordination 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 but the lines are somewhat broader. Both hyperfine and ring current shifts have been observed for paramagnetic deoxymyoglobin. However, since oxymyoglobin is diamagnetic, all resolved resonances have to be shifted by ring-current fields.

Using the X-ray coordinates of myoglobin, determined by Kendrew and coworkers, and empirical expressions to simulate the local ring-current fields, the approximate nmr positions of the different amino acid residues were calculated. The comparison of the calculated and experimental spectra indicated that the protein structures are quite similar in single crystals and in solution. Furthermore some of the

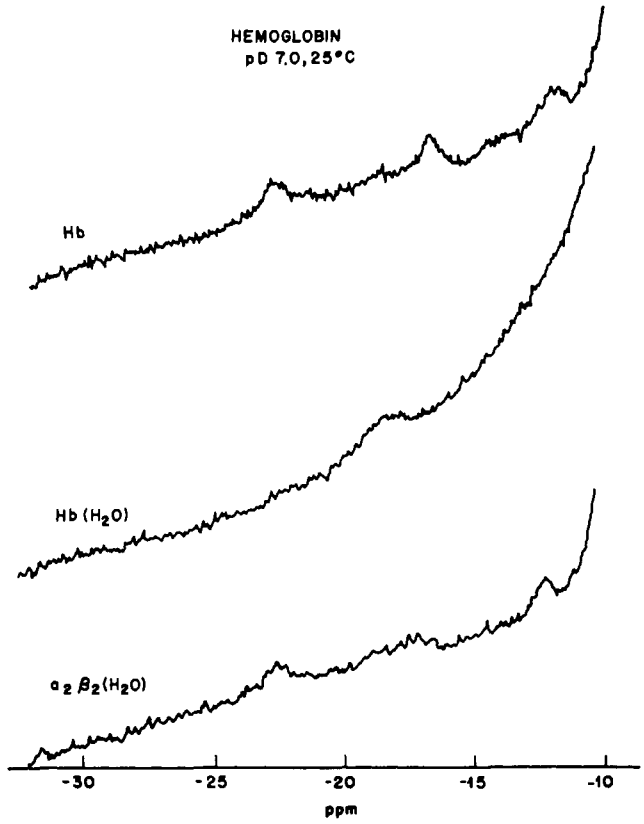

Figure 9. Comparison of the hyperfine shifted resonances at low fields of deoxyhemoglobin (no ligands bound), ferrihemoglobin (completely ligated), and partially ligated Hb[α_2,$\beta(H_2O)_2$]. The spectrum of the partially ligated hemoglobin is a superposition of the spectra of Hb and Hb(H_2O). These spectra were recorded over a period of several hours, employing a computer of average transients (Shulman *et al.*, 1969b). Reprinted with permission of: *Science* 165, 251 (1969)

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 the resonances in the spectra of oxy- and deoxymyoglobin it was apparent that oxygenation does change the structure of the myoglobin molecule (Shulman *et al.*, 1970). Even though these changes, which are estimated from the nmr data to be of the order of 0.2 Å, may be too small to be resolved in X-ray studies, they are observed in the nmr spectra as shifts of more than 100 Hz.

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. The comparison of the low-field resonances of completely unligated deoxyhemoglobin (Hb) and completely ligated ferrihemoglobin [Hb(H_2O)] to those of the partially ligated form Hb[α_2,β^{III}-(H_2O)$_2$] (Figure 9) showed that the spectrum of the partially ligated hemoglobin is a superposition of the spectra of Hb and Hb(H_2O). 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 chain, and not caused by direct heme–heme interactions (Shulman *et al.*, 1969b).

Recent nmr studies on mixed states of hemoglobin have

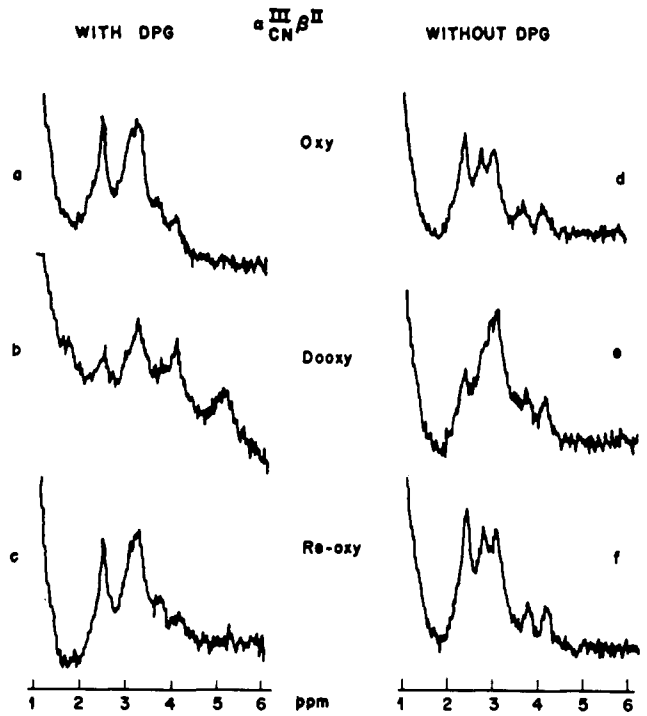

Figure 10. Effect of DPG on nmr spectra of $(\alpha^{III}CN\ \beta^{II})_2$ mixed state hemoglobin in high-field region. (a) $(\alpha^{III}CN\ \beta^{II}O_2)_2$ with DPG; (b) $(\alpha^{III}CN\ \beta^{II})_2$ with DPG; (c) b was reoxygenated; (d) $(\alpha^{III}CN\ \beta^{II}O_2)_2$ without DPG; (e) $(\alpha^{III}CN\ \beta^{II})_2$ without DPG; (f) e was reoxygenated in 0.2 *M* bis–Tris buffer pH 7.6 at 15° C. The ratio of DPG to hemoglobin tetramer was 2 (Ogawa and Shulman, 1971). Reprinted with permission of: *Biochem. Biophys. Res. Commun.* 53, 9 (1971)

shed some light on the mechanism of oxygenation (Ogawa and Shulman, 1971).

In mixed state cyano plus oxyhemoglobins, only the paramagnetic cyanoferric hemes give resonance in the low-field region between −10 and −25 ppm in D_2O buffer. α and β subunits in the cyanoferric forms give slightly different spectra and they can be distinguished in the spectrum of $Hb^{III}CN$ which is a superposition of the spectra of α and β subunits in the fully ligated mixed state hemoglobins.

The deoxygenation of $(\alpha^{II}O_2\beta^{III}CN)_2$ in phosphate buffer at pH 7.2 in D_2O did not show any appreciable changes in the paramagnetically shifted resonance lines of the $\beta^{III}CN$ subunits. However, definite changes in the nmr spectrum of $(\alpha^{III}CN\ \beta^{II}O_2)_2$ were observed upon deoxygenation. When $(\alpha^{III}CN\ \beta^{II}O_2)_2$ in phosphate buffer at pH 7.1 was deoxygenated, all the paramagnetically shifted lines of $\alpha^{III}CN$ subunits in the observable region of resonance (−10 to −25 and +2 to +6 ppm) showed substantial shifts. This indicates that the α and β subunits in hemoglobin behave differently, as one would have expected, since the β subunit is known to be flexible and the α to be a rather rigid protein.

At pH 7.6 {0.2 *M* bis–Tris [bis(2-hydroxyethyl)imino Tris(hydroxymethyl)methane]buffer} the spectrum of the $\alpha^{III}CN$ subunits in $\alpha^{III}CN\ \beta^{II}O_2$ is not affected by deoxygenation but changed upon the addition of twofold molar excess of 2,3-diphosphoglycerate (DPG) (Figure 10). This spectrum change was also observed at the lower pH values without DPG. At an intermediate pH value, *e.g.*, 7.3 (0.07 *M* phosphate buffer) at 15° C, a mixture of the changed and unchanged spectra of the $\alpha^{III}CN$ subunit was obtained.

Since no appreciable line broadening of the two different nmr spectra was observed, the lifetimes of the two states corresponding to these two spectra must be longer than ~6 msec. This spectral change is different in its character from the one induced by the pH variation of the totally ligated state of hemoglobin in phosphate buffer. Those changes are continuous and titratable, indicating fast exchange in which changes due to protonation or phosphate ion binding are very rapid compared with the frequency shift induced in the nmr lines. In that case the lifetimes must be less than 1 msec. Hence the spectral change observed upon adding DPG to $\alpha^{III}CN\ \beta^{II}$ is probably not caused by a conformational change directly induced by the binding of DPG to this mixed state.

These observations suggest that the deoxygenated $(\alpha^{III}CN\ \beta^{II})_2$ mixed state hemoglobin can exist in two structural forms, one similar to totally ligated hemoglobin and the other similar to but not necessarily identical with the totally deoxygenated hemoglobin. At least the specific binding site of DPG in the latter form should have a structure quite similar to that in hemoglobin. These observations are consistent with the report that the specific binding sites of DPG are located in the β subunits (Benesch *et al.*, 1968). Since DPG preferentially binds to the deoxy quaternary structure (Benesch *et al.*, 1968), the nmr spectral changes induced by DPG can be considered to represent transitions between two different quaternary structures which are similar to those of oxy- and deoxyhemoglobin. Since the stabilization of deoxy structure by DPG leads to the reduced oxygen affinity of hemoglobin, the two structural forms should have an important role in the oxygenation process of hemoglobin.

This finding seems to corroborate the allosteric transition model, and the authors proposed a modified allosteric transition model. Since the hemes and the heme–ligand bond are not likely to be responsible for the 2 to 3 kcal/mol of interaction energy, the large difference in oxygen affinity between the two forms of hemoglobin has to come from the protein part and especially from the interfaces of the subunits. It is likely to be the case that ligand-induced tertiary structural changes extend to the interfaces of the subunits where those changes are more strongly stabilized in one quaternary structure than in the other, and this leads to a large difference in oxygen affinity between the two structures. In this modified allosteric model, both tertiary and quaternary structures play important roles for the cooperative oxygen binding and also most probably for the binding of allosteric effectors like DPG.

CONCLUSIONS

In attempting to demonstrate briefly the scope and limitations of the nmr method as applied to proteins, many worthwhile studies have been neglected. The applicability of nmr method to a wide range of problems has been established beyond any doubt and the studies of subtle structural and dynamic aspects of proteins in solution seem to be the areas to which it could be best directed.

It is clear that useful information of the 0 to −8 ppm spectral region will not be possible even with the probable future availability of even higher polarizing fields. Resonances affected by ring-current fields and contact shifts have been very useful in elucidating protein structure and interactions. The only general method by which peaks in the spectrum can be assigned would appear to reside in isotope substitution—the selective deuterium replacement procedures such as those carried out by Jardetzky and coworkers (Markley *et al.*, 1968a,b) on a nuclease to simplify the pmr spectrum and the selective C^{13} enrichment for the as yet rela-

tively unexplored but inevitably rewarding C^{13} spectroscopy of proteins. The isotope substitution method requires the synthesis of a protein in which all the amino acids except one, two, or three are deuterated, or only few are labeled with C^{13}; the spectrum will then consist of the resonances of these amino acids. It is clear that with the preparation of a series of such isotopically substituted analogs the entire nmr spectrum can be analyzed in terms of amino acid components, at least in principle.

It can be categorically said that significant advances in relating nmr data to the subtleties of protein structure is possible only with the prior and concurrent X-ray results. The nmr and X-ray approaches are complementary in nature and this courtship will last forever.

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