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Nuclear Magnetic Resonance in Protein Research

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Introduction

Over the last two decades, investigations of structure-function relations in proteins have been based mainly on the structural information obtained from the elucidation of the amino acid sequences by chemical methods[1], and from X-ray studies of the molecular conformations in protein single crystals[2]. More recently different methods have been employed to complement the data on the covalent structure and the single crystal conformations with studies of the molecular conformations in solution, and of the dynamic aspects of the protein molecules in their biological roles. Today, after some quite spectacular advances in instrumentation, nuclear magnetic resonance (NMR) spectroscopy holds a prominent position among the techniques used for solution studies of proteins. In this survey, the principles underlying the applications of NMR to proteins will be outlined and illustrated with some typical experiments.

NMR techniques have been applied to a variety of different aspects of protein research. These include the molecular conformations in solution, the dynamics of protein conformation, the hydration of protein molecules, protein denaturation, allosteric effects in proteins, structural and kinetic aspects of the interactions between enzymes and substrates or inhibitors, intermolecular interactions between different proteins, proteins and nucleic acids, and proteins and lipids, e.g. in biological membranes. Even though this list gives only a selection of the prominent themes in the current literature, and does not include any of the analytical work, e.g. to test the purity and homogeneity of protein preparations, it may serve to illustrate the multitude of current and potential future applications of NMR spectroscopy in the protein field.

Nature has endowed proteins with a number of different nuclei which can in principle be observed in NMR experiments (Table). In practice, however, because of high sensitivity for NMR detection, which is given approximately by the product of isotope abundance and relative sensitivity (Table), is a crucial prerequisite for studies of macromolecules, biological applications were, until approximately two years ago, essentially restricted to ^{1}H-NMR. With the introduction of Fourier transform techniques, the observation of less abundant and less sensitive nuclei has also become practicable. Combined with the preparation of proteins enriched in certain isotopes, e.g. ^{2}D, ^{13}C, ^{15}N or ^{19}F, this provides now, from the technical point of view, for a greatly enlarged variety of potential applications of NMR methods.

Historically, the first NMR experiments with proteins were described in the late 1950's. Very early on two different approaches were proposed, which are

[1] *Handbook of Protein Sequences* (Ed. L. R. CROFT; Joynson-Bruvvers Ltd., Oxford, England 1973).

[2] R. E. DICKERSON and I. GEIS, *The Structure and Action of Proteins* (Harper and Row, New York 1969).

NMR nuclei in proteins

Isotope	Nuclear spin I (in multiples of $h/2\pi$)	Natural abundance (%)	Relative sensitivity for equal number of nuclei at constant field	NMR frequency at 10 kilogauss (MHz)	Electric quadruple moment Q (in multiples of $e \times 10^{-24}$ cm^2)
^{1}H	$^{1}/_{2}$	99.98	1.000	42.57	
^{2}D	1	0.015	9.65×10^{-3}	6.53	2.77×10^{-3}
^{13}C	$^{1}/_{2}$	1.11	1.59×10^{-2}	10.70	
^{14}N	1	99.63	1.01×10^{-3}	3.08	7.1×10^{-2}
^{15}N	$^{1}/_{2}$	0.37	1.04×10^{-3}	4.31	
^{17}O	$^{5}/_{2}$	0.04	2.91×10^{-2}	5.77	-4×10^{-2}
^{19}F	$^{1}/_{2}$	100	8.33×10^{-1}	40.05	
^{33}S	$^{3}/_{2}$	0.74	2.26×10^{-3}	3.27	-6.4×10^{-2}
^{35}Cl	$^{3}/_{2}$	75.4	4.70×10^{-3}	4.17	-7.9×10^{-2}
^{37}Cl	$^{3}/_{2}$	24.6	2.71×10^{-3}	3.47	-6.21×10^{-2}

now generally referred to as 'high resolution NMR', and 'relaxation enhancement studies'. In a high resolution NMR experiment one observes the nuclei of the protein, and derives structural information from the NMR parameters of the macromolecule. As a consequence of the high molecular weight, one encounters two principal difficulties in these experiments, i.e. the relatively small molar concentration attainable in solutions of even the smallest common proteins, and the limited resolution of the NMR spectra which consist of the mutually overlapping resonances of the several hundred to several thousand protons or carbon atoms in the protein molecule. Therefore, even though the proton NMR spectra of proteins could be observed as early as 1957[3], it was only after the introduction of superconducting magnets into NMR spectrometers around 1967[4], and more recently of the Fourier transform method[5], that high resolution NMR became an attractive technique for studies of proteins. With the presently available instrumentation, high resolution NMR is second only to single crystal X-ray methods in the amount of details available on the molecular conformations in proteins, and has thus become a most valuable technique for studies in solution. In a relaxation enhancement experiment one observes the influence of the macromolecule on the NMR of one of the low molecular weight components, most commonly the solvent. From these data, inferences can be drawn on structural and kinetic aspects of the interactions between the macromolecules and the observed ligand. Since the latter can be present in high molar concentrations, the requirements for sensitivity and resolution of the NMR spectrometer are frequently much less stringent than for high resolution work, and could be met by the equipment available 15 years ago. Correspondingly accounts of relaxation enhancement experiments have been quite common in the literature since 1958.

NMR parameters and protein conformation

The NMR experiment[6]. A nucleus with a spin I = 1/2 (Table) can occur in two different spin states described by the magnetic quantum numbers $m = 1/2$ (or α), and $m = -1/2$ (or β). In the absence of an external magnetic field, the two spin states are energetically degenerate and hence equally populated (Figure. 1) In a NMR experiment the sample is placed in a magnetic field H_o where the α- and β-spins have different energy (Figure 1), and as a consequence their relative populations are given according to the Boltzmann distribution law by

$$\frac{n_\beta}{n_\alpha} = e^{-\frac{\Delta E}{Tk}} \qquad (1)$$

where n_α and n_β are the number of nuclei in the respective levels. Because of this population difference the sample becomes magnetized, and a net absorption of energy is observed during irradiation with an electromagnetic field of suitable frequency. This resonance frequency is in the radiofrequency region (Table), and for a magnetic field H_o is given in hertz (Hz) by

$$\nu = \frac{\Delta E}{h} = \frac{\gamma}{2\pi} H_o (1-\sigma) \qquad (2)$$

Here h is the Planck constant, γ the magnetogyric ratio which is a constant for a given nucleus, and σ the shielding factor which accounts for the chemical surroundings of the observed nucleus.

NMR Parameters[6]. The utility of the NMR method results primarily from the sensitive variation of the resonance frequency with chemical structure and conformation of the molecules and their environment, which is accounted for by σ in equation (2), and usually referred to as the chemical shift. In practice the chemical shifts, δ, are measured with respect to a standard reference compound, and given in parts per million (ppm) of the resonance frequency ν_o. For diamagnetic organic molecules, proton chemical shifts cover a spectral region of approximately 15 ppm from the reference tetramethylsilane (TMS), or a derivative thereof, to lower field at constant radiofrequency ν (2), and carbon-13 chemical shifts extend to approximately 200 ppm to low field from TMS.

Figure 2 shows that the proton NMR in alanine which are separated by different chemical shifts, are further split into multiplets by spin-spin coupling between the protons bound to neighboring carbon atoms. The extent of the multiplet splitting is given by the spin-spin coupling constants J.

Through the radio-frequency irradiation of the sample during the NMR experiment, the Boltzmann distribution (1) of the spin states is disturbed, and in the aftermath of the irradiation, the system tends to return to the equilibrium population of spins α and β. This relaxation is an exponential process with a characteristic time constant T_1, the so-called longitudinal relaxation time.

As will be further discussed in the following, the NMR parameters δ, J, and T_1 are closely related to the size, structure, conformation and dynamic characteristics of the observed molecules.

High resolution NMR spectra of proteins in random coil forms. The NMR chemical shifts in diamagnetic organic materials are primarily determined by the covalent

[3] M. SAUNDERS, A. WISHNIA and J. G. KIRKWOOD, J. Am. chem. Soc. *79*, 3289 (1957).
[4] F. A. NELSON and H. E. WEAVER, Science *146*, 223 (1964).
[5] R. R. ERNST and W. A. ANDERSON, Rev. scient. Instrum. *37*, 93 (1966).
[6] For introductory textbooks see e.g. F.A. BOVEY, *Nuclear Magnetic Resonance Spectroscopy* (Academic Press, New York 1969); T. C. FARRAR and E. D. BECKER, *Pulse and Fourier Transform NMR* (Academic Press, New York 1971).

structure of the molecules. Some typical proton chemical shifts in amino acids are those of the aliphatic methyl groups at around 1 ppm (Figure 2), the alipatic methylene protons at 1.5–3.0 ppm, the C_α-protons at 3.5 to 4.5 ppm, the aromatic protons at around 7 ppm, and the amide protons at 7 to 9 ppm. The chemical shifts are further affected by the magnetic susceptibility of their environment in the solution. The starting point for high resolution NMR experiments of proteins are therefore the spectra of the individual amino acid residues observed in linear oligopeptides, where the amino acid side chains are readily acessible to the solvent (Figure 3A). For oligopeptides dissolved in D_2O, these spectra are qualitatively very similar to that of alanine shown in Figure 2. In particular, because the labile hydrogen atoms, i.e. those bonded to O, N, or S, are, within a few minutes, exchanged with deuterium atoms of the solvent, only the NMR of protons bound to the carbon atoms will be observed. Standard proton chemical shifts for amino acid residues in D_2O had been tabulated by McDONALD and PHILLIPS[7], and corresponding carbon-13 data have been collected in D_2O and dimethyl-sulfoxide[8].

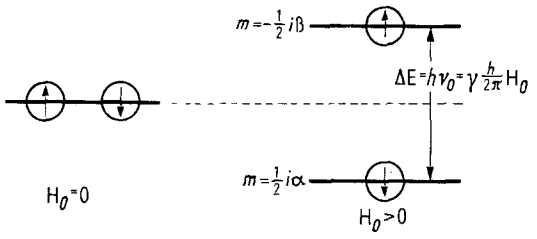

Fig. 1. Magnetic energy levels for a nucleus with spin I = 1/2 with and without application of an external magnetic field.

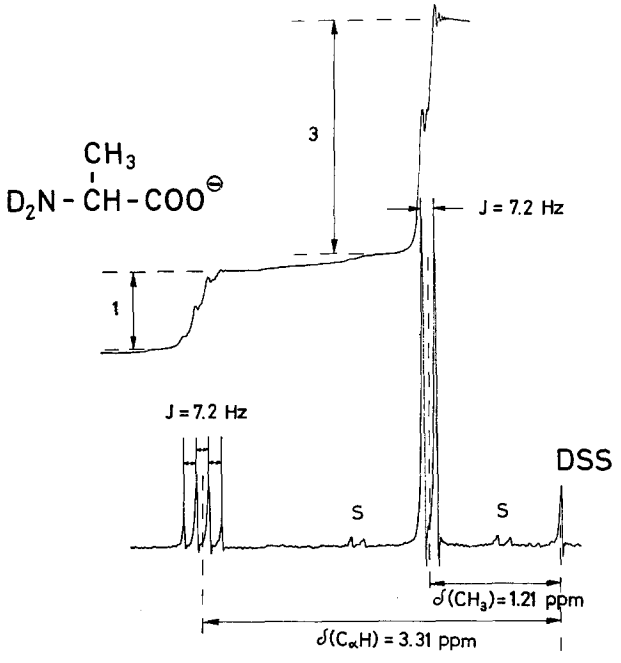

Fig. 2. Proton NMR spectrum at 60 MHz of a 0.5-*M* solution of L-alanine in D_2O, *p*D = 13.0, T = 35°. DSS, a water soluble derivative of tetramethylsilane, is used as an internal reference. The resonance assignments can be derived from the relative signal intensities in the integrated spectrum (upper trace), and from the multiplet structures. In this example, the NMR parameters δ and J can be obtained from the line positions, as indicated by the arrows. The weak lines labelled *s* are the spinning side bands of the methyl doublet resonance. The scale is in parts per million (ppm) with respect to DSS, where positive numbers indicate shifts ot lower field at constant radio frequency (equation 2).

In the context of the present discussion, a protein molecule is taken to be in a 'random coil form' when all the amino acid side chains stick freely out into the solvent (Figure 3A). From what has been said above, one would expect that the NMR spectrum of a random coil protein should very nearly correspond to a hypothetical spectrum obtained through addition of the resonances of the individual amino acid residues in the polypeptide chain[7]. This is illustrated by the Figures 4 and 5. Figure 4 shows the proton NMR spectrum of a human polypeptide hormone, calcitonin M. This peptide (Figure 5) has been synthesized by chemical methods[10], and its physiological role in the regulation of the blood calcium level has been extensively investigated[11]. NMR studies have shown that calcitonin M in aqueous solution is predominantly in an extended random coil form[12]. In the spectral regions of the aliphatic amino acid side chains between 0 and 3 ppm, and the aromatic side chains between 6 and 9 ppm, the experimental spectrum A and the computed spectrum B in Figure 4 are nearly identical. This shows that all the labile hydrogen atoms in calcitonin M had been replaced by deuterium atoms of D_2O, and that the different amino acid residues of one kind, e.g. the 3 phenylalanines in positions 16, 19, and 22 (Figure 5), are magnetically essentially equivalent. Here the methyl resonances of the five threonyl residues 6, 11, 13, 21, and 25 at approximately 1.1 ppm (the second line from the right in Figures 4 A and B) are the only apparent exception, in that they are slightly shifted with respect to each other in the spectrum 4 A[12]. Figure 6 shows a corresponding ^{13}C-NMR experiment. The basic pancreatic trypsin inhibitor (BPTI) is a 'miniprotein' which consists of one polypeptide chain with 58 amino acid residues[13]. In dimethyl-sulfoxide solution, BPTI is in a random coil form, as evidenced by the ^{1}H-NMR[14]. In the spectral regions of the aliphatic and aromatic amino

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[10] P. SIEBER, B. RINIKER, M. BRÜGGER, B. KAMBER and W. RITTEL, Helv. chim. Acta *53*, 2135 (1970).

[11] *Calcitonin 1969*, Proceedings of the Second International Symposium (Heinemann Ltd., London 1970).

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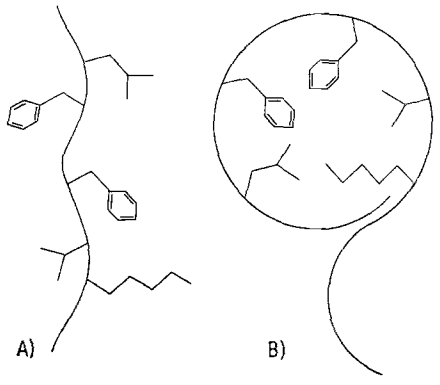

Fig. 3. Schematic representation of a segment of the polypeptide chain in an extended random coil form (A) and a globular conformation (B) of a protein molecule.

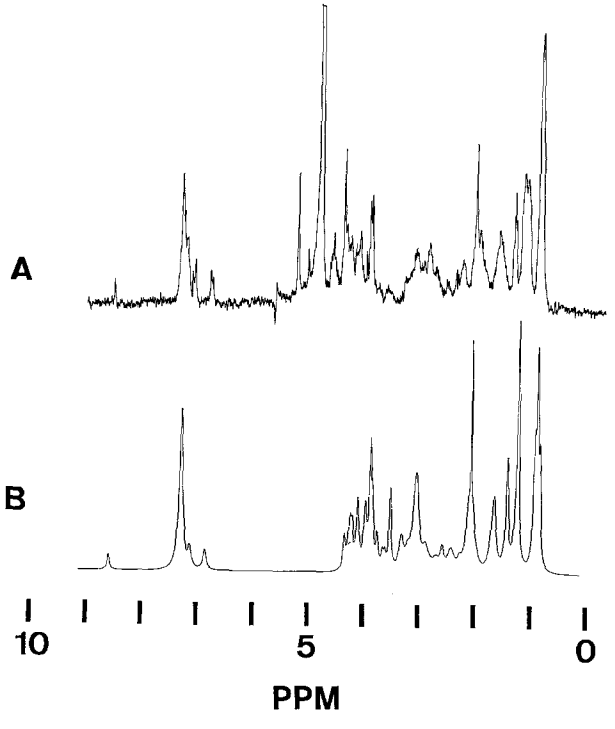

Fig. 4. Proton NMR spectrum at 220 MHz of calcitonin M in a D_2O-solution, *p*D = 5.0. A) Experimental spectrum. B) Hypothetical spectrum computed on the basis of the amino acid composition (Figure 5) as the sum of the resonances of the individual amino acid residues[7]. The strong narrow lines between 4 and 6 ppm in spectrum A correspond to the resonance of the residual protons of the solvent, and its spinning side bands. Proton NMR spectra recorded at 220 MHz or higher frequencies are recorded on spectrometers equipped with a superconducting solenoid as the source of the polarizing magnetic field H_0.

1 5 10
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-
15 20 25
Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-
30
Gly-Ala-Pro-NH_2

Fig. 5. Amino acid sequence in calcitonin M[8].

acid side chains from 0 to 30 ppm, and from 100 to 160 ppm, respectively, the ^{13}C-NMR spectrum of the random coil protein (Figure 6c) agrees again quite closely with the hypothetical spectrum computed by adding together the NMR of the constituent amino acid residues (Figure 6B)[8].

High resolution NMR spectra of native globular proteins. In Figure 7 the proton NMR spectrum of reduced cytochrome c (A and B) in neutral aqueous solution is compared with the hypothetical spectrum of the random coil polypeptide chain computed from the resonances of the constituent amino acid residues (C). Cytochrome c is a globular protein of molecular weight 12,500 which consists of 1 polypeptide chain with 104 amino acids, and 1 heme group (Figure 8). In its physiological role as an electron transport protein in the respiratory chain, it shuttles forth and back between the reduced diamagnetic state (Fe^{2+}, S = O) and the paramagnetic oxidized state (Fe^{3+}, S = 1/2), where S is the electron spin. The proton NMR spectrum of the oxidized cytochrome c is shown in Figure 8. It is readily apparent from the inspection of Figures 7 and 8 that there are quite dramatic NMR spectral differences between the hypothetical random coil form and the globular forms in both oxidation states of the protein. The resonances shifted to positions outside the spectral region from 0 to 10 ppm, which contains all the resonances of the individual amino acid residues (Figure 7 C)[7], are particularly worth noting. From the discussion in the preceding section, it is quite evident that these new spectral features arise as a consequence of the globular conformation of the protein. They are the major source of conformational information in the proton NMR spectra of this protein[15–17].

The manifestation of the protein conformation in the proton NMR spectra is due to interactions through space between different segments of the polypeptide chain. As is shown schematically in Figure 3 B, some of the amino acid side chains in the interior of a globular protein are no longer freely accessible to the solvent, and are instead in close contact with other amino acids. Across the protein molecule, the different amino acid residues of a given kind are thus in different micro-environments characterized by different local magnetic susceptibilities, and hence their chemical shifts may be different. This dispersion of the chemical shifts of structurally identical groups leads on the one hand to the

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[17] K. WÜTHRICH, Proc. natn. Acad. Sci. *63*, 1071 (1969).

characteristic poorly resolved features in the region between 1 and 9 ppm of the protein spectra (Figures 7 and 8). On the other hand it can also lead to the appearance of well resolved lines outside the spectral range in which the resonances of diamagnetic organic molecules are usually observed. This is best illustrated by the chemical shifts arising from the local ring current fields in aromatic rings[6].

If an external field H_o is applied perpendicular to the plane of an aromatic ring, it will induce a local ring current field H_R which opposes H_o in the areas above and below the ring plane, and reinforces it in the ring plane outside the contours of the molecule (Figure 9). This large diamagnetic anisotropy can cause sizeable upfield or downfield shifts of the NMR of neighboring nuclei. Thus the resonance of a methyl group located near the plane of an aromatic ring in the three-dimensional structure of a protein will appear at higher field H_o at fixed radio frequency, i.e. it will be shifted to the right in the NMR spectrum. Ring current shifts near aromatic amino acids can be as large as 2 ppm, near the porphyrin ring (Figure 8) in hemoproteins as large as 5 ppm[16]. Since in random coil peptides, the aliphatic methyl protons are observed at around 1 ppm (Figure 2) and the methylene protons at 1.5 to 3 ppm, it is readily seen that ring current fields can in principle produce resonances at high fields from DSS in the spectra of proteins. In the reduced cytochrome c (Figure 7), all the resonances between 0 and –4 ppm have been shifted upfield by the aromatic amino acids and the porphyrin ring. In particular, the line at –3.3 ppm corresponds to the methyl protons, and the lines at –3.7, –2.7, and –1.9 ppm to 3 of the 4 methylene protons of the methionyl side chain bound to the heme iron (Figure 8)[17].

The extent and the direction of the ring current shifts arising in the immediate surroundings of an aromatic molecule depend solely on the relative coordinates (d and θ in Figure 9) of the aromatic ring and the observed nuclei. Hence the ring current shifted resonances are sensitive probes for changes of the molecular conformation near the aromatic rings[7]. In hemoproteins[16] the porphyrin ring current effects are of particular interest because the heme group is an integral part of the active site in these molecules.

In view of the limited spectral resolution even at the highest presently available magnetic fields H_o, the detailed interpretation of the proton NMR in proteins has usually been limited to relatively few resonance lines in outstanding positions. In addition to ring current shifted lines, these included proton resonances of histidyl[18] and tryptophanyl[7] residues, which have both been found or postulated in the active sites of numerous enzymes, and resonances in paramagnetic metalloproteins which can be largely shifted by interactions with the electron spin of the paramagnetic center (Figure 8)[16].

Comparison of the carbon-13 NMR spectra of BPTI in the globular native and the denatured random coil form (Figure 6) shows that the protein conformation is also manifested in the ^{13}C chemical shifts. In particular it can be seen that different amino acid residues of a given type, which give rise to a single sharp line in the computed and the observed random coil spectra (Figure 6, B and C), are magnetically not equivalent in the globular protein (A, e.g. observe the line at around 115 ppm which corresponds to the ε aromatic carbon atoms of each of the 4 tyrosyl residues in BPTI).

In addition to the chemical shifts, the molecular conformation of peptide chains can also be manifested in the proton-proton spin-spin coupling constants. Of the torsion angles ϕ_i and ψ_i, which are used to describe the backbone conformations in polypeptide chains[2], ϕ can in principle be determined from measurements of the coupling constant $J_{HNC_\alpha H}$ between the amide proton and the C_α proton. The dependence of $J_{HNC_\alpha H}$ on ϕ, which obeys a Karplus-type relation[6], was the basis of numerous conformational studies in cyclic oligopep-

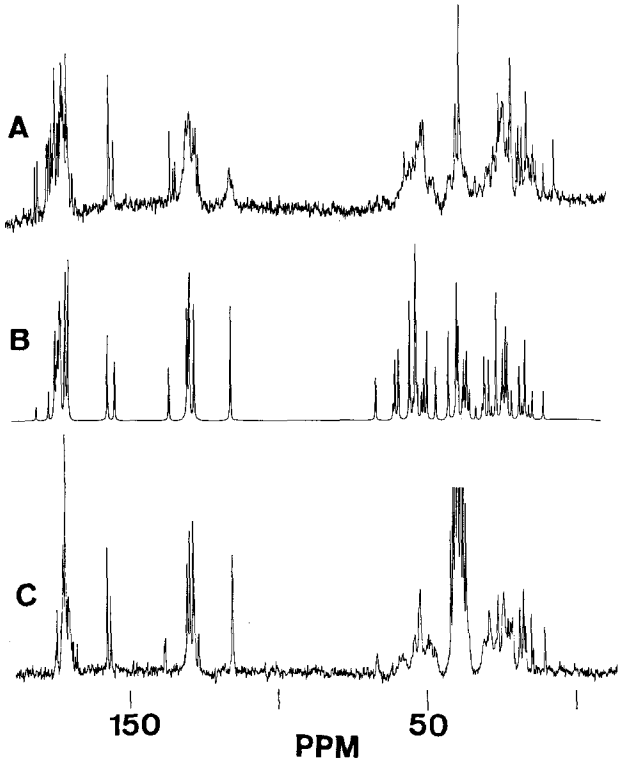

Fig. 6. Proton noise-decoupled natural abundance ^{13}C Fourier transform NMR spectra at 25.14 MHz of the basic pancreatic trypsin inhibitor. A) Native protein in D_2O solution. B) Hypothetical spectrum computed on the basis of the amino acid composition by addition of the resonances of the individual amino acid residues in DMSO[8]. C) Denatured protein in DMSO solution.

[18] G. C. K. Roberts and O. Jardetzky, Adv. Protein Chem. *24*, 447 (1970).

tides[19,20]. In the proton NMR spectra recorded in D_2O solutions (Figures 4, 7 and 8), this source of information is in most cases lost because the amide protons are replaced by deuterium. With the most advanced high resolution proton NMR spectrometers at present available, it has in certain cases been possible to measure particular ones of the parameters $J_{HNC_\alpha H}$ in H_2O solutions of proteins. This indicates that it may soon be practicable to obtain complementary information on the protein conformations from the analysis of the spin-spin coupling constants.

Protein conformations in single crystals and in solution

When the single crystal atomic coordinates of a protein are available, and the local magnetic fields of the individual components are known, the conformation-dependent proton NMR chemical shifts which would arise if the molecular conformations in the crystal and in solution were identical, can in principle be computed. Comparison of the calculated chemical shifts with the observed spectra then provides a criterion for the investigation of the relations between the single crystal and solution conformations.

In practice such an analysis will depend most heavily on those resonance lines which are shifted to extreme positions at high or low field as a consequence of the folding of the protein. Ring current shifted high field lines have been extensively investigated in different proteins[7]. Descriptions of the local ring current fields (Figure 9) are available for benzene rings[6] and porphyrin rings[16]. With d and θ (Figure 9) determined by the single crystal atomic coordinates, the ring current shifts can be computed for all the atoms in the protein. Similar procedures can be applied to assess the effects of the local dipolar field in the vicinity of a paramagnetic center, e.g. the heme groups in paramagnetic hemoproteins. Detailed comparative studies of single crystal and solution conformations have been reported for lyozyme [21] and cytochrome b_5[22].

When a close correspodance between the protein conformations in the crystal and in solution is evidenced by the above procedures, some of the largely shifted and well resolved resonance lines (Figures 7 and 8, spectral regions outside the range from 0 to 10 ppm) can in general be assigned to specific amino acid residues. These can then be used as probes for conformational changes in well-defined regions of the molecule, e.g. during interactions of an enzyme with substrates, inhibitors, or other effector molecules.

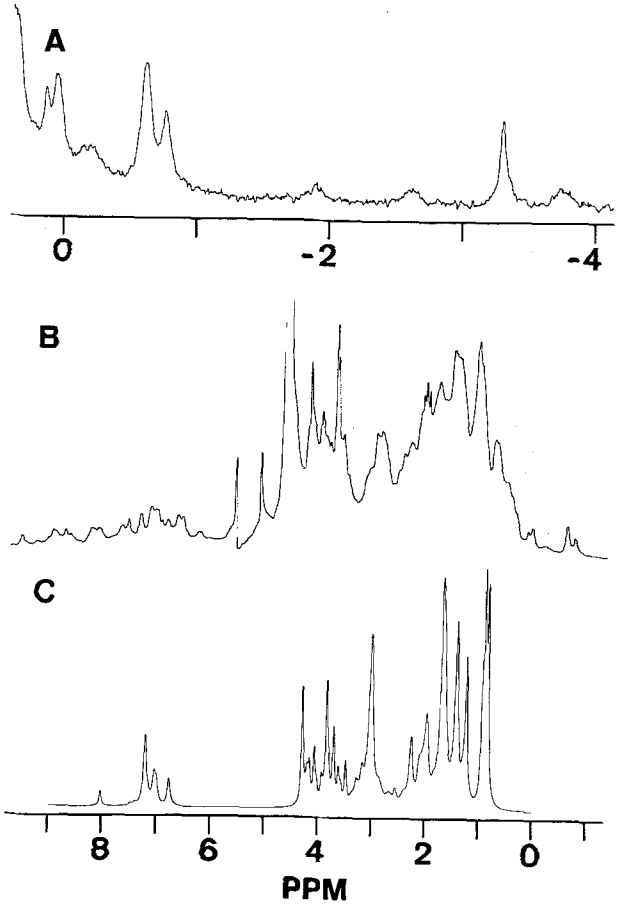

Fig. 7. Proton NMR spectrum at 220 MHz of reduced guanaco cytochrome c in a neutral solution in D_2O. A) and B) Experimental spectrum. C) Hypothetical computed spectrum of the random coil polypeptide chain. The horizontal and vertical scales are different in A, where the resonance at −3.3 ppm corresponds in intensity to 3 protons, and B, where the resonances between 0 and 10 ppm correspond to approx. 650 protons. The 5 sharp lines between 3.5 and 6.0 ppm in the experimental spectrum correspond to the resonance of the residual water protons and its spinning side bands.

Protein denaturation

The NMR spectral differences between globular and random coil proteins can be used for investigations of protein denaturation[7]. In Figure 10, this is illustrated with some experiments with BPTI[14]. The hypothetical sum of the NMR of the amino acid residues in this protein (Figure 10 C) contains no resonances at higher field than 1 ppm, between 5 and 7 ppm, and at lower field than 8 ppm. In the native BPTI (Figure 10 A), these spectral regions contain numerous quite well resolved resonance lines. At 83°, the proton NMR of the amino acid side chains (Figure 10 B, spectral regions from 0 to 3, and 5 to 10 ppm) agree quite closely with the resonances computed for a random coil form of the polypeptide chain (Figure 10 C), indicating that BPTI has

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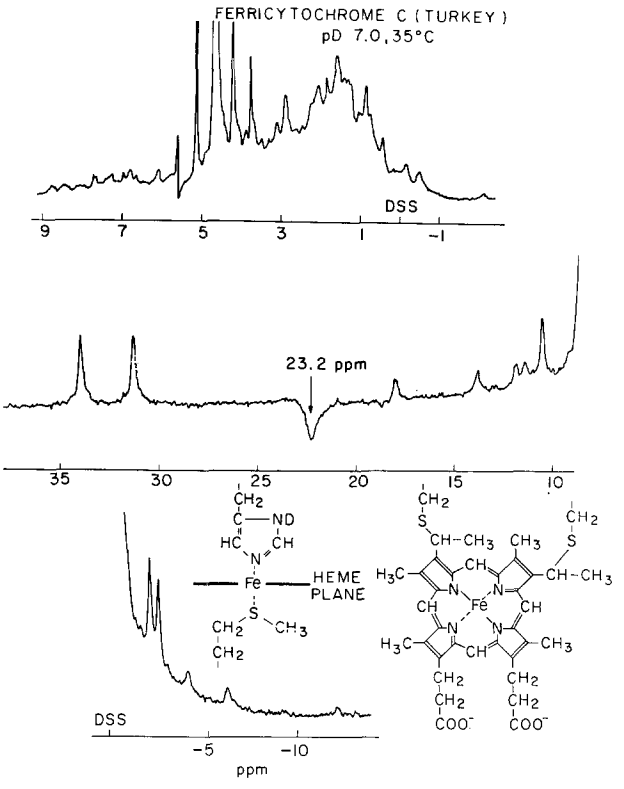

Fig. 8. High resolution proton NMR spectrum at 220 MHz of a 0.01-*M* solution of ferricytochrome c in D_2O. The sharp lines between 4 and 6 ppm correspond to the HDO resonance and its first and second spinning side bands. Different vertical and horizontal scales are used for the regions from –1 to 9 ppm, and from 0 to –10 and 10 to 35 ppm. The 2 lines between 30 and 35 ppm correspond to 3 protons each, while the resonances between 0 and 9 ppm correspond to approximately 650 protons. For technical reasons the high field resonance at –23.2 ppm appears as an inversed line in the low field region. The structures of the heme group of cytochrome c, which is an iron porphyrin complex, and of the axial ligands of the heme iron, are shown at the bottom.

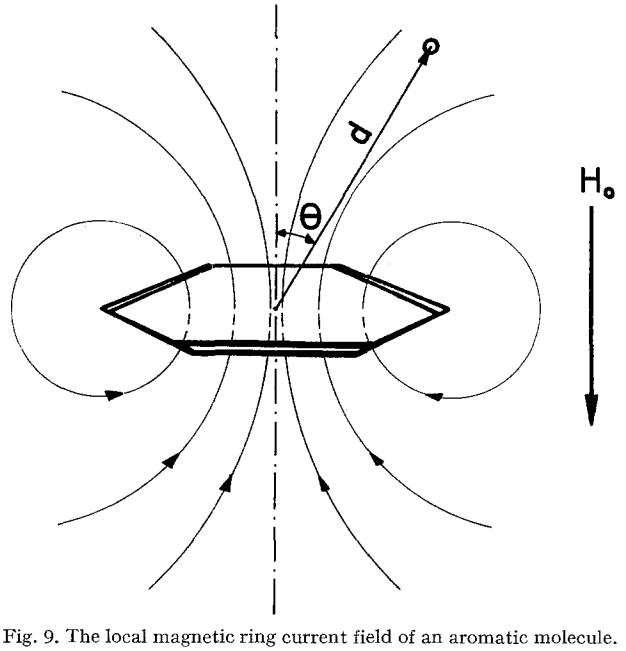

Fig. 9. The local magnetic ring current field of an aromatic molecule. H_0 is the external polarizing field. For a given ring the field strength at any point in space is determined by the position (d, θ) relative to the aromatic molecule.

been denaturated under these conditions of temperature and solvent medium. The reappearance of the resonances at 0 ppm, and between 5 and 7 ppm in the spectrum D (Figure 10) shows that the thermal denaturation was essentially reversible. The resonances between 8 and 11 ppm in Figure 10A do not reappear in spectrum D because they correspond to amide protons which had been replaced by deuterium atoms of the solvent when the protein was in the denatured form.

A particular advantage of high resolution NMR for denaturation studies is that in general the behaviour of a number of different resolved lines, which had in favorable cases be assigned to specific amino acid residues, can be observed during the denaturation. In addition to monitoring the overall course of the denaturation process, one can thus in principle simultaneously investigate the degree of unfolding of the polypeptide chain in various different regions of the protein molecule.

Dynamics of protein conformations

When random coil oligopeptides and polypeptide chains are dissolved in D_2O, the amide protons are within a few minutes replaced by deuterium of the solvent. In globular proteins, part of the labile hydrogen atoms can be buried in the interior of the molecule where they are not readily accessible to the solvent. Studies of the proton exchange rates can then yield information on the dynamics of the protein conformation, i.e. on the frequency of the occurrence of certain events in which interior parts of the globular molecule would temporarily be exposed to the solvent[23].

In the proton NMR spectra, the exchange of the labile hydrogen atoms can be observed in the disappearance of the corresponding resonance lines. If the latter are involved in intramolecular hydrogen bond formation with electronegative groups in the interior of the molecule, their resonances can be shifted sufficiently far downfield to be observable as individual resolved lines. The spectrum of BPTI in Figure 10A contains numerous resonances of slowly exchanging amide protons between 8 and 11 ppm.

An experiment in which the variations of the intensity of the low field resonances of a BPTI solution in D_2O had been measured over a period of several months, is represented in Figure 11. Comparing the spectra 11 A–C, one finds several resonances corresponding to protons which are replaced by deuterium within a few minutes to several hours, others which are exchanged within days, and lines where no change in intensity was noticeable after several months[14]. It is a particular attraction of the NMR method that the exchange rates

[23] A. Hvidt and S. O. Nielsen, Adv. Protein Chem. *21*, 287 (1966).

of individual protons, which can, in favorable cases, be assigned to specific locations in the protein[14,24,25], can be determined. When BPTI was dissolved in H_2O instead of D_2O, additional resonances of labile protons with life times with respect to chemical exchange of the order of approx. 10^{-3} sec to 1 min could be observed[25].

Intramolecular segmental motions in the time domain 10^{-8} to 10^{-11} sec can be studied by measurement of the nuclear spin relaxation times T_1. Using Fourier transform methods, T_1 can be obtained for the individual ^{13}C-resonances in the spectrum of a protein (Figure 6)[26]. The ^{13}C relaxation time T_1 for a given molecular structure is mainly determined by the rotational tumbling in the solution[6]. For proteins the relevant correlation time is determined by the combination of the overall rotational tumbling of the macromolecule and the intramolecular segmental motions. ^{13}C relaxation times can therefore be employed to compare the segmental motions of individual amino acid side chains in the globular and the random coil forms of the polypeptide chain[26]. Experiments of interest can, for example, include comparisons of the segmental mobility of the amino acids in the active site of an enzyme in the presence and absence of substrates, inhibitors, or other effector molecules.

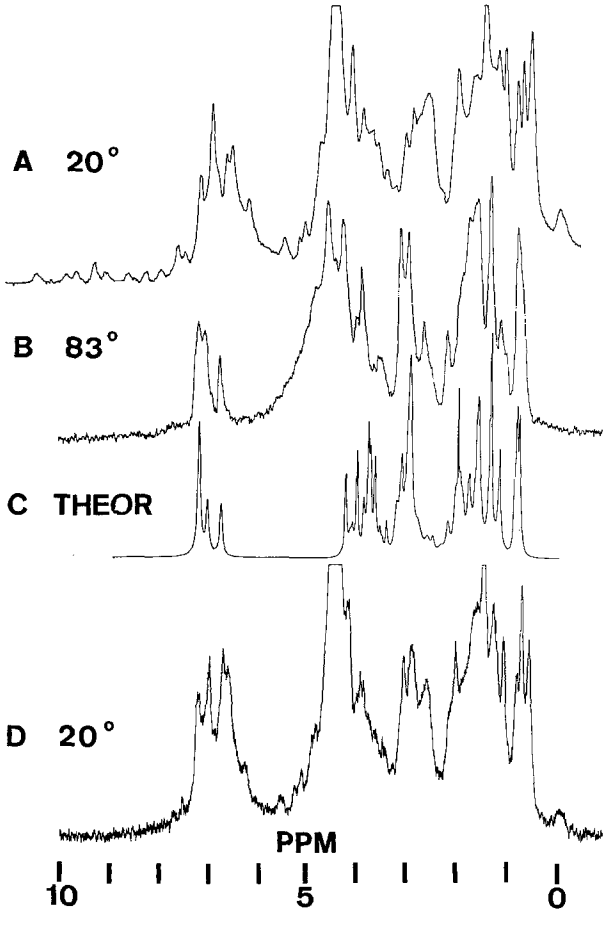

Fig. 10. Spectral changes in the proton NMR spectrum at 220 MHz during the thermal denaturation of the basic pancreatic trypsin inhibitor (BPTI) in D_2O solution containing 0.01-*M* of the protein and 6-*M* guanidinum chloride, $pD = 7.1$. A) Native BPTI at 20°. B) Denatured BPTI at 83°. C) Hypothetical computed spectrum of random coil BPTI. D) Same sample as in B, after cooling down to 20°.

Enzyme-substrate interactions

The dependence of the nuclear spin relaxation times on the rotational tumbling of the molecules is also at the basis of the so-called relaxation enhancement studies, which have for many years attracted the interest of enzymologists. In introducing this technique, let us for simplicity of presentation assume that the relaxation rate, $1/T_1$, is proportional to a so-called 'correlation time' τ_c. The effective correlation time determined by the rotational tumbling is of the order 10^{-11} sec for a small molecule like H_2O, and 10^{-9} to 10^{-8} sec for protein molecules with molecular weights from 10,000 to 50,000. It follows that the relaxation rate for the protons of a water molecule bound to the protein in such a way that its rotational motions are restricted to those of the hydrated protein is enhanced compared to that of the free water molecules. This relaxation enhancement is particularly dramatic for the nuclei of low molecular weight ligands attached to the metal ions in paramagnetic metalloproteins. The nuclear spin relaxation arising from the interactions with the unpaired electrons is then in most cases by far the most dominant contribution to the observed relaxation effects, and the relaxation rates of the nuclei bound to the protein, $1/T_{1pr}$, can be described by

$$\frac{1}{T_{1pr}} = C \cdot \frac{1}{r^6} \cdot f(\tau_c) \qquad (3)$$

where C is a constant, r the distance between the paramagnetic centre and the observed nuclei, and $f(\tau_c)$ accounts for the dependence on the effective correlation time. When the ligands bound to the metal ion are rapidly exchanging with the bulk of the ligands in the solution, the relaxation enhancement is averaged over the entire ligand population in the system. One then observes the NMR signal of the bulk of the ligand molecules in the presence and absence of the metalloprotein. The measured enhancement of the relaxation rate, $(1/T_1 - 1/T_{1L})$, where T_1 and T_{1L} are respectively the relaxation times of the ligand nuclei in the presence and absence of the protein, can be described by

$$\frac{1}{T_1} - \frac{1}{T_{1L}} = \frac{P}{T_{1pr}+\tau_{pr}} \qquad (4)$$

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[26] A. ALLERHAND, D. DODDRELL, V. GLUSHKO, D. W. COCHRAN, E. WENKERT, P. J. LAWSON and R. F. N. GURD, J. Am. chem. Soc. *93*, 544 (1971).

P accounts for the protein concentration and the number of ligand binding sites, T_{1pr} is the relaxation time of the bounds ligand (3), and τ_{pr} the life time with respect to chemical exchange of the ligand molecules bound to the protein. An important advantage of this type of experiment is that the ligands can be present in large excess, so that solutions with quite low molar concentrations of paramagnetic proteins can be investigated.

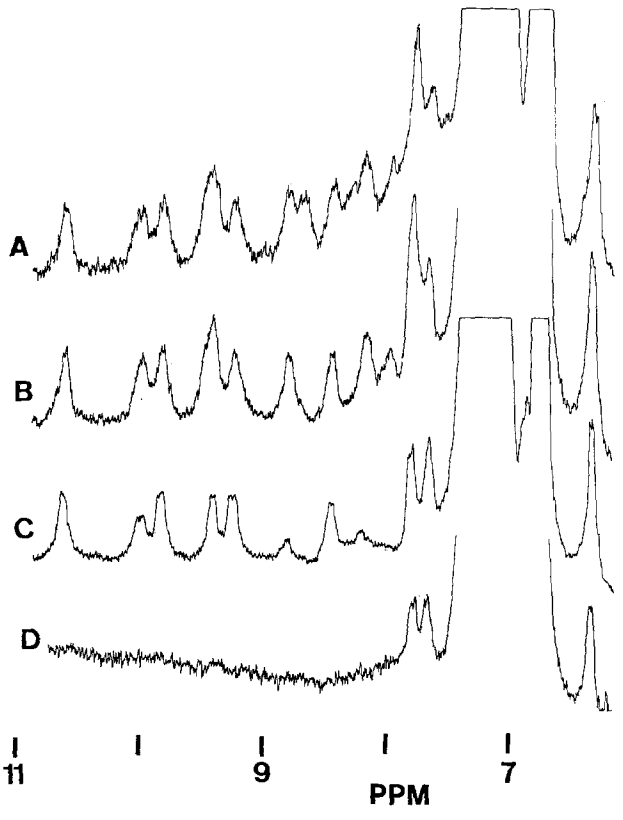

Fig. 11. Spectral region from 7 to 11 ppm of the proton NMR spectrum at 220 MHz of BPTI at different times after preparation of a 0.01-*M* solution in D_2O, *p*D = 7.3. A) After 25 min at 22°; B) After 150 min at 22°; C) After 660 h at 22°; D) After standing at 85° for a few minutes.

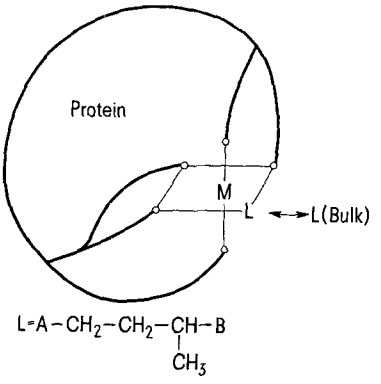

Fig. 12. Schematic representation of a situation which may arise in a solution containing a metalloprotein and an excess of a low molecular weight ligand L. It is assumed that L contains 2 potential metal binding sites A and B. Ligand molecules are rapidly exchanged between the binding site on the paramagnetic metal ion and the bulk of the solution.

A typical system which might be studied by relaxation enhancement experiments is presented schematically in Figure 12. On the basis of equations (3) and (4), a variety of qualitatively informative experiments can be developped. If one finds that $\tau_R \ll T_{1pr}$ in equation (4), the relaxation enhancement is determined by T_{1pr}. Because of the dependence of T_{1pr} on the sixth power of r, one can then decide from the extent of the relaxation enhancement whether the ligands are directly attached to the metal ion (as in Figure 12), or if the latter is, on the contrary, buried in the interior of the protein. Because of the dependence on $1/r^6$ (equation 3), one can in principle also determine the metal binding site in the ligand (Figure 12). When a system contains more than one potential ligand, e.g. H_2O and a substrate or an inhibitor, relaxation enhancement studies should in principle reveal when the different ligands compete for the metal binding sites. Overall, relaxation enhancement experiments have already had quite an impact on the elucidation of the mechanisms of action of certain metal ion dependent enzymes [27,28]. The application of the method was extended to include a variety of diamagnetic metalloenzymes after it had been found that the diamagnetic bivalent metal ions in certain proteins could be replaced by the paramagnetic Mn^{2+} without complete loss of the enzymatic activity [27,28].

Zusammenfassung

Die magnetische Kernresonanzspektroskopie hat sich im Laufe der letzten Jahre zu einer attraktiven Methode für Studien der Molekülkonformationen von Proteinen in Lösung entwickelt. In mancher Hinsicht bildet sie eine ideale Ergänzung der Einkristallröntgenuntersuchungen. In der vorliegenden Arbeit wird versucht, die Beziehungen zwischen Proteinstruktur, Proteinkonformation und den verschiedenen NMR-Parametern anschaulich darzustellen. Anschliessend werden einige ausgewählte Themenkreise etwas eingehender diskutiert: Vergleich der Proteinkonformationen in Lösung und in Einkristallen, Denaturierung von Proteinen, dynamische Aspekte von Proteinkonformationen in Lösung, Studien von Enzym-Substratwechselwirkungen [29].

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