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REVIEW ARTICLE

The study of conformational states of proteins by nuclear magnetic resonance

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Introduction

N.m.r. is now becoming widely applied to study protein conformations in solution, the internal dynamics of these conformations, and the nature and rates of conformational changes. In this article we examine the general principles behind these studies, outline some of the approaches which can be adopted, and look at a selection of results.

The conformation of a protein

It is now well established that protein molecules, whether in solution or crystals, are not rigid and that at least some of the conformational motions, see Table 1, have functional significance (Gurd & Rothgeb, 1979; Williams, 1979; Jardetzky, 1980; Porter *et al.*, 1983; McCammon & Karplus, 1983). Fluctuations occur about some average structure, and a significant region of conformational space can be explored by individual residues. One result of the existence of these fluctuations is that the precise meaning of the 'conformation' of a protein must be defined carefully. To illustrate some of the problems involved in this, one can consider first a simple molecule that exhibits well defined conformational fluctuations. A mono-substituted cyclohexane such as fluorocyclohexane exists in solution in two non-degenerate conformations, one having the substituent in an axial and the other in an equatorial configuration. Each of these conformations is defined closely, despite the existence of small fluctuations (e.g. vibrations), for example by specifying internuclear distances and angles. Given a suitable reference frame, co-ordinates can be defined for the atoms in each conformation and these provide an entirely satisfactory description of the two conformations. The conformations do, however, interconvert rapidly in solution; in fact an n.m.r. experiment at room temperature does not show distinct resonances from nuclei in the two conformations, but only averages of these (Jensen *et al.*, 1969). Directly from the experiment under these conditions one can define an 'average structure' for the molecule in solution in terms of average torsion angles or atomic co-ordinates. This does not, however, correspond to any real state of the molecule; the molecule does not adopt a structure intermediate between axial and equatorial configurations, except transiently when interconverting. In addition, the nature of the 'average structure' will differ depending on which parameters are used to define the conformations; an average of atomic co-ordinates will not produce the same result as the average of torsion angles or internuclear distances. A proper definition of the conformational state of cyclohexanes therefore requires the analysis of the n.m.r. data in terms of the two known and distinct conformations and their relative populations. For many small molecules the existence of a larger number of rapidly interconverting distinct conformers makes such conformational analysis in solution difficult. In these cases it may be possible to use diagrams to represent the various states which contribute to the overall molecular shape rather than just draw an average structure (which will have chemically unrealistic bond lengths and angles) but these representations must be used with caution (Barry *et al.*, 1971).

If we assume that distinct conformations of a protein are those corresponding to the classical rotameric states about bonds, the number of possible conformations becomes enormous. Since many of the rotamers equilibrate very rapidly this approach to conformational analysis is not possible and it is necessary to resort to some way of illustrating the average structure. The significance of any average structure of a protein deduced from experimental data depends critically on the differences between different conformations of the protein and on their populations. When the conformational differences (e.g. in atomic co-ordinates or torsion angles) are small, or the populations of very different conformations are small, the average structure (in terms of atomic co-ordinates or torsion angles) can be a good description of the overall molecular 'conformation' since it will be very close to a single true conformation. The assumption of such an average

Table 1. Protein conformational motions of known or possible function

Functional property	Motion
Ion movement in channel	Very fast movement, local around ion 'site'
Electron transfer	Fast relaxation of conformation around site
Enzyme catalysis	(*a*) Movement of side-chains with substrate transformation (*b*) Binding adjustments, induced fit
Allosteric co-operativity	Segmental shifts especially of helices (slow)
Triggering by ions	Segmental shifts especially of helices (intermediate rate)
Binding of surfaces, docking	Adjustment of surface groups (fast)
Two-dimensional diffusion on membranes or DNA	Adjustment of surface groups, e.g. lysine (fast)

Abbreviation used: n.O.e., nuclear Overhauser effect.

structure is implicit in most X-ray diffraction studies of globular proteins. [Experimental and theoretical studies of globular proteins suggest that typical r.m.s. fluctuations in atomic positions are of the order of 0.1 nm (1 Å) and in torsion angles of the order of 20° (McCammon & Karplus, 1983; Dobson & Karplus, 1985). Structural parameters defined by different techniques (e.g. X-ray diffraction and n.m.r.) often correlate well within these limits (see below).] In other cases such a description of a protein in terms of average co-ordinates or torsion angles will be inadequate for a detailed stereochemical discussion. This is likely to be the case, for example, for surface residues of folded proteins or for an unfolded denatured protein where many widely different conformations are interconverting rapidly. For the latter, only rather generalized statements are likely to be possible (for example that the protein has a certain fraction of its residues in helical structures).

A given conformational state (e.g. native, denatured, bound, unbound) is, in general, an ensemble of conformations, observed experimentally as an average whether the protein is in a crystal or in solution. A protein conformational change must then be seen as a change to another ensemble of conformations. We can present this view of a protein in a diagram similar to that used for small molecule conformations (Fig. 1). Each distinct state will be represented by a different average structure which, except for coincidence, will have a different energy. The Figure divides conformational space such that any single conformation attributed to a given state falls in a somewhat limited space (a two-state model, for example, would indicate that in a population of molecules any real single conformation would be attributed to one of these two states). The limits of any one state will vary with the compactness of the protein fold and will generally include those conformations which are reached by rapid fluctuations about the average structure.

The differences between the average structures of different conformational states may be substantial, such as those, for example between R and T haemoglobin (Dickerson & Geiss, 1983), or more dramatically between native and denatured states of proteins. The magnitude of the difference in terms of the overall protein co-ordinates is not by itself the criterion for the definition of different states. Thus, for example, the existence of both *cis* and *trans* conformations of a single proline in either a native or denatured protein could result in the experimental identification of two states, even if the r.m.s. difference in overall average structures were to be small compared with the r.m.s. fluctuations in each state. The importance of such different states of proteins has been demonstrated most clearly in studies of the folding of a number of proteins where the rates of interconversion of *cis* and *trans* prolines in the unfolded state can be the rate-limiting step (Kim & Baldwin, 1982). The significance of the protein ensemble of states has also been clearly demonstrated, for example with ribonuclease where the rate of *cis–trans* isomerization in the folded protein differs significantly from the rates found in unstructured peptides (Cook *et al.*, 1979). If this situation is represented by a diagram of the type shown in Fig. 1 the barrier to the transition between two states of a protein

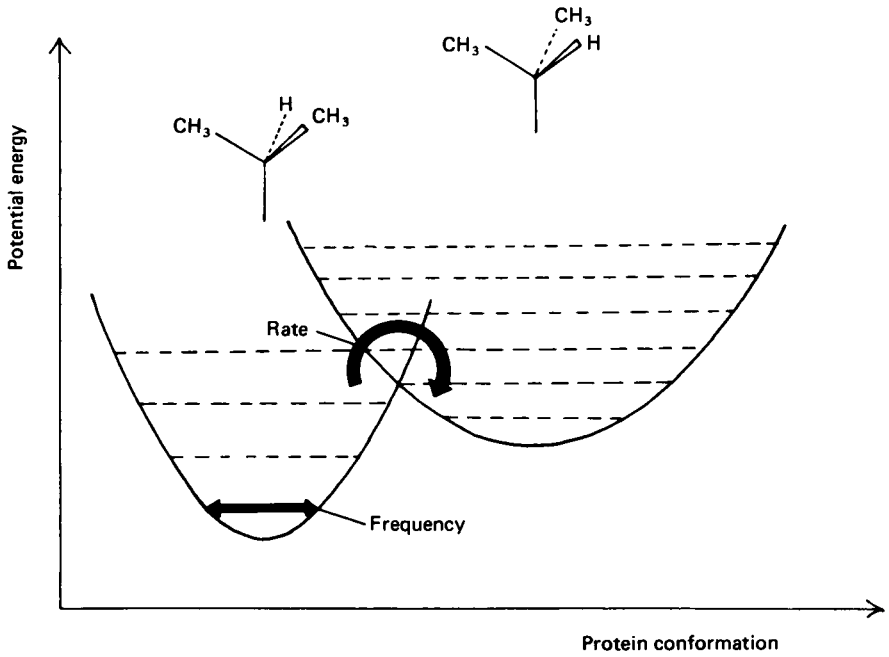

Fig. 1. Schematic representation of a protein molecule which has two conformational states, say A and B; the conformational states consist of very many individual conformations which are interconverting at high frequency

A could represent, for example, a native protein and B a denatured (unfolded) protein in which a much wider conformational space is found within a given energy range. This would represent a gross conformational change. A barrier to interconversion exists which determines the rate A→B and represents the need to pass through high energy conformations in passing from one conformational state to the other. In the Figure we have represented a very small conformational change, a rotameric reorganization of a particular valine, as also requiring a rearrangement energy arising from the co-operativity of the whole protein structure, which locks the valine side chain in one or another configuration.

can be seen as dependent on the influence of the different conformations of a proline residue on the potential surface of the protein as a whole.

In those cases where differences between different average structures are not large compared with the fluctuations then considerable care must be taken as to whether or not a conformational change can be said to occur. For example, the average structures of a protein in a crystal and in solution could differ because some of the conformational fluctuations that occur in the latter may be restricted by packing effects in the former. Similarly, binding of a small molecule to a protein could restrict conformational fluctuations in the region of binding. It does not seem reasonable to describe these as conformational changes if the average structure changes simply as a result of the changes in the magnitude of dynamical events. As a consequence, differences between conformational ensembles may have to be defined somewhat arbitrarily in a given study, generally by the relationship of observables to the property of the protein which is said to be affected by the conformational change. Some proteins, for example cytochrome *c*, crystallize with more than one molecule in the unit cell in such a way that there are readily seen conformational differences between them (Takano & Dickerson, 1981). As far as the crystal is concerned there are two different conformational states of cytochrome *c*, but as soon as the crystal is dissolved these are indistinguishable. Only some average of the two conformations observed in the crystal can be compared with experimental data in solution. If the oxidation state of cytochrome *c* is changed a new crystal form appears; it is not possible to oxidize or reduce the protein in the crystal. Observable structure parameters in solution change with the oxidation state (see below) and hence we consider that this is a significant conformational change (Williams *et al.*, 1985*a*).

N.m.r. provides a very detailed experimental basis for investigating the structure and dynamics of proteins in solution (Campbell & Dobson, 1979; Jardetzky & Roberts, 1981; Dobson, 1982; Wagner, 1983). It provides information about molecular conformations largely through local magnetic interactions; hence the average structure of a molecule is defined largely in terms of distances and angles between nuclei close to each other either through bonds (sequence) or through space (fold). For a macromolecule this approach requires considerable detail if an *a priori* study is to be made, although the observation of conformational differences in local regions may be quite straightforward.

If different conformations or conformational states of a protein exist in equilibrium with each other the effect on the n.m.r. spectrum will depend on the rate of interconversion between them (Campbell & Dobson, 1979). If the interconversion is slow on the n.m.r. timescale (usually about 10^{-3} s) separate resonances may be observed for the different conformers. In such cases the states can be distinguished and it should be possible directly to determine their populations and the rates of their interconversion. If the rate of interconversion is rapid on the n.m.r. timescale, the observed n.m.r. parameters will be an average of those for the different conformations. [The nature of this average is quite different from that observed in diffraction experiments. The latter essentially observe an instantaneous superposition of the scattering from all molecules in the crystal, which occurs because different molecules in the crystals have different conformers or because the molecules change their conformation during the period where signals are superimposed during detection. In n.m.r., the averaging depends on the phase coherence of the spins, a process which has a relatively long timescale (usually ms). The fundamental timescale in X-ray diffraction is determined by the frequency of the radiation, about 10^{20} s^{-1}.] In such a case information about the interconversion rates and the populations and nature of the interconverting forms will only be obtained indirectly from detailed analysis of n.m.r. parameters as described below.

N.m.r. and protein conformation

In order to use n.m.r. to define protein conformation it is necessary first to assign resonances to specific nuclei in the molecule. It is now possible to make extensive assignments of ^1H and ^{13}C resonances of proteins with M_r values of up to about 20000; this topic is outside the scope of this article but has been discussed elsewhere in detail (Campbell & Dobson, 1979; Wagner & Wüthrich, 1982). Then, it is necessary that the parameters obtained from a given n.m.r. experiment can be related to specific internuclear distances and angles. The basis for such relationships comes from theoretical considerations and from detailed studies of the spectra of molecules of known structure. Here, we consider the most important of these for ^1H n.m.r. spectroscopy of proteins. Although ^{13}C and other nuclei have been studied in proteins these have in general not provided information about molecular conformation but about the dynamical behaviour of specific residues (Howarth & Lilley, 1978; Gurd & Rothgeb, 1979; Richarz *et al.*, 1980). The most important n.m.r. parameters for protein conformational studies are the chemical shifts of resonances, the spin–spin coupling constants for protons separated by three bonds, and the nuclear Overhauser effects between specific pairs of protons.

The chemical shift of a proton resonance can usually be obtained directly from the n.m.r. spectrum with considerable accuracy. Chemical shifts are extremely sensitive to the average structure of the protein and can be used to characterize a given conformational state of the molecule and to detect any changes in conformation. The variety of factors affecting the shifts, however, makes it difficult to use them directly in a quantitative manner for determining structure. The dominant factor determining the chemical shifts of a proton resonance in a protein is usually the chemical nature of the group concerned. The contributions of interest, however, are those that arise because of the spatial environment of an atom in a protein (Perkins, 1982). These contributions can be estimated from the differences between chemical shifts observed in a protein and those observed for the resonances of the same amino acid in an unstructured polypeptide. One important effect in diamagnetic proteins is the shift induced by aromatic rings, the ring current shift. This shift depends on the angle θ between the perpendicular to the ring and the vector joining the centre of the ring and the nucleus whose resonance is being observed. The distance dependence is the reciprocal cube of the length of this vector (r^{-3}). This appears to be the major contribution to shifts of resonances of methyl groups, but shifts of other protons are more complicated to analyse. Those of H^{α} resonances are, for example, strongly affected by the presence of the nearby carbonyl group, and those of H^N resonance by hydrogen bonding.

Taking all these factors into consideration, reasonable correlations have been obtained between chemical shifts observed for proteins in solution, and those calculated on the basis that the average structures are those defined in X-ray crystal structures (Perkins, 1982; Hoch *et al.*, 1982; Pardi *et al.*, 1983; Dalgarno *et al.*, 1983).

Spin–spin coupling between nuclei manifests itself in the splitting of resonances into multiplets or in the appearance of cross-peaks in two-dimensional correlated n.m.r. experiments (Bax, 1982; Wider *et al.*, 1984). The coupling is transmitted via the electrons of chemical bonds and the magnitude falls off rapidly with the number of bonds involved. The most important couplings in ^1H n.m.r. spectroscopy of proteins are three-bond couplings because these can be related to the bond torsion angles. The magnitude of the coupling constant is given by an expression of the form:

$$^3J = A\cos^2\theta + B\cos\theta + C$$

where θ is the H—X—Y—H torsion angle, and A, B and C are empirical constants (Bystrov, 1976). In the proteins where data have so far been analysed, a very good correlation has been found between observed H^N–H^α couplings and those calculated from torsion angles obtained from X-ray diffraction studies (Delepierre *et al.*, 1984; Pardi *et al.*, 1984). Good correlations have also been found for coupling constants dependent on side-chain torsion angles, though these are more susceptible to the effects of conformational fluctuations (see below).

The nuclear Overhauser effect (n.O.e.) is a relaxation effect which depends on dipolar coupling between nuclear spins (Noggle & Schirmer, 1971). The effects between pairs of protons can be observed either by intensity changes in selective irradiation experiments or by detecting cross-peaks in a suitable two-dimensional experiment (Wider *et al.*, 1984). The magnitudes of n.O.e. effects are dependent on the distances (r) between pairs of protons; in the simplest case this dependence is as r^{-6}. Good correlations between experimental n.O.e.s and those anticipated from internuclear distances derived from crystal structures have been observed for proteins such as lysozyme (Poulsen *et al.*, 1980), and provided that the time dependence of the effects are studied and that consideration is given to the effects of motional averaging, quantitative estimates of distances can be made (Olejniczak *et al.*, 1981, 1984).

The high degree of correlation (essentially to the limits of accuracy of the measurements and calculations) generally found between these different n.m.r. parameters and those predicted from the average structures determined in crystals for proteins such as lysozyme, basic pancreatic trypsin inhibitor and cytochrome *c* (note that these proteins are quite rigid) suggests that, except for surface residues, the conformational fluctuations are limited, and that the average structure is a valuable concept for these proteins. The overwhelming importance of the average structure in determining n.m.r. parameters has also been demonstrated by analysis of the results of molecular dynamics simulations (Hoch *et al.*, 1982; Dobson & Karplus, 1985). Nevertheless, analysis of the averaged n.m.r. parameters has provided important information about the nature of the fluctuations of even internal residues. This has been of particular value in characterizing motions of the aromatic rings of tyrosine and phenylalanine in many proteins (see, for example, Campbell *et al.*, 1976), whilst more general information about molecular dynamics has come from analysis of nuclear relaxation rates (Campbell & Dobson, 1979). In addition, the rates of solvent exchange of individual labile ^1H atoms for ^2H, monitored by measurements of the intensities of proton signals as a function of time following dissolution in ^2H_2O, have provided important data about local and co-operative motions within proteins. Details of these studies are to be found in several recent reviews (Woodward *et al.*, 1982; Wagner, 1983; Englander & Kallenbach, 1984). Recently, considerable progress has been made towards the definition of the overall conformational states of small proteins without recourse to information from diffraction studies. The use of n.O.e. data is the key to these studies, because it is possible to observe using two-dimensional methods a very large number of effects which depend on distances between specific pairs of protons within the protein. N.O.e.s observed in this way are sensitive to internuclear separations of up to about 0.45 nm, and it is possible to construct a reasonably accurate average structure for a protein from enough of these distances even if the distances themselves are not very well defined (Williamson *et al.*, 1984; Kaptein *et al.*, 1985). The n.O.e. data can be supported by other information, particularly ^3J values which provide information about individual torsion angles. It is, of course, a very important feature of both n.O.e. data and spin–spin coupling constants that specific interactions between defined pairs of nuclei are determined directly, and so can be used independently of any other structural data. Once an outline structure is available for examination, yet further information such as chemical shifts can be used to test the proposal. Undoubtedly the importance of these studies will increase, particularly for those proteins that cannot readily be crystallized in an environment of biological interest (e.g. membrane proteins). The examination of as much data as possible is clearly desirable, particularly when the extent of conformational flexibility is in question. The wider the range of different parameters consistent with a given average structure the more probable it becomes that this is an adequate description of the protein. At the present time these very detailed studies can only be managed for M_r values of less than 15000.

We have discussed up to this point only diamagnetic systems. If a paramagnetic centre is present, as for example in a metalloprotein such as cytochrome *c* (Williams *et al.*, 1985*b*), or can be introduced, as has been done for example with lysozyme (Dobson & Williams, 1977), additional conformational information may be obtained. The shift and relaxation effects of the paramagnetic centre are often large and readily measured, because of the large magnetic moment of an unpaired electron. This enables the relative positions of residues over a wide region of a protein to be related to a single point, the paramagnetic centre, and complements the more local information available using the procedures discussed above.

When the different parameters are not consistent, within reasonable limits, with such an average structure of a protein the way to proceed is less clear. In some cases it may be possible to seek for two conformations which we can combine to represent the average structure, as would be the case for fluorocyclohexane. This solution to the problem has clear dangers but if all available n.m.r. parameters are consistent with this description it is as

good a description as n.m.r. can give. Alternative approaches are to attempt to define a distribution of conformations, for example by use of theoretical calculation to suggest likely populations of different states, which can then be tested against the n.m.r. data. N.m.r. techniques are being combined with molecular dynamics simulations in order to test and develop this approach to conformational analysis (Dobson & Karplus, 1985).

Some specific examples

In this section we discuss briefly three specific aspects of the study of conformational states of proteins by n.m.r. In the first, we look at the effect of a change of redox state of a metalloprotein using cytochrome *c* as an example. In the second, the nature of ligand-induced conformational change is examined using dihydrofolate reductase as an example. In the third, the contribution that n.m.r. can make to the study of the interconversion of folded and unfolded states is explored using lysozyme as an example.

A change of redox state: cytochrome *c*. The assignment of the ^{1}H n.m.r. spectrum of cytochrome *c* is proceeding steadily, and at present more than half of the residues have assigned resonances enabling their conformational states to be explored (Moore *et al.*, 1985; Williams *et al.*, 1985*a*). We shall concentrate here on internal residues and consider all of surface residues to be relatively flexible. The average structures of the protein in the Fe(II) and Fe(III) states are different from each other both in crystals and in solutions, and the ensembles have some conformational properties that differ between the two states.

A clear example of the alteration of the average structure in solution with redox state change is revealed by the spin–spin coupling between H^{α} and H^{β} of Thr-78. In the reduced state the value of the coupling constant is less than 4.0 Hz, but in the oxidized state it is more than 8.0 Hz (Williams *et al.*, 1985*a*). This means that the rotamer population has changed significantly, and this must be a manifestation of a change in the ensemble conformation of the whole protein as discussed above. [Interestingly, this change in torsion angle for Thr-78, and some of the changes detected by n.O.e. effects, are not observed in the crystal structures of the protein in different redox states.] Other changes are revealed by comparison of n.O.e.s in the different states, showing changes in inter-residue distances. A conformational change of the type indicated by the schematic diagram of Fig. 1 accompanies the change of oxidation state. The changes in different parts of the molecule are probably linked together in the whole protein, as for example in the R and T states of haemoglobin, by small changes in the interaction of helices (Takano & Dickerson, 1981). Similar changes have also been proposed in calmodulin and insulin where it is necessary to rotate helices in order to explain changes in protein surfaces. [A general reference to these studies is Williams (1985).] A two-state model can be used to describe the switch between the two states (although not for the kinetics of the interconversion) and we can examine the significance of the conformational change on this basis.

Cytochrome *c* is an electron transfer protein and any conformational change such as we have found will effectively reduce the rate of electron transfer unless there is coupling between the changes of the states of the iron and the protein conformation. This implies that the conformational switch has to be relatively fast, say with a relaxation time of 10^{-3} s or less. The n.m.r. studies have indicated that the environment of the haem, at least on the side co-ordinated to His-15, is highly constained, and significant dynamic processes on this timescale do not occur (Fig. 2). Elsewhere in the protein information about the dynamics has been deduced from the rates of flip of the aromatic rings of phenylalanine and tyrosine about their C^{β}–C^{γ} bonds. The rates for Phe-10, Tyr-46, Tyr-48, Tyr-67 and Tyr-97 are all slow, less than approx. 10^2 s^{-1}. This implies that the activation energies for rotation are some 100 kJ·mol^{-1} and that fluctuations of this type, or in the regions of these groups, are likely to have no significance for the kinetics of electron transfer. Other rings, however, are observed to flip rapidly. This is the case for Phe-36, Phe-82 and Tyr-74, and implies that in these regions conformations of the protein significantly different from the average structure are more readily accessible. These residues are all in the region of the side of the haem co-ordinated to Met-80 (Fig. 2). This suggests that significant motion of the protein chain between Tyr-74 and Phe-82 could be occurring in this part of the molecule. This region includes Thr-78, referred to earlier as undergoing a conformational change with redox state change. The conformational mobility in this region of the protein may allow the rapid conformational change needed for the electron transfer process. The proposal of a more labile region of cytochrome *c* in this region is consistent with other information about the protein. It has, for example, been observed that cytochrome *c* in the Fe(III) form can rapidly be attacked by CN^- and other reagents which displace Met-80 from the haem. The rate of substitution is independent of the attacking group, showing that it proceeds through an activated conformational state. N.m.r. studies hence show that the conformational state change generated by CN^- is similar in kind to that associated with a change in redox state to the Fe(II) protein.

Further n.m.r. studies of cytochrome *c* are designed to search for hinge-bending modes which lead to the opening of the haem crevice, and to attempt to describe the conformational states of surface residues. It is hoped that the ideas put forward for cytochrome *c* will provide a model for reactions of other haem proteins, such as cytochrome *P*-450. Undoubtedly in the latter case conformational changes are larger, but many factors could be similar, in particular (1) the coupling of local conformational changes between the haem group and the rest of the protein, (2) the change in the relative positions of helices and (3) the relative conformational rigidity of one side of the haem compared with the other.

Ligand-induced conformational changes: dihydrofolate reductase. Ligand-induced conformational changes have been widely observed, and invoked, in studies of proteins and enzymes (see, e.g., Steitz *et al.*, 1983). Dihydrofolate reductase from *Lactobacillus casei* has been studied in detail by n.m.r., observing not only ^{1}H resonances of the protein but also those of other nuclei including ^{13}C, ^{15}N and ^{19}F incorporated into ligands. Two recent studies of this protein demonstrate the influences of ligands on the equilibrium distribution of conformational states, and how n.m.r. studies can describe these.

Clore *et al.* (1984) studied the binding of 3′,5′-

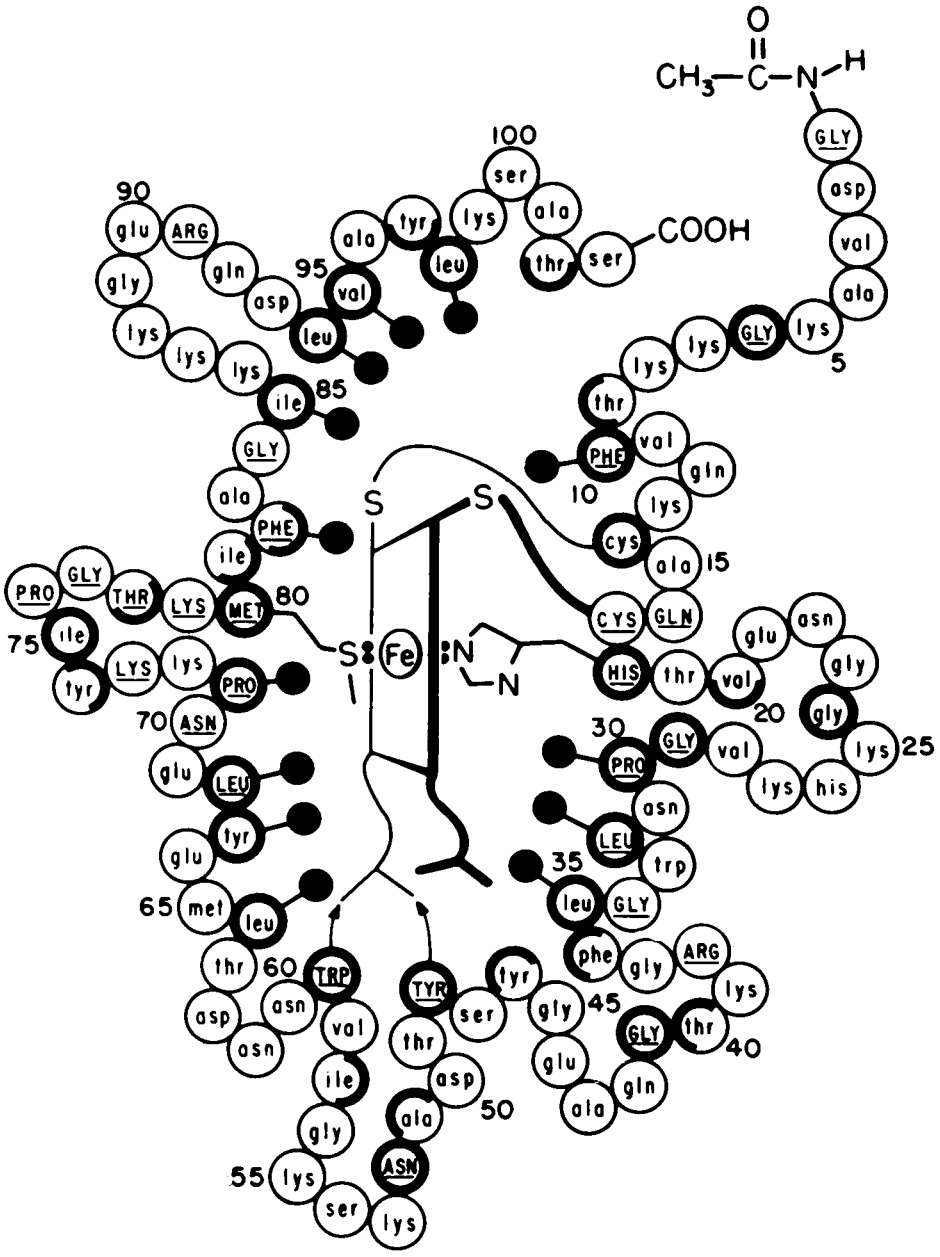

Fig. 2. Diagramatic representation of the structure of cytochrome *c*

Capital letters show invariant residues, heavy circles or parts of circles the degree of burial, and filled circles the haem contact residues. Very many of the exposed residues are conformationally mobile; most of the haem contact residues flip very rarely, but there are special regions of conformation mobility probably connected to reactivity, e.g. Phe-82, or to antigenicity, e.g. Ile-57.

difluoromethotrexate to *L. casei* dihydrofolate reductase by ^{19}F n.m.r. Using analysis of the resonance linewidths the benzoyl ring of the bound difluoromethotrexate was observed to flip about its symmetry axis at a rate of $7.3 \times 10^3\ s^{-1}$ at 298 K. Addition of the coenzymes $NADP^+$ or NADH to form the enzyme–difluoromethotrexate–coenzyme ternary complex increased the rate of ring flipping by a factor of 2.7 as well as causing substantial changes in the ^{19}F chemical shifts. These observations clearly imply some changes in the distribution of conformational states but do not give precise information on the nature or populations of these states.

In another study of dihydrofolate reductase, Birdsall *et al.* (1984) characterized two co-existing conformational states of the enzyme–trimethoprim–$NADP^+$ complex. Two approximately equal populations of slowly interconverting conformational states were observed. These states were characterized by two sets of ^1H and ^{31}P chemical shifts for the coenzyme and by separate shifts of particular residues on the protein. The relative populations of the two states was observed to vary between 0.4 and 2.3 when various trimethoprim analogues were bound to the protein. For the enzyme–trimethoprim–$NADP^+$ complex, the rate of interconversion has been measured (Gronenborn *et al.*, 1981), and

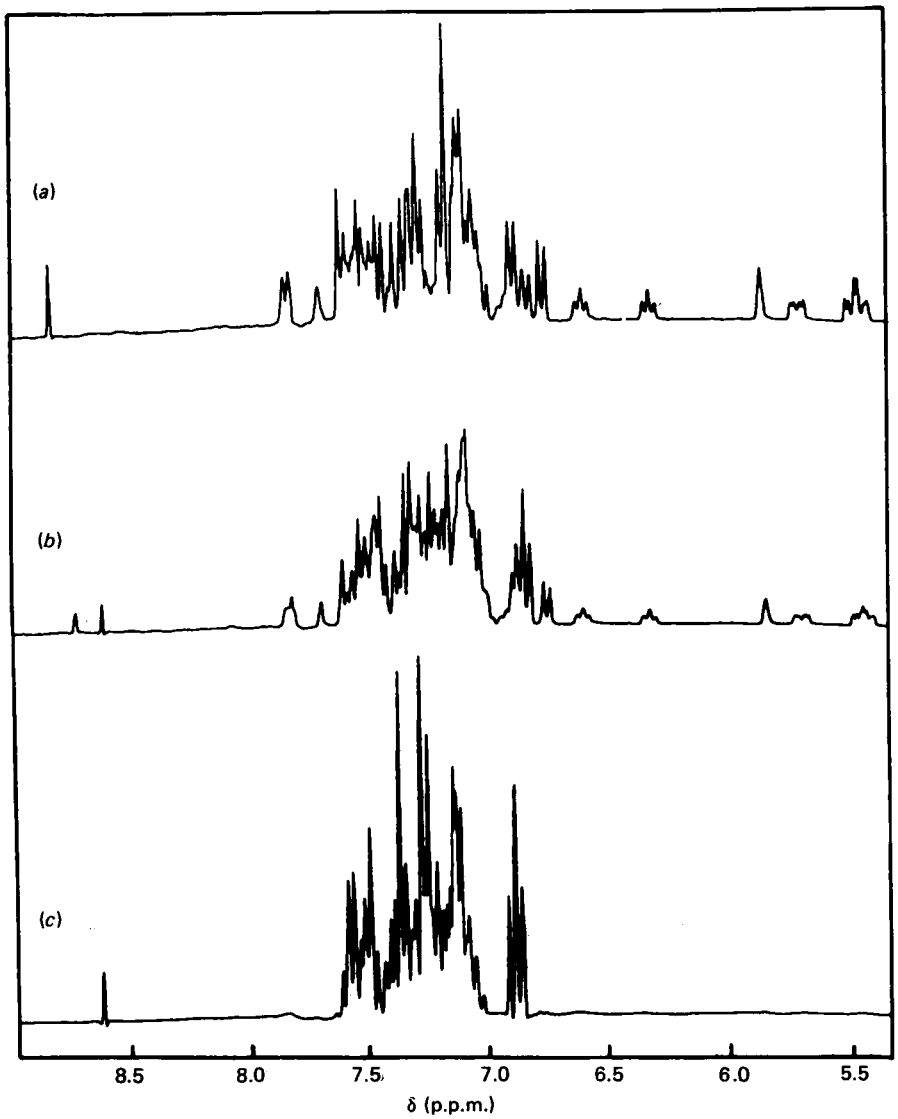

Fig. 3. Low-field regions of 300 MHz ^1H n.m.r. spectra of 5 mM-lysozyme in ^2H_2O at pH 3.8

Resolution has been enhanced by Gaussian multiplication, and labile ^1H atoms exchanged for ^2H atoms. The top spectrum, at 65 °C, is of native lysozyme and the bottom spectrum, at 82 °C, is of denatured lysozyme. The middle spectrum, at 77 °C which is close to the mid-point of denaturation, shows the superposition of the spectra of both native and denatured states. [From Dobson & Evans (1984).]

it is interesting in the context of this review that the nature of the two conformations remains the same for all the trimethoprim analogues, just the proportions changing. This indicates that the nature of the conformational equilibrium is largely determined by the protein structure. Each state of the protein could be characterized and n.O.e. measurements were used to determine the conformation of the bound ligands. In one conformation the nicotinamide ring of the coenzyme appears to swing away from the enzyme surface into solution. In addition ^{13}C and ^{15}N experiments demonstrated the protonation states of the trimethoprim in the two conformations. This is a good example of the important point that if the n.m.r. resonances of two distinct conformers can be observed separately then it is possible to define these conformers, at least in relative terms. If this is not the case, as in the first dihydrofolate reductase example, then the information obtained is less precise.

Folding and unfolding of a protein: lysozyme. The level of assignment of the ^1H n.m.r. spectrum of lysozyme from hen egg white is similar to that of cytochrome *c* (Delepierre *et al.*, 1984). This has permitted detailed studies of the structure and dynamics of the native state of the protein to be made (Dobson, 1982), including investigation of conformational changes associated with inhibitor binding (Dobson & Williams, 1975). Recently, the n.m.r. studies have been extended to explore the interconversion of the folded and unfolded states of the protein, and to begin to define the conformational properties of the unfolded protein (Dobson *et al.*, 1984; Dobson & Evans, 1984).

Fig. 3 shows part of the ^1H n.m.r. spectrum of lysozyme at different temperatures; the resonances between 6.0 and 8.8 p.p.m. are all from aromatic protons. Large differences are clearly observed between the spectrum of the protein above and below the temperature at which denaturation

occurs, and much of the dispersion of the chemical shifts characteristic of the folded state has been lost on unfolding. The spectra also show that the rate of interconversion of the two states is sufficiently slow that, when both exist in significant concentrations, the n.m.r. spectrum is a sum, rather than an average, of the spectra characteristic of the different states. Because of this slow exchange, analysis of the intensities of resonances corresponding to the different states has permitted the denaturation temperature and the enthalpy of denaturation to be measured for individual residues in different regions of the protein (Wedin *et al.*, 1982). The close similarity of the values obtained demonstrates directly the high degree of co-operativity of the process under these conditions. [That intermediates exist between folded and denatured states, has been shown by n.m.r. in the case of ribonuclease (Benz & Roberts, 1975).] No direct information, however, can be obtained from experiments of this type about the kinetics of interconversion of the two states or about the nature of the unfolded state. The experimental approaches that we have adopted to provide this information are based on magnetization transfer experiments.

The most straightforward magnetization transfer experiment involves selective irradiation at the resonant frequency of a given nucleus for a fixed length of time prior to accumulating the n.m.r. spectrum. If interconversion takes place between different states of the molecule, and provided the nucleus in question has a different resonant frequency in each state, the saturation caused by the irradiation can be transferred to the resonance of the nucleus in the different state. An alternative approach is to use two-dimensional n.m.r.; the technique is identical to that used to observe n.O.e.s. Here, cross-peaks correlate the frequencies of resonances in the different states. Both these approaches have proved successful in studying the folding and unfolding of lysozyme.

A quantitative analysis of the magnetization transfer effects can provide detailed kinetic information. In the case of the irradiation experiment, for example, increasing the length of time of irradiation increases the extent of magnetization transfer. From a combination of such time dependencies it has proved possible to obtain independent values for both the folding and unfolding rates of individual residues of lysozyme, and to determine apparent activation energies for the reactions (Fig. 4). The very similar values obtained for different residues show again the highly co-operative nature of the folding and unfolding reactions and demonstrate the validity of the method (Dobson & Evans, 1984).

One of the immediate results of magnetization transfer experiments of the type described here is that the correlation between resonances in the folded and unfolded states enables assignments to be made in the unfolded state spectrum of resonances of those protons assigned in the spectrum of the folded state (Dobson *et al.*, 1984). Because of the complexity of the unfolded state, and the absence of structural information about it, these assignments would be essentially impossible without this procedure. Assignment of the spectrum of the unfolded protein is an essential step towards the detailed characterization of its conformational properties. Conformational analysis of the type discussed previously would, in principle, permit a description of the various structures involved. In practice the analysis would be

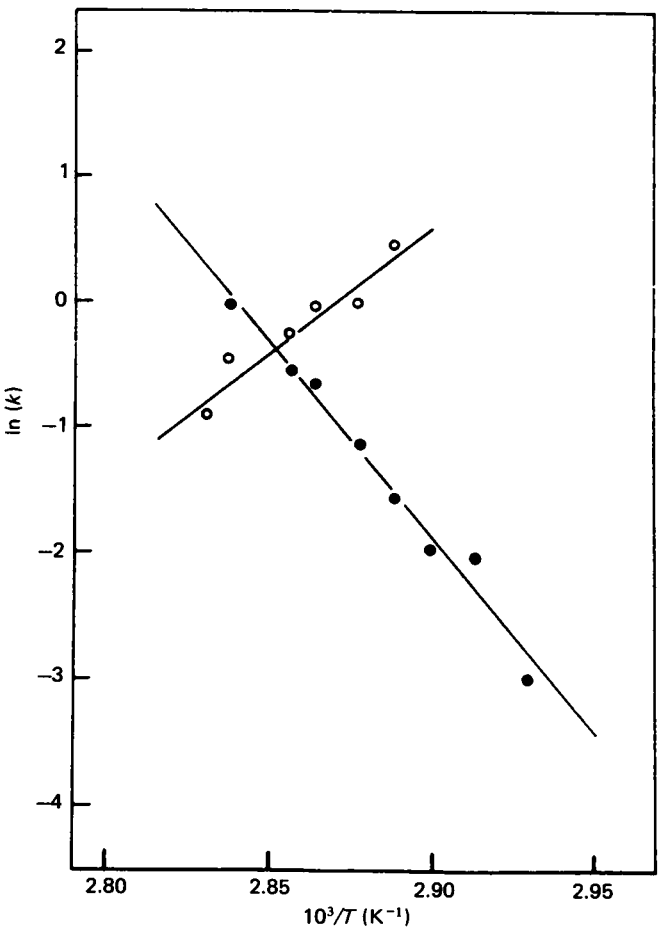

Fig. 4. Temperature dependence of the unfolding (●) and folding (○) rates of lysozyme at pH 3.8

The values were derived from a study of the time developments of saturation transfer between resonances of His-15 in the folded and unfolded states. Closely similar values were obtained from experiments involving resonances of Cys-64 and Met-105, situated in different regions of the protein structure. The ratio of the two rate constants at each temperature agrees closely with the equilibrium constant for the denaturation process measured independently from resonance intensities. [From Dobson & Evans (1984).]

extremely complex. An alternative approach that we are exploring involves complementing such a study with one based on the combination of the kinetic measurements referred to above with results from n.m.r. studies of hydrogen exchange. At temperatures close to the unfolding transitions, the most rapid route for exchange with solvent for labile hydrogens buried in the folded structure may be via the unfolded state (Wedin *et al.*, 1982). Measurement of the exchange kinetics of individual hydrogens, observed by the decrease in the intensities of resonances in the spectrum of the native protein, in conjunction with the kinetics of folding and unfolding for individual residues, will then provide information about the lability of hydrogens in the unfolded state. This information should enable regions of persistent structure in the unfolded state to be identified. These may represent localized elements of secondary

structure which subsequently combine, leading to more extensive folding.

The approach outlined here for lysozyme should be applicable to other proteins, provided that conditions can be chosen such that the interconversion between folded and unfolded states is fast enough to compete with spin-lattice (T_1) relaxation. In practice this is likely to mean that rates in the range of 0.1–10 s^{-1} can be investigated. One system of particular interest under study is staphylococcal nuclease where interconversion of more than one folded form with the unfolded form can be studied using these methods (Dobson *et al.*, 1985).

Summary

By the use of examples, mainly of rather rigid proteins, we hope to have shown that conformational analysis of proteins is a problem that is not simply related to the conformational analysis of small molecules. The primary difficulties with proteins are (1) the multitude of possible conformers, (2) the complex dynamical behaviour and (3) the degree of co-operativity within the molecules. Any experimentally derived structural description of a protein is an attempt to represent some average of a complex time dependence. N.m.r. techniques have now reached the point where it is possible to use them to describe many detailed structural features of small globular proteins in solution and to detect and to describe conformational changes in such proteins. In addition, analysis is becoming possible of much less ordered regions of polypeptides, such as are found in less compact proteins, of for example myosin, histones and virus coat proteins, or in denatured states. The limits to the detailed conformational analysis of such proteins are likely to be ones of reality rather than method but the description of the properties shown in Table 1 is by its very nature an extremely important problem in conformational analysis of dynamic macromolecules.

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