Experimental NMR techniques for studies of biopolymers

Ad Bax

National Institutes of Health, Bethesda, Maryland, USA

NMR technology has advanced significantly during the past year. Four-dimensional NMR spectroscopy is a feasible approach for unraveling the extremely crowded NOE spectra of medium-sized proteins. Alternatively, the use of selective deuteration also shows promise in this area. New three-dimensional NMR techniques can be used for accurate measurement of multiple-bond J couplings and improved methods are appearing for measurement of heteronuclear relaxation times, providing a more accurate characterization of internal protein dynamics.

Current Opinion in Structural Biology 1991, **1**:1030–1035

Introduction

During the second half of the 1980s, NMR has become firmly established as a technique with which to determine the solution structure of small proteins, up to ~12 kD (for a recent review, see [1•]). For larger proteins, the increase in the number of proton resonances and the increase in the line width of these resonances cause problems that frequently cannot be overcome for the standard two-dimensional (2D) ^{1}H NMR experiments. Many of the recent methodological developments are aimed at increasing the molecular-weight barrier of proteins that can be solved by NMR. Most of the new approaches, therefore, rely on the incorporation, either selectively or uniformly, of the stable isotopes ^{2}H, ^{13}C and ^{15}N. Many of the recently reported three- and four-dimensional techniques that rely on the use of these isotopes have been described in detail by Clore and Gronenborn [2,3,4•]. For proteins, nucleic acids, and oligosaccharides that cannot easily be labeled isotopically, homonuclear ^{1}H three-dimensional (3D) NMR appears to present the only hope for successful studies of larger systems.

Information about molecular structure in solution is derived from two kinds of NMR parameters: the nuclear Overhauser effect (NOE) provides information about interproton distances; and, homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{15}N or ^{1}H-^{13}C) J couplings contain information about torsion angles. J couplings can also aid the interpretation of NOE spectra by providing stereospecific assignments of non-equivalent C_{β} methylene protons. In the past year, several methodological advances relating to measurement of these couplings have appeared.

Frequently, the main interest lies not in determining the entire protein structure, but in studying the conformation of a flexible ligand when tightly bound to a protein, and the conformation of the protein in the vicinity of the bound ligand. Recently, the use of isotopic labeling has led to significant advances in this area, and solid-state NMR developments also appear promising.

NMR relaxation times can provide information about the local dynamics in a protein. Although ^{1}H relaxation times frequently are difficult to interpret (because small uncertainties in local geometries can have large effects on relaxation times), relaxation of the stable isotopes ^{15}N and ^{13}C is much less sensitive to these effects. The relative ease with which many proteins can be isotopically enriched has led to a surge in the popularity of studying protein dynamics. However, techniques previously used in the measurement of these relaxation times have recently been shown to yield small but systematic errors that can be overcome by altering the experimental approach.

In this review, I focus on the recent methodological developments outlined above and restrict myself to those techniques that I believe to be of the most immediate importance to the study of the structure, function or dynamics of biopolymers.

Homonuclear three-dimensional NMR

The problem of spectral overlap in homonuclear 2D ^{1}H spectroscopy can be alleviated by increasing the spectral dimensionality to three. Although original 3D experiments focused on a combination of J connectivity and NOE techniques [5,6], a recent preliminary report by Breg *et al.* [7•] has indicated that a 3D experiment con-

Abbreviations

COSY—correlated spectroscopy; **2D**—two-dimensional; **3D**—three-dimensional; **NOE**—nuclear Overhauser effect; **NOESY**—NOE spectroscopy; **H(CA)NHN**—α-proton (via α-carbon) to nitrogen to amide proton correlation; **HN(CO)CA**—amide proton (via carbonyl) to α-carbon correlation; **HOHAHA**—homonuclear Hartman–Hahn spectroscopy; **ROESY**—rotating frame Overhauser enhancement spectroscopy; **TOCSY**—total correlation spectroscopy.

Current Biology Ltd ISSN 0959-440X

taining two NOE transfer steps may be of considerable value in obtaining resonance assignments and structural information in proteins. It is anticipated that the 3D NOE-NOE experiment can yield more accurate ^1H-^1H distance information because the effect of spin diffusion on NOE cross-peak intensity can be satisfactorily quantified in this type of experiment. The fact that the spectral overlap in this experiment is reduced compared with that derived from 2D NOE spectroscopy (NOESY) specrtra should make this approach useful in the study of not only proteins but also nucleic acids and carbohydrates, where the problems of spectral overlap can be extremely severe.

Heteronuclear three- and four-dimensional NMR

Where one wishes to study proteins that can be isotopically enriched, highly sensitive heteronuclear 3D NMR techniques can be used, which typically yield well resolved spectra. In contrast with homonuclear 3D NMR, it is important to note that in heteronuclear 3D NMR the number of resonances is usually unchanged relative to its 2D ^1H-^1H counterpart; the information obtained from the 2D and from the heteronuclear 3D spectrum are of a similar nature but the overlap in the 3D spectrum is greatly reduced. In the case of homonuclear 3D NMR, the resonances of interest experience two independent mixing processes and the number of resonances is therefore much larger and resonance intensity is correspondingly lower. However, the homonuclear 3D spectrum can contain important additional information, e.g. spin diffusion data [8], not obtainable from heteronuclear 3D spectra.

Heteronuclear 3D and 4D NMR experiments can be used to generate resonance assignments, to obtain ^1H-^1H NOE-based distance constraints, or to obtain torsion angle constraints from the measurement of J couplings. New methods have been developed for each of these applications.

Assignment techniques

For proteins which can be labeled uniformly with ^{15}N and ^{13}C, a backbone assignment approach has been described which relies exclusively on J coupling connectivity and not on conformation-dependent NOEs [9•]. The experimental techniques used in this study have been described elsewhere in detail by Kay *et al.* [10]. Because magnetization is transferred mainly through relatively large J couplings, which are resolvable for proteins as large as 30 kD, the new methods provide an extremely straightforward approach for assigning the backbone ^1H, ^{13}C and ^{15}N resonances in proteins of a substantial size. In the past year, two new methods have been added to the arsenal of triple-resonance techniques that can be used for obtaining backbone assignments.

One technique, α-proton (via α-carbon) to nitrogen to amide proton correlation (H(CA)NHN) [11•], provides intra-residue connectivity between the amide proton, the amide ^{15}N and the H_α proton resonance by transferring magnetization via the heteronuclear one-bond couplings and using C_α as a relay nucleus. The H(CA)NHN spectrum provides information similar to the HN-H_α fingerprint region of a ^1H-^1H correlated spectroscopy (COSY) or homonuclear Hartman–Hahn spectroscopy (HOHAHA)/total correlation spectroscopy (TOCSY) spectrum, but resonances are dispersed in a third dimension according to the ^{15}N amide chemical shift, so decreasing spectral overlap. In addition, apart from intra-residue HN-H_α J connectivity, inter-residue connectivity between H_α of residue i and the amide of $i+1$ is frequently also observable. These correlations result from transfer of magnetization via the inter-residue $^2J_{N\text{-}C\alpha}$ couplings which are only marginally smaller than intra-residue $^1J_{N\text{-}C\alpha}$ values.

An additional new triple-resonance experiment, amide proton (via carbonyl) to α-carbon correlation (HN(CO)CA) [12], provides connectivity between the amide proton and ^{15}N of residue i, and the C_α resonance of $i-1$. The experiment appears more robust, especially for proteins larger than 20 kD, than the H(CA)NHN experiment, which transfers magnetization via the rapidly relaxing C_α nucleus. When interpreting the amide proton to α-carbon correlation spectrum, the HN(CO)CA data unambiguously distinguish intra-residue from sequential connectivities.

For obtaining side-chain assignments in ^{13}C-labeled proteins, several powerful schemes for establishing J connectivity have recently been introduced [13•,14,15,16•]. These so-called carbon-carbon-proton and proton-carbon-carbon-proton techniques rely on transferring magnetization in several steps, one step involving ^{13}C-^{13}C magnetization transfer via the one-bond $^1J_{CC}$ couplings, and one or two steps transferring magnetization from ^{13}C to ^1H, or *vice versa*. Magnetization can be transferred from one carbon to another either via a COSY mechanism [14,15], or by isotropic mixing of ^{13}C magnetization [13•,16•]. In the latter case, expected resonance intensities have been calculated for each amino acid [17,18•] assuming that the $^1J_{CC}$ couplings are independent of the side-chain conformation.

Nuclear Overhauser effect techniques

^1H-^1H NOE spectra of proteins that are isotopically enriched can be separated in a third dimension (either the ^{15}N or ^{13}C chemical shift) by means of 3D ^{15}N- or ^{13}C-separated NOESY techniques that are now becoming popular in a large number of laboratories. In these 3D spectra, ^1H-^1H NOE cross-peaks usually are separated in the third dimension by the chemical shift of the heteronucleus attached to the proton that is detected during the acquisition period. For larger proteins, however, even in the 3D spectrum, substantial overlap can remain. Moreover, even in the absence of resonance overlap, interpretation can sometimes be ambiguous if the second

proton, for which the chemical shift of its attached ^{13}C or ^{15}N is not measured, resonates in a dense region of the proton NMR spectrum. Although, in principle, such problems can be solved with a combination of several 3D experiments [19•], the most logical solution extends the dimensionality of the NOESY spectrum to four, separating the ^{1}H-^{1}H NOE interaction in two orthogonal dimensions corresponding to the chemical shifts of the two heteronuclei to which the protons are attached. Four-dimensional experiments have been described for studying interactions between amide and carbon-attached protons [20•] and for the study of NOE interactions between carbon-attached protons [21••,22•]. The recently released solution structure of the protein interleukin-1β (17.4 kD) demonstrates that the new methodology can generate high-resolution structures for proteins in the 15–20 kD size range [23•]. Other examples of even larger proteins are expected to follow.

Measurement of J couplings

A number of new or modified techniques for measuring J couplings have been proposed during the past year. Wagner and coworkers [24•] have described a new technique for measuring HN-H_α J couplings, which essentially amounts to a modified, ^{1}H-coupled, amide proton to nitrogen to α-carbon 3D correlation. This technique requires uniformly $^{13}C/^{15}N$-labeled protein and works even if the ^{1}H-^{1}H J coupling is much smaller than the ^{1}H line width. Its accuracy is determined mainly by the precision with which frequencies of individual resonances in a 3D spectrum can be measured.

A technique which provides an approximate measure for the intra-residue three-bond ^{15}N-H_β J couplings has been described by Wuthrich and coworkers [25•]. This coupling is of interest as it characterizes the C_α-C_β torsion angle, χ_i, and helps in making stereospecific assignments of non-equivalent H_β methylene protons. Clore *et al.* [26•] have described how information on the χ_1 angle and stereospecific assignments can be obtained from measuring the relative intensities of HN-H_α and HN-H_β cross peaks in a 3D ^{15}N-separated HOHAHA spectrum, combined with distances measured from a 3D ^{13}C-separated rotating frame Overhauser enhancement spectroscopy (ROESY) spectrum.

Study of protein–ligand interactions

The potential for isotopic labeling of biosynthetically synthesized drugs allows the entire array of heteronuclear multi-dimensional experiments, previously applied to proteins, now to be used for the study of ligand conformation in protein–ligand complexes. Impressive examples of this kind of technology are independent studies by the groups of Wuthrich [27••] and Fesik [28••] on the conformation of cyclosporin A when bound to cyclophilin. Both groups showed that protein-bound cyclosporin has a conformation which is dramatically different from that of either its free solution structure or its X-ray crystal structure. Wider *et al.* [29] showed that isotope-filtering of the ligand signals can also simplify the protein spectrum, and the ligand–ligand, ligand–protein, and protein–protein interactions can be extracted separately from the same set of experimental data.

Simplification of protein NMR spectra by selective deuteration

Conceptually, the simplest way to simplify 2D ^{1}H NMR spectra of proteins is to remove selectively a large fraction of the ^{1}H resonances by deuteration of a number of amino acids. This approach, initially explored by LeMaster and Richards [30], has been used by Arrowsmith *et al.* [31•] in obtaining resonance assignments and secondary structure of the Trp repressor, a symmetric dimer of 107 residues per monomer. Intermolecular NOE interactions were identified by preparing dimers for which the deuteration pattern for the two monomeric halves were different. The most remarkable feature of this study, which distinguishes it from a number of other impressive selective deuteration studies, was that assignments were obtained without recourse to scalar couplings. Although the reliability of such an assignment procedure remains a matter of debate, if generally applicable, it presents an approach for studying proteins significantly larger than even the Trp repressor, provided that a larger number of more extensively deuterated samples are prepared.

Protein dynamics

The availability of isotopically enriched proteins has provided a unique opportunity to study in detail and with high sensitivity the local dynamics in a protein at the site of the isotopic label. In recent years, 2D NMR techniques have been developed for measuring ^{15}N and ^{13}C T_1 and T_2 relaxation times together with their heteronuclear NOEs [32,33]. Using the model-free approach of Lipari and Szabo [34], these values can be readily interpreted in terms of order parameters and correlation times. However, as recently pointed out by Boyd *et al.* [35•], the cross correlation terms between chemical shift anisotropy and dipolar coupling can lead to significant errors in the T_1 measurement unless special precaution is taken to suppress the cross correlation. A similar problem exists in the measurement of T_2 values, and a useful procedure for correctly measuring the T_2 values has been developed by Kay *et al.* [36•].

Other interesting developments

It is now well accepted that water can be an integral part of a macromolecular structure. Such tightly bound water molecules can be identified in the NMR spectrum,

even though they remain in fast exchange with free water [37,38]. Until recently, however, no NOE interactions between protein and surface water protons had been identified, although such interactions were expected to have a lifetime of many nanoseconds, based on other biophysical studies which suggested relatively slow diffusion rates parallel and perpendicular to the protein surface. Otting *et al.* [39•] have now shown that a combination of 3D NOESY-TOCSY and ROESY-TOCSY experiments can identify direct water–protein NOEs at the protein surface. Their work on basic pancreatic trypsin inhibitor indicates that even at 4 °C, the water protons at many of the surface hydration sites interact with a particular protein proton for significantly less than ~1 ns.

Although developments in biological solid-state NMR in recent years have not appeared with the same explosive pace of liquid-state NMR developments, important progress is being made in this area. Of particular interest is the possibility of being able to measure accurately ^{13}C-^{13}C distances in the solid phase using rotational resonance magic angle spinning NMR, as recently demonstrated by Creuzet *et al.* [40••]. The method requires selective labeling with ^{13}C at two sites and is, therefore, not a stand-alone structure determination technique in its present form. Instead, it is most useful in answering detailed questions about specific sites in a macromolecule.

Further methodological advances are anticipated, both in solution and in solid-state NMR of biopolymers. In high-resolution NMR, most of the advances in future years are expected to come from developments in software which are needed to work through the vast amounts of data being generated by the multi-dimensional NMR experiments. Together with further refinements of existing techniques and a possible hybridization of ^{2}H and $^{13}C/^{15}N$ labeling strategies, it may become possible to increase the molecular weight of biosynthetically obtained biopolymers well beyond the present limits. For systems that cannot be enriched isotopically, the future appears less bright and magnetic fields stronger than currently available will be essential if we are to extend the present molecular-weight limits by a significant margin.

Acknowledgement

I thank Dennis Torchia and Ingrid Pufahl for many valuable suggestions during the preparation of this review.

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A Bax, Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, Maryland 20892, USA.