# High-Resolution Nuclear Magnetic Resonance Applied to Biophysics and Molecular Biology: Highlights and Challenges

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***Abstract*—The development and success of nuclear magnetic resonance (NMR) methods in biological research depend on high-field superconducting magnets with outstanding homogeneity and excellent long term stability. In biological and biomedical research NMR provides a means for studying crucial questions at a molecular level, including three-dimensional structures at atomic resolution, dynamics and folding as well as a drug discovery and drug design. Today, NMR with biological macromolecules in solution is one of the principal experimental techniques of structural biology, despite the fact that very large molecules cannot readily be investigated. With recent NMR methological and biochemical advances this size limit could be extended several times.**

***Index Terms*—Drug design, NMR spectrometer, polypeptide, protein structure, superconducting magnets, TROSY.**

## I. Introduction

In THE half century since its discovery, nuclear magnetic resonance (NMR) has become the single most powerful form of spectroscopy in both chemistry and structural biology. The dramatic technical advances over the past 10–15 years, which continue apace, have established NMR as a major technique in biological sciences. Physicists may take the view that not much is new in NMR, since its principles were described 40 years ago [1]. However, this view ignores how those principles are applied in practice to a range of problems in biology and chemistry, which has required exceptional efforts integrating chemical physics, instrumentation development, new algorithms, and stable isotope labeling strategies.

The first NMR spectrum of a protein was recorded about 10 years after the discovery of NMR signals, but it was another 25 years before the technology had advanced to the point where the resonances of an entire protein could be assigned to individual atoms and the prospect of a complete structure determination by NMR became feasible [2], [3]. NMR spectroscopy began to make major contributions to biology only when it developed the capacity to solve macromolecular structures. In molecular biology and pharmaceutical research, atomic resolution three-dimensional (3-D) structures of biological macromolecules provide the necessary basis for a detailed understanding of molecular functions and intermolecular interactions, and a foundation for protein engineering and drug design.

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NMR measurements not only provide structural data but reach much further and can supply information on dynamics, conformational equilibria, folding of proteins and intra-as well as intermolecular interactions [4]–[7]. Among many other applications these capabilities have led to a powerful method for drug discovery in pharmaceutical industry [8], to a technique for detailed studies of structural and dynamic aspects of the hydration shell of macromolecules [7], and to the discovery of residual structure in the assumed completely unfolded reference state in protein folding studies [9].

Nevertheless, 3-D structures of proteins are by far the most prevalent applications, and protein NMR spectroscopy in structural biology will comprise the bulk of this article. The application in structural biology has not only made NMR such a major technique for biological studies, but also utilizes many concepts and principles used in NMR applied in molecular biology and biophysics and is thus a good example to discuss the methods exploited in solution NMR of biological molecules [7], [10].

The foundations of successful applications of NMR are spectra that resolve the resonance lines of individual nuclei in the molecules under investigation. The necessary resolution is obtained with multidimensional NMR experiments measured at high magnetic fields. A prerequisite for the detailed analysis of the enormous information content of multidimensional NMR spectra are individual assignments of resonances to specific atoms in the molecule. For this task general methods based on multidimensional NMR measurements are available.

With increasing molecular size it becomes harder to obtain satisfactory resolution and sensitivity, due to fast relaxation of the signal. Up to now only very few detailed NMR studies were performed with molecules larger than 30–40 kDa. New methods will be discussed which improve sensitivity with large molecular systems by the use of the interference between different relaxation mechanisms, a technique that is realized in TROSY (transverse relaxation-optimized spectroscopy) [11], [12]. TROSY works best at high polarizing magnetic fields ($\sim$22 teslas) and enables the recording of high-resolution NMR spectra of macromolecules and multimolecular assemblies with molecular weights larger than 100 kDa [13], [14].

## II. NMR With Biological Macromolecules

In NMR measurements with biological macromolecules the omnipresent hydrogens, i.e., the proton nuclei with their spin 1/2, play a dominant part. In most measurements the proton

signal is recorded, due to its high sensitivity. Further, interactions of the protons contribute a major part of the information content of the NMR spectra. A complete analysis of the appropriate NMR spectra of a macromolecule provides the basis for a detailed analysis of its structural and dynamic properties at atomic resolution. However, usually proton resonances cover a very small frequency range ($\sim$10 ppm of the $^1$H resonance frequency), and with increasing size of the molecules an individual observation of the many proton resonances gets more and more difficult even in multidimensional spectra. A detailed analysis of molecular properties at atomic resolution based exclusively on $^1$H NMR is possible only for molecular weights smaller 10 kDa.

When the resonances can no longer be separated in a $^1$H spectrum, the heavy atoms to which the protons are attached are used for the separation. Most protons in biological macromolecules are directly attached to carbons or nitrogens, and the resonance frequencies of these nuclei are used to distinguish hydrogen protons with overlapping resonance frequencies. Unfortunately, the natural abundance of the necessary spin 1/2 nuclei $^{15}$N and $^{13}$C is only 0.37% and 1.1%, respectively, far too low to obtain an acceptable sensitivity of the experiments. Thus the molecules must be isotope enriched with $^{15}$N and/or $^{13}$C to at least 90%.

Spectra of large molecules not only suffer from signal overlap. With increasing molecular weight faster relaxation will result in poor sensitivity. Relaxation leads to broad signals and unreasonably long measuring times for decent spectra. It had long been realized that substituting deuterons for protons would lengthen the relaxation times, since protons are usually the major source of relaxation. Thus macromolecules deuterated to a certain degree will give greater signal than the corresponding protonated molecules. This difference becomes important for systems larger than about 25 kDa. Since protons are the main source of information, samples with differing degrees of deuteration may be necessary. Recently new methods were introduced which reduce relaxation using NMR techniques based on interference effects of different relaxation mechanisms. Details of this approach will be discussed later in this article.

A typical biological macromolecule (e.g., a protein) generates hundreds or thousands of NMR resonance lines in a $^1$H spectrum. These must be distinguished from one another if NMR analysis is to succeed. A conventional one-dimensional (1-D) spectrum depending on one frequency variable does not have the necessary resolving power. For successful applications of NMR in biological sciences the development of multidimensional NMR experiments was mandatory [15]. Further, the interpretation of NMR data requires correlations between different nuclei, which are implicitly contained in 1-D spectra but often difficult to extract. Multidimensional NMR spectra provide both increased resolution and correlations that are easy to analyze. Fig. 1 shows a two-dimensional NMR spectrum in which the $^1$H and the attached $^{15}$N nucleus in an NH-group are correlated with each other and give rise to one resonance in the 2-D [$^{15}$N,$^1$H] spectrum. The two frequency axes indicate the $^1$H and the $^{15}$N resonance frequencies (chemical shifts) of the NH-group. In Fig. 1 the individual NH-groups of a protein are represented by one resonance line each. This spectrum provides a unique fingerprint of the protein and contains information on the state of

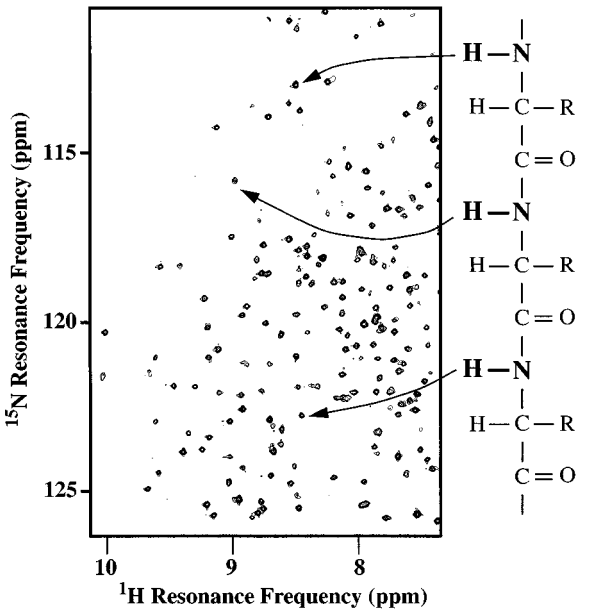


Fig. 1. Two-dimensional (2-D) NMR spectrum showing through-bond correlations between nitrogen ($^{15}$N) and attached hydrogen ($^1$H) nuclei of amide groups in a protein. Every amide group gives rise to one resonance in the 2-D spectrum. The locations of the amide groups in the polypeptide backbone are sketched in the chemical formula on the right hand side of the figure. The arrows indicate that each peak in the NMR spectrum corresponds to one NH-moiety. The axes of the NMR spectrum indicate the resonance frequencies of the hydrogen ($^1$H) and the nitrogen ($^{15}$N) nuclei (adapted from [3]).

the protein. For example, from the spread of the resonances and the completeness of their number it can be concluded that the protein has a three-dimensional fold and does not have exceptional dynamic features.

A key feature exploited in NMR spectroscopy is the fact that the individual nuclear spins interact with each other. Different nuclei in a molecule can thus be correlated. Nuclear interactions are either "direct," through-space, or "indirect," through-bond. Through-space interactions are the basis for the nuclear Overhauser effect (NOE), which for example permits distance measurements between hydrogen nuclei. Through-bond interactions are transmitted via polarization of bonding electrons and known as scalar spin–spin coupling or $J$ coupling. Multidimensional NMR experiments that correlate spins via $J$ coupling are referred to as COSY-type experiments, and those based on NOEs as NOESY-type experiments [7]. The detailed analysis of spectra of biological macromolecules is based on through-bond and through-space correlations, which can directly be extracted from multidimensional NOESY and COSY experiments [3]. The spectrum shown in Fig. 1 is an example of a COSY-type experiment.

Most spectra measured with biological macromolecules have two or three dimensions. The spectra can have only proton dimensions or combinations of other nuclei with protons. For example, a 3-D spectrum can be obtained by correlating the amide groups represented in the spectrum of Fig. 1 with the $\alpha$-carbon nuclei attached to $^{15}$N. The $\alpha$-carbon corresponds to the carbon where the group R is attached in Fig. 1. The different resonance frequencies of these carbon nuclei are used to spread the resonances from the 2-D plane into a third dimension.

## III. NMR in Structural Biology

The application of NMR in structural biology of proteins shall serve as a very successful example for the use of NMR in biological sciences. With the near completion of the human genome project the method will find an even broader field of applications since all the proteins coded by the genes will now be studied. In many cases it will be necessary to know the 3-D structure of a protein to understand its function. The two main techniques that can provide structures of macromolecules at atomic resolution are X-ray diffraction and nuclear magnetic resonance. Whereas X-ray crystallography requires single crystals, NMR measurements are carried out in solution under nearly physiological conditions. The important role that NMR plays in structural biology is illustrated by the fact that there are more than 2000 NMR structures in the protein data bank [16], including proteins, nucleic acids, protein-nucleic acid complexes, and carbohydrates.

Since the publication of the first complete NMR solution structure of a protein in 1985 [17], tremendous technological advances have brought nuclear magnetic resonance spectroscopy to the forefront of structural biology. Innovations in magnet design, electronics, pulse sequences, data analysis, and computational methods are facilitating the study of systems of ever-increasing complexity and molecular weight.

## IV. NMR Structure Determination

The principles of a NMR structure determination can be summarized as follows (Fig. 1): preparation of the protein solution, the NMR measurements, the assignment of NMR signals to individual atoms in the molecule, identification of conformational constraints (e.g., distances between hydrogen atoms), and the calculation of the 3-D structure on the basis of the experimental constraints. These five steps are briefly described in the following.

### *A. Protein Solution*

An efficient protein structure determination by NMR requires a highly purified protein preparation. The pH, ionic strength, and temperature often can be adjusted to mimic physiological conditions or to further optimize the solubility, since concentrations of at least 0.3 mM are required. For molecular weights larger than 10 kDa isotope enrichment of $^{15}$N and/or $^{13}$C nuclei to a level close to 100% is required for an efficient structure determination. For stable isotope labeling the protein is usually overexpressed in a bacterial system by growing the bacteria in a minimal medium containing $^{13}$C and or $^{15}$N labeled nutrients. For systems larger than 30 kDa deuteration and/or specific isotope labeling may be necessary [18].

### *B. NMR Measurements*

The foundations of NMR structural studies are high quality NMR spectra recorded with good sensitivity and spectral resolution. The necessary multidimensional NMR spectra are measured on NMR instruments operating with magnetic fields larger than 14 T, which corresponds to a proton resonance frequency of 600 MHz or higher. Fortunately, the chemical shift is very sensitive to the microenvironment of a particular nucleus. Thus, in a folded protein resonances from multiple copies of the same amino acid can be distinguished due to the conformation-dependent frequency shifts.

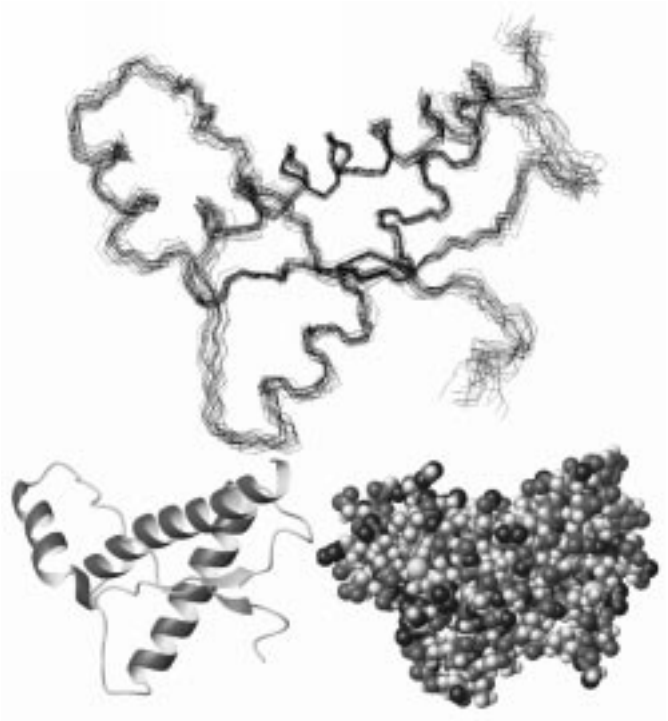

Fig. 2. Different representations of the NMR structure of the folded domain of the human prion protein in solution [25]. Top: Bundle of 20 NMR structures defining the conformational space of the protein that is obtained from the structure calculation. Only the polypeptide backbone is shown without any sidechains. Bottom: Ribbon (left) and atom (right) representation of the average structure calculated from the bundle of structures (top).

Most NMR measurements are performed in aqueous solution. The $^{1}$H resonance of water is located in the center of the $^{1}$H frequency range and interferes with the recording of $^{1}$H protein spectra. Since many NMR measurements with biological macromolecules rely on the presence of exchangeable protons, especially NH protons, the use of $D_2O$ is counterproductive. Measuring the $^{1}$H spectrum of a protein at a typical concentration of 1 mM dissolved in $H_2O$ requires special NMR techniques to massively reduce the enormous water resonance [7]. A prerequisite for a successful application of such techniques is a highly homogeneous static magnetic field across the sample.

### *C. Assignments*

For a detailed analysis of the information content of NMR spectra nearly complete assignments of signals in the spectra to individual atoms in the molecule are a requirement. The assignment procedure for protein spectra takes advantage of the sequential arrangement of the amino acids in a polypeptide chain. The spins in the individual amino acids are grouped into spin systems using COSY-type spectra, and spin systems belonging to adjacent amino acids are connected using triple resonance [$^{1}$H–$^{13}$C–$^{15}$N]-COSY (Fig. 4) and/or [$^{1}$H–$^{1}$H]-NOESY spectra. A comparison of the sequentially ordered spin systems with the known amino acid sequence of the linear polypeptide permits the assignment of the sequence position to every spin system, and thus complete sequence specific resonance assignments are obtained [3], [19].

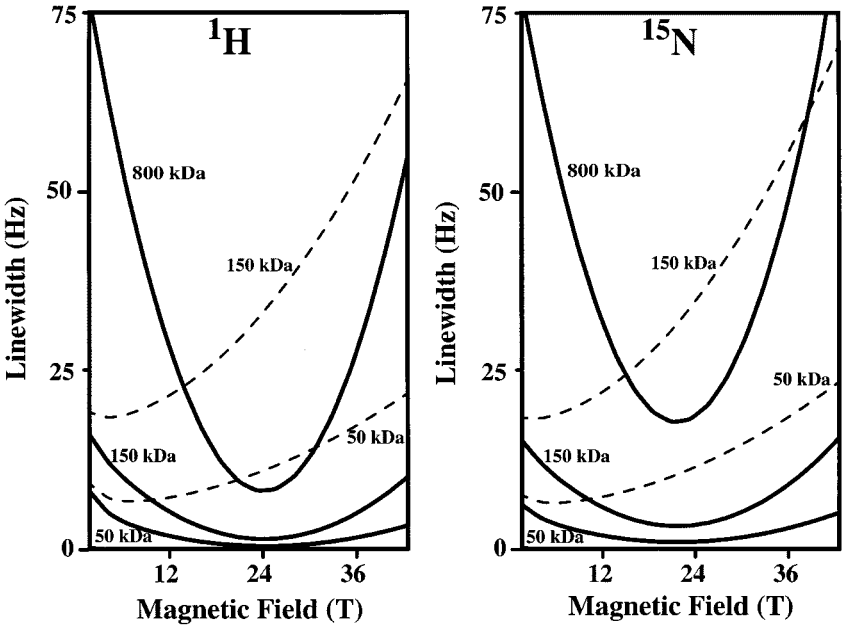


Fig. 3. Dependence on the static magnetic field of the full resonance linewidth at half height for NH groups in TROSY experiments calculated for three cases representing spherical proteins with molecular weights of 50 kDa, 150 kDa and 800 kDa. Relaxation due to spins outside the $^{15}$N–$^{1}$H moiety was ignored, which is best approximated by a deuterated protein in $H_2O$ solution. The solid and broken lines indicate the linewidth obtained with TROSY and conventional experiments, respectively (the broken line for 800 kDa lies outside the range shown). Left: linewidth of the amide $^{1}$H; right: linewidth of the amide $^{15}$N.

### *D. Conformational Constraints*

For the determination of the structure of a macromolecule geometric conformational information in the form of distances and/or torsion angles of chemical bonds have to be derived from the NMR data. Through-space correlations provide the basis for geometric information and are measured via the nuclear Overhauser effect (NOE). The NMR method for protein structure determination relies on a dense network of distance constraints derived from NOEs between nearby hydrogen atoms in the protein [20]. NOEs connect pairs of hydrogen atoms separated by less than 0.5 nm. In contrast to COSY-type experiments the nuclei involved in the NOE correlation can belong to amino acid residues that may be far apart along the protein sequence but close in space.

Additional conformational constraints can be obtained from scalar coupling constants. Further, chemical shift data, especially from $^{13}$C, provides information on the type of local structure elements [21], and hydrogen bonds can be detected via through-bond interactions [22]. When NOEs are scarce, e.g., in partially deuterated proteins, additional constraints can be obtained from residual dipolar couplings, which are related directly to the orientation of the internuclear vectors between the interacting nuclei relative to the molecular frame [23], [24]. Most proteins in solution give rise to only extremely small dipolar couplings, and to enhance the effect they are weakly aligned by adding proper additives to the aqueous solution.

### *E. Structure Calculation*

There are several methods available which calculate the Cartesian coordinates of the spatial molecular structures that are consistent with the set of constraints obtained from the NMR data [20]. It must be kept in mind that the experimental constraints do not uniquely describe one exact 3-D structure, because NMR-derived constraints typically describe a range of possible values and not all possible distances can be determined, e.g., those larger than 0.5 nm are missing. For the structure calculation the NMR constraints are supplemented by the known chemical structure of the protein under investigation. The NMR structure calculation starts with a random, extended conformation of the protein with steric restrictions represented by the van der Waals radii of the atoms [20].

The structure calculation is repeated many times to determine an ensemble of structures consistent with the input data set. A good ensemble of structures minimizes violations of input constraints and samples the complete conformational space allowed by the constraints. For this reason NMR structures are represented by a bundle of structures (Fig. 2) that determines the precision of the structure determination. For practical work an average or representative structure is derived from the bundle (Fig. 2) [20].

## V. NMR With Large Molecules

The foundations of successful NMR studies are high quality spectra with good sensitivity and resolution. When studying larger molecules in solution these basic requirements become harder to fulfill. Large molecules tumble more slowly, which results in faster spin–spin relaxation and consequently broader lines in the NMR spectrum. Thus the corresponding spectra show poor resolution and sensitivity. In practice, it becomes very hard to determine structures from proteins that have molecular weights above 30–40 kDa. Important advances in extending this size limit have recently been made with the introduction of novel NMR techniques and new biochemical approaches [14]. Most importantly relaxation in large molecules could be reduced with TROSY (transverse relaxation-optimized

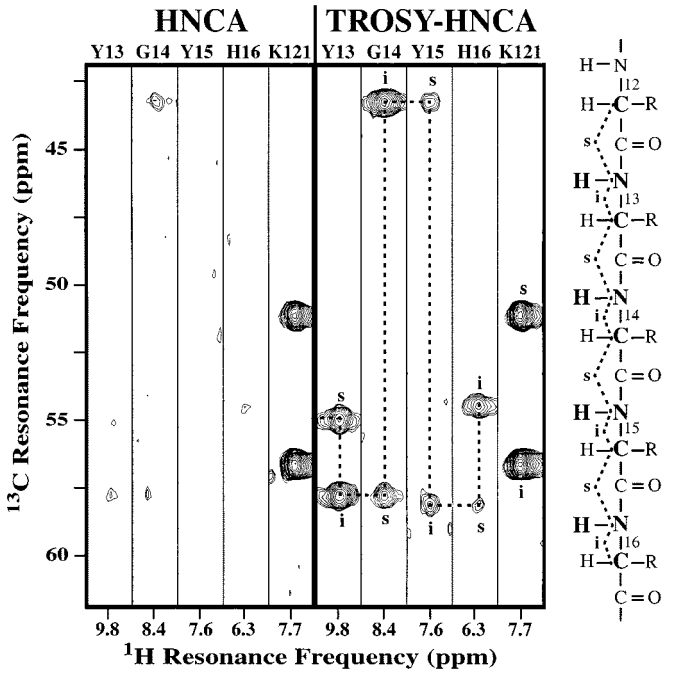


Fig. 4. Comparison of a 3-D TROSY-HNCA and a conventional 3-D HNCA spectrum. Both spectra were measured with identical experimental conditions with an aqueous solution of a protein of 110 kDa molecular weight [13]. The HNCA experiment correlates the chemical shifts of the $^{1}$H and the $^{15}$N of the amide group with the two $\alpha$-carbons separated by one and two bonds. This is indicated by the schematic drawing on the right. Only small parts, strips, of the complete 3-D spectrum are shown. For the presentation the 3-D spectrum was separated into a stack of [$^{1}$H,$^{13}$C]-planes along the $^{15}$N dimension. From these [$^{1}$H,$^{13}$C]-planes 130 Hz wide strips were cut out which run parallel to the $^{13}$C axis. The strips are centered about the chemical shift of the amide proton attached to the $^{15}$N represented by the plane. From both HNCA spectra, five strips are shown which represent the H–N–C$^{\alpha}$ correlations in the four sequential amino acids Tyr 13, Gly 14, Tyr 15, His 16 and in the C-terminal Lys 121, as indicated at the top of each strip by the one-letter amino acid symbol. The intraresidual correlation is marked with $i$ and the sequential correlation with $s$. The broken lines indicate the connections of the intraresidual and sequential HNCA correlations in the spectrum and the chemical formula, respectively.

spectroscopy [11], but also the potentially limiting spectral crowding is addressed by biochemical methods [26].

TROSY promises a several-fold increase of the molecular size accessible with solution NMR. The method makes use of the fact that for some NMR transitions field dependent minima in the relaxation are obtained. For example, for $^{15}$N–$^{1}$H moieties measured at a static magnetic field of $\sim$24 T (corresponding to a 1H frequency between 900 and 1000 MHz) a substantial reduction of transverse relaxation can be achieved for both the $^{15}$N and the $^{1}$H nuclei (Fig. 3). TROSY observes exclusively these slowly relaxing transitions. Theory predicts that TROSY experiments with suitably isotope-labeled systems can yield informative data for particles with molecular weights of several hundred thousand Daltons (Fig. 3).

Here the 3-D HNCA experiment shall serve as an example for the application of the TROSY technique [27]. The HNCA experiment correlates the resonance frequencies of the $^{1}$H and the $^{15}$N nuclei of an amide group with the intraresidual and the sequentially preceding $\alpha$-carbon nuclei. The correlation of each NH moiety with the two neighboring $\alpha$-carbons (Fig. 4) allows sequential ordering of the amide groups, which finally results in sequence-specific assignments. Fig. 4 shows a comparison of a 3-D TROSY-HNCA and a conventional 3-D HNCA spectrum, which were measured with a 110 kDa oligomeric protein under identical experimental conditions [13]. The tremendous differences in signal intensities in favor of the TROSY-type spectrum are obvious. Only small parts, strips, of the complete 3-D spectrum are shown. In each strip two peaks are expected which correspond to the two $\alpha$-carbon nuclei adjacent to the amide group represented by the strip. In Fig. 4 the strip for Lys 121 is included as a control. As expected on the basis of the theory, the two spectra show similar signal intensities only for this residue, which is located at the flexible C-terminus of the polypeptide chain and is thus much less affected by relaxation than the nuclei in the core of the protein.

For larger molecules there are more NMR active nuclei and hence more resonance lines in the NMR spectra; this increases spectral overlap. New isotope labeling techniques promise to alleviate the problem with spectral crowding [26]. Complementing protein fragments are separately biosynthesized in bacteria with and without isotope labeling, respectively, and then combined to form a complete, segmentally labeled protein. The solution structure of only the labeled domain can then be determined by NMR. By labeling a different protein domain in each series of separate experiments, the structure of the entire large protein may be obtained using TROSY techniques, even for proteins that would otherwise produce overcrowded NMR spectra.

There are interesting biological systems that do not suffer from spectral crowding and are affected only by fast relaxation. These systems can readily be investigated by TROSY-type NMR experiments. Such systems include symmetric oligomeric proteins, or isotope-labeled proteins contained in unlabeled large particles, such as nucleic acid complexes, detergent micelles or lipid vesicles. Sequence-specific NMR assignments for such large structures have already been obtained [13], and TROSY-based NOESY-type experiments [28] for the collection of structural constraints are also available. Thus with this approach it also becomes feasible to determine the structures of smaller membrane proteins in micelles using solution NMR. With this potential NMR is again at the forefront of structural biology, where membrane proteins are the next frontier.

## VI. Conclusion

NMR finds widespread applications in biophysics and molecular biology. Although this article focuses on structure determination in solution using NMR, the methods, highlights, challenges and limitations discussed are representative for many applications in biology. There are some key requirements that must be fulfilled to permit successful NMR studies of biological macromolecules at atomic resolution. The molecule under investigation must be highly purified, soluble to a concentration of at least 0.3 mM without aggregation, and for molecular weights larger than 5 to 10 kDa stable isotope enrichment is required.

Although in this article little consideration was given to compounds other than peptides and proteins, the same methodology can be used with other biological macromolecules, in particular

nucleic acids and their complexes with proteins and drugs. In addition, although not discussed in this review, NMR offers unique means of probing molecular motions on time scales of picoseconds to nanoseconds and microseconds to milliseconds [5], [6].

At the present time only a few atomic resolution NMR studies of biological macromolecules with a molecular weight larger than 30 kDa have been performed. One can anticipate, however, that in the not too distant future many more NMR studies of larger molecules and molecular complexes will become available by the widespread use of TROSY, novel NMR experiments and creative isotope labeling schemes. In addition, development of higher magnetic fields and improved spectrometer hardware will result in gains in resolution and sensitivity and will further increase the potential of NMR and guarantee continued excitement in biomolecular NMR studies in the immediate and more distant future.

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