Multidimensional nuclear magnetic resonance methods for protein studies

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Nuclear magnetic resonance (NMR) technology has advanced significantly over the past few years. After the initial development of three- and four-dimensional NMR spectroscopy, several important improvements on these experiments have been made; these offer significantly higher sensitivity and improved resolution, resulting in additional structural constraints. Other methodological improvements now make it possible to obtain complete resonance assignments, even in partially denatured proteins, opening new windows for studying the protein folding problem.

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Introduction

During the second half of the 1980s, ^{1}H NMR has become firmly established as a technique for determining the solution structure of small proteins, up to ~100 residues. For larger proteins, there were two major problems: first, the number of ^{1}H resonances increases with the number of residues; and second, the resonance line widths increase in proportion to the rotational correlation time of the protein, which is directly related to its size. Once the ^{1}H line width exceeds the size of the relatively small ^{1}H–^{1}H J couplings (5–10 Hz), J connectivity information which is critical in homonuclear ^{1}H studies becomes inaccessible. Both resonance overlap and the line width problem become limiting factors for proteins larger than about 10 kDa. The introduction of three- and four-dimensional NMR, combined with isotopic labeling of he protein, has alleviated the overlap problem by dispersing the crowded two-dimensional spectra into higher-dimensional frequency space [1]. The heteronuclear one-bond J couplings are significantly larger than the three-bond ^{1}H–^{1}H J couplings and provide an efficient means for correlating adjacent atoms. This so-called triple-resonance J connectivity approach has been used successfully for obtaining complete assignments of the backbone resonances of uniformly ^{13}C- and ^{15}N-enriched proteins as large as 269 residues [2,3].

The solution structure of a protein is derived primarily from the pairwise proton distance information contained in the strength of ^{1}H–^{1}H nuclear Overhauser effect (NOE) interactions. The three- and four-dimensional methods measure the same NOEs as the original two-dimensional techniques, but with significantly less spectral overlap. An alternative approach to reduce the overlap problem is to deuterate a set of amino acids and to study the protons of the remaining residues via two-dimensional NMR. This can, however, require a large number of samples for obtaining a complete set of NOE constraints. The use of selective deuteration and $^{13}C/^{15}N$ labeling can actually be complementary, as was recently exemplified by the study of the trp repressor DNA complex, with a total molecular weight of nearly 37 kDa [4•]. Grzesiek *et al.* [5•] proposed to use ^{13}C and ^{15}N labeling together with random fractional deuteration and high power ^{2}H decoupling to obtain line narrowing of the ^{13}C resonances, thereby making it possible to correlate sequential amides in calcineurin B via a multi-step magnetization transfer process.

Numerous improvements have been made in the past few years on the earliest triple resonance experiments. The introduction of pulsed field gradients in high resolution NMR has played a particularly important role in many of these developments. Development of a 'self-shielded' gradient coil assembly for high-resolution probeheads, modeled after a pivotal invention in magnetic resonance imaging, has dramatically improved the gradient pulse recovery times by reducing eddy currents. This technological breakthrough has found numerous important applications in protein NMR. Therefore, before reviewing the improvements and developments of individual techniques, I first briefly discuss the different ways pulsed field gradients are having an impact on protein NMR.

Pulsed field gradients

In virtually all sophisticated multidimensional NMR experiments, the aim is to transfer magnetization from one type of nucleus to another, via a set of well defined

Abbreviations

NMR—nuclear magnetic resonance; **NOE**—nuclear Overhauser effect; **NOESY**—nuclear Overhauser spectroscopy.

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multiple steps that involve J coupling and/or NOE interactions. A problem with many of these experiments is that pathways other than the desired one can result in spurious resonances (artefacts) in the final spectrum. In two-dimensional NMR, this problem was usually solved by repeating the experiment many times with different phases of radiofrequency pulses, and combining the acquired signals in such a way that the desired magnetization would add up, whereas the signals from spurious pathways would destructively interfere. This so-called phase-cycling process thus required the experiment to be repeated many times, even if the inherent sensitivity of the experiment was sufficiently high that no signal averaging was needed.

In many of the complex three- and four-dimensional NMR experiments, the multitude of different possible pathways is frequently even higher than for two-dimensional NMR and the need for extended phase cycling would result in extremely lengthy data accumulation times; insufficient phase cycling would result in spectra containing many confusing spectral artefacts. It has long been recognized that pulsed field gradients can take over the role of phase cycling, but the time needed for restabilizing the static magnetic field after the application of a strong gradient pulse had been forbiddingly long for most applications. The introduction of so-called self-shielded gradient coil assemblies, which are designed to cause maximum field gradients inside the sample volume but minimal magnetic fields in the metallic probe body, offers much faster recovery of the static magnetic field and permits the use of very short and intense magnetic field gradients. Many years ago, pulsed field gradients were used to select a certain type of magnetization transfer pathway, thereby automatically rejecting the spurious pathways that would otherwise lead to artefacts. The first applications in protein NMR, using the improved self-shielded gradient coils, also relied on this principle [6,7]. However, a major problem with these so-called pathway selection techniques was that only a single pathway could be selected whereas in most cases there are two 'mirror-image' pathways that both contribute equally to the desired signal. The elimination of one of these two pathways made this type of gradient experiment inherently less sensitive than its phase-cycled counterpart.

Pathway rejection

An alternative technique, which does not suffer from the sensitivity loss mentioned above, reduces spectral artefacts by introducing field gradients in the pulse scheme so that they do not have any net effect on the desired magnetization pathways, but do destroy the undesired magnetization components [8,9]. For example, during an interval when the desired magnetization is temporarily aligned parallel to the static magnetic field, a brief gradient pulse will only affect the spurious magnetization that precesses around the magnetic field. As many as a dozen gradient pulses may be required to selectively kill all undesired magnetization components during the various stages of the NMR pulse scheme. The first example of this approach showed that high quality triple resonance three-dimensional spectra, correlating the amide ^{1}H and ^{15}N of one residue with the $^{13}C^{\alpha}$ of the preceding one in a 20 kDa calmodulin–peptide complex, could be obtained in a matter of a few hours rather than a few days [8]. Virtually all of the pulse schemes developed during the past year rely heavily on the use of pulsed field gradients for reducing the complexity of the required phase cycle.

Pathway election à la Kay

Most multidimensional NMR experiments rely on the fact that the detected signal is sinusoidally modulated in amplitude when the duration of one of its time periods is systemically incremented. This means that whenever the modulation sinusoid crosses zero, no signal is detected. Rance and co-workers [10] demonstrated several years ago that this signal loss (by $2^{1/2}$, on average) could be recovered if the experiment is modified to yield modulation of the phase rather than the amplitude of the detected signal. This must be done in such a way that both mirror-image pathways mentioned above contribute to the final signal. A clever adaptation of this technique by Kay *et al.* [11] proposes the use of a pulsed field gradient pathway selection procedure that offers the sensitivity enhancement of the Rance method, and the high artefact suppression an the simplicity of the original pathway selection procedures. Unfortunately, the Kay trick only works for cases where the heteronucleus has just a single proton attached. This includes the important backbone amide signals, but excludes CH_2 and CH_3 groups. A recent adaptation by Griesinger and co-workers [12•] allows Kay's coherence pathway selection procedure to be extended to methyl and methylene signals, but without earning the $2^{1/2}$ enhancement in sensitivity. An excellent degree of suppression of the otherwise extremely intense H_2O signal can be obtained with the coherent pathway selection gradient approach and may remove the need to prepare a sample dissolved in D_2O; this opens the possibility to record all NMR data on a single sample.

Water suppression

Kay's pathway selection procedure is only suitable for a limited, albeit important, set of protein NMR experiments. In the remaining experiments, alternative methods need to be used for reducing the intensity of the H_2O signal. The oldest but least desirable solution attempts to saturate the water NMR transition by using a weak radiofrequency field, tuned to the H_2O resonance, during the period between successive scans when the protein magnetization recovers. Saturation of protein signals that resonate in the immediate vicinity of H_2O and cross relaxation between water and protein can dramatically reduce the intensity of the entire

proton NMR spectrum. Techniques that use composite excitation pulses, such as the popular jump-and-return pulse pair combination, do not excite the water signal, but have a poor excitation profile for the protein resonances. The introduction of pulsed field gradients, however, makes it possible to use a simple but effective variant of this approach. The so-called WATERGATE scheme excites all resonances equally but includes a refocussing (180°) pulse which has a narrow gap in its excitation profile at the H_2O frequency [13]. Pulsed field gradients then select only magnetization that experiences the refocusing pulse, dephasing the intense H_2O signal. Most of the heteronuclear pulse schemes already include a 180° pulse before detection, and the scheme can be modified to include the WATERGATE solvent suppression without adding any delays.

Water conservation techniques

Even in experiments where the water is not selectively presaturated, the H_2O magnetization is usually scrambled during data acquisition. The relaxation time of water is long compared with that of the protein, and the H_2O magnetization has insufficient time during the short delay between consecutive scans to reach its thermal equilibrium value. Consequently, water magnetization in these experiments remains attenuated relative to the protein's nuclear magnetization. Magnetization exchange between the protein and the solvent, which occurs via rapid exchange of labile protein protons and cross relaxation processes, can considerably attenuate the protein signals. With the introduction of pulsed field gradients it has now become possible to adapt many of the NMR pulse schemes in such a way that the H_2O magnetization is returned to its equilibrium position, parallel to the static magnetic field, at the end of each individual scan [14•]. This so-called 'water flip-back' adaptation can yield up to two-fold improvements in sensitivity compared with other non-saturation experiments.

Resonance assignment techniques

When relying exclusively on J connectivity, the original triple resonance backbone assignment procedure [15] encounters ambiguities whenever a $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ or a ^{15}N–^{1}H correlation cannot be distinguished from another C^{α} or amide correlation at the resolution available in the three- or four-dimensional NMR spectrum. Several solutions to this problem have been proposed. For example, the $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ degeneracy can be resolved by recording a spectrum that correlates the intraresidue amide and carbonyl signals, thus 'jumping' over the degenerate C^{α} resonance [16]. Another solution is presented by a so-called (HB)CBCACO(CA)HA experiment that records the intraresidue correlations between H^{α}, the C^{α} and C^{β} carbons and the carbonyl resonance [17•]. The degeneracy in the $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ correlations in this case is lifted by the additional C^{β} resonance. To date, however, the most widely used solution appears to be the so-called CBCA(CO)NH experiment [18]. This experiment, originally demonstrated for the 31 kDa homodimer interferon-γ, is relatively tolerant for large line widths. It records a correlation between the amide signal, observed during data acquisition, an the C^{α} and C^{β} resonances of the preceding amino acid. The combination of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts also characterizes the type of amino acid that precedes each amide, and thereby provides critical information during the backbone assignment process [19•]. Somewhat less sensitive variants of the CBCA(CO)NH experiments can correlate the H^{α} and H^{β} proton signals with the amide of the next residue [19•], or with the entire set of aliphatic side chain resonances [20–23]. This latter type of experiment, which works well for smaller proteins, or if there is significant internal protein mobility, has been used to obtain complete assignments in a largely denatured protein, where all but the amide signals are severely degenerate [24•]. This type of experiment therefore opens the possibility for detailed studies of the residual structure in partially unfolded proteins.

Intraresidue connectivity between the C^{β} and amide signals can be obtained with the CBCANH [25] or HN(CA)CB [26•] experiment. In both cases the magnetization is transferred via the one-bond $^{13}C^{\alpha}$–^{15}N J coupling, which is a relatively inefficient process, particularly in larger proteins for which the $^{13}C^{\alpha}$ line width can be significantly larger than this ~11 Hz J value. Weak correlations to the C^{α} and C^{β} of the preceding residue can be observed as a result of transfer via the two-bond (~7 Hz) $^{13}C^{\alpha}$–^{15}N inter-residue J coupling. The CBCANH experiment is more sensitive to the protein line width problem than HN(CA)CB, and the latter experiment can still yield useful results for proteins that are slightly too large for CBCANH. Numerous interesting extensions and modifications of the CBCANH, CBC(CO)NH and other triple resonance experiments have also been proposed over the past year, but most of these are not yet considered part of the essential protein pulse sequence arsenal.

One potentially important development not mentioned above, however, is the incorporation of heteronuclear Hartmann–Hahn cross polarization in many of the two- and three-dimensional experiments. This approach [27,28,29•,30] is claimed to yield spectra that are superior to the more commonly used pulsed magnetization transfer methods. Several of the cross polarization based schemes are also less sensitive to spurious pathways that give rise to artefacts, and Zuiderweg and co-workers [29•] demonstrated that high quality HCCH-TOCSY spectra can be recorded with a minimal four-step cycle, even without using pulsed field gradients.

Structural constraints

Interproton distances, as derived from interproton NOEs, constitute the cornerstones to protein structure determination by NMR. The utility of homonuclear ^1H–^1H J couplings in structure determination is also well established. Several other parameters are also becoming increasingly recognized as potentially useful in determining the solution structure of a protein. For example, the one-bond $^{13}C^{\alpha}$–$^1H^{\alpha}$ J coupling exhibits a characteristic dependence on the ϕ and ψ backbone angles [30,31] and has been used as a constant during structure calculations [31]. Similar attempts are underway for the ^{13}C and ^{15}N chemical shifts, particularly after Oldfield and co-workers [32••] demonstrated that reasonable values for these chemical shifts can be calculated on the basis of the protein structure. Gronenborn and Clore [33] noted a characteristic 1–2 ppm upfield C^{α} and a downfield C^{β} shift for the N-cap residues in helix-capping boxes. This type of capping box has a well defined geometry and its identification on the basis of ^{13}C chemical shifts may be a useful diagnostic tool for identification of hydrogen bond acceptors. It is also tempting to use the well defined N-cap backbone angles as structural constraints. Berg and co-workers [34] demonstrated that the orientation and magnitude of the paramagnetic shift tensor can be determined in a cobalt-substituted zinc finger. This type of information may serve as a precise constraint during structure refinement. The same approach may be applicable to other metal binding proteins, provided that metal substitution does not significantly alter the conformation of the protein.

Improved J coupling measurement techniques

Redfield and co-workers [35•] demonstrated that the agreement between measured H^N–H^{α} J couplings and the values predicted from the X-ray structure of turkey egg white lysozyme, using the well known Karplus relation, dramatically improves with increasing resolution of the X-ray structure. The rms difference between the measured values and the values predicted from a 1.5 Å structure was as little as 0.8 Hz. This suggests that it is desirable to measure this NMR parameter as accurately as possible. In favorable cases the J values can be measured from careful inspection and deconvolution of the H^N–H^{α} COSY cross peak structure [35•]. Recent alternative approaches to measure this important parameter include a technique, referred to as HNHA [36•], which yields the J value from the H^N–H^{α} cross peak to H^N diagonal peak intensity ratio. Although, on average, HNHA is a reasonably sensitive technique its ability to measure small J values is limited by the H^N line width, a limitation which does not apply to the alternative triple resonance E.COSY variant for measuring this coupling [37].

^{13}C–^{13}C and ^{15}N–^{13}C J couplings

Three-bond J couplings between ^{13}C nuclei and between ^{13}C and ^{15}N are considerably smaller than ^1H–^1H J couplings, but they exhibit the same Karplus type of dependence on the intervening torsion angle. In proteins uniformly enriched with ^{13}C and ^{15}N, these couplings can be measured provided that one of the ^{13}C carbons corresponds to a methyl group, which, due to its rapid rotation, has a narrow ^{13}C line width. For rapidly tumbling proteins (smaller than ~10 kDa) the methods are also applicable to non-methyl carbons. The ^{13}C–^{13}C J couplings can be measured in a manner similar to the above mentioned HNHA experiment, by quantitating the intensity of an in-phase COSY-type cross peak relative to the corresponding diagonal peak intensity [38]. The utility of an E.COSY-based technique for measuring this type of coupling has also been demonstrated but appears less straightforward as the splitting must be measured in one of the indirectly detected dimensions [39]. The simplest method for measuring long range ^{13}C–^{13}C and ^{13}C–^{15}N J couplings merely quantitates the loss in magnetization when dephasing caused by the coupling is active, versus when it is not. This method is most useful for measuring J couplings between aliphatic and carbonyl carbons [40], and between the backbone amide ^{15}N and sidechain and aliphatic carbons [41•]. As a bonus, this latter experiment also identifies the ^1H–^{13}C correlations for Arg–C^{δ}, Lys–C^{ε}, Asn–C^{β}, Gln–C^{γ}, and Pro–C^{δ}, which have relatively large one- and two-bond couplings to ^{15}N. Together, the three-bond ^{13}C–^{13}C and ^{13}C–^{15}N J couplings yield unambiguous stereospecific assignments of valine methyl groups, and allow the sidechain torsion angle χ_1 to be determined in Val, Thr and Ile residues. When surface residues such as Arg and Lys are subjected to extensive internal motions they exhibit rotamer-averaged C^{γ}–C' J couplings of 2–2.5 Hz, providing positive evidence for their side chain mobility [40].

Improved NOE measurement techniques

The introduction of pulsed field gradients has resulted in several important improvements of the four-dimensional pulse schemes. Kay and co-workers [42••] applied their pathway selection gradient approach to $^{15}N/^{13}C$ separated NOESY and demonstrated that clean spectra could be obtained by using a simple two-step phase cycle. Possibly even more importantly, the method also offers a significant gain in sensitivity, approaching $2^{1/2}$, over the original scheme. Suppression of artefacts and minimization of the phase cycle are even more important in the pivotal $^{13}C/^{13}C$-separated four-dimensional NOESY experiment. Using the standard pathway rejection approach [8], Vuister *et al.* [43•] proposed a HMQC-NOESY HMQC and a HMQC-NOESY-HSQC pulse scheme for recording such four-dimensional spectra with a two-step phase cycle. The latter

experiment is generally preferred if a sufficiently short ^{13}C 90° pulse width (<12 μs at 150 MHz) is available.

Separation of intra- from intermolecular NOEs

Symmetric homodimeric protein spectra are deceptively simple, as the spectra of the two halves of the dimer are equivalent. But analysis of their NOESY spectra can actually be more challenging than for single chain proteins as it can be difficult to distinguish inter- from intramolecular NOEs. This ambiguity can be resolved experimentally by preparing the protein monomers in two different forms: one preparation at natural abundance ^{13}C, and one highly enriched in ^{13}C. If the dimer is reconstituted from a denatured 50/50 mixture of monomers, a 50% fraction of mixed heterodimers is obtained in which one monomer is natural abundance ^{13}C, whereas the other monomer is highly ^{13}C-enriched. Isotope filtering techniques can then be used to highlight interactions between ^{13}C-attached and ^{12}C-attached protons, i.e. intermolecular NOEs. The approach has been used successfully for two homodimeric proteins [44•,45] and proved critical in breaking the symmetry in the oligomerization domain of p53 [46].

Simultaneous ^{13}C,^{13}C- and ^{13}C,^{15}N-separated 4D NOESY

Boelens *et al.* [47] demonstrated that high quality ^{1}H–^{15}N and ^{1}H–^{13}C correlations can be obtained simultaneously, yielding a significant saving in data acquisition time. The simultaneously executed experiments can be optimized individually and yield results that are comparable to those obtained when four-dimensional spectra are recorded separately. An added advantage of this type of approach may be that the spectra are truly recorded under identical conditions, and the chemical shifts observed in the different spectra are therefore not influenced by small changes in sample temperature, concentration, or a range of other factors which affect the reproducibility of peak positions.

Protein hydration

Although X-ray crystal structure of proteins solved at sufficiently high resolution show an abundance of water molecules on the protein surface, and a much smaller number in the protein's interior, crystallography only provides information on the relative occupancy of a given position, and not on the time that a water remains in that position. For residence times shorter than the rotational correlation times of the protein, the relative rates of NOE buildup in the rotating and laboratory frames can settle this question.

For tightly bound waters, with residence times that are on the order of the protein rotational correlation time or longer, other experiments are needed to estimate their residence times. For the tightly bound internal waters in basic pancreatic trypsin inhibitor, Wüthrich and co-workers previously established an upper limit for the lifetime of the internal waters at 50 ms. Using a very elegant new pulsed field gradient experiment, relying on the known differences of the translational diffusion rates of the trypsin inhibitor and the surrounding solvent, the same group was able to lower this upper limit to ~1 ms [48•].

Using the sensitivity-enhanced water-flip-back method for studying NOE interactions between solvent and bound water, Clore and co-workers [49•] found numerous weak interactions between water- and solvent-exposed residues in a complex of the erythroid transcription factor GATA-1 and its cognate DNA fragment. For most of the surface hydration sites, the rotating frame NOE spectra are considerably more intense than laboratory frame NOE data which vary between weak positive and weak negative values. This suggests lifetimes for the observed surface waters in the 0.1–1 ns range. No hydration water is observed in the major groove at the hydrophobic interface between the protein and the DNA bases.

Conclusions

NMR methods for protein structure determination have undergone important technical improvements during the past few years. In particular, the introduction of pulsed field gradient technology has led to a dramatic improvement in spectral quality and to a reduction of measurement times for well-behaved proteins smaller than ~20 kDa. Pulsed field gradients also have greatly facilitated the observation of protein–solvent interactions and allows for better quantitation of the lifetime of tightly bound solvent molecules. Once the newer technology finds its way into application laboratories, it is anticipated that both the number and the precision of protein structures solved by NMR will increase significantly.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
- • of special interest
- •• of outstanding interest

1. Bax A, Grzesiek S: **Methodological Advances in Protein NMR.** *Acc Chem Res* 1993, **26**:131–138.

2. Fogh RH, Schipper D, Boelens R, Kaptein R: **^{1}H, ^{13}C and ^{15}N NMR Backbone Assignments of the 269 Residue Serine Protease PB92 from *Bacillus alcalophilus.*** *J Biomol NMR* 1994, **4**:123–128.

3. Remerowski ML, Domke T, Gorenewegen A, Pepermans HAM, Hilbers CW, van de Ven FJM: **^{1}H,^{13}C and ^{15}N NMR Backbone Assignments and Secondary Structure of the 269-Residue Protease Subtilisin 309 from *Bacillus lentus.*** *J Biomol NMR* 1994, **4**:257–278.

4. • Zhang H, Zhao D, Revington M, Lee W, Jia X, Arrowsmith CH, Jardetzky O: **The Solution Structure of the trp Repressor–Operator DNA Complex.** *J Mol Biol* 1994, **229**:735–746.

The structure of the 37 kDa complex of the trp repressor homodimer and a symmetric consensus 20 bp DNA operator fragment is determined by using a combination of selective deuteration and heteronuclear NMR experiments.

5. • Grzesiek S, Anglister J, Ren H, Bax A: **^{13}C Line Narrowing by ^{2}H Decoupling in $^{2}H/^{13}C/^{15}N$ Enriched Proteins. Application to Triple Resonance 4D J Connectivity of Sequential Amides.** *J Am Chem Soc* 1993, **115**:4369–4370.

Random fractional deuteration at 50% and 85%, combined with high power ^{2}H decoupling results in significant lengthening of the ^{13}C T_2 values, allowing multi-step magnetization transfer between sequential backbone amides.

6. John BK, Plant D, Heald SH, Hurd RD: **Efficient Detection of $C_\alpha H$-HN Correlations in Proteins Using Gradient-Enhanced ^{15}N HMQC-TOCSY.** *J Magn Reson* 1991, **94**:664–669.

7. Davis AL, Boelens R, Kaptein R: **Rapid Acquisition of Three-Dimensional Triple-Resonance Experiments Using Pulsed Field Gradient Techniques.** *J Biomol NMR* 1992, **2**:395–400.

8. Bax A, Pochapsky SS: **Optimized Recording of Heteronuclear Multi-Dimensional NMR Spectra Using Pulsed Field Gradients.** *J Magn Reson* 1992, **99**:638–643.

9. Wider G, Wüthrich K: **A Simple Experimental Scheme Using Pulsed Field Gradients for Coherence-Pathway Rejection and Solvent Suppression in Phase-Sensitive Heteronuclear Correlation Spectra.** *J Magn Reson Ser A* 1993, **102**:239–241.

10. Palmer AG, Cavanagh J, Wright PE, Rance M: **Sensitivity Improvement in Proton-Detected Two-Dimensional Heteronuclear Correlation NMR Spectroscopy.** *J Magn Reson* 1991, **93**:151–170.

11. Kay LE, Keifer P, Saarinen T: **Pure Absorption Gradient Enhanced Heteronuclear Single Quantum Correlation Spectroscopy with Improved Sensitivity.** *J Am Chem Soc* 1992, **114**:10663–10665.

12. • Schleucher J, Schwendinger M, Sattler M, Schmidt P, Schedletzky O, Glaser SJ, Sørensen S, Griesinger C: **A General Enhancement Scheme in Heteronuclear Multidimensional NMR Employing Pulsed Field Gradients.** *J Biomol NMR* 1994, **4**:301–306.

The method of [11] can be extended to CH_2 and CH_3 groups, albeit without the sensitivity advantage of [10].

13. Piotto M, Saudek V, Sklenar V: **Gradient-Tailored Excitation for Single-Quantum NMR Spectroscopy of Aqueous Solutions.** *J Biomol NMR* 1992, **2**:661–665.

14. • Grzesiek S, Bax A: **The Importance of not Saturating H_2O in Protein NMR Application to Sensitivity Enhancement and NOE Measurements.** *J Am Chem Soc* 1993, **115**:12593–12594.

By ensuring that the slowly relaxing water magnetization is returned to the positive z axis at the beginning of data acquisition, significant sensitivity improvements can be obtained in many types of protein NMR experiments conducted in H_2O solution.

15. Ikura M, Kayh LE, Bax A: **A Novel Approach for Sequential Assignment of ^{1}H, ^{13}C, and ^{15}N Spectra of Larger Proteins: Heteronuclear Triple-Resonance NMR Spectroscopy. Application to Calmodulin.** *Biochemistry* 1990, **29**:4659–4667.

16. Clubb RT, Thanabal V, Wagner G: **A Constant-Time Three-Dimensional Triple-Resonance Pulse Scheme to Correlate Intraresidue $^{1}H^{N}$, ^{15}N, and $^{13}C'$ Chemical Sifts in ^{15}N–^{13}C-Labeled Proteins.** *J Magn Reson* 1994, **97**:213–217.

17. • Kay LE: **Pulsed-Field Gradient-Enhanced Three-Dimensional NMR Experiment for Correlating $^{13}C_{\alpha/\beta}$, $^{13}C'$ and $^{1}H_\alpha$ Chemical Shifts in Uniformly ^{13}C-Labeled Proteins Dissolved in H_2O.** *J Am Chem Soc* 1993, **115**:2055–2057.

Using the pulsed field gradient method of [11], a high quality correlation of C_α, C_β and C′ with H_α can be obtained in H_2O solution.

18. Grzesiek S, Bax A: **Correlating Backbone Amide and Sidechain Resonances in Larger Proteins by Multiple Relayed Triple Resonance NMR.** *J Am Chem Soc* 1992, **114**:6291–6293.

19. • Grzesiek S, Bax A: **Amino Acid Type Determination in the Sequential Assignment procedure of Uniformly $^{13}C/^{15}N$ Enriched Proteins.** *J Biomol NMR* 1993, **3**:185–204.

A program that relies on statistical analysis of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts is used to predict the type of amino acid involved. Simple modifications of the constant-time HSQC experiment can be used to selectively observe the C^{β}–H^{β} correlations for aromatic residues or for Asn/Asp.

20. Montelione G, Lyons BA, Emerson SD, Tashiro M: **An Efficient Triple Resonance Experiment Using Carbon-13 Isotropic Mixing for Determining Sequence-Specific Resonance Assignments of Isotopically Enriched Proteins.** *J Am Chem Soc* 1992, **114**:10974–10975.

21. Grzesiek S, Anglister J, Bax A: **Correlation of Backbone Amide and Aliphatic Sidechain Resonances in $^{13}C/^{15}N$-Enriched Proteins by Isotropic Mixing of ^{13}C Magnetization.** *J Magn Reson Ser B* 1993, **101**:114–119.

22. Logan TM, Olejniczak ET, Xu RX, Fesik SW: **Side Chain and Backbone Assignments in Isotopically Labeled Proteins from Two Heteronuclear Triple Resonance Experiments.** *FEBS Lett* 1992, **314**:413–418.

23. Lyons BA, Montelione GT: **An HCCNH Triple-Resonance Experiment Using Carbon-13 Isotropic Mixing for Correlating Backbone Amide and Side-Chain Aliphatic Resonances in Isotopically Enriched Proteins.** *J Magn Reson Ser B* 1993, **101**:206–209.

24. • Logan TM, Olejniczak ET, Xu RX, Fesik SW: **A General Method for Assigning NMR Spectra of Denatured Proteins Using 3D HC(CO)NH-TOCSY Triple Resonance Experiments.** *J Biomol NMR* 1993, **3**:225–231.

The J connectivity approach described in [22] is extended by correlating the backbone amide with the side chain protons, using (H)C(CO)NH-TOCSY and correlating the intraresidue H^{N} and sidechain resonances via TOCSY-HSQC. Together, these experiments provide sufficient information for the assignment of FKBP, denatured in 6.3 M urea.

25. Grzesiek S, Bax A: **An Efficient Experiment for Sequential Backbone Assignment of Medium Sized Isotropically Enriched Proteins.** *J Magn Reson* 1992, **99**:201–207.

26. • Wittekind M, Mueller L: **HNCACB, a High-Sensitivity 3D NMR Experiment to Correlate Amide-Proton and Nitrogen Resonances with Alpha- and Beta-Carbon Resonances in Proteins.** *J Magn Reson Ser B* 1993, **101**:201–205.

An extension of the HNCA experiment can be used to provide intraresidue connectivity to C^{β} in a manner that is frequently more sensitive than that described in [23,25].

27. Ernst M, Griesinger C, Ernst RR, Bermel W: **Optimized Heteronuclear Cross Polarization in Liquids.** *Mol Phys* 1991, **74**:219–252.

28. Richardson JM, Clowes RT, Boucher W, Domaille PJ, Hardman CH, Keeler J, Laue ED: **The Use of Heteronuclear Cross Polarization to Enhance the Sensitivity of Triple-Resonance NMR Experiments. Improved 4D HCNNH Pulse Sequences.** *J Magn Reson Ser B* 1993, **101**:223–228.

29. • Majumdar A, Wang H, Morshauser RC, Zuiderweg ERP: **Sensitivity Improvement in 2D and 3D HCCH Spectroscopy Using Heteronuclear Cross-Polarization.** *J Biomol NMR* 1993, **3**:387–397.
High quality HCCH-TOCSY spectra can be obtained using heteronuclear cross polarization. The use of cross polarization reduces the number of spurious pathways and a four-step phase cycle is enough to obtain clean spectra.

30. Vuister GW, Delaglio F, Bax A: **An Empirical Correlation Between $^1J_{C\alpha-H\alpha}$ and Protein Backbone Conformation.** *J Am Chem Soc* 1992, **114**:9674–9675.

31. Mierke DF, Golic Grdadolnik S, Kessler H: **Use of One-Bond C^α–H^α Coupling Constants as Restraints in MD Simulations.** *J Am Chem Soc* 1992, **114**:8283–8284.

32. •• De Dios AC, Pearson JG, Oldfield E: **Secondary and Tertiary Structural Effects on Protein NMR Chemical Shifts: An *Ab Initio* Approach.** *Science* 1993, **260**:1491–1495.
A comparison of ^{15}N and ^{13}C chemical shift values in staphylococcal nuclease and the result of *ab initio* calculations indicate that chemical shifts can be predicted with reasonable accuracy on the basis of a high resolution protein structure.

33. Gronenborn AM, Clore GM: **Identification of N-Terminal Helix Capping Boxes by Means of ^{13}C Chemical Shifts.** *J Biomol NMR* 1994, **4**:455–458.

34. Harper LV, Amann BT, Vinson VK, Berg JM: **NMR Studies of a Cobalt-Substituted Zinc Finger Peptide.** *J Am Chem Soc* 1993, **115**:2577–2580.

35. • Bartik K, Dobson CM, Redfield C: **^1H-NMR Analysis of Turkey Egg-White Lysozyme and Comparison with Hen Egg-White Lysozyme.** *Eur J Biochem* 1993, **215**:255–266.
Measured coupling constants are compared with those predicted from X-ray crystal structures at various degrees of resolution and refinement. At high resolution (1.5 Å) the agreement between measured and X-ray predicted J values is 0.8 Hz. At lower crystallographic resolution and R factor the agreement is considerably worse.

36. • Vuister GW, Bax A: **Quantitative J Correlation — A New Approach for Measuring Homonuclear 3-Bond $J(H^NH^\alpha)$ Coupling Constants in N-15-Enriched Proteins.** *J Am Chem Soc* 1993, **115**:7772–7777.
The HNHA experiment simultaneously provides intraresidue H^NH^α coupling for residues with resolved $^1H^N$-^{15}N correlations.

37. Weisemann R, Rüterjans H, Schwalbe H, Schleucher J, Bermel W, Griesinger C: **Determination of H^N,H^α and H^N,C′ Coupling Constants in ^{13}C,^{15}N-Labelled Proteins.** *J Biomol NMR* 1994, **4**:231–240.

38. Bax A, Max D, Zax D: **Measurement of Multiple-Bond ^{13}C–^{13}C J Couplings in a 20-kDa Protein–Peptide Complex.** *J Am Chem Soc* 1992, **114**:6923–5925.

39. Schwalbe H, Rexroth A, Eggenberger U, Geppert T, Griesinger C: **Proteins — A New Method for the Stereospecific Assignment of γ-Methyl Groups in Valine Residues.** *J Am Chem Soc* 1993, **115**:7878–7879.

40. Grzesiek S, Vuister GW, Bax A: **A Simple and Sensitive Experiment for Measurement of J_{CC} Couplings Between Backbone Carbonyl and Methyl Carbons in Isotopically Enriched Proteins.** *J Biomol NMR* 1993, **3**:487–493.

41. • Vuister GW, Wang AC, Bax A: **Measurement of 3-Bond Nitrogen-Carbon J Couplings in Proteins Uniformly Enriched in N-15 and C-13.** *J Am Chem Soc* 1993, **115**:5334–5335.
A simple two-dimensional difference experiment provides quantitative $^3J_{CN}$ values.

42. •• Muhandiram DR, Xu GY, Kay LE: **An Enhanced-Sensitivity Pure Absorption Gradient 4D N-15, C-13-Edited NOESY Experiment.** *J Biomol NMR* 1993, **3**:463–470.
Use of the gradient trick of [11] provides improved sensitivity and superb water suppression in $^{13}C/^{15}N$ separated 4D NOESY.

43. • Vuister GW, Clore GM, Gronenborn AM, Powers R, Garrett DS, Tschudin R, Bax A: **Increased Resolution and Improved Spectral Quality in Four-Dimensional 13C/13C/Separated HMQC-NOESY-HMQC Spectra Using Pulsed Field Gradients.** *J Magn Reson Ser B* 1993, **101**:210–213.
Purging unwanted magnetization by using the methodology of [8] permits recording of the four-dimensional spectrum with a two-step phase cycle, yielding far higher resolution than previously possible with this experiment.

44. • Folkers PJM, Folmer RHA, Konings RNH, Hilbers CW: **Overcoming the Ambiguity Problem Encountered in the Analysis of Nuclear Overhauser Magnetic Resonance Spectra of Symmetric Dimer Proteins.** *J Am Chem Soc* 1993, **115**:3798–3799.
A simple combination of mixed isotopic labeling together with ^{13}C editing and reverse filtering can be used to observe selectively the intermolecular NOEs in a symmetric homodimer.

45. Burgering MJM, Boelens R, Caffrey M, Breg JN, Kaptein R: **Observation of Inter-Subunit Nuclear Overhauser Effects in a Dimeric Protein — Application to the Arc Repressor.** *FEBS Lett* 1993, **330**:105–109.

46. Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E, Gronenborn AM: **High-Resolution Solution Structure of the Oligomerization Domain of p53 by Multi-Dimensional NMR.** *Science* 1994, **265**:386–391.

47. Boelens R, Burgering M, Fogh RH, Kaptein R: **Time Saving Methods for Heteronuclear Multidimensional NMR of (^{13}C,^{15}N) Doubly Labeled Proteins.** *J Biomol NMR* 1994, **4**:201–214.

48. • Wider G, Götsch V, Wüthrich K: **Self-Compensating Pulsed Magnetic Field Gradients for Short Recovery Times.** *J Magn Reson Ser [A]* 1994, **108**:255–258.
A combination of a positive and negative gradient pulse, separated by a 180° radiofrequency pulse provides very rapid recovery of the static magnetic field. These combinations could be used to establish that the lifetime of the tightly bound waters in BPTI is less than 1 ms.

49. • Clore GM, Bax A, Omichinski JG, Gronenborn AM: **Localization of Bound Water in the Solution Structure of a Complex of the Erythroid Transcription Factor GATA-1 with DNA.** *Structure* 1994, **2**:89–94.
Using sensitivity-enhanced water-flip-back methodology of [14], many side-chain interactions are observed for solvent exposed residues.

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