APPLICATIONS FOR MEASURING SCALAR AND RESIDUAL DIPOLAR COUPLINGS IN PROTEINS

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APPLICATIONS FOR MEASURING SCALAR AND RESIDUAL DIPOLAR COUPLINGS IN PROTEINS

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Abstract

Nuclear magnetic resonance spectroscopic structure determination of proteins has been under rapid development during the last decade. The size limitation impeding structural studies of biological macromolecules in solution has increased from 10 kDa to 30 kDa thanks to exploitation of $^{15}\text{N}/^{13}\text{C}$ enrichment. Perdeuteration of non-exchangeable protons has pushed this limit even further, allowing backbone resonance assignment of 40 to 50 kDa proteins. Most recently, transverse relaxation optimized spectroscopy (TROSY) has been demonstrated to lengthen $^{15}\text{N}$ and $^{1}\text{H}^{\alpha}$ spin transverse relaxation times significantly, especially in large perdeuterated proteins, thus extending the size limit beyond 100 kDa systems. However, determination of structurally important nuclear Overhauser enhancements (NOE) suffers from perdeuteration, due to the lower density of proton spins available, eventually leading to imprecise protein structures. Very recently, residual dipolar couplings have been used to supplement NOE information, enabling accurate molecular structures to also be obtained with perdeuterated proteins. This thesis focuses on the measurement of the structurally important $^{3}J$-coupling between $^{1}\text{H}^{\alpha}$ and $^{1}\text{H}^{\beta}$ spins, and determination of residual dipolar couplings by utilizing the novel spin-state-selective subspectral editing together with the TROSY methodology. This approach allows precise measurement of a large number of dipolar couplings in larger protonated or perdeuterated proteins.

Keywords: NMR, TROSY, coupling.
One man’s constant is another man’s variable –A. J. Perlis
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Helsinki, October, 2000

Perttu Permi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Proton</td>
</tr>
<tr>
<td>H'</td>
<td>Deuterium</td>
</tr>
<tr>
<td>¹³C</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>¹⁵N</td>
<td>Nitrogen-15</td>
</tr>
<tr>
<td>Cα</td>
<td>Alpha carbon</td>
</tr>
<tr>
<td>C'</td>
<td>Carbonyl carbon</td>
</tr>
<tr>
<td>COSY</td>
<td>COrelation SpectroscopY</td>
</tr>
<tr>
<td>CSA</td>
<td>Chemical Shift Anisotropy</td>
</tr>
<tr>
<td>CT</td>
<td>Constant-Time</td>
</tr>
<tr>
<td>DD</td>
<td>Dipole-Dipole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy riboNucleic Acid</td>
</tr>
<tr>
<td>DQ</td>
<td>Double-Quantum</td>
</tr>
<tr>
<td>DSCT</td>
<td>Double-Semi-Constant-Time</td>
</tr>
<tr>
<td>E.COSY</td>
<td>Exclusive Correlation SpectroscopY</td>
</tr>
<tr>
<td>EIN</td>
<td>N-terminal domain of Enzyme 1</td>
</tr>
<tr>
<td>F₁</td>
<td>First indirectly detected dimension in N-dimensional spectroscopy</td>
</tr>
<tr>
<td>F₂</td>
<td>Second indirectly detected dimension in N-dimensional spectroscopy</td>
</tr>
<tr>
<td>F₃</td>
<td>Directly detected dimension in 3-dimensional spectroscopy</td>
</tr>
<tr>
<td>Hα</td>
<td>Alpha proton</td>
</tr>
<tr>
<td>HB-GAM</td>
<td>Heparin Binding Growth Associated Molecule</td>
</tr>
<tr>
<td>HCA II</td>
<td>Human Carbonic Anhydrase</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Coherence</td>
</tr>
<tr>
<td>HMSQC</td>
<td>Heteronuclear Multiple/Single Quantum Coherence</td>
</tr>
<tr>
<td>Hν</td>
<td>Amide proton</td>
</tr>
<tr>
<td>HNCA</td>
<td>Amide proton to nitrogen to alpha carbon experiment</td>
</tr>
<tr>
<td>HNCO</td>
<td>Amide proton to nitrogen to carbonyl carbon experiment</td>
</tr>
<tr>
<td>HN(CO)CA</td>
<td>Amide proton to nitrogen to alpha carbon via carbonyl carbon experiment</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>INEPT</td>
<td>Insensitive Nuclei Enhancement Polarization Transfer</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MQ</td>
<td>Multiple Quantum</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Enhancement</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Enhancement Spectroscopy</td>
</tr>
<tr>
<td>PEP</td>
<td>Preservation of Equivalent Pathways</td>
</tr>
<tr>
<td>PFG</td>
<td>Pulsed Field Gradient</td>
</tr>
<tr>
<td>ROE</td>
<td>Rotating frame Overhauser Enhancement</td>
</tr>
<tr>
<td>S'CT</td>
<td>Spin-State-Selective Coherence Transfer</td>
</tr>
<tr>
<td>S'E</td>
<td>Spin-State-Selective Excitation</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure/Activity Relationship</td>
</tr>
<tr>
<td>States-TPPI</td>
<td>States-Time Proportional Phase Incrementation</td>
</tr>
<tr>
<td>SCT</td>
<td>Semi-Constant-Time</td>
</tr>
<tr>
<td>SQ</td>
<td>Single Quantum</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Rotational correlation time</td>
</tr>
<tr>
<td>$t_1$</td>
<td>First incremented delay in N-dimensional spectroscopy</td>
</tr>
<tr>
<td>$t_{1,max}$</td>
<td>Duration of the longest time increment</td>
</tr>
<tr>
<td>$t_2$</td>
<td>Second incremented delay in N-dimensional spectroscopy</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal or spin-lattice relaxation time</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TROSY</td>
<td>Transverse Relaxation Optimized Spectroscopy</td>
</tr>
<tr>
<td>ZQ</td>
<td>Zero Quantum</td>
</tr>
<tr>
<td>U</td>
<td>Uniformly</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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References
1 Introduction

Structure determination of biological macromolecules, such as proteins and nucleic acids, is crucial to understanding their biological function. NMR spectra provide a wealth of information on molecular structure. In addition, NMR spectroscopy facilitates studies of conformational changes induced by ligand binding in protein-ligand, protein-DNA, and protein-protein interactions, providing insights into structural biology. This information is pivotal for modern knowledge-based drug design and protein engineering, and for their applications in biotechnology.

The three-dimensional structure of the molecule, in terms of interatomic distances and angular geometries, is present in NMR spectra through different kinds of restraints, all sensitive to local bonded geometry or orientation of the molecule. Distance restraints, i.e. information on the proximity of particular nuclei, are determined from NOE/ROE intensities, which are inversely proportional to interatomic distances. Dihedral restraints are extracted from scalar couplings to supply information on torsional angles. Chemical shift restraints, which are readily available after assignment of NMR signals, enable loose definition of backbone dihedral angles $\phi$ and $\psi$. Projection restraints, which define the orientations of different interatomic vectors with respect to each other, can be used as long-range structural restraints. These can be measured, for example, from cross-correlated relaxation rates (Reif et al. 1997), from diffusion anisotropy by measuring $T_1/T_2$ ratios for $^{15}$N and $^{13}$C (Tjandra et al. 1997), or from residual dipolar couplings (Tolman et al. 1995). In addition, recently measured $J$-couplings across hydrogen bonds have become valuable constraints for structure determination (Dingley & Grzesiek 1998).

The present thesis focuses on different NMR methods devised for measurement of scalar and residual dipolar couplings in the protein backbone. Scalar couplings (or $J$-couplings) provide important structural information as they relate to dihedral angles via Karplus equations (Karplus 1959; Bystrov 1976), and their magnitude and sign reflect the local bonded geometry. There are three torsion angles in the polypeptide chain, which can be determined by NMR spectroscopy. The most important of these is the $\phi$ angle, which defines the dihedral between intraresidue amide and alpha protons. The size of this coupling constant is usually indicative of whether the corresponding amino acid residue resides in the $\alpha$-helical or $\beta$-sheet region (Wüthrich 1986). Scalar coupling information is
very useful in protein structure refinement as a structural constraint for defining the angle between two bond vectors, e.g. N-H and C-H.

Dipolar couplings have been used for structure determination in small molecules since the 1960's (Saupe & Englert 1963), but their applicability for protein structure determination was demonstrated only very recently (Tolman et al. 1995; Tjandra et al. 1996; Tjandra & Bax 1997). The information content in dipolar couplings is quite different to that obtained from scalar interactions. Unlike scalar interaction, dipolar coupling is not a molecular constant. It originates from the through-space magnetic interaction between nuclei. Thus, dipolar interactions depend on interatomic distances and on the orientation of the internuclear vector with respect to the applied magnetic field (Saupe & Englert 1963).

In high-resolution NMR, dipolar couplings average to zero in solution, owing to the isotropic tumbling of molecules. However, minute residual dipolar couplings can be measured from macromolecules due to their intrinsic magnetic susceptibility anisotropy, and consequently, their small degree of alignment with the magnetic field (Tolman et al. 1995). A larger alignment emerges by dissolving the molecule in an anisotropic medium (Bax & Tjandra 1997). This enables a more reliable measurement of residual dipolar couplings.
2 Aims of the study

This thesis focuses on the development of NMR methods suitable for the measurement of scalar and dipolar couplings in biological macromolecules, particularly in proteins. In section 4, based on study I, a method designed for the measurement of the structurally important $^3J_{HN\alpha}$ coupling constant is described. In section 5, based on studies II-IV, the focus shifts to methods suitable for the measurement of several dipolar couplings observable in anisotropic media. The goal has been to develop pulse sequences appropriate for measuring coupling constants with reasonable accuracy, convenience, and sensitivity. Most of the pulse sequences described in this thesis are modifications of those pulse sequences presented in original papers I-IV. For instance, many pulse sequences are revised to take advantage of gradient selection and inclusion of $^{15}N$ steady-state magnetization, in order to improve artefact suppression and overall sensitivity.
3 Problems associated with measurement of coupling constants in proteins

Traditional one-dimensional $^1$H NMR spectroscopy is sufficient to provide information on chemical shifts and coupling constants in small organic molecules. Owing to the long transverse relaxation times, proton resonances have very narrow line widths in comparison with the inhomogeneity of the magnetic field. Hence, relatively small coupling constants can often be measured with high accuracy from a well-dispersed spectrum.

In the case of biological macromolecules, the situation is very different for two reasons: increased spectral overlap and faster transverse relaxation of protons. The number of proton resonances in the same rather limited chemical shift range increases rapidly as the protein size increases. For example, a 10-kDa protein has approximately 100 amide proton resonances in the chemical shift range of 3 to 4 ppm. In the case of a 30-kDa protein, the number of resonances is on the order of 300. In addition, as the isotropic molecular rotational correlation time, $\tau_c$, increases with increasing molecular weight, the line width is dictated by the shorter transverse relaxation time of the corresponding spin instead of magnetic field inhomogeneity. Ultimately, the line width is larger than the coupling constant of interest. These two dilemmas make it impossible to obtain structurally important vicinal proton-proton coupling constants simply from a 1D proton spectrum.

An obvious solution to the overlap problem is to increase the dimensionality of the spectrum. For example, a two-dimensional phase-sensitive COSY experiment (Marion et al. 1983) has been successfully used with small proteins. The dispersion of the proton signals in the COSY spectrum is to the power of two better than in the 1D proton spectrum. However, applicability of the phase-sensitive COSY is limited to small proteins, because in the case of determination of $J_{\alpha,N}$, the cross-peak intensity between $'H^N$ and $'H^\alpha$ is low due to self-cancellation of antiphase splitting in the presence of broad lines. Furthermore, the magnitude of the coupling constant is not accurate because the antiphase splitting greatly overestimates the true coupling constant (Neuhaus et al. 1985). For this reason, several line-fitting techniques have been developed for the extraction of the true coupling constant in the phase-sensitive COSY experiment (Neuhaus et al. 1985).
One major limitation of homonuclear experiments stems from the rapid transverse relaxation of protons. This reduces their usability for protein structure determination due to coherence transfer inefficiency in multi-dimensional experiments. Proton relaxation is dominated by the large dipole-dipole (DD) interaction between protons in the protein main-chain and side-chains. Consequently, the proton line width increases rapidly with increasing rotational correlation time. As can be seen from Figure 1a, the proton line width is close to 10 Hz when the correlation time is of the order 10 ns. In contrast, the line width of the backbone amide nitrogen is less than 5 Hz (Figure 1b). It is then obvious that the development of heteronuclear correlation experiments, which allow a greater dispersion of NMR signals with higher coherence transfer efficiency, is indispensable for larger proteins.

Fig. 1. Protein resonance line widths as a function of isotropic molecular rotational correlation time $\tau_c$. (a) Plots are shown for (-) $^1$H spins, (…) $^1$H spins directly bonded to $^{13}$C, and (- - -) $^1$H spins directly bonded to $^{15}$N spin. (b) Line widths for $^{13}$C and $^{15}$N spins. Plots are shown for (---) $^{13}$C, (- - -) $^{13}$C-H, (· · · · · · ·) $^{15}$N, and (…) $^{15}$N'H coherences (Cavanagh et al. 1994).
Introduction of isotopic labeling in protein NMR spectroscopy in the late 1980’s improved the sensitivity and resolution of protein NMR spectra. Thus, magnetically inactive $^{12}\text{C}$, and spin-$\frac{1}{2}$ nuclei $^{13}\text{C}$ and $^{15}\text{N}$. In addition, the isotope enrichment facilitated the measurement of several, also heteronuclear, coupling constants in the protein main-chain. More importantly, new techniques were devised to determine coupling constants in general. It is now possible to measure finite couplings, without the need to resolve them directly, by utilizing for instance, E.COSY (Griesinger et al. 1986), multiple-quantum coherence principles (Braunschweiler et al. 1983) and quantitative J-correlation (Blake et al. 1992). The $^{13}\text{N}$, $^{13}\text{C}$ double labeling of protein samples has increased the size limit of NMR-feasible proteins from 10 kDa to the 20-30 kDa limit. Table 1 represents typical transverse relaxation times found for $^{1}\text{H}$, $^{15}\text{N}$, and $^{13}\text{C}$ nuclei in a protein backbone at a 30-kDa size regime.

**Table 1. Transverse relaxation times for different spins in the 29 kDa, uniformly $^{15}\text{N}^{13}\text{C}$ and $^{15}\text{N}^{13}\text{C}^{2}\text{H}$ labeled, HCA II protein at 600 $^{1}\text{H}$ frequency. (Farmer & V enters 1999).**

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>$T_{2,\text{exp}}$ (ms)</th>
<th>$T_{2,\text{calc}}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}\text{C}$α</td>
<td>124.0</td>
<td>18.0</td>
</tr>
<tr>
<td>$^{13}\text{C}$'</td>
<td>47.0</td>
<td>45.0</td>
</tr>
<tr>
<td>$^{15}\text{N}$</td>
<td>52.0</td>
<td>49.0</td>
</tr>
<tr>
<td>$^{15}\text{N}(\text{H})$</td>
<td>43.0</td>
<td>-</td>
</tr>
<tr>
<td>$^{1}\text{H}$ (α-helix)</td>
<td>24.0</td>
<td>21.0</td>
</tr>
<tr>
<td>$^{1}\text{H}$ (β-sheet)</td>
<td>29.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*Measured $T_2$ values for perdeuterated HCA II, †Calculated $T_2$ values for protonated HCA II.

To increase the protein size further, rapid transverse relaxation of heteronuclei must be taken into account as well. Transverse relaxation times for aliphatic carbons with proteins at the 20-30 kDa regime have been shown to be as short as 10-20 ms (Gardner & Kay 1998) (Table 1). It is inevitable that this seriously limits sensitivity and resolution in the experiments that record chemical shift or relay magnetization via aliphatic carbons. A very efficient solution to this problem is perdeuteration, that is, replacement of non-exchangeable protons with deuterium. This decreases the carbon relaxation rate over 10-fold, since the dipole-dipole relaxation between $^{13}\text{C}$ and its directly bound $^{2}\text{H}$ is much smaller than that between $^{13}\text{C}$ and $^{1}\text{H}$, owing to the 6.5 times smaller magnetogyric ratio of $^{2}\text{H}$. Perdeuteration is beneficial for $^{15}\text{N}$, $^{1}\text{H}$ correlation experiments as well, since the relaxation rate of the amide proton decreases approximately twofold, due to the disappearance of homonuclear dipolar relaxation caused by aliphatic protons. It has been shown that 40% of the transverse relaxation rate of the amide proton arises from the dipolar contribution with the aliphatic protons (Markus et al. 1994). Amide nitrogens and carbonyl carbons are, however, much less affected by perdeuteration (Table 1).

Unfortunately, the improved sensitivity and resolution does not come without a cost. As mentioned earlier, NOE information available from non-exchangeable protons is reduced or completely lost (in the case of fully perdeuterated proteins), which results in low-resolution protein structures (Venters et al. 1995; Gardner & Kay 1998). Problems associated with a phenomenon referred to as spin diffusion are, however, less serious.
because of the decreased proton density (Gardner & Kay 1998; Farmer & Venters 1999). Thus, a separation between protons \( i \) and \( j \) can be determined more accurately thanks to the absence of magnetization relay via mutual spin \( k \). Furthermore, it permits the use of longer NOE mixing times, allowing the measurement of larger distances than would be possible in protonated systems (Venters et al. 1995).

Chemical shift anisotropy (CSA) is a significant source of \( T_2 \) relaxation for \(^{15}\text{N}\) and \(^{13}\text{C}\) spins in proteins. As the CSA interaction increases with increasing magnetic field, it reduces the gain in sensitivity and resolution obtained by the use of the highest magnetic fields. The second important source of relaxation is dipole-dipole (DD) interaction between spins. It has been commonly known for several years that the interference between chemical shift anisotropy (CSA) and dipole-dipole (DD) relaxation mechanisms results in differently relaxing \(^{15}\text{N}-\text{H} \) multiplet components (Goldman 1984). Pervushin and co-workers recently showed that at proton frequencies near 1.1 GHz, almost complete cancellation of transverse relaxation effects within a \(^{15}\text{N}-\text{H} \) moiety is expected for one of the four multiplet components in fully \(^{15}\text{N}-\text{H} \) coupled 2D experiment. By using a certain spin-state edited experiment for the selection of the most slowly relaxing \(^{15}\text{N}-\text{H} \) multiplet component (TROSY, transverse relaxation optimized spectroscopy), significant improvement in sensitivity and resolution in \(^{15}\text{N}-\text{H} \) detected experiments, especially with larger perdeuterated protein samples, can be achieved (Pervushin et al. 1997). Interestingly, in addition to \(^1\text{H} \) spin relaxation, use of perdeuterated samples with TROSY selection also dramatically decreases the relaxation rate of \(^{15}\text{N} \) spin. Remote protons have been shown to correspond with 75% of the residual \( T_2 \) of \(^{15}\text{N} \) relaxation (Pervushin et al. 1997). Therefore, TROSY selection concomitantly with perdeuteration is most effective for large \( \beta \)-sheet proteins, where there are only a few routes to amide nitrogen relaxation. For \( \alpha \)-helical proteins, the TROSY effect is somewhat less dramatic because of the proximity of \(^1\text{H} \)(i-1) and \(^1\text{H} \)(i+1) spins to the \(^{15}\text{N} \)(i) spin. In highly perdeuterated \( \beta \)-sheet proteins, it may also be advantageous to prevent the formation of \( \text{NC} \), antiphase coherence by coherent carbon decoupling, since longitudinal \(^{13}\text{C} \) spin relaxation forms a significant source of residual relaxation for \(^{15}\text{N} \) \( T_2 \). Table 2 shows the average \( T_2 \) relaxation times of the TROSY peak for the backbone \(^{15}\text{N} \) spin in the uniformly \(^{13}\text{N}^{13}\text{C}\) labeled protein EIN, and the corresponding \( T_2 \) for the \(^{15}\text{N}^{13}\text{C}^2\text{H} \) labeled sample (Kontaxis et al. 2000).

<table>
<thead>
<tr>
<th>Protein</th>
<th>( T_2 ) (ms)</th>
<th>( T_2 ) (ms)</th>
<th>( T_2 ) (ms)</th>
<th>( T_2 ) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIN-(^{15}\text{N}^{13}\text{C}^2\text{H} ) (800 MHz)</td>
<td>131.0</td>
<td>27.0</td>
<td>51.0</td>
<td>52.0</td>
</tr>
<tr>
<td>EIN-(^{15}\text{N}^{13}\text{C}^2\text{H} ) (600 MHz)</td>
<td>118.0</td>
<td>33.0</td>
<td>60.0</td>
<td>57.0</td>
</tr>
<tr>
<td>EIN-(^{15}\text{N} ) (800 MHz)</td>
<td>79.0</td>
<td>22.0</td>
<td>52.0</td>
<td>35.0</td>
</tr>
<tr>
<td>EIN-(^{15}\text{N} ) (600 MHz)</td>
<td>72.0</td>
<td>28.0</td>
<td>57.0</td>
<td>39.0</td>
</tr>
<tr>
<td>UBI-(^{15}\text{N}^{13}\text{C}^2\text{H} ) (800 MHz)</td>
<td>200.0</td>
<td>45.0</td>
<td>74.0</td>
<td>74.0</td>
</tr>
<tr>
<td>UBI-(^{15}\text{N}^{13}\text{C}^2\text{H} ) (600 MHz)</td>
<td>185.0</td>
<td>55.0</td>
<td>93.0</td>
<td>93.0</td>
</tr>
<tr>
<td>UBI-(^{15}\text{N}^{13}\text{C} ) (800 MHz)</td>
<td>111.0</td>
<td>40.0</td>
<td>73.0</td>
<td>59.0</td>
</tr>
<tr>
<td>UBI-(^{15}\text{N}^{13}\text{C} ) (600 MHz)</td>
<td>104.0</td>
<td>46.0</td>
<td>87.0</td>
<td>67.0</td>
</tr>
</tbody>
</table>
According to Table 2, it can be seen that the transverse relaxation time of the $^{15}$N spin shortens with respect to increasing magnetic field strength due to increasing cross-correlation between CSA and DD in the usual $^{15}$N-HSQC experiment, where slow and fast relaxing spin-states are interchanged by a $180^\circ$($^1$H) pulse applied during $t_1$. Consequently, the $^{15}$N line width increases with increasing magnetic field strength. On the other hand, line width of the TROSY component becomes narrower as a function of polarizing magnetic field strength. Interestingly, the transverse relaxation of $^{15}$N is largely affected by remote $^1$H spins in the regular HSQC experiment, resulting in a broader line for the $^{15}$N spin than for that in the corresponding refocused INEPT experiment. In contrast, the transverse relaxation of the $^{15}$N spin in perdeuterated proteins does not receive any major contribution from the $^1$H spin flips, yielding comparable line widths between the regular HSQC and the refocused INEPT experiments. It can also be seen that the $^{15}$N transverse relaxation time in perdeuterated proteins can be almost twice as long as that in protonated samples due to cancellation of remote, aliphatic proton interaction with the $^{15}$N nucleus.

The so-called TROSY implementation (Pervushin et al. 1997; Andersson et al. 1998; Meissner et al. 1998; Salzmann et al. 1998; Yang & Kay 1999; Rance et al. 1999), together with deuterium labeling and utilization of directional information available through dipolar couplings, will increase the size limit of proteins applicable for structure determination much further than was predicted earlier.
4 Methods for determination of scalar and dipolar couplings in proteins

Isotopic labeling of proteins has enabled the development of series of new heteronuclear experiments, which are more suitable for coupling constant measurements than the corresponding homonuclear experiments. There are several experimental approaches, which can be divided roughly into four groups. The first group of experiments relies on the measurement of coupling constants directly from in- or antiphase splittings. The second group consists of experiments that use double- and zero-quantum (DQ/ZQ) spectroscopy for determination of couplings from DQ/ZQ splittings. The third group of experiments is designed for the creation of an E.COSY pattern, from which the small coupling constant of interest can be measured with the aid of a larger coupling. The fourth group is based on the quantitative $J$-correlation principle, where the intensity of two cross-peaks, or diagonal peak and cross-peak are compared. Recently introduced spin-state-selective filtering can be combined with $J$-resolved, DQ/ZQ, and E.COSY experiments to assist in the measurement of couplings. The following sections outline the basic principles, advantages, drawbacks, and limitations of the aforementioned approaches.
4.1 J-resolved experiments

The simplest way to construct a multi-dimensional NMR experiment with coupling constant information is to record a two-dimensional correlation map in which the coupling constant of interest can be measured either from the indirectly or the directly detected dimension from an in- or antiphase splitting. In protein NMR spectroscopy, this approach is successful in the determination of large one-bond couplings between, for example, $^{13}\text{C}^\alpha$ and $^1\text{H}^\alpha$, and $^{15}\text{N}$ and $^1\text{H}^\text{N}$. The J-resolved spectroscopy may fail if the coupling of interest is small compared with the line width, which is often the case with structurally important $J$-couplings. To determine these relatively small couplings from J-resolved experiments, special means have to be employed. These are discussed in some detail in Section 5.1.1.

In addition to simplicity, an obvious advantage of J-resolved experiments is that they are not as prone to systematic errors as experiments in which couplings are extracted from cross-peak intensities. Since the coupling constant of interest is measured from the frequency difference between the corresponding resonances, instead of the relative intensities, J-resolved methods are not apt to errors influencing signal intensity. Furthermore, systematic errors that do occur originate from the same spin operator(s) throughout the experiment. Regrettably, J-resolved experiments are sensitive to the effects of differential relaxation and cross-correlation effects, whenever separation of multiplet components is small compared with line width. Effects of cross-correlation can, however, be recognized from a small asymmetry in the line shape.

The most serious drawback of the J-resolved experiments is their inherent nature to increase spectral crowding. For instance, a typical 25 kDa protein has approximately 200 main chain NH cross-peaks that can be well dispersed in the usual $^{15}\text{N}$-HSQC spectrum at high magnetic field. However, if the proton coupled $^{15}\text{N}$-HSQC spectrum is recorded, the number of cross-peaks increases to 400, and consequently, it is more than likely that the resulting spectrum will exhibit serious overlap. Fortunately, it is possible to measure $J$-couplings without additional cross-peaks due to $J$-splitting by utilizing spin-state-selection (vide infra).

4.2 Double- and zero-quantum experiments

Double- and zero-quantum coherence (DQ/ZQ) techniques (Braunschweiler et al. 1983; Rexroth et al. 1995; Permi et al. 1999a) require at least a three-spin system A, M, and X. Let us assume that A and M correspond to $^{13}\text{N}$ and $^{13}\text{C}'$, which are both coupled to a common passive spin X, corresponding to $^1\text{H}^\text{N}$ (Figure 2). If it is possible to invoke DQ/ZQ coherence between spins A and M, followed by a free precession period, the DQ coherence evolves with the sum of the chemical shifts of A and M. More importantly, DQ coherence evolves with the sum of the couplings $J_{AX}$ and $J_{MX}$. The corresponding ZQ coherence evolves with the difference in the chemical shifts of A and M, whereas the coupling evolves with the difference of $J_{AX}$ and $J_{MX}$. 
It is now obvious that $J_{AX} = 0.5 \times (J_{DQ} + J_{ZQ})$ and $J_{MX} = 0.5 \times (J_{DQ} - J_{ZQ})$, where $J_{DQ}$ and $J_{ZQ}$ are DQ and ZQ splittings, respectively. In this example, $J_{DQ}$ would correspond to $J_{NN} + J_{NC}'$ at the chemical shift of $\omega_{NC}'$, and $J_{ZQ}$ would be $J_{NN}' - J_{NC}'$ at the chemical shift of $\omega_{NC}'$. Addition of $J_{DQ}$ and $J_{ZQ}$ would give $J_{NN}$, whereas subtraction yields $J_{NN}'$.

The DQ/ZQ experiments have numerous advantages over the traditional single-quantum coherence-based methods. The most important of these is a good tolerance against the effects of differential relaxation. The differential relaxation of in-phase and antiphase magnetization results in a decrease of the apparent coupling constants. The error in the experimental coupling is inversely proportional to the magnitude of the coupling. Thus, it would be advantageous to measure small coupling from large splitting. As $J_{DQ} \neq J_{ZQ}$, (if $J_{AX}$ and $J_{MX} \neq 0$), the effect of differential relaxation will be eliminated as it is inversely proportional to the magnitude of the DQ and ZQ splittings (Rexroth et al. 1995). Additionally, the DQ/ZQ experiments give information on the relative sign of the coupling constants. Since $J_{DQ}$ evolves with the sum of two couplings and $J_{ZQ}$ with the difference, it is rather straightforward to extract the relative signs of $J_{AX}$ and $J_{MX}$. This is very useful in the case of residual dipolar couplings where dipolar contributions can be either positive or negative, depending on the relative orientation of internuclear bond vectors. Finally, the random measurement error is smaller than for the $J$-resolved experiments since the coupling constant of interest is extracted from two splittings, and the sum or difference is divided by two. A minor complication is the assignment of cross-peaks now resonating at shifts differing from the usual single-quantum coherence chemical shift frequencies.

### 4.3 E.COSY experiments

The principle of the exclusive correlation spectroscopy, i.e. E.COSY type experiments, was first presented by Griesinger (et al. 1986) and several applications for the measurement of different couplings in proteins have since been demonstrated (Seip et al.
1994; Weisemann et al. 1994; Wang & Bax 1995; Löhr & Rüterjans 1997). The key idea is to measure an unresolved coupling with the aid of a larger coupling that is resolved in a dimension orthogonal to the small coupling. In order to obtain the E.COSY or tilted cross-peak pattern, three magnetically active nuclei are needed, i.e. a three-spin system AMX, in which $J_{AM}$ is the small coupling about to be measured. In proteins, this type of spin-system contains, for instance, the amide proton ($^1H^N$), $\alpha$-proton ($^1H^\alpha$), and $\alpha$-carbon ($^{13}C^\alpha$). When $^1H^\alpha$ couples with its directly bound $^{13}C^\alpha$ during the indirect detection period ($t_1$) and with $^1H^N$ during the acquisition, the familiar E.COSY pattern arises (Figure 3).

![Diagram](image)

**Fig. 3.** A schematic representation of the E.COSY principle for the measurement of coupling constants.

The E.COSY pattern originates from a superposition of two or more subspectra. In this particular case, two subspectra are superimposed, i.e. magnetization that is absorptive antiphase with respect to the $^1H^\alpha$ spin at $t_1$ and $t_3$, and magnetization that is absorptive in-phase with respect to $^1H^\alpha$ at $t_1$ and $t_3$ (Figure 3). A simple product operator description (Sørensen et al. 1983) of these two magnetization components is as follows:

$$
H^N H^\alpha \sin(\omega_\alpha t_1) \sin(\pi J_{C^\alpha H^\alpha} t_1) \\
H^N \cos(\omega_\alpha t_1) \cos(\pi J_{C^\alpha H^\alpha} t_1)
$$
The resulting spectrum consists of two cross-peaks for each $^1$H-$^1$C correlation, corresponding to $^1$H in the $|\alpha>$ and $|\beta>$ spin states. These two cross-peaks are separated by $J_{\alpha,\alpha}$ in the $F_1$-dimension, whereas separation in the orthogonal $F_2$-dimension corresponds to $J_{\alpha,\beta}$. It is then obvious that the displacement of the $|\alpha>$ and $|\beta>$ spin states in the $F_2$-dimension is easily measured if the states are well separated in the $F_1$-dimension. The E.COSY pattern also provides information on relative signs of the couplings measured in the orthogonal dimensions. A positive tilt, i.e. the direction of the line connecting two E.COSY multiplet components is from the bottom left corner to the upper right corner, results if the couplings in the $F_1$ and $F_2$ dimensions have the same sign. A negative tilt, that is, the slope connecting the multiplet components is from the bottom right corner towards the upper left corner, indicates that the couplings in the $F_1$- and $F_2$-dimensions have opposite signs (Otting et al. 1996).

It is noteworthy that the E.COSY pattern emerges only if the passive spin is left unperturbed or it is inverted between two evolution periods. If the passive spin has a short longitudinal relaxation time compared with the duration of the experiment, a systematic decrease of the splitting caused by coupling is conceivable due to passive spin flips. This is usually problematic only if the passive spin is a proton, since $^{13}$C and $^{15}$N $T_1$ relaxation times increase as protein size increases (Wang & Bax 1995). Pulse imperfections may also cause a collapsed E.COSY pattern. For example, any imperfection in the 180° pulse used for decoupling of a passive spin during another indirect evolution period will lead to a partial collapse of the E.COSY pattern, since the spin-state of the passive spin will not be properly inverted. The E.COSY-based methods are usually most suitable for coupling constant measurements if the coupling to be measured is more than an order of magnitude smaller than the transverse relaxation rate of the observed nucleus (Wang & Bax 1995).

### 4.4 J-correlation experiments

The third set of experiments for measuring couplings relies on the principle referred to as quantitative J-correlation (Blake et al. 1992; Vuister & Bax 1993). In this case, the coupling constants are extracted from either (i) the relative intensities of two cross-peaks in two separate experiments, or (ii) the intensity ratio of diagonal peak and cross-peak in a single experiment. The former can be obtained by allowing the evolution of the coupling of interest during a fixed delay in the first experiment (referred to as the J-modulated experiment), whereas the coupling is effectively decoupled in the second experiment (referred to as the reference experiment). There is a simple trigonometric relation between the cross-peak intensities in the J-modulated and reference experiments, i.e. the reference experiment has the intensity of 1, whereas the J-modulated experiment has a cos($\pi JT$) dependence on the signal intensity. In the latter case (ii), a reference experiment is not necessary since the method is based on the coherence transfer efficiency during a finite delay in an out-and-back-type experiment. The intensity of the diagonal peak has a cosinusoidal dependence, and that of the cross-peak a sinusoidal dependence on the coherence transfer efficiency. Hence, the coupling constant of interest can be calculated from this intensity ratio of cosine- and sine-modulated magnetization (see Section 5.1.4).
4.5 Spin-state-selective filtering

The J-resolved, DQ/ZQ and E.COSY type experiments can be combined with a technique referred to as spin-state-selective filtering, first presented by Meissner (et al. 1997a-b). Spin-state-selective filtering separates the $\alpha$- and $\beta$-states of the corresponding doublet into two subspectra, thus preventing the increase of the resonance overlap due to coupled evolution. Two separate experiments are recorded, one with the corresponding doublet in-phase and the other one with an antiphase splitting (Figure 4). When the two subspectra are added together, a single resonance line, corresponding to either the doublet’s $\alpha$-state or $\beta$-state, is observed. On the other hand, when the in- and antiphase data sets are subtracted, a spectrum containing the other spin-state of the doublet is obtained.

![Figure 4. A schematic representation of spin-state-selective subspectral editing for the measurement of coupling constants.](image)

Figure 5 represents different spin-state-selective filter elements. The function of the filter elements can be described using the product operator description. Initially in-phase magnetization $I_y$ evolves under scalar (+dipolar) coupling $J_{IS}$ during the $S^3E$ element (A) (Meissner et al., 1997a) and the antiphase filter elements (B-D) (Andersson et al. 1998; IV). The filter delay in the $S^3E$ element (A) is matched to $1/(4J_{IS})$, and thus cosine and sine modulated terms are equal in their intensity. At this point, the $S^3E$ applies two 90°(I) pulses, either in the same or opposite phase, to invert or to preserve the sign of one of the magnetization terms. When these two experiments are stored separately, they can be added or subtracted to yield the up- and downfield components of the corresponding $J_{IS}$ doublet.
The filter element in Figure 5B (Andersson et al. 1998) allows subspectral editing by recording two data sets with and without $J_{IS}$ decoupling. This filter element is less sensitive to variation in coupling constants than the $S'E$ element since the filter delay is matched to $1/(2J_{IS})$. Thus, one data set is recorded without a 180° editing pulse on S spin (in-phase filter element), and another data set is recorded in the presence of the 180°(S) editing pulse (antiphase filter element). Additionally, a 90°(S) purge pulse is applied during the antiphase filter to remove any residual cosine-modulated magnetization, which would otherwise contribute to the line shape as a dispersive magnetization component.

The filter element in Figure 5C (Andersson et al. 1998) achieves subspectral editing in a similar way, but two 180° pulses are applied during the in-phase filter element. The function of these pulses is to average the relaxation rates of the two doublet components in the presence of DD/CSA cross-correlated relaxation (vide infra). The filter element in Figure 5D (IV) is mostly similar to the element in 5C, but it can be used for in-phase I magnetization. It employs pulsed field gradient z-filtering (PFG-z) by preserving the desired magnetization component in a longitudinal zz-order during the pulsed field gradient. This purges any undesired magnetization components. It should be noted that a 90° pulse prior to the gradient pulse is applied both in the in- and antiphase (90° phase shift between the in- and antiphase elements) filter elements in order to select the desired magnetization component.

The $S'CT$ filter element (Figure 5E) (Meissner et al. 1997b) was designed to obtain spin-state selection concomitantly with coherence transfer. It is thus obvious that $S'CT$ element requires antiphase 2$I_S$ at the start. Its applicability to IS, I,S, and I,S editing is somewhat limited because $S'CT$ necessitates different filter delays for CH, CH$_2$, and CH$_3$ moieties (Andersson et al. 1998).

An obvious advantage of subspectral editing with respect to spin-states is that each subspectrum consists of either the upfield or downfield components of a doublet, thus there is no increase in spectral crowding due to coupling evolution. A second aspect concerns the overlapping doublet components. If the in-phase doublet is not resolved to the baseline, the apparent splitting will underestimate the true coupling since the peaks shift towards each other due to partial overlap of line shapes. In spin-state-selective experiments, the peak placements are not affected by each other owing to the subspectral editing of upfield and downfield components. The practical advantage of this is that it is possible to measure couplings in the case of rapid transverse relaxation. Consider, for instance, the 3D-HNCO(α/β-NC’-J) experiment (II; Permi et al. 2000), where $J_{nc}$ is 15
Hz. In order to resolve a 15 Hz coupling adequately, at least a 60 millisecond collection period is needed. In a three-dimensional experiment, the experimental time would then increase considerably. However, by utilizing spin-state-selective filtering, the acquisition time in the carbonyl dimension can be shortened significantly because the partially resolved doublet components are separated in two subspectra. The sensitivity of the spin-state-selective experiment is inherently somewhat lower than the corresponding $J$-resolved experiment owing to the additional delays or radio-frequency pulses. However, other factors that can be achieved by the spin-state-selective filtering must also be considered. First, contrary to the usual $^{15}N$-$^{13}C'$-coupled HNCO spectrum, it provides improved resolution since spectral overlap does not increase. Second, owing to the shorter acquisition time needed in the $^{13}C'$-dimension, the sensitivity of the experiment is improved with respect to $^{13}C$ transverse relaxation. Third, the $\alpha/\beta$-filtered HNCO spectrum can also be used for backbone assignment because it resembles the ordinary HNCO spectrum (Ikura & Bax 1990), thus reducing the total number of spectra needed for structure determination.

Numerous reasons lead to insufficient subspectral editing, such as $J$-mismatch, differential relaxation, cross-correlation, and pulse imperfections. Let us first focus on $J$-mismatch. If the filter is not exactly matched to the true coupling value, the observed signal has a dispersive contribution because the undesirable magnetization component leaks through the filter. The dispersive component can be efficiently removed, i.e. purged, using various techniques for the selection of the desired coherence pathway (vide supra). However, the amplitude of the desired signal also diminishes due to its \(\sin(\pi J \tau)\) dependence, where \(J\) is the true coupling and \(\tau\) is the delay to which the filter is matched \((\tau = 1/nJ, \text{ where } n \text{ is usually } 1, 2, 4, \text{ or } 8)\). The $J$-mismatch is problematic only in cases in which there is a large variation in couplings. For example, the $^1J_{NH}$ couplings in proteins, in the isotropic phase, are usually within the range of 91 to 95 Hz, and very good to excellent subspectral editing can be obtained with filters selective to the $^1J_{NH}$ coupling. The same also holds true for the $^1J_{CC\alpha}$ and $^1J_{NC}'$ couplings, which are in the range of 51 to 55 Hz, and 14 to 16 Hz for the majority of amino acid residues in isotropic phase, respectively. Figure 6 illustrates $J$-leaking profiles for the filters sensitive to $^1J_{NH}$, $^1J_{CC\alpha}$, and $^1J_{NC}'$ couplings in the isotropic phase. However, the situation can be somewhat different in the presence of a large residual dipolar contribution to the scalar coupling. Dipolar contributions to the $^1J_{NH}$ splitting as large as 30 Hz are usual and $J$-crosstalk due to $J$-mismatch is likely to occur, i.e. the subspectrum corresponding to the doublet in the $\alpha$-state also receives a contribution from the undesired doublet component corresponding to the $\beta$-state, and vice versa. The corresponding maximal dipolar contribution to the $^1J_{NC}'$ splitting can be as large as 3 Hz, and $J$-crosstalk appears when $^1J_{NC}'$ differs from the canonical 14-16 Hz. Fortunately, $J$-crosstalk can be almost completely removed from the subspectra by taking appropriate linear combinations of the in- and antiphase spectra (Meissner et al. 1998b; Ottiger et al. 1998; Sørensen et al. 1999; II-IV).
Fig. 6. Intensity (%) of the minor component with respect to the principal component as a function of $J_{\text{NH}}$, $J_{\text{C'C}}$, and $J_{\text{NC'}}$ couplings. Plots for the filters matched on $J_{\text{NH}}$, $J_{\text{C'C}}$, and $J_{\text{NC'}}$ were calculated using 94, 55, and 15 Hz as nominal values, respectively.

The differential relaxation can also affect subspectral editing. If the relaxation rate of the in-phase coherence differs from that of antiphase coherence, this results in an amplitude imbalance between the in- and antiphase spectra and eventually leads to $J$-crosstalk. Thus, to prevent $J$-crosstalk due to differential relaxation, the time period during which differential relaxation is effective should be kept to a minimum. In practice, one should avoid using long coherence transfer delays or mixing periods between the spin-state-selective filter and the evolution period for the coupling constant of interest.

Cross-correlation between CSA and DD relaxation mechanisms may lead to amplitude imbalance between the in- and antiphase time domain spectra. A careful inspection of the in-phase filter elements shown in Figure 5B and 5C reveals that the only difference between them is the lack of a 180°(S) pulse in the former, and the use of a pair of 180°(S) pulses in the latter. At first glance, this may not seem to be a significant difference since the coupling between I and S is effectively decoupled in both schemes. However, relaxation interference between spin I CSA and I-S DD interactions is not averaged in the former scheme. Eventually, this results in an amplitude imbalance between the in-phase and the antiphase spectra. On the other hand, the scheme in Figure 5C interchanges $\alpha$- and $\beta$-states of the spin S during the filter element, and averages the cross-correlated relaxation during the in-phase filter. This can be realized by considering the effect of the 180°(I) pulse alone on $I_z$ (CSA) and $I_z S_z$ (DD) operators, and the concomitant 180° pulses.
on I and S spins. Hence, in the middle of the filter element in Figure 5B, the 180°(I)
inverts both the I, and I,S, operators, and thus, the cross-correlated relaxation mechanism
between CSA and DD interactions is not averaged. In the case of 180° pulses applied
both to I and S spins in the filter element of the Figure 5C, the cross-correlated relaxation
is averaged owing to inversion of I, whereas the I,S, operator is not inverted.
5 Applications

The following sections, 5.1 and 5.2, focus on the methods devised for measuring $^3J_{HN\alpha}$, and dipolar couplings between different nuclei. These data probe the molecular structure of the polypeptide backbone.

5.1 Methods for measuring $^3J_{HN\alpha}$ coupling constants in $^{15}$N and $^{15}$N/$^{13}$C-labeled protein samples

The $^3J_{HN\alpha}$ coupling is among the most useful coupling constants in proteins. Therefore, there are many pulse sequences for the measurement of $^3J_{HN\alpha}$, based on the principles summarized in the previous chapter. The experiments based on quantitative $J$-correlation are described here more thoroughly.

5.1.1 $J$-resolved experiments for measuring $^3J_{HN\alpha}$

A phase-sensitive COSY is suitable for small proteins, but the cancellation of antiphase cross-peaks is a problem for large proteins with broad lines. Therefore, it is helpful to measure the couplings from in-phase splittings. For smaller proteins with favorable relaxation rates, $^3J_{HN\alpha}$ coupling constants can be determined most efficiently and conveniently from in-phase multiplets in a $^{15}$N-HSQC type spectrum, either from the directly detected dimension by a line-fitting technique (Szyperski et al. 1992), or directly from the $^{15}$N-dimension, when accordion style spectroscopy (Bodenhausen & Ernst 1981) is used (Heikkinen et al. 1999). A limiting factor in both of these cases is the rapid transverse relaxation of proton single-quantum coherence, and consequently the applicability of $^{15}$N-HSQC and MJ-HSQC experiments is somewhat limited.

The problems arising from rapid transverse relaxation can be alleviated if multiple-quantum coherence between the amide proton and nitrogen is formed. Due to the absence of dipolar interaction between $^1H$ and $^15$N spins in the $^1H$-$^15$N multiple-quantum spin-state, this coherence relaxes much more slowly than the corresponding amide proton
single-quantum coherence. For example, the protein staphylococcal nuclease (SNase), with a correlation time of 9 ns at 37°C, has been shown to have a $T_2,_{\text{H N}}/T_2,_{\text{MQ}}$ ratio of 0.27 at a $^1$H frequency of 500 MHz (Kay & Bax 1990). A very simple approach employs the familiar HMQC experiment for the determination of $J_{\text{H N}}$, which offers good sensitivity and robustness, due to a small number of RF pulses. In addition, it is applicable to $^{15}$N-enriched samples. Unfortunately, due to a mixture of absorptive and dispersive line shapes, it is necessary to correct the measured couplings to obtain accurate coupling constants (Kay & Bax 1990). A slight modification of the HMQC is the HMQC-J scheme (Kay et al. 1989; Kay & Bax 1990). The HMQC-J experiment differs from HMQC only by the implementation of an additional 90°($^1$H) pulse immediately before the acquisition period. The pulse serves as a purge pulse for the dispersive antiphase magnetization that arises from the homonuclear coupling evolution during $t_1$. The pulse has no influence on the desired cosine-modulated magnetization, but it transfers dispersive sine-modulated magnetization to the $\alpha$-proton frequency. It is then possible to measure the coupling in the $^{15}$N-dimension from the in-phase splitting of the $^{15}$N, $^1$H$^N$ cross-peak with an absorptive line shape.

Using a $J$-multiplied, pseudo-constant-time evolution period for the coupling and the $^{15}$N chemical shift evolution, the $J_{\text{H N}}$ coupling can also be rapidly measured by the JM-HMQC experiment (Permi et al. 1999b) that is a modification of the CT-HMQC-J experiment (Kuboniva et al. 1994). The advantage over the conventional HMQC-J experiment is greater accuracy due to a multiplication of $J_{\text{H N}}$, because the error in the measured couplings is divided by $1+\kappa$, where $\kappa$ is the $J$-multiplication factor. The factor $\kappa$ is incremented in concert with $t_1$ to downscale chemical shift evolution with respect to coupling evolution, i.e. $\kappa$ is a multiple of $t_1$. In addition, fewer time increments are required to resolve the couplings adequately, allowing more transients per increment. However, this will not affect signal averaging, as fewer increments are used for the same experiment time. One should be aware that in neither the HMQC-J nor the JM-HMQC experiment is the signal purely absorptive, due to homonuclear $J$-modulation during the polarization transfer steps. Therefore, line width-dependent corrections should be applied for accurate determination of $J_{\text{H N}}$. On the other hand, selective decoupling of the alpha-proton can easily be employed during polarization transfer steps in order to prevent formation of spurious antiphase magnetization before the $t_1$ evolution period (Permi et al. 1999b).

### 5.1.2 DQ/ZQ experiments for measuring $J_{\text{H N}}$

It is also possible to determine $J_{\text{H N}}$ by DQ/ZQ spectroscopy. If a mixed DQ/ZQ coherence is created between $^1$H$^N(i)$ and $^{13}$C$^\alpha(i)$ spins, and chemical shift evolution and coupling to the $^1$H$^\alpha$ spin are allowed to take place simultaneously during $t_1$, the $J_{\text{H N}}$ couplings can be measured from the HNCA-type correlation spectrum (Rexroth et al. 1995). Thus, $J_{\text{H N}}$ can be extracted from DQ and ZQ multiplet patterns, which are split by $-145 + J_{\text{H N}}$ Hz and $-145 - J_{\text{H N}}$ Hz in the $^{13}$C$^\alpha$-dimension, respectively. Although this approach is rather insensitive to the effects of differential relaxation, it is not very suitable for large proteins due to rapid relaxation of the $^{13}$C$^\alpha$ spin. On the other hand, by
utilizing a semi-constant time TROSY evolution (Permi et al. 2000) during \( t_r \), it is possible to decrease the time period during which \(^{13}\text{C}^{\alpha}\) and \(^1\text{H}^N\) are in transverse plane, while preserving high resolution in the \(^{15}\text{N}\)-dimension.

5.1.3 Methods using the E.COSY principle

One of the first E.COSY-type experiments used for determination of coupling constants in \(^{15}\text{N}/^{13}\text{C}\)-labeled proteins was the HNCA-J experiment (Wagner et al. 1991). The pulse sequence is based on HNCA (Kay et al. 1990). During the \( t_1 \) evolution period, the \(^{13}\text{C}^{\alpha}\) spin is allowed to couple to its directly bound \(^1\text{H}^\alpha\) spin by a large ~145 Hz coupling. Subsequently, the magnetization is transferred back to the amide proton, without perturbing the \(^1\text{H}^\alpha\) spin-state. As \(^1\text{H}^N\) couples to the passive \(^1\text{H}^\alpha\) spin during acquisition, \(^1\text{H}^\alpha\) acts as a passive spin during the evolution periods \( t_1 \) and \( t_3 \), resulting in the E.COSY pattern. As long as the large \(^{1}J_{\text{C}^{\alpha}{\text{H}^{\alpha}}}\) coupling is resolved in the \( F_1 \)-dimension, the small \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) coupling can be measured from the orthogonal \( F_3 \)-dimension. As mentioned earlier, a partial collapse of the E.COSY pattern due to \(^1\text{H}^\alpha\) spin flips between the \( t_1 \) and \( t_3 \) periods will lead to too small values of the measured \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) coupling constants. This effect can be reduced by shortening the time periods of the \(^{13}\text{C}-^{15}\text{N}\) and \(^{15}\text{N}-^{1}\text{H}\) back-INEPT steps.

5.1.4 Quantitative \(J\)-correlation

The very first method for determination of \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\), which exploited quantitative \(J\)-correlation, was the HNHA experiment (Vuister & Bax 1993). It correlates the \(^1\text{H}^N\), \(^{15}\text{N}\), and \(^1\text{H}^\alpha\) spins in a three-dimensional spectrum, permitting extraction of \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) from the intensity ratio of diagonal peak and cross-peak. The essential idea behind the HNHA experiment is to use a constant time period \( (T) \) during which the \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) coupling is allowed to evolve. While a part of the magnetization is subsequently transferred to the \(^1\text{H}^\alpha\) spin, another part remains on the \(^1\text{H}^N\). The transferred magnetization resonates at the \(^1\text{H}^\alpha\) frequency during the period \( t_2 \) and corresponds to the cross-peak. The other part of the magnetization resonates at the \(^1\text{H}^N\) frequency, corresponding to the diagonal peak in the spectrum. At the end of the period \( t_2 \), the magnetization is transferred back to the \(^1\text{H}^N\) spin and is refocused with respect to the \(^1\text{H}^\alpha\) and \(^{15}\text{N}\) spins prior to acquisition. Because ‘diagonal peak’ has \( \cos(\pi J_{\text{H}^N{\text{H}^{\alpha}}}T) \) dependence on signal intensity and the ‘cross-peak’ has \( \sin(\pi J_{\text{H}^N{\text{H}^{\alpha}}}T) \) dependence, \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) can be extracted from the intensity ratio of the diagonal peak and the cross-peak based on the simple equation

\[ I_c / I_d = \tan(\pi J_{\text{H}^N{\text{H}^{\alpha}}}T) \]

It should be noted that the determination of the \(^{3}J_{\text{H}^N{\text{H}^{\alpha}}}\) couplings of the individual \(^1\text{H}^\alpha\) spin in glycines is also obtained if \(^1\text{H}^\alpha\) shifts are not degenerate. The HNHA scheme has proven to be a robust method, but a few limitations exist for large proteins. Although cross-peaks are virtually free from overlap, the dispersion of diagonal peaks is somewhat
limited despite the three dimensions of the HNHA experiment. In fact, the dispersion of
diagonal peaks relies entirely on the two-dimensional \(^{15}\text{N}-^{1}\text{H}\) correlation map. Sufficient
resolution in the \(^{15}\text{N}\)-dimension in the HNHA experiment requires a rather long
measurement time, even with minimal phase cycling.

Recently, Ponstingl and Otting (1998) introduced a rapid method for the measurement
of \(^{1}J_{\text{H}N_{\alpha}}\) in \(^{15}\text{N}\)-labeled protein samples. The basic idea in their CT-HMQC-HA
experiment is rather simple (Figure 7A). Two constant-time HMQC experiments are
recorded. A semi-selective \(^{1}\text{H}\) decoupling is applied during the course of the pulse
sequence in one experiment, whereas \(^{1}J_{\text{H}N_{\alpha}}\)-modulation is allowed in the other
experiment. This results in two \(^{15}\text{N}-^{1}\text{H}\) correlation spectra, in which the intensity ratio
between cross-peaks is determined by \(\cos(2\pi^{1}J_{\text{H}N_{\alpha}}\tau)\), and \(2\tau (=4T+4\Delta)\) is the length
of the \(J\)-modulation period. As the period \(2\tau\) is known, \(^{1}J_{\text{H}N_{\alpha}}\) can be determined by using a
simple trigonometric relation

\[\frac{I_{d}}{I_{m}} = \cos(2\pi^{1}J_{\text{H}N_{\alpha}}\tau),\]

where \(I_{d}\) and \(I_{m}\) are the cross-peak intensities in the decoupled and \(J\)-modulated
experiments, respectively. The experiment has high sensitivity and it allows a rapid
measurement of the \(^{1}J_{\text{H}N_{\alpha}}\) coupling constants. However, it is obvious that the resolution
in the \(^{15}\text{N}\)-dimension is constrained by the delay \(4T\). To avoid ambiguities in cross-peak
intensities, the delay \(2\tau\) should not be chosen in such a way that the period \(2\tau\) is >
\(1/(^{1}J_{\text{H}N_{\alpha}})\). In practice, \(J\)-modulation periods exceeding 80-90 ms should be avoided.
Thus, the available resolution in the \(F_{1}\)-dimension depends on the magnitude of the
coupling constants as well. For example, choosing the \(4(T+\Delta)\) period equal to 50 ms
would cause the disappearance of cross-peaks from residues having \(^{1}J_{\text{H}N_{\alpha}}\) equal to 10
Hz. This allows an acquisition time of 40 ms in the \(F_{1}\)-dimension, which is insufficient for
larger or helical proteins. Of course, it is possible to increase the length of the \(J\-
modulation period, but this is rather 'costly' from the relaxation point of view.
Additionally, cross-peaks of certain residues will disappear from the \(J\)-modulated
spectrum, owing to the \(\cos(2\pi^{1}J_{\text{H}N_{\alpha}}\tau)\) dependence.
Fig. 7. Pulse sequences of the (A) CT-HMQC-HA and (B) IM-HSQC experiments for the determination of $J_{HN\alpha}$ coupling constants in $^{15}$N($^{13}$C) labeled proteins. Narrow and wide bars denote 90° and 180° pulses, respectively. The delays employed are: $\Delta = 1/(4J_{HN})$; $4T+4\Delta = 2\tau = J$-modulation delay. (A) Phase cycling: $\phi_1 = x; \phi_2 = 16(x), 16(y), 16(-x), 16(-y); \phi_3 = x, y, -x, -y; \phi_4 = 4(x), 4(y), 4(-x), 4(-y); \phi_{rec} = 2(2(2(x, -x), 2(-x, x)), 2(2(-x, x), 2(x, -x)))$. $\phi_1$ is incremented in the usual States-TPPI manner for quadrature detection in F. (Marion et al. 1989). (B) Phase cycling: $\phi_1 = 2(x), 2(y), 2(-x), 2(-y); \phi_2 = x, -x; \phi_3 = x; \phi_4 = 8(x), 8(-x); \phi_{rec} = 2(x, 2(-x) x), 2(-x, 2(x) -x)$. $\phi_2$ is incremented in the usual States-TPPI manner for quadrature detection in F. Two spectra for each experiment are recorded in an interleaved manner, with and without alpha proton decoupling during the $J$-modulation delay, $2\tau$. Semi-selective alpha proton decoupling can be achieved either by applying a selective decoupling field, e.g., G3 pulse cascade (Emsley & Bodenhausen 1990), or two selective inversion pulses to the alpha proton region. $^{15}$N is decoupled during acquisition by WALTZ-16 decoupling field (Shaka et al. 1983). Efficient water suppression can be obtained using the WET scheme (Smallcombe et al. 1995).

If separate time periods for the $J$-modulation and $^{15}$N chemical shift evolution are used, the signal in the F$_1$-dimension can be acquired independently from the $J$-modulation period. Figure 7B illustrates the pulse sequence of the Intensity-Modulated HSQC (IM-HSQC) experiment for the measurement of $J_{HN\alpha}$ in $^{15}$N-labeled protein samples (I). The basic idea is the very same as in the CT-HMQC-HA experiment. Thus, two separate experiments are recorded: $J_{HN\alpha}$ is allowed to evolve in the $J$-modulated experiment,
whereas it is effectively decoupled in the reference experiment during time period \(2\tau\). The \(^1\text{H}\) magnetization is subsequently transferred to its directly bound \(^{15}\text{N}\), whose chemical shift is detected in a manner analogous to the \(^{15}\text{N}-\text{HSQC}\) experiment (Bodenhausen & Ruben 1980). Eventually the desired coherence is transferred back to the amide proton, whose chemical shift is detected during the acquisition. In summary, two \(^{15}\text{N}-\text{HSQC}\)-type spectra result, in which \(^1J\text{NN}\) couplings can be extracted by comparison of cross-peak intensities between the \(J\)-modulated and the reference experiments. Figure 8 clearly emphasizes the gain in resolution obtained with the IM-HSQC over the CT-HMQC-HA experiment. For large proteins, with unfavorable relaxation properties, it is judicious to exploit the slower relaxation rate of \(^{15}\text{N}-\text{H}\) multiple-quantum coherence and concatenation of \(J\)-modulation and \(^{15}\text{N}\) chemical shift labeling periods then in the SCT-HMQC-HA experiment (Aitio & Permi 2000).

Fig. 8. A selected region of the (A) \(J\)-modulated CT-HMQC-HA and the (B) corresponding IM-HSQC spectrum of 1.1 mM U-\(^{15}\text{N}/^{13}\text{C}\) HB-GAM in 95%/5% H\(_2\)O/D\(_2\)O, pH 4.7, 30°C, recorded on the Varian INOVA 500 spectrometer. Experimental parameters IM-HSQC (CT-HMQC-HA): \(t_1\) max = 141.1 (29.4) ms, \(t_2\) = 128 (128) ms, number of transients = 32 (32). Data were zero-filled to 2K (4K) in \(F_1\) (\(F_2\)) dimensions in both experiments and apodized using a cosine bell weighting function in both dimensions.

The longitudinal relaxation rate of the \(^1\text{H}\) spin (or passive spin in general) causes the phenomenon called differential relaxation, which may alter the multiplet pattern or distort the cross-peak intensities, leading to systematic errors in the measured coupling values (Abragam 1961; Harbison 1993; Rexroth et al. 1995). Due to the rapid spin flip of the \(^1\text{H}\) spin, the \(^1\text{H}\) in-phase and antiphase magnetizations relax at different rates in the HNHA and CT-HMQC-HA experiments, as well as in the IM-HSQC experiment. The difference in the relaxation rates of the in-phase and antiphase coherences is
approximately proportional to the selective longitudinal relaxation rate of the $^1\text{H}^\alpha$ spins (Vuister & Bax 1993). Analogously, the passive $^1\text{H}^\alpha$ spin changes its spin-state between the $t_1$ and $t_3$ acquisition periods in the HNCA-$J$ experiment, leading to partial collapse of the E.COSY pattern. Altogether, the differential relaxation results in a systematic decrease of the apparent $J_{\alpha N_{\alpha}}^\alpha$ coupling. If the period used for the $J$-modulation in the HNHA, CT-HMQC-HA, or IM-HSQC experiments constitutes a considerable fraction of $T_{1,\alpha}$, a systematic error in the measured coupling constant, develops. Furthermore, as the differential relaxation affects the magnitude of the measured couplings by an amount depending on the length of the $J$-modulation period used, it is advantageous to use the shortest possible delay for the coupling evolution. Thus, the shorter the delay used for the $J$-modulation, the smaller the correction factor needed. On the other hand, the larger the coupling, the smaller the effect of differential relaxation, and consequently, the smaller the correction factor required. The effects of differential relaxation can be corrected for the measured $J_{\alpha N_{\alpha}}^\alpha$ values in the HNHA, CT-HMQC-HA, and IM-HSQC experiments if $T_{1,\alpha}$ is known (Vuister & Bax 1993; Kuboniwa et al. 1994; Ponstingl & Otting 1998). The correction provides coupling constants with higher accuracy for larger proteins. It should be noted that the DQ/ZQ-HNCA experiment is insensitive to the effects of differential relaxation. This can be understood by realizing that the absolute error in $J$, induced by the differential relaxation, is inversely proportional to the magnitude of $J$. As the effect of differential relaxation on $J_{\alpha N_{\alpha}}^\alpha$ is inversely proportional to the magnitude of the DQ and ZQ splittings, the DQ/ZQ-HNCA method compensates for the effects of differential relaxation.

5.2 Methods for measuring scalar and dipolar couplings in dilute liquid crystal medium

There are three subsequent stages in protein structure determination. (1) Assignment of backbone resonances to the sequentially specific sites. (2) Identification of secondary structure elements. (3) Determination of a protein fold or a complete tertiary structure.

The assignment procedure can be performed, for example, by using HNCACB and HN(CO)CACB experiments (Salzmann et al. 1999) with either protonated or perdeuterated samples. Identification of the protein secondary structure is based on chemical shifts (Wishart & Sykes 1994), $J$-couplings, secondary structure specific NOE correlations (Wüthrich, 1986), and hydrogen bonds (Dingley & Grzesiek 1998).

Determination of a protein fold relies on long-range distance constraints, which are readily available from NOE intensities. However, the intensity of NOE signals is proportional to the inverse sixth power of the interatomic distance ($1/r^6$ dependence), and it falls off rapidly as the distance between protons increases, limiting its applicability to serving as long-range distance information. As a result, the largest long-distance NOE intensities are usually found between side-chain protons. Unfortunately, these protons are much more tedious to assign than protons in the main-chain. Moreover, owing to the limited range of NOE intensities (< 5-6 Å), it is inevitably difficult to gain information on the relative orientation of domains. Additionally, as perdeuteration is usually preferred for larger proteins, owing to the gain in sensitivity and resolution, it dramatically decreases
the available NOE information originating from aliphatic protons. Obviously then, obtaining good quality structure for perdeuterated, yet modular proteins, by using NOE information alone is laborious (Kay & Gardner 1997).

It has been shown recently that other type of structural information obtained from residual dipolar couplings can supplement the NOE information in order to recognize a protein fold (Tjandra et al. 1996; Tjandra et al. 1997b; Bax & Tjandra 1997; Tjandra & Bax 1997; Annila et al. 1999). Usually, for diamagnetic molecules there is hardly any dipolar contribution in scalar couplings in the high-resolution NMR spectrum due to motional averaging. However, when a certain molecule with anisotropic magnetic susceptibility is placed into a very high magnetic field, molecular tumbling is not completely isotropic because the molecule has a preference for a particular orientation. Consequently, a residual dipolar contribution appears. Hence, the measured splitting is caused by both the scalar and residual dipolar couplings ($J+D$). Although this phenomenon has been used in the NMR of small molecules for several years (Lounila & Jokisaari, 1982), it has been demonstrated on proteins only recently. Tolman and co-workers determined residual dipolar couplings ranging from -1 to 5 Hz for $^1$H-$^{15}$N spin pairs at a 17.6 T magnetic field for the paramagnetic protein cyanometmyoglobin (Tolman (et al. 1995). Bax and co-workers measured very small $^1$H-$^{15}$N and $^1$H-$^{13}$C dipolar couplings (< 0.3 Hz), which were in very good agreement with the crystal structure, for the small diamagnetic protein ubiquitin (Tjandra et al. 1996). Similar results were also obtained for the GATA-1 protein-DNA complex (Tjandra et al. 1997b). Evidently, the method is not particularly suitable for diamagnetic proteins as the induced dipolar contributions are very small. It is applicable for systems with relatively large magnetic susceptibility, e.g. nucleic acids, protein-nucleic acid complexes, and metal binding proteins.

Fortunately, a method was found to induce a weak and tunable alignment while maintaining the high resolution and sensitivity of the normal isotropic spectrum. By dissolving the protein into a dilute nematic liquid crystal medium consisting of oriented phospholipid particles (bicelles), it is possible to induce a weak alignment of the protein (Bax & Tjandra 1997; Tjandra & Bax 1997). This has enabled the measurement of a large number of residual dipolar couplings in the protein backbone, including those between $^{15}$N(i)-$^{13}$C'(i-1) and even $^1$H'(i)-$^{13}$C'(i-1). Unfortunately, proteins exist which interact destructively with bicelles, and consequently with this type of liquid crystal (Clore et al. 1998). Very recently, certain virus particles and membrane fragments have been shown to orientate in the magnetic field, providing an alternative liquid crystal media suitable for many proteins (Clore et al. 1998; Hansen et al. 1998; Koenig et al. 1999).

The dipolar contribution between amide proton and nitrogen will not necessarily provide precise information for structure calculation. If the internuclear bond vector is nearly parallel to the applied magnetic field, small changes in the vector orientation will not markedly affect the size of the dipolar coupling. The situation is very different when the bond-vector is orientated close to the magic angle ($54.77^\circ$). In this case, small changes in vector orientation will produce significant variation in dipolar couplings. Therefore, it is obvious that error functions are non-linear. It would be beneficial to retrieve dipolar couplings from different orientations, but in practice this is restricted primarily due to a lack of liquid crystals orienting in different directions with respect to the applied magnetic field, and somewhat by the planarity of the peptide bond.
Constructive use of several heteronuclear dipolar couplings measurable in the polypeptide backbone will not only improve the quality of the structure but will also provide a wealth of information on backbone dynamics. The utilization of tunable alignment via liquid crystal media enables assessment of residual dipolar couplings between $^{15}\text{N}(i)-^{13}\text{C}'(i-1)$, $^{15}\text{N}(i)-^{13}\text{C}^\alpha(i-1)$, $^{13}\text{C}'(i-1)-^{13}\text{C}^\alpha(i-1)$, $^{13}\text{C}^\beta(i-1)-^{13}\text{C}^\alpha(i-1)$, and $^{13}\text{C}'(i-1)$, in addition to $^1\text{H}-^{15}\text{N}$. Figure 9 shows the maximal predicted dipolar contributions to different scalar couplings when the dipolar contribution to one-bond $^1\text{H}-^{15}\text{N}$ splitting is around 25 Hz. As emphasized in Figure 9, up to nine residual couplings are present in the protein main-chain, which can be measured with reasonable precision from perdeuterated samples.

**Fig. 9. The estimated maximal dipolar contributions to various scalar couplings between different nuclei in the protein main-chain when the maximal contribution to $^1J_{\text{NH}}$ is 25 Hz in a dilute liquid crystal.**

Dipolar couplings can basically be measured using the existing experiments devised for the determination of scalar coupling constants. However, due to a non-existing conformational degree of freedom, many of the one- and two-bond scalar couplings have not earlier attracted much attention. Thus, there is a lack of optimized pulse sequences for the measurement of those couplings that were previously regarded as structurally insignificant. One guideline in this thesis was to devise a set of pulse sequences enabling a convenient measurement of several dipolar couplings from two-dimensional spectra superficially resembling the familiar $^{15}\text{N}$-HSQC, a spectrum that is usually available at an early stage of the structure determination procedure. Convenience is a desirable feature, especially when several different couplings are to be measured. Accordingly, many of the pulse sequences are designed to utilize spin-state-selective filtering (II-IV), which is advantageous for minimizing spectral crowding due to coupling. However, for large or highly $\alpha$-helical proteins, the $^{15}\text{N}-^1\text{H}$ correlation map may be insufficiently resolved, and for that reason spin-state-selective, two-dimensional $^{13}\text{C}'-^1\text{H}$ and especially three-dimensional $^{13}\text{C}'-^{15}\text{N}-^1\text{H}$ correlation-based methods are also taken into use.

Several spectroscopic reasons in favor of measuring couplings from the $^{15}\text{N}$, $^1\text{H}$ correlation spectra exist. 1) Among the nuclei in the backbone of proteins, amide protons and nitrogens usually provide the best dispersion of chemical shifts, that is, minimal cross-peak overlap. 2) The amide nitrogen and proton relax more slowly in protonated
samples than aliphatic carbons and protons do. Furthermore, transverse relaxation optimized spectroscopy (TROSY), which exploits the destructive relaxation interference between $^{15}$N chemical shift anisotropy (CSA) and $^{15}$N-$^1$H dipolar interaction, offers significant improvement in resolution and sensitivity in high magnetic fields, especially with perdeuterated samples (Pervushin et al. 1997). 3) Heteronuclear polarization transfer steps are more easily and precisely controlled with nitrogen than with carbon because no significant coupling exists between nitrogens in the protein backbone. 4) The water signal can be effectively suppressed without disturbing the $^1$H signals of the solute.

There are also practical advantages in measuring couplings from the $^{15}$N, $^1$H correlation spectra. Several dipolar couplings measured conveniently from two-dimensional $^{13}$C-$^1$H correlation spectra give insight into conformational changes induced by ligand-binding, in a manner similar to the structure/activity relationship (SAR) by NMR studies in which binding epitopes can be localized from changes in chemical shifts (Shuker et al. 1996; Hajduk et al. 1997). As amide nitrogen and proton chemical shifts are very sensitive to changes in chemical environment and conformation, it is conceivable that changes in residual dipolar couplings induced by ligand-binding could also be observed concomitantly with chemical shift changes to reveal conformational changes.

### 5.2.1 Pulse sequences for determination of $^1J_{NC}$ and $^2J_{HN/C}$ couplings

The simplest way of determining $^1J_{NC}$ and $^2J_{HN/C}$ coupling constants is to record a two-dimensional $^{13}$C-coupled $^{15}$N-HSQC spectrum (Delaglio et al. 1991). Because the backbone $^{15}$N nuclei are coupled to three different carbon spins, i.e. $^{13}$C'(i-1), $^{13}$Cα(i-1), and $^{13}$Cα(i), it is necessary to decouple the $^{13}$Cα spins to measure the $^1J_{NC}$ splitting adequately. Thus, $^1J_{NC}$ is measured from the indirectly detected dimension, whereas $^2J_{HN/C}$ can be measured from the orthogonal, i.e. proton, dimension in a familiar E.COSY fashion provided that the $^{13}$C spin-state is left untouched between the $t_1$ evolution period and acquisition. When this pulse sequence is combined with the spin-state-selective IPAP-HSQC (Ottiger et al. 1998), three different coupling constants can be measured from the same experiment (Wang et al. 1998). This pulse sequence has good sensitivity and the appearance of a $^{15}$N, $^1$H correlation map, but it creates doublets in the spectrum and thus increases spectral overlap. However, this can be avoided by inserting a spin-state-selective filter, sensitive to the $^{13}$C'-spin-state, prior to the $t_1$ evolution period [II, IV]. Figure 10 shows the gradient-selected, sensitivity-enhanced versions of the HN($\alpha$/$\beta$-NC'-J) and HN($\alpha$/$\beta$-NC'-J)-TROSY pulse schemes, which enable simultaneous measurement of $^1J_{NC}$, $^2J_{HN/C}$, and $^2J_{SSS}$ couplings from a two-dimensional $^{15}$N, $^1$H correlation spectrum superficially resembling $^{15}$N-HSQC or TROSY. In this triple-spin-state-selective experiment, the magnetization is initially transferred from the amide proton to its directly bound nitrogen using the familiar INEPT step. Subsequently, with the aid of a spin-state-selective filter element, either antiphase or in-phase $^{15}$N magnetization with respect to the $^{13}$C' spin is created. During the following $t_1$ period, the $^{15}$N chemical shift is recorded with simultaneous evolution of $^{15}$N-$^{13}$C' coupling. Ultimately, by inserting a generalized TROSY scheme (Andersson et al. 1998; Weigelt 1998) prior to the acquisition period, the
most slowly relaxing $^{15}\text{N}$ and $^1\text{H}$ multiplet components can be selected, thus providing a $^{15}\text{N}$, $^1\text{H}$ correlation spectrum with high sensitivity and resolution.

Fig. 10. Pulse schemes of the (A) HN($\alpha\beta$-NC'-J) and (B) HN($\alpha\beta$-NC'-J)-TROSY experiments for the measurement of $^1J_{\text{NC'}}$ and $^2J_{\text{HN}}$ couplings from the $^{15}\text{N}$, $^1\text{H}$ correlation spectrum. Narrow and wide bars denote 90º and 180º pulses, respectively, whereas selective 90º pulses for water are denoted by half-ellipses. $^{13}\text{C'}$ 180º pulses are applied with a strength of $\delta/\sqrt{3}$, where $\delta$ is the frequency between centers of the $^{13}\text{C'}$ and $^{13}\text{C}$ regions. Aliphatic carbons are selectively decoupled during $t_1$ with the semi-selective SEDUCE-1 decoupling scheme (McCoy & Mueller 1992). Alternatively, an 180º pulse selective for $\alpha$-carbons can be used. If the gradient-selected, sensitivity-enhanced HSQC is used, $^{15}\text{N}$ is decoupled during acquisition using the WALTZ-16 decoupling field (Shaka et al. 1983). The delays employed are: $\Delta = 1/(4J_{\text{sub}})$; $T_a = 1/(4J_{\text{NC'}})$; $\delta$ = gradient duration + recovery delay. (A) Phase cycling for the in-phase experiment: $\phi_1 = x, -x; \phi_2 = x; \phi_3 = 2(y), 2(-y); \phi_4 = x; \phi_{\text{rec}} = x, -x$. For the antiphase experiment, the phases of the $\phi_1$ and $\phi_2$ pulses are incremented by 90º. Frequency discrimination in $F_2$ is obtained using the PEP sensitivity-enhanced gradient selection (Kay et al. 1992; Schleucher et al. 1993) by inverting the sign of the $G_i$ gradient pulse together with the inversion of $\phi_3$. (B) Phase cycling for the in-phase experiment: $\phi_1 = x, -x; \phi_2 = x; \phi_3 = y; \phi_4 = x; \phi_5 = 2(y), 2(-y); \phi_{\text{rec}} = x, -x$. For the in-phase experiment, $\phi_5$ is incremented by 90º. For the axial peak suppression, $\phi_1$, $\phi_3$, and $\phi_5$ are incremented in the usual States-TPPI manner.
(Marion et al. 1989). Quadrature detection and TROSY selection in $F_3$, is obtained by collecting two data sets, (I): $\phi_2 = y; \phi_3 = x$, (II): $\phi_2 = -y; \phi_3 = -x$, with simultaneous change in the gradient polarity (Weigelt 1998).

This approach provides obvious advantages: 1) The spectral overlap diminishes. 2) As we separate the $\alpha$- and $\beta$-spin-states of the $^1J_{NC}'$ doublet into different subspectra, i.e. remove the overlap, the measured coupling is not an underestimate of the true coupling. The above-mentioned statements are valid only if an adequate subspectral editing is obtained, i.e. corresponding $\alpha$- and $\beta$-spin-states are well separated into the two subspectra. As can be seen in Figure 6, good suppression of the undesired multiplet component is achieved for $^1J_{NC}$ couplings in the range between 11.5 and 18.5 Hz. Within this range, the principle component is at least 30 times larger than the undesired minor component. We can thus obtain a very good filtering in isotropic phase, where the variation of the $^1J_{NC}'$ couplings is negligible with respect to the filtering capability. In the anisotropic phase, especially when strongly orienting Pf1 phages (Hansen et al. 1998) are exploited, insufficient subspectral editing is expected for some residues. However, this can easily be avoided by scaling the corresponding in- or antiphase spectra before the reconstruction of subspectra. Figure 11 shows overlaid subspectra recorded using the pulse sequence shown in Figure 10B from the uniformly $^{15}$N, $^{13}$C labeled human cardiac troponin C (cTnC), which has a molecular weight of 18 kDa (161 residues). Dipolar contribution to $^1J_{HNC}'$ can clearly be seen in the $F_2$-dimension by observing the direction of the slope connecting the E.COSY multiplets.

**Fig. 11.** Expansion of the HN($\alpha/\beta$-NC'-J) spectrum recorded from U-($^{15}$N, $^{13}$C) 18 kDa cTnC

(0.5 mM) in a dilute liquid crystal at 40ºC, $t_{\text{max}} (t_r) = 71$ (128) ms, 16 transients. The upfield and downfield multiplet components are shown overlaid. $^1J_{+D}$ and $^1J_{+D}$ can clearly be measured along the $^{15}$N- and $^1$H-dimensions, respectively. The spectrum was recorded.
on the Varian Unity 600 NMR spectrometer. The data were zero-filled to 2048x2048 points prior to Fourier transformation, and phase-shifted squared sine-bell window functions were applied in both dimensions.

It is also possible to measure $^{1}J_{NC}$ and $^{2}J_{HN}$ couplings from a HNCO-type spectrum by utilizing a short filter element matched to the $^{1}J_{HN}$ coupling. Ottiger (et al. 1998) have devised a pulse sequence in which the $^{2}J_{HN}$ coupling can be measured from a spin-state-selective two-dimensional H(N)CO spectrum. In their approach, $^{2}J_{HN}$ is measured from the $^{13}C$-dimension. Advantages of measuring $^{2}J_{HN}$ from the $^{13}C$ rather than from the proton dimension are more favorable relaxation properties and the absence of large proton-proton dipolar contributions in the anisotropic phase if protonated samples are used. Unfortunately, the $^{13}C$ chemical shift range is rather small (~10 ppm), and owing to its large chemical shift anisotropy, relaxation properties are not optimal at the highest magnetic fields. Kay and co-workers have used TROSY-based accordion spectroscopy (Bodenhausen & Ernst 1981) to measure $^{1}J_{NC}$ and $^{2}J_{HN}$ from 3D-HNCO spectrum (Yang et al. 1999). In this case, $^{1}J_{NC}$ and $^{2}J_{HN}$ are determined from the $^{15}N$- and $^{1}H$-dimensions from the slowly relaxing cross-peak. However, as the $^{15}N$-$^{13}C$ coupling is not refocused, the couplings are measured from an antiphase splitting, and errors similar to those found in the phase-sensitive COSY are likely to occur in unfavorable cases. In addition, spectral crowding increases since the number of peaks is doubled.

Figure 12 illustrates two-dimensional H(α/β-NC’-J)CO and corresponding 3D-HNCO(α/β-NC’-J) pulse schemes (II; Permi et al. 2000) for determination of $^{1}J_{NC}$ coupling either from spin-state-selective $^{13}C$-H’ or $^{13}C$-$^{15}N$-$^{1}H$ correlation spectrum, respectively.
Fig. 12. Pulse sequences of the (A) H(\(\alpha/\beta\)-NC\(^{-}\)J)CO and (B) HNCO(\(\alpha/\beta\)-NC\(^{-}\)J) experiments for measuring \(J_{\text{NC}}\) couplings from the two (three)-dimensional \(^{13}\)C', (\(^{15}\)N), \(^{1}H\) correlation spectra. Narrow and wide bars denote 90° and 180° pulses, respectively, whereas selective 90° pulses for water are denoted by half-ellipses. Aliphatic carbons are selectively decoupled during \(t_1\) with the semi-selective SEDUCE-I decoupling scheme (McCoy & Mueller 1992). Alternatively, a 180° pulse selective for \(\alpha\) carbons can be used. The WALTZ-16 sequence (Shaka et al. 1983) was used to decouple \(^1H\) during heteronuclear coherence transfer and \(^{15}\)N during acquisition. \(^{13}\)C 90° (180°) pulses were applied with a strength of \(\delta/\sqrt{15}\) \((\delta/\sqrt{3})\), where \(\delta\) is the frequency between centers of the \(^{13}\)C' and \(^{13}\)C\(^{\alpha}\) regions. All \(^{13}\)C' pulses were applied on-resonance and \(^{13}\)C\(^{\alpha}\) pulses off-resonance with phase modulation by \(\delta\). The vertical arrow indicates the position of the off-resonance compensation pulse. The delays employed are: \(\Delta = \tau = 1/(4J_{\text{NH}}); T_1 = 1/(4J_{\text{NC}'\alpha}); T_2 = 1/(4J_{\text{NC}'\alpha}); \lambda \geq 0; \delta = \text{gradient duration} + \text{recovery delay.}\) (A) Phase cycling for the in-phase experiment: \(\phi = x, -x; \phi_1 = 2(y), 2(-y); \phi_2 = 4(x), 4(-x); \phi_{\text{rec.}} = x, 2(-x), x.\) For the antiphase experiment, the phase of \(\phi\) is incremented by 90°. Frequency discrimination in \(F_1\) is achieved by incrementing \(\phi\) according to the States-TPPI protocol (Marion et al. 1989). (B) Phase cycling for the in-phase experiment: \(\phi = y; \phi_1 = x, -x; \phi_2 = 2(y), 2(-y); \phi_3 = 4(x), 4(-x); \phi_{\text{rec.}} = x, 2(-x), x.\) For the antiphase experiment, phase of the \(\phi\) pulse is incremented by 90°. Frequency discrimination in \(F_1\) is achieved by incrementing \(\phi\) according to the States-TPPI protocol. Frequency discrimination in \(F_2\) is obtained using the PEP sensitivity-enhanced gradient selection (Kay et al. 1992). The echo and anti-echo signals are collected separately by inverting the sign of the \(G_2\) gradient pulse together with the inversion of \(\phi\). In addition to echo/anti-echo selection, \(\phi_1\) and \(\phi_{\text{rec.}}\) are inverted according to the States-TPPI protocol for axial peak suppression. A 90° pulse on the
carbonyl carbon after the t₁ period serves as a purge pulse for the undesired dispersive magnetization component arising from the 1/(2\(J_{NC'}\)) mismatch (III-IV).

In both schemes, the magnetization is transferred from \(^1\)H\(^\alpha\) to \(^{13}\)C\(^\alpha\) via directly bound \(^{15}\)N, and overall sensitivity is improved by employing proton decoupling during the \(^{13}\)N-\(^{13}\)C\(^\alpha\) INEPT steps (Figure 12). Subsequently, proton single-quantum coherence is excited and edited with respect to \(^{15}\)N. Editing is based on the large and uniform \(J_{HN}\) coupling, providing an excellent subspectral editing with respect to the \(J\)-mismatch (Figure 6). Thus, at the beginning of the t₁ period, magnetization is in the form of 4\(^1\)H\(^\alpha\)-\(^{13}\)C\(^\alpha\)-\(^{15}\)N, whereas it is in the form of 2\(^1\)H\(^\alpha\)-\(^{13}\)C\(^\alpha\)-\(^{15}\)N in one experiment, whereas it is in the form of 2\(^1\)H\(^\alpha\)-\(^{13}\)C\(^\alpha\) in the other. After labeling the \(^{13}\)C\(^\alpha\) chemical shift and \(J_{NC}\) coupling frequencies during the t₁ evolution period, the magnetization is transferred back to the amide proton. In the 3D-HNCO(\(\alpha/\beta\)-NC\(^\alpha\)-J)-TROSY scheme (Permi et al. 2000), \(^1\)N chemical shift is detected during \(^{13}\)C\(^\alpha\)-\(^{15}\)N back-transfer in the usual constant-time manner, utilizing the gradient-selected sensitivity-enhancement scheme. Eventually, after a post-acquisitional addition and subtraction of the corresponding in- and antiphase data sets, the cross-peaks appear at \(\omega_{C'}(i-1) + \pi J_{CN}, \omega_{N}(i)\) and \(\omega_{C'}(i-1) \cdot \pi J_{CN}, \omega_{N}(i)\) in 2D, and \(\omega_{C'}(i-1) + \pi J_{CN}, \omega_{N}(i)\) and \(\omega_{C'}(i-1) \cdot \pi J_{CN}, \omega_{N}(i)\) in 3D data sets, respectively. The separation of cross-peak placements in the F₂-dimension between the two subspectra yields the \(J_{NC}\) couplings.

The improvement over the pulse sequence presented by Kay and co-workers is the spin-state-selective filtering utilized in the \(^{13}\)C-dimension. Hence, overlapping \(^1\)N-\(^{13}\)C doublet components are separated into two subspectra. Although transverse relaxation of \(^{13}\)C is faster than that of \(^1\)N in larger proteins, \(\alpha/\beta\)-filtering establishes the use of a shorter acquisition time in the \(^{13}\)C-domain. It is also beneficial to scale \(J_{NC}\) coupling up with respect to the \(^{13}\)C chemical shift in order to reduce the experimental time (at the cost of transverse relaxation, of course). Therefore, the number of t₁ increments can be reduced in the 3D version of the experiment. This also enables a more precise measurement of variations in \(J_{NC}\), and reduces random measurement errors since the measured coupling is divided by 1+κ. For large, perdeuterated proteins, the measurement of \(J_{NC}\) is better carried out using the 3D HNCO(\(\alpha/\beta\)-NC\(^\alpha\)-J)-TROSY scheme (Permi et al. 2000).

**5.2.2 Determination of \(J_{C'\alpha}\) coupling**

Relatively large dipolar contributions, i.e. 5-6 Hz, can be expected for one-bond scalar coupling between \(^{13}\)C\(^\alpha\)(i-1) and \(^{13}\)C\(^\alpha\)(i-1) in the protein backbone. The scalar coupling varies between 51-55 Hz, but to the best of our knowledge, no dependence on backbone local conformation has been reported. The most obvious way of measuring this coupling is to record a \(^{13}\)C-HSQC spectrum, in which the coupling between \(^{13}\)C\(^\alpha\) and \(^{13}\)C is allowed to evolve during the t₁ evolution period. This evolution period is usually implemented as a constant-time to decouple the relatively large couplings between aliphatic \(^{13}\)C\(^\alpha\) and \(^{13}\)C\(^\beta\). This is not very suitable for larger protonated protein samples due to the rapid relaxation of the \(^{13}\)C\(^\alpha\) spins and the constant-time nature of the experiment. Clearly, neither is the method applicable to perdeuterated samples. Alternatively, starting from the \(^1\)H\(^\alpha\)
magnetization, one could record a two-dimensional $^{13}\text{C}$-$^1\text{H}$ correlation spectrum, in which the $^{1}J_{c\alpha}$ coupling evolves concomitantly with the $^{13}\text{C}$ chemical shift during $t_1$. However, as the dispersion of resonances in the $^{13}\text{C}$-dimension is rather limited, the resulting 2D-spectrum may have severely overlapping signals.

As already mentioned, a $^{15}\text{N}$, $^1\text{H}$ correlation map usually gives best results when considering minimum overlap in a two-dimensional spectrum. Thus, it is advantageous to record the $^{15}\text{N}$, $^1\text{H}$ correlation spectrum, in which one is able to measure the $^{1}J_{c\alpha}$ coupling from cross-peak displacements in the $F_1$-dimension. Several slightly different approaches can be used, one of which is illustrated in Figure 13. In a simple HN($\alpha/\beta$-COCA-J) experiment (III; Figure 13A), the $^1\text{H}$ magnetization is first transferred to its preceding $^{13}\text{C}$ spin. At this point, the half-filter sensitive to the $^{13}\text{C}$ spin-state is inserted into the pulse sequence to create either $N_z C'_y$ or $N_z C'_x C''$ coherence for the in-phase and antiphase experiment, respectively. Subsequently, a mixed DQ/ZQ coherence between $^{15}\text{N}$ and the preceding $^{13}\text{C}$ is created by applying a 90° pulse for $^{15}\text{N}$ (time point $a$ in Figure 13A). During the following $t_1$ evolution period, the $^{15}\text{N}$ chemical shift evolves concomitantly with the $^{1}J_{c\alpha}$ coupling. Ultimately, the magnetization is transferred back to $^{15}\text{N}$ single-quantum coherence and is brought back to the amide proton. Eventually, after addition and subtraction of the in-phase and antiphase data sets and their corresponding quadrature counterparts, correlations at $\omega_n(i) + \pi J_{c\alpha}$, $\omega_n(i)$ and $\omega_n(i) - \pi J_{c\alpha}$, $\omega_n(i)$ appear. It is then obvious that $^{1}J_{c\alpha}$ can be measured from the cross-peak displacement in the $F_1$-dimension between the two subspectra.
Fig. 13. Pulse sequences of the (A) HN(\(\alpha\beta\delta\gamma\alpha\beta\\cdot\cdot\cdot\alpha\beta\delta\gamma\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\
decouple $^1$H during heteronuclear transfer and $^{15}$N during acquisition in non-TROSY experiments. The delays employed are: $\Delta = 1/(4J_{\alpha\text{rec}})$; $T_\gamma = 1/(4J_{\text{rec}})$; $T_\alpha = 1/(4J_{\text{rec}}^C)$. (A) Phase cycling scheme for the in-phase experiment is $\phi_1 = x, -x; \phi_2 = 2(x), 2(-x); \phi_3 = x; \phi_4 = x; \phi_5 = 4(x), 4(-x); \phi_6 = x, 2(-x), x$. For the antiphase experiment, $\phi_1$ and $\phi_2$ are incremented by 90°. Frequency discrimination in $F_1$ is obtained using the PEP sensitivity-enhanced gradient selection (Kay et al. 1992). The echo and anti-echo signals are collected separately by inverting the sign of $G_s$ gradient pulse together with inversion of $\phi_4$. (B) Phase cycling for the in-phase spectrum: $\phi_1 = y; \phi_2 = x; \phi_3 = y; \phi_4 = 4(x), 4(-x); \phi_5 = 2(x), 2(-x); \phi_6 = x, 2(-x), x$. For the antiphase experiment, $\phi_1$ and $\phi_2$ are incremented by 90°. Delays as in (A) except for $T_s = 1/(4J_{\text{rec}})$ + $T_\gamma/2 - \Delta/2 - \kappa t/4$; $T_s' = 1/(4J_{\text{rec}}') - T_\gamma/2 - \Delta/2 + \kappa t/4$. $0 \leq \kappa \leq T_\gamma/4$; $\delta = \text{gradient duration + recovery delay}$. Frequency discrimination in $F_1$ is obtained using the sensitivity-enhanced TROSY scheme with gradient selection (Weigelt 1998). The echo and anti-echo signals are collected separately by inverting the sign of $G_s$ gradient pulse together with inversion of $\phi_4$. (C) The phase cycling scheme for $\cos(\pi J_{\alpha\text{rec}}/4) \cos(\alpha_1 t)$ modulated data is $\phi_1 = x; \phi_2 = x, -x; \phi_3 = 2(x), 2(-x); \phi_4 = 4(x), 4(-x); \phi_5 = x; \phi_6 = x, 2(-x), x$. For $\sin(\pi J_{\alpha\text{rec}}/4) \sin(\alpha_1 t)$ modulated data, $\phi_1$ is incremented by 90°. Frequency discrimination in $F_1$ is obtained using the PEP sensitivity-enhanced gradient selection. The echo and anti-echo signals are collected separately by inverting the sign of $G_s$ gradient pulse together with inversion of $\phi_4$.

An alternative approach is to utilize the double-semi-constant-time scheme (DSCT) to evolve part of the $^{15}$N chemical shift during the $^{15}$N-$^1$C out- and $^1$H-$^{15}$N back-transfer steps. By combining this approach with the sensitivity-enhanced TROSY scheme, more sensitive spectra for larger proteins can be obtained (IV). Alternatively, one could first record the coupling between $^1$C and $^{13}$C under $^1$C single-quantum coherence and monitor the $^{15}$N chemical shift under $^{15}$N single quantum coherence during the $^1$C-$^{15}$N back-transfer step, by employing the semi-constant-time TROSY scheme in order to obtain sufficient resolution in the $^{15}$N dimension. Additional sensitivity improvement is obtained by concatenating the first $^{15}$N, $^1$H spin-state-selective filter element in the TROSY scheme with the $^1$C-$^{15}$N back-INEPT step (Salzmann et al. 1999; Permi et al. 2000; Figure 13B). Regrettably, use of both out- and back- $^{15}$N-$^1$C INEPT steps for $^{15}$N shift evolution results in concomitant downscaling of $^1J_{\alpha\text{rec}}$ and increases the error in the measured coupling. However, improvement in overall sensitivity may compete with the downscaling of apparent splitting and thus, no clear distinction between the accuracy of the two experiments can be made. One interesting aspect is the function of the last 90° pulse on $^1$C following the $^1$C-$^{15}$N back-INEPT step. This pulse purges the undesired dispersive magnetization components arising from the J-mismatch of delay $2T_s$, used to refocus $^1J_{\text{sc}}$ (III, IV). In most cases, due to relaxation, the delay $2T_s$ is set shorter than $1/(2J_{\text{sc}})$, and as a result, the dispersive antiphase term also contributes to the detected signal. Therefore, it is essential to use a purge pulse on $^1$C to yield an absorptive line shape. It may also be advantageous to apply selective decoupling of either the $^1$C out and/or $^1$C spins during acquisition (Yang & Kay 1999). This is because two- and three-bond dipolar contributions between $^1$H($^i$) and $^1$C($^i$-$^j$-$^k$) spins can be quite large in the anisotropic medium, and decoupling of these spins may improve the sensitivity of the experiments, even with larger proteins.

When considering large or highly alpha-helical proteins where resonance overlap is likely to occur more frequently, the need for a three-dimensional experiment is inevitable. The most obvious way to achieve sufficient resonance dispersion is then to record a HNCO-type experiment where $^1$C is allowed to couple to its preceding $^1$C during $t_1$. 
Kay and co-workers have successfully applied this approach with a TROSY implementation for two large, perdeuterated proteins (Yang et al. 1999). However, analogously to their HNCO-TROSY scheme for the measurement of $^{1}J_{NC}$ and $^{2}J_{HN}$ couplings, the $^{13}C$α coupled HNCO-TROSY experiment creates doublets in the spectrum, which may degrade a number of adequately separated cross-peaks. Thus, to achieve a minimum resonance overlap, it is advantageous to record an α/β-filtered HNCO-experiment (III; Permi et al. 2000; Figure 13C). Analogously to the 3D-HNCO(αβ-NC$'$-J) scheme, post-acquisitional addition and subtraction of the in- and antiphase data sets result in two spectra with correlations at $\omega_{x}(i-1) + \pi J_{cc} \alpha$, $\omega_{x}N(i)$ and $\omega_{x}(i-1) - \pi J_{cc} \alpha$, $\omega_{x}(i)$, $\omega_{y}N(i)$, respectively. This ensures a minimum resonance overlap and allows direct extraction of $^{13}C-^{13}C$ coupling constants by subtracting cross-peak frequencies in the F$_1$-dimension in the two subspectra. Since the $^{13}C-^{13}C$ coupling is large (~53 Hz) and the spin-state-selective filtering is utilized, $t_{1,max}$ can be sufficiently short, i.e. 20 ms. Without spin-state separation, a limited acquisition time for the $^{1}J_{CC} \alpha$ coupling would result in $^{13}C-^{13}C$ doublets, which are not resolved to the baseline. Consequently, the separation of doublet components would underestimate the true coupling values.

![Fig. 14. A representative 2D-plane from the HNCO(αβ-CC$'$-J)-TROSY spectrum recorded from the 30.4 kDa protein E2. The corresponding upfield and downfield $^{1}J_{CC} \alpha$ multiplet components are shown overlaid. A 1D trace is taken at the $^{13}C$ chemical shift of 176.0 ppm. The spectrum was recorded on the Varian Unity INOVA 600 NMR spectrometer using 4 transients per FID from 1.0 mM U-$^{15}N$, $^{13}C$ and 80% $^{2}H$-labeled E2, 95%/5% H$_2$O/D$_2$O, 40 °C, t$_{1,max}$, t$_{2,max}$, (t$_{3}$) = 17, 18, (64) ms. Resolution in the F$_1$-domain was doubled using forward...](image-url)
linear prediction. Data were zero-filled to 128x512x512 data matrices and apodized with shifted squared sine-bell functions in all dimensions.

For larger proteins, measurement of \( ^1J_{Cc,\alpha} \) is best carried out by using the transverse-relaxation-optimized HNCO(\( \alpha/\beta-C'-C\alpha - J \))-TROSY scheme (Permi et al. 2000). The TROSY version is essentially similar to the non-TROSY version, excluding a few modifications. In this case, the time period for \( ^{15}N \) frequency labeling is implemented in a manner similar to the semi-constant-time (SCT) TROSY evolution (IV). The \( ^{13}C'-^{15}N \) back-INEPT step and the first spin-state-selective filter element of the generalized TROSY scheme are concatenated to obtain an optimum sensitivity (Permi et al. 2000). In addition, for the selection of the most slowly relaxing \( ^{15}N-^1H \) multiplet component, it is possible to take advantage of the gradient- and sensitivity-enhanced TROSY implementation (Weigelt 1998). This enables minimal phase cycling needed for the coherence selection, and also provides for excellent water suppression.

The corresponding cross-peaks in the HNCO(\( \alpha/\beta-C'-C\alpha - J \))-TROSY experiment, after addition and subtraction, appear at \( \omega_{C'}(i-1) + \pi J_{C'C\alpha}, \omega_{N}(i) - \pi J_{NH}, \omega_{H\beta}(i) + \pi J_{NH} \), and \( \omega_{C'}(i) - \pi J_{C'C\alpha}, \omega_{N}(i) + \pi J_{NH} \), respectively. Therefore, \( ^1J_{C'C\alpha} \) couplings can be measured analogously to the HNCO(\( \alpha/\beta-C'-C\alpha - J \)) experiment, but from the most slowly relaxing \( ^{15}N-^1H \) multiplet component. A three-dimensional HNCO(\( \alpha/\beta-C'-C\alpha - J \))-TROSY spectrum recorded from the 30.4 kDa (286 amino acid residues), uniformly \( ^{15}N/^{13}C \) and 80% \(^2H\)-labeled protein, E2, is shown in Figure 14. The in- and antiphase data sets were recorded in an interleaved manner over 18 hours at a 600 MHz \(^1H \) frequency.

### 5.2.3 Access to \( ^1J_{N\alpha}, ^2J_{HNC\alpha}, ^2J_{NCA}, \) and \( ^3J_{HNC\alpha} \)

Determination of \( ^1J_{N\alpha} \) and \( ^2J_{HCA} \) from proteins is a difficult task because both intra- and interresidual couplings between the amide nitrogen and \(^{13}C\alpha \) are comparable, and rather small in magnitude. The intraresidual coupling varies between 7 and 12 Hz, whereas interresidual \( ^2J_{HCA} \) shows a discrepancy between 4 and 9 Hz (Bystrov 1976). If a \(^{15}N\)-HSQC spectrum without \(^{13}C\alpha \) decoupling during the t\(_1\) evolution period (Delaglio et al. 1991) is recorded, the cross-peaks split into a doublet of doublets in the \(^{15}N\)-dimension. This is due to the coupling of \(^{15}N(i) \) to both the \(^{13}C\alpha(i) \) and \(^{13}C\alpha(i-1) \) spins. Analogously to the case of \( ^1J_{N\alpha} \), it is necessary to decouple the \(^{13}C\alpha \) spins from \(^{15}N \) during t\(_1\) in order to maintain the simplified multiplet structure. In addition, if the spin-state of \(^{13}C\alpha \) is not perturbed, the \(^{15}N, ^1H \) cross-peaks show a tilted E.COSY pattern because both the intra- and interresidual \(^{13}C\alpha \)'s act as passive spins during the t\(_1\) and t\(_2\) periods. In practice, a triplet-like cross-peak is found due to the overlapping \( \alpha- \) and \( \beta- \)states of the corresponding doublet of doublets. Even in small proteins, it is difficult to resolve the two center lines of the multiplet. Recording the corresponding TROSY spectrum, with the most slowly relaxing multiplet component, provides narrower line widths, but adequate separation of the multiplet components is still difficult in most cases, as can be seen in Figure 15, illustrating the \(^{15}N, ^1H \) cross-peak from Lys63 in ubiquitin (IV).
Fig. 15. Expansion of the Lys63 $^{15}$N, $^1$H cross-peak recorded using the \{\(^{13}\)C$\alpha$\}$-^{15}$N-TROSY experiment. The spectrum was recorded on the Varian Unity 600 NMR spectrometer from 1.0 mM U-(\(^{15}\)N, \(^{13}\)C) ubiquitin, 90/10% H$_2$O/D$_2$O, 25°C, $t_{\text{max}} (t_1) = 222$ (128) ms. Data were zero-filled to 4kx4k data matrices and apodized with shifted squared sine-bell functions in both dimensions. The data were processed using a squared cosine bell weighting functions in both dimensions.

One possibility is to make use of accordion-style $J$-multiplication, as illustrated in Figure 16. The pulse sequence is a simple modification of the \{\(^{13}\)C$\alpha$\}$-^{15}$N-TROSY. In this case, the $^{15}$N chemical shift is recorded during the $t_1$ period, whereas the coupling between $^{13}\text{C}\alpha$ and $^{15}$N evolves simultaneously for $\kappa t_1$, where $\kappa$ is the multiplication coefficient. This approach necessitates, however, that the $^{15}$N spin relaxes at a favorable rate due to the long period of time during which the $^{15}$N spin is in the transverse plane.

Fig. 16. The $J$-multiplied \{\(^{13}\)C$\alpha$\}$-^{15}$N-TROSY experiment for the measurement of $J_{\text{nc.}}$, $J_{\text{nc.}}$, $J_{\text{nn.}}$, and $J_{\text{nnc.}}$ couplings in $^{15}$N, $^{13}$C, (H)-labeled proteins. Narrow and wide bars denote 90° and 180° pulses, respectively, whereas selective 90° pulses for water are denoted by half-ellipses. $^{13}$C 180° pulses were applied with a strength of $\delta/\sqrt{3}$, where $\delta$ is the frequency between centers of the $^{13}$C and $^{15}$C$\alpha$ regions. Aliphatic carbons are selectively decoupled during $t_1$ with the semi-selective SEDUCE-I decoupling scheme (McCoy & Mueller 1992). Alternatively, a 180° pulse selective for $\alpha$-carbons can be used. The delays employed are: $\Delta = 1/(4J_{\text{nn.}})$; $\delta =$ gradient duration + recovery delay; $\kappa \geq 0$. Phase cycling: $\phi_1 = \pi$, $\phi_2 = \pi$; $\phi_3 = \pi$; $\phi_4 = \pi$; $\phi_5 = \pi$.
\( \phi_{rec} = x, -x. \) Quadrature detection and TROSY selection in F\(_{1}\) is obtained by collecting two data sets, (I): \( \phi_2 = x; \phi_3 = y, \) (II): \( \phi_2 = -x; \phi_3 = -y, \) with simultaneous change in the gradient polarity (Weigelt 1998).

It is obvious that the multiplet pattern could be simplified if one of the \( ^{13}\text{C}^\alpha \) spins coupled to the \( ^{15}\text{N} \) spin could be selectively decoupled. This approach is not suitable for proteins due to numerous \( ^{13}\text{C}^\alpha \) spins, therefore, an alternative method is required to obtain a simplified multiplet pattern. We have used the HN(\( \alpha/b\)-NC\(^\alpha\)-J)-TROSY experiment (IV; Figure 17), allowing exploitation of spin-state-selective filtering sensitive to the \( ^{13}\text{C}^\alpha(i-1) \) spin-state. The pulse sequence in Figure 17 has been modified similar to the one presented in Figure 13B to obtain higher sensitivity.

Fig. 17. The pulse scheme of the HN(\( \alpha/b\)-NC\(^\alpha\)-J)-TROSY experiment for determination of \( J_{\text{NC}^\alpha}, J_{\text{NC}^\alpha}, J_{\text{HN}^\alpha}, \) and \( J_{\text{HN}^\alpha,\text{a}} \) couplings in \( ^{15}\text{N}/^{13}\text{C}/(\text{2H})\)-labeled protein samples. The delays employed are: \( \Delta = 1/(4 J_{\text{NC}^\alpha}); T_a = 1/(4 J_{\text{HN}^\alpha}) \cdot -\Delta; T' = 1/(4 J_{\text{HN}^\alpha,\text{a}}); 0 \leq \kappa \leq T'/T_{\text{max}}. \) Phase cycling for the in-phase spectrum: \( \phi_1 = x, -x; \phi_2 = x; \phi_3 = y; \phi_4 = 2(x), 2(-x); \phi_5 = 4(x), 4(-x); \phi_{rec} = x, 2(-x), x; \) for the antiphase spectrum, \( \phi_4 \) is incremented by 90°. The arrow indicates the position of the Bloch-Siegert compensation pulse in the antiphase filter. Frequency discrimination in F\(_{1}\) is obtained using the sensitivity-enhanced TROSY scheme with gradient selection. The echo and anti-echo signals are collected separately by inverting the sign of G\(_s\) gradient pulse together with inversion of \( \phi_2 \) and \( \phi_3. \) Resolution in the \( ^{15}\text{N} \)-dimension is improved by implementing an evolution period for the \( ^{15}\text{N} \) chemical shift and the \( ^{15}\text{N}^-\text{C}^\alpha \) couplings in a semi-constant time manner. Narrow and wide bars denote 90° and 180° pulses, respectively, whereas selective 90° pulses for water are denoted by half-ellipses. \( ^{13}\text{C} 90°(180°) \) pulses are applied with a strength of \( \delta/\sqrt{15} (\delta/3), \) where \( \delta \) is the frequency between centers of \( ^{13}\text{C} \) and \( ^{13}\text{C}^\alpha \) regions. All \( ^{13}\text{C} \) pulses are applied on-resonance and \( ^{13}\text{C}^\alpha \) pulses off-resonance with phase modulation by \( \delta. \)

Initially, magnetization is transferred from the \( ^1\text{H}^\alpha \) spin to \( ^{13}\text{C}^\alpha, \) as in the TROSY-type HNCO scheme. The subsequent spin-state-selective filter element is employed to edit the \( ^{13}\text{C}^\alpha(i-1) \) spin-state in order to create \( 4^1\text{H}^\alpha_N^\alpha C^\alpha \) coherence in the in-phase experiment and \( 8^1\text{H}^\alpha_N^\alpha C^\alpha C^\alpha \) coherence in the corresponding antiphase experiment (time point a). During the following \( ^{13}\text{C}^-\text{H}\) back-INEPT step, which is implemented as a semi-constant time, the \( ^{15}\text{N} \) chemical shift evolves simultaneously with couplings to \( ^{13}\text{C}^\alpha(i) \) and \( ^{13}\text{C}^\alpha(i-1) \) on the most slowly relaxing \( ^{15}\text{N}^-\text{H} \) multiplet component. Post-acquisitional addition and subtraction yields two subspectra, in which the centers of visible doublets are separated by \( J_{\text{NC}^\alpha}. \) The individual doublet components in either subspectrum are separated by \( J_{\text{HN}^\alpha,\text{a}}. \) Figure 18 clearly illustrates this spectral simplification, showing previously overlapping...
doublet components overlaid with thick and thin contours. Two additional couplings, namely $J_{H^N,N^C}$ and $J_{H^N,N^C}$, are readily available from this HN(αβ-NC$α$-J) experiment in the $^1$H dimension. A familiar E.COSY pattern emerges since the $^{13}$C$α$ spin acts as a common passive spin to both the $^1$N and $^1$H spins during the $t_1$ and acquisition periods, respectively. Thus, $J_{H^N,N^C}$ can be determined from cross-peak displacement in the $F_1$-dimension between two subspectra, whereas $J_{H^N,N^C}$ can be measured from the tilt between doublet components in the $F_2$-dimension within each subspectrum. Analogously to the former HN(αβ-C$C^α$-J) schemes, to preserve the purely absorptive line shape in $F_1$ and $F_2$, an additional 90° pulse on $^{13}$C is applied prior to the acquisition period.

Fig. 18. A selected $F_2$-$F_3$ plane from the 3D-HNCO(αβ-NC$α$-J)-TROSY experiment recorded from the 30.4 kDa, uniformly $^{15}$N/$^{13}$C and 80% $^2$H-labeled protein, E2. The spectrum was recorded on the Varian Unity INOVA 600 NMR spectrometer using 8 transients per FID from 1.0 mM U-(15N, $^{13}$C) and 80% $^2$H-labeled E2, 95%/5% H$_2$O/D$_2$O, 40 °C, $t_{max}$, $t_{max}$, (t$_3$) = 12, 35, (56) ms. Resolution in the $F_1$-domain was doubled using forward linear prediction. Data were zero-filled to 128x1kx1k data matrices and apodized with shifted squared sine-bell functions in all dimensions. The upfield and downfield $J_{H^N,N^C}$ multiplet components are shown overlaid. The visible splittings for the $J_{H^N,N^C}$ and $J_{H^N,N^C}$ couplings were multiplied by a factor of 4.

The HN(αβ-NC$α$-J)-TROSY scheme can be modified to obtain the three-dimensional HNCO(αβ-NC$α$-J)-TROSY experiment (Permut et al. 2000), which provides improved resolution necessary for highly α-helical proteins. In this case, the $^{13}$C chemical shift is
recorded during the \( t_1 \) period. The \( \alpha/\beta \)-filter is inserted after the \( t_1 \) evolution period and for the in-phase (antiphase) experiment, the \( 4H^N C^\alpha \) \( (8H^N C^\alpha C^\alpha) \) coherence is created before the \( t_2 \) evolution period, which is again implemented in a semi-constant-time TROSY manner. However, due to the relatively small size of the \( ^1J_{NC}^\alpha \) coupling, an additional spin-echo period must be inserted for the \( ^1N-^1C^\alpha \) coupling evolution to reduce the number of increments needed in the \( F_2 \)-dimension. Hence, the \( ^1J_{NC}^\alpha \) and \( ^2J_{NC}^\alpha \) couplings scale up by \( 1+\lambda \) \( (\lambda \geq 0) \) with respect to the \( ^1N \) chemical shift. After addition and subtraction of the in- and antiphase data sets and their corresponding quadrature counterparts, cross-peaks are found at \( \omega \) \( C^\alpha \) \( (i-1) \), \( \omega \) \( N \) \( (i) \) + \( \pi \) \( J_{NH} \) + \( (1+\lambda) \) \( \pi \) \( J_{NC}^\alpha \), \( \omega \) \( N \) \( (i) \) - \( \pi \) \( J_{NH} \) - \( (1+\lambda) \) \( \pi \) \( J_{NC}^\alpha \), \( \omega \) \( H^N \) \( (i) \) + \( \pi \) \( J_{NH} \) - \( \pi \) \( J_{HC}^\alpha \), \( \omega \) \( H^N \) \( (i) \) + \( \pi \) \( J_{NH} \) + \( \pi \) \( J_{HC}^\alpha \), for the two- and three-dimensional subspectra, respectively.

## 5.2.4 Insight to side-chains, \( ^1J_{C\alpha C\beta} \)

Up to this point, we have obtained a wealth of data from which the orientation of several internuclear vectors in the protein backbone can be derived. It is obvious that it is crucial to gain information on the orientation of side-chains, as well. A logical approach is to measure the one-bond couplings between the \( ^{13}C^\alpha \) and \( ^{13}C^\beta \) spins. This is readily available from a \( ^{13}C \) HSQC spectrum. However, several reasons limit the applicability of a \( ^{13}C \) HSQC method to larger proteins. First, the high transverse relaxation rates of the \( ^{13}C^\alpha \) and \( ^1H^\alpha \) spins dramatically reduce the intensity and resolution of the \( ^{13}C \) HSQC spectrum. Second, the \( ^1H^\alpha \) spins resonate close to the water signal, which hampers the interpretation of data. Third, if perdeuterated samples are used, it is clear that this method cannot be used. We can alleviate these problems with a HNCO -based TROSY-type experiment, as shown in Figure 19 (Permi et al. 2000).

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**Fig. 19.** The pulse sequence of the HNCO(C^\alpha C^\beta -J)-TROSY experiment for the measurement of \( ^1J_{C\alpha C\beta} \) couplings in \( ^{13}N/^{13}C/^{1}H \)-labeled proteins. The delays employed are: \( \Delta = 1/(4J_{NH}) \); \( T_a = 1/(4J_{NC}) \); \( T_b = 1/(4J_{NC}) - \Delta \); \( T_c = 1/(4J_{HC}) \); \( 0 \leq \kappa \leq T/c_{max} \); \( \lambda \geq 0 \); \( 0 \leq \mu \leq T/c_{max} \). Phase cycle: \( \phi_1 = y \); \( \phi_2 = x \); \( \phi_3 = y \); \( \phi_4 = 2x \); \( \phi_5 = x \); \( \phi_6 = 4x \); \( \phi_rec = x \); \( 2x \); \( x \). The arrow indicates the position of the Bloch-Siegert compensation pulse in the antiphase filter. Frequency discrimination in \( F_1 \) is obtained using the sensitivity-enhanced TROSY scheme.
with gradient selection. The echo and anti-echo signals are collected separately by inverting the sign of $G_s$ gradient pulse together with inversion of $\phi_2$ and $\phi_3$. The resolution in the $^{15}$N-dimension is improved by implementing an evolution period for the $^{15}$N chemical shift and $^{15}$N-$^{13}$C$^{\alpha}$ couplings in a semi-constant time manner. Narrow and wide bars denote 90° and 180° pulses, respectively, whereas selective 90° pulses for water are denoted by half-ellipses. $^{13}$C 90° (180°) pulses are applied with a strength of $\delta_1/\sqrt{15}$ ($\delta_1/\sqrt{3}$), where $\delta_1$ is the frequency between centers of the $^{13}$C′ and $^{13}$C$^{\alpha}$ regions. All $^{13}$C pulses are applied on-resonance and $^{13}$C$^{\alpha}$ pulses off-resonance with phase modulation by $\delta_1$.

Initially, magnetization is transferred from the $^1$H spin to the preceding $^{13}$C$^{\alpha}$ spin. Depending on the sample, we can use two different schemes to record $^{1}J_{\alpha\beta}$. Consider first a protonated sample. During the following $t_1$ evolution period, $^{1}J_{\alpha\beta}$ evolution is allowed to take place, while the large $^{1}J_{\alpha\beta\gamma}$ and the chemical shift of $^{13}$C′ are refocused by inserting a 180° ($^{13}$C$^{\alpha}$/C$^{\beta}$) pulse in the middle of $\lambda t_1$. Instead of $^{13}$C′, we record the $^{13}$C$^{\alpha}$ chemical shift during the following $^{1}J_{c\alpha}$ refocusing delay. This period is implemented in a semi-constant time manner in order to obtain sufficient resolution in the $F_1$-dimension. Eventually, the $^{15}$N chemical shift is incremented during the $t_2$ evolution period by using the semi-constant-time TROSY scheme (IV; Permi et al. 2000). It should be noted that, by using this approach, the 180° ($^1$H) pulse during the $t_1$ time for $^{1}J_{\alpha\beta\gamma}$ refocusing can be omitted. As this pulse would interchange the fast and slow relaxing $^{13}$N-$^1$H multiplet components, the presented scheme is preferred. On the other hand, the $^{13}$C$^{\alpha}$ chemical shift can be recorded without mixing the $^{13}$N-$^1$H spin-states if we apply during $t_1$ a semi-selective $^1$H$^\alpha$ decoupling or, alternatively, a spin-locking. Finally, the $^{13}$C$^{\alpha}$, $^{15}$N, $^1$H correlation map results, where the multiplets in the $F_1$-dimension are resolved by $\lambda^*$ $^{1}J_{\alpha\beta}$, as illustrated in Figure 20 (Permi et al. 2000).

Fig. 20. A representative portion of the HNCO($^{13}$C$^{\alpha}$,$^{15}$N)-TROSY spectrum of ubiquitin. Cross-peaks are shown for K6, I13, L67, and V70 residues at the $^{15}$N cross-section of I13. The cross-peaks are split by apparent $2^{*}J_{\alpha\beta}$ in the $F_1$-dimension ($\lambda = 2$). The spectrum was recorded on the Varian Unity INOVA 500 NMR spectrometer using 24 transients per FID
from 1.0 mM U-(\textsuperscript{15}N, \textsuperscript{13}C) ubiquitin, 90%/10% H\textsubscript{2}O/D\textsubscript{2}O, 30\textdegree C, t\textsubscript{1,max}, t\textsubscript{2,max}, (t\textsubscript{s}) = 37.6, 18.8, (64) ms. The data were post-processed to a 1024x128x1024 matrix prior to Fourier transformation, and phase-shifted squared sine-bell window functions were applied in all dimensions.

For perdeuterated samples, a scheme in which the \textsuperscript{13}C\textsubscript{α} chemical shift is recorded simultaneously with \textsuperscript{1}J\textsubscript{Cα,Cβ}, is preferred. In this case, it is also possible to use \textit{J}-scaling by inserting an additional spin-echo period for the \textsuperscript{1}J\textsubscript{Cα,Cβ} evolution. As the relaxation rate of the \textsuperscript{13}C\textsubscript{α} spin is rather long in perdeuterated samples, this implementation improves the accuracy in data analysis, thanks to the properly resolved coupling. Additionally, since the measured coupling is divided by 1+\lambda, more precise values with respect to random measurement error can be obtained. Subsequently, magnetization is transferred back to \textsuperscript{15}N via the \textsuperscript{13}C\textsubscript{α} spin, and the \textsuperscript{15}N chemical shift is recorded during the SCT TROSY period.
6 Conclusions

Several different approaches have been proposed to increase the protein size limit in solution NMR spectroscopy. Heteronuclear triple resonance experiments, enabled through the isotopic enrichment of carbon and nitrogen atoms in protein samples, have rapidly extended this size limit from 10 to 30 kDa during the last decade. The $^{15}\text{N}/^{13}\text{C}$-enrichment, combined with perdeuteration, allows the assignment of proteins in the size regime of 40 to 50 kDa. Very recently, transverse relaxation optimized spectroscopy has pushed the size limit even further, and as a result, backbone resonance assignment has been successfully obtained for a 110 kDa octamer (Salzmann et al. 2000). However, protein structure determination is still a very time-consuming procedure, especially in the case of assignment of NOEs. On the other hand, the use of perdeuterated samples suppresses a major part of the information available from NOESY spectra for global fold determination, ultimately leading to loose models. It is therefore inevitable that special means are needed to speed up global fold recognition and to complement long-range distance information lost through the perdeuteration procedure. Use of residual dipolar couplings serves as a powerful solution to both problems.

It has been shown that relatively precise structures can be determined by utilizing residual dipolar couplings with the aid of a small quantity of NOE information (Clore et al. 1998b; Mueller et al. 2000). Thus, the aim should be placed on the structure determination of highly perdeuterated or site-specifically protonated proteins, where the transverse relaxation times of $^{15}\text{N}/^{13}\text{C}\alpha$ spins can be rather long when the TROSY approach is used. Thus, the protein backbone assignment can be retrieved using spectra with high sensitivity and resolution. The measurement of dipolar couplings can be accomplished in a relatively short time compared with traditional NOE analysis. The measurement of changes in the residual dipolar couplings supplemented with chemical shift changes due to ligand binding can provide unique and valuable insight into the structural biology of protein and protein complexes. The directional information, available through several residual dipolar couplings, can be obtained as soon as the protein backbone assignment procedure is accomplished. The experiments presented can supply up to nine residual dipolar couplings in protonated or perdeuterated proteins. Two-dimensional spin-state-selective $^{15}\text{N}, ^1\text{H}$-detected experiments can be very handy in structure activity relationship (SAR) by NMR studies (Shuker et al. 1996; Hajduk et al.
Thus, the wealth of information available through residual dipolar couplings can be used concomitantly with chemical shift changes as an indicator of conformational changes induced by ligand binding. The spin-state-selective $^{15}\text{N}$, $^1\text{H}$-detected experiments are the most suitable ones for large proteins, owing to the inherently good dispersion of the $^{15}\text{N}$, $^1\text{H}$ correlation map. Furthermore, selection of the most slowly relaxing (TROSY) component, especially in perdeuterated proteins, improves the sensitivity of the $^{15}\text{N}$, $^1\text{H}$-detected experiments considerably. For the largest or highly helical proteins, it may be necessary to record three-dimensional HNCO-type experiments, combined with spin-state-selective subspectral editing and TROSY selection. This approach also provides minimal resonance overlap in larger $\alpha$-helical proteins, which otherwise often exhibit overcrowded spectra.

Development in experimental methodology and new labeling techniques, as well as technical advancements in NMR instrumentation, have not only increased the protein size limit but have also enabled more convenient and reliable structure determination of smaller proteins. The proposed IM-HSQC experiment enables precise and efficient determination of the structurally most important three-bond $J$-coupling between $^1\text{H}^N$ and $^1\text{H}^\alpha$ spins from $^{15}\text{N}$-HSQC-type spectra. It is also applicable to protein samples with minimum isotope labeling, i.e. $^{15}\text{N}$-enrichment.
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