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^{1}H-filtered correlation experiments for assignment and determination of coupling constants in backbone labelled proteins

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Abstract

The implementation of [^{13}Cα,^{13}C′,^{15}N,^{2}Hα] labelled amino acids into proteins allows the acquisition of high resolution triple resonance experiments. We present for the first time resonance assignments facilitated by this new labelling strategy. The absence of $^{1}J_{C\alpha,C\beta}$ couplings enables us to measure $^{1}J_{C\alpha,C'}$ scalar and $^{1}D_{C\alpha,C'}$ residual dipolar coupling constants using modified HNCA experiments which do not suffer from sensitivity losses characteristic for ^{13}C constant time experiments.

Introduction

HNCA and HN(CO)CA experiments are important tools for the backbone assignment of ^{15}N,^{13}C labelled proteins. Using the Cα chemical shifts in order to establish sequential connectivities, these experiments provide intra- and inter-residue correlations. However, they suffer from poor resolution in the crucial Cα dimension. In order to prevent the resolution of the homonuclear $^{1}J_{C\alpha,C\beta}$ scalar coupling, the Cα acquisition time is usually limited to 10 ms (Grzesiek and Bax, 1992). Consequently, this approach frequently does not allow unambiguous identification of correlations. Elimination of this splitting would possibly remove many ambiguities in the resonance assignment and lead to higher resolution and sensitivity.

In an indirectly detected dimension, homonuclear coupling can be removed with a constant time (CT) acquisition scheme (Vuister and Bax, 1992; Santoro and King, 1992). In an HNCA or HN(CO)CA experiment, CT-Cα acquisition eliminates the $^{1}J_{C\alpha,C\beta}$ coupling (Yamazaki et al., 1994). This approach has a major drawback: the time period during which transverse Cα magnetisation is present is increased to approximately 27 ms. For medium sized proteins this means a significant reduction or even elimination of magnetisation during the course of the experiment. It is well known that deuteration dramatically improves the situation by increasing the transverse relaxation time of Cα and makes ^{13}C-CT-HNCA and ^{13}C-CT-HN(CO)CA experiments much more sensitive. However, for large proteins the signal attenuation is still significant, as demonstrated by a TROSY ^{13}C-CT-HNCA experiment of a 110 kDa octamer. In this example a reduction of the signal to noise ratio (S/N) by a factor of three was observed when compared to an experiment with conventional ^{13}C acquisition (Salzmann et al., 1999).

In principle, selective decoupling (Kupce and Wagner, 1995) or selective refocusing (McCoy, 1995) of Cβ overcomes this limitation. However, these approaches introduce further undesirable complications. Firstly, the analysis of available chemical shift data indicates overlap of certain combinations of residues. For example, it is in general impossible to decouple serine Cβ atoms selectively. Secondly, the composite

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pulse trains of decoupling sequences introduce decoupling sidebands and an undesirable Bloch-Siegert shift on Cα resonances (Matsuo et al., 1996), which might result in additional complications in automated assignment protocols. It has been shown that the effect of the homonuclear $^1J_{C\alpha,C\beta}$ coupling in uniformly labelled proteins can be diminished by splitting the carbon evolution time into separate periods. Each period is incremented to the extent that the evolution of the coupling remains tolerable. However, the sensitivity of this method is affected by the nonlinear coupling evolution and a gradient echo required for the combination of the evolution periods (Baur et al., 1998).

Here we demonstrate an alternative approach using proteins that are isotopically enriched exclusively in the backbone N, Cα and C′ atoms. This permits optimal resolution and sensitivity to be obtained in triple resonance experiments, since non CT detection schemes can be used for acquisition in indirect detected dimensions. The acquisition time can be extended to a point that is limited only by the ^{13}Cα transverse relaxation time. We synthesised backbone ^{13}C, ^{15}N labelled Phe, Leu, Glu, Asn, Val, Lys, Ala, Ile, Asp and Gln stereoselectively by chemical means starting from ^{13}C, ^{15}N labelled glycine as described (Giesen et al., 2001). In order to minimize the synthetic effort 50% of the Cα atoms were deuterated. Two samples of partially backbone labelled human ubiquitin were then obtained from these amino acids by biochemical means. [^{13}Cα, ^{13}C′, ^{15}N, 50% ^{2}Hα] labelled Val, Phe, Leu and [^{13}Cα, ^{13}C′, ^{15}N, 50% ^{2}Hα] labelled Val, Glu, Lys, Asn, Asp, Gln, Ala and Ile were incorporated into two separate samples. ^{13}Cα,^{1}Hα-correlation spectra and ^{15}N,^{1}H-correlation spectra confirmed the desired labelling pattern. Scrambling of ^{13}C or ^{15}N into side chains was not observed. However, we detected Phe and Tyr resonances in V,E,K,N,D,Q,A,I backbone labelled ubiquitin as a result of isotopic scrambling.

NMR experiments

Two backbone labelled samples of ubiquitin (V,F,L backbone labelled and V,E,K,N,D,Q,A,I backbone labelled) in 200 µl solution (H_2O:D_2O = 9:1, phosphate buffer, pH = 5, 30 °C, concentration 0.8 mM) were used. All spectra were recorded on a 500 MHz Varian Unity Inova spectrometer. The ^{1}H-filtered 2D-HNCA and 2D-HN(CO)CA experiments were acquired with spectral widths of 4000 Hz and 3000 Hz and acquisition times of 128 ms and 42 ms in the ^{1}H and ^{13}C dimensions, respectively. The $J_{C\alpha,C'}$ modulated HNCA experiment was acquired with the same acquisition parameters and a constant time period for the evolution of the coupling of T = 11 ms and delays Δ = 1, 2,..,11 ms. The acquisition time was 6 h per spectrum.

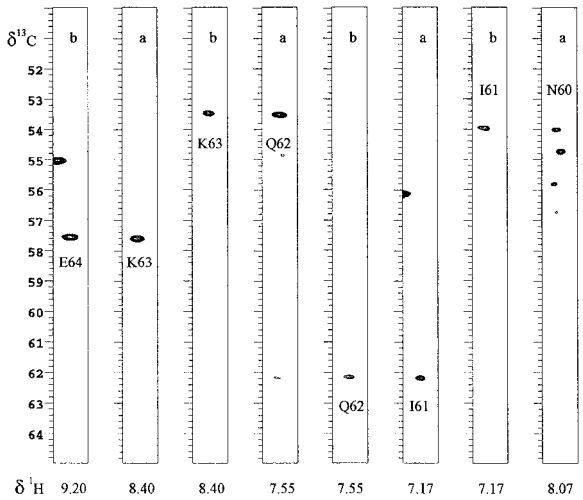

Figure 1. Selected strip plots for the assignment of residues E64 to N60 of V,E,K,N,D,Q,A,I backbone labelled ubiquitin. (a) ^{1}H-filtered HNCA and (b) ^{1}H-filtered HN(CO)CA. Due to the high resolution obtained from the backbone labelled protein two dimensional $^1H^N$,^{13}Cα-spectra were used for assignment.

Results and discussion

Figure 1 shows selected HNCA and selected HN(CO)CA strip plots taken from 2D spectra of V,E,K,N,D,Q,A,I backbone labelled ubiquitin. Due to Cα deuteration and the absence of the $^1J_{C\alpha,C\beta}$ coupling it was possible to record these spectra with high resolution in the carbon-dimension. The significant one bond deuterium isotope shift of approximately 0.4 ppm causes a doubling of signals and increases the likelihood of overlap (data not shown). Therefore, the resonances arising from Cα spins that are one bond coupled to protons as opposed to deuterons are suppressed by a ^{1}Hα-filter (Figure 2). Carbon magnetisation arising from ^{13}Cα,^{1}Hα pairs evolves over a period 2ξ ($=1/2J_{CH} \approx 3.6$ ms) into an antiphase term which is converted into longitudinal multispin order and not retransformed into observable magnetisation. For optimal results one would work with fully ^{2}H labelled proteins. This would remove the need for the ^{1}H-filter in the pulse sequence. On the other hand, pro-

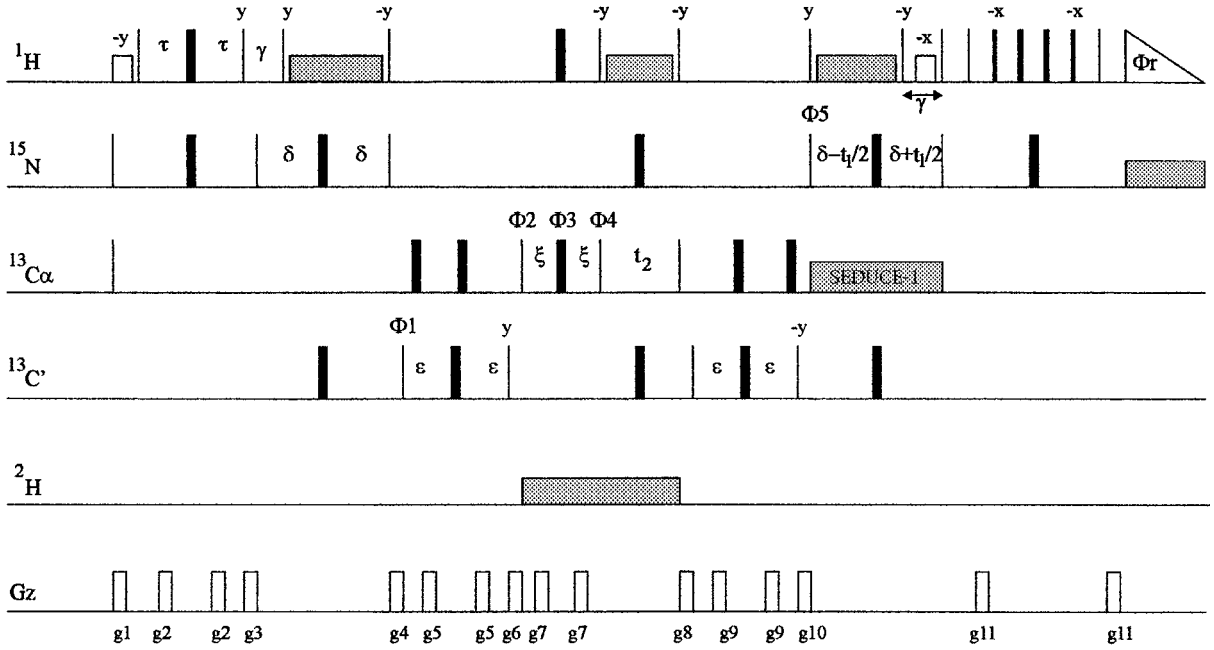

Figure 2. Pulse scheme for acquisition of two- and three-dimensional HN(CO)CA spectra with suppression of correlations to protonated Cα. Narrow bars represent 90° pulses and wide bars represent 180° pulses, with phase x unless indicated. The power and duration of Cα pulses are adjusted to give a null in their excitation at the carbonyl carbon frequency and vice versa. The final six proton pulses are symmetrical 3-9-19 refocusing pulses for watergate water suppression (Sklenar et al., 1993). Phase cycling: Φ_1 = x, −x; Φ_2 = x, x, −x, −x; Φ_3 = x; Φ_4 = y; Φ_5 = x; Φ_r = x, −x, −x, x. Quadrature detection is obtained by altering the phases Φ_2, Φ_3 and Φ_4 for t_2-dimension and Φ_5 for t_1-dimension in a States-TPPI manner (Marion et al., 1989). The delay ξ is set to $1/(4^1J_{C,H})$, and the other delays and pulsed field gradients in the sequence are set according to the scheme described by Matsuo et al. (1996). ^{2}H decoupling is achieved using a 900 Hz field with WALTZ profile. A SEDUCE-1 (McCoy and Mueller, 1992) decoupling field with 28 ppm bandwidth centred in the Cα region is applied during t_1.

tonation of the Cα-position allows the measurement of $^1J_{C\alpha,H\alpha}$ and $^1J_{C\alpha,C'}$ coupling using a [^{1}H,^{13}C]-HSQC approach. In the present and in previous studies backbone labelled amino acids with ca. 50% deuteration of the Cα position were used for the expression of the proteins. This enabled us to work with a single sample for Cα-deuterated and Cα-protonated experiments.

Triple resonance spectra of uniformly ^{13}C labelled proteins with high resolution in the indirect detected Cα-dimension are frequently obtained from Cα constant time experiments. In general, those experiments suffer from a significant S/N-reduction caused by T_2-relaxation of the Cα-magnetisation during the constant time period. Obviously, such a CT acquisition period is not required for backbone labelled proteins. The S/N-gain achieved by using a non CT acquisition scheme for backbone labelled proteins compared to CT acquisition can be estimated by:

$$G = \frac{\int_0^{t_{max}} e^{-t/T_2} dt}{\int_0^{t_{max}} e^{-T/T_2} dt} \quad (1)$$

T is the constant time period, T_2 is the Cα transverse relaxation time and t the acquisition time. Figure 3 shows the S/N-gain for backbone labelled proteins as a function of the transverse Cα relaxation time. This figure indicates a significant S/N-gain for systems with T_2 below 60 ms.

For fully ^{13}C labelled proteins the $^1J_{C\alpha,C'}$ coupling is frequently measured from the signal splitting in the C′-dimension of Cα-coupled HNCO (Ottiger and Bax, 1998a), TROSY-HNCO (Yang et al., 1999) or spin state selective HNCO (Permi et al., 2000) experiments. For high magnetic fields this approach becomes unfavourable because of the effective chemical shift anisotropy relaxation of the carbonyl atom. Relaxation times of ^{13}Cα and ^{13}C′ nuclei in uniformly

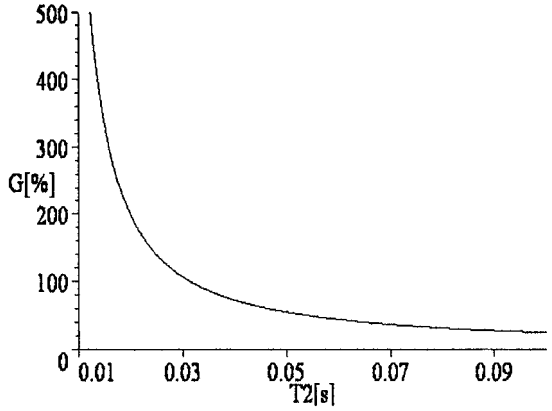

Figure 3. Estimated S/N-gain for non constant time acquisition (backbone labelled protein) compared to constant time acquisition (uniformly ^{13}C labelled protein) as a function of T_2 (Cα). Constant time period is $T = 26.6$ ms, acquisition time in Cα dimension is $t_{max} = 10$ ms.

^{13}C,^{15}N labelled and ^{2}H,^{13}C,^{15}N labelled proteins (Venters et al., 1996; Permi et al., 2001) indicate that for deuterated proteins it should be advantageous to measure the $^{1}J_{C\alpha,C'}$ coupling by observing the splitting of the Cα-signal instead of the C′-signal. Straightforward estimations of T_2(Cα) and T_2(C′) for [^{2}Hα,^{13}Cα,^{13}C′,^{15}N] labelled proteins as a function of the correlation-time τ_C and magnetic field strength indicate a dramatic decrease of the transverse relaxation time for carbonyl carbons with increasing field strength, whereas the transverse relaxation time for deuterated Cα is only mildly affected.

Whereas in fully ^{13}C labelled proteins the measurement of $^{1}J_{C\alpha,C'}$ or $^{3}J_{C\alpha,C\alpha}$ couplings from an HNCA experiment is complicated by passive couplings (i.e. $J_{C\alpha,C\beta}$, $J_{C\alpha,C\gamma}$, etc.), the absence of NMR active side chain carbons in backbone labelled proteins allows the measurement of the $^{1}J_{C\alpha,C'}$ couplings by direct observation of the Cα-signal splitting in a C′-coupled HNCA experiment. During the course of this experiment, the spin state of the carbonyl carbon remains undisturbed. It is well known that under this condition the $^{3}J_{HN,C'}$ couplings are observed as a displacement of the components along the proton dimension (Seip et al., 1994, Weisemann et al., 1994). Thus, the coupled HNCA experiment allows the simultaneous measurement of $^{1}J_{C\alpha,C'}$ and $^{3}J_{HN,C'}$ coupling constants.

We measured $^{1}J_{C\alpha,C'}$ couplings using the HNCA and HNCO approach and obtained a pairwise rmsd of 0.37 Hz (40 values). The C′-coupled, ^{1}H-filtered HNCA experiment can be performed easily in anisotropic media for measurement of residual dipolar couplings. In our hands, the change of the Cα,Hα-coupling constant due to dipolar contributions in diluted liquid crystal media does not affect the efficiency of the ^{1}H-filter significantly. Figure 4 shows strip plots for F4 in V,F,L backbone labelled ubiquitin in water, 5% and 7.5% DHPC/DMPC liquid crystal media. In this example the coupling is measured twice by observing the H_i^N,$C\alpha_i$ (F4) and the H_{i+1}^N,$C\alpha_i$ (V5) cross peaks. With the HNCA approach residual dipolar coupling data for residues followed by proline can be measured, which is not possible with the HNCO experiment.

The determination of single-bond J splittings from the separation of doublets is useful for well resolved spectra of small proteins. However, due to the doubling of resonance lines it becomes more difficult to resolve peaks as the protein size increases. This problem can be alleviated somewhat by collecting spectra in which each individual doublet component is collected separately (Ottiger et al., 1998b). Another approach for the measurement of the $^{1}J_{C\alpha,C'}$ couplings involves the introduction of a $^{1}J_{C\alpha,C'}$ intensity modulation into the HNCA experiment (Figure 5). The main advantages of intensity modulated methods over direct measurement of coupling constants from signal splitting are a reduction in the complexity of the spectra and a high reproducibility of the data obtained (Tjandra et al., 1996). It has been demonstrated that intensity modulation type experiments allow the accurate determination of various one bond coupling constants, e.g., $^{1}J_{C\alpha,H\alpha}$ (Hitchens et al., 1999) or $^{1}J_{N,H}$ (Tolman et al., 1996). We implemented a constant time modulation scheme for the measurement of the $^{1}J_{C\alpha,C'}$ coupling and simultaneous chemical shift labelling in a manner similar to the $^{1}J_{C,H}$ modulated HSQC described by Bax et al. (1996). The constant time period for the evolution of the $^{1}J_{C\alpha,C'}$ coupling is set to 11 ms. This allows the acquisition of intensity modulated data up to the first minimum at 1/(2 $^{1}J_{C\alpha,C'}$) for the cosine-type modulation function (9 ms for $^{1}J_{C\alpha,C'} = 55$ Hz). The intensity of a cross-peak in this constant time J-modulated HNCA experiment is proportional to $\cos[2\pi^{1}J_{C\alpha,C'}(T - \Delta)]$, where T is the constant time period and Δ is a variable delay. $^{1}J_{C\alpha,C'}$ is determined by optimising the fit between $I_0 \cos[2\pi^{1}J_{C\alpha,C'}(T - \Delta)]$ and the observed intensities. A numerical simulation shows that for small dephasing delays used in these experiments the long range $^{3}J_{C\alpha,C\alpha}$ has no significant effect on the obtained coupling.

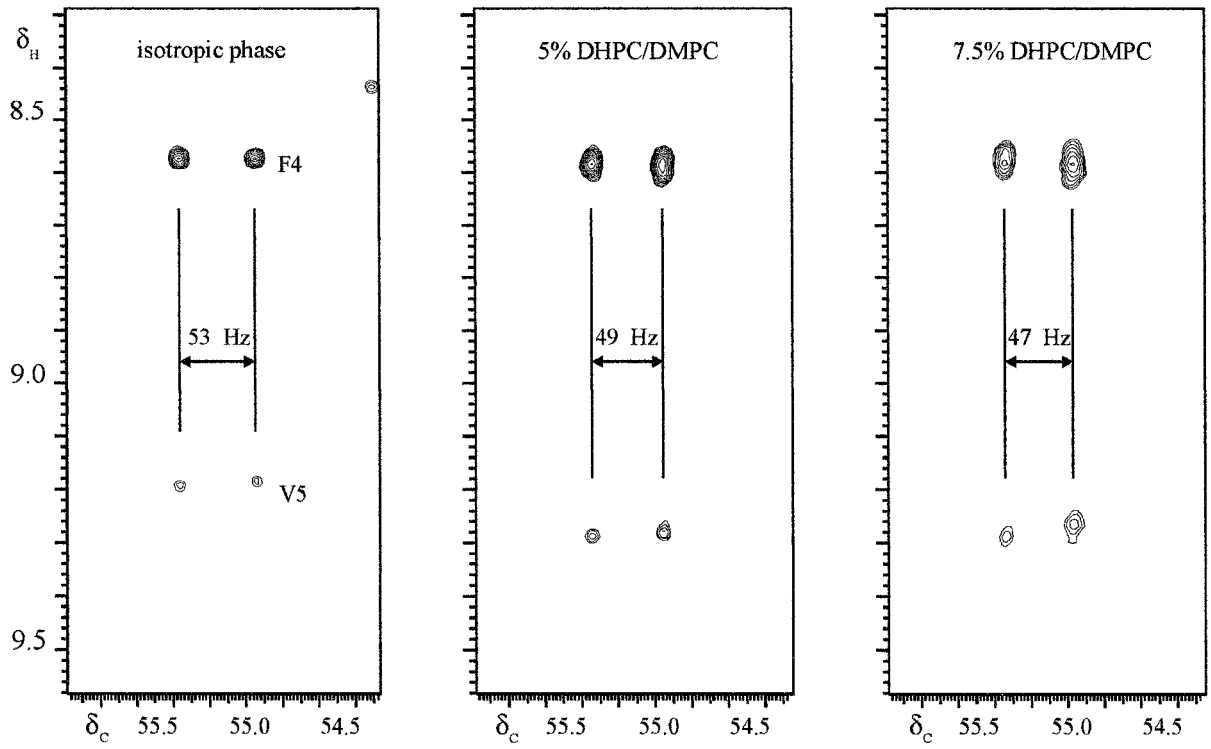

Figure 4. Selected regions of a C′-coupled ^{1}H-filtered HNCA of V,F,L backbone labelled ubiquitin, dissolved in water (30 °C, 90% H_2O, 10% D_2O), 5% DHPC/DMPC (38 °C) and 7.5% DHPC/DMPC (38 °C) liquid crystal medium. The cross peaks are the $H_i^N C\alpha_i$ correlation for F4 and the $H_j^N C\alpha_{j-1}$ correlation for V5. Both cross peaks show the same Cα, C′ splitting for residue F4.

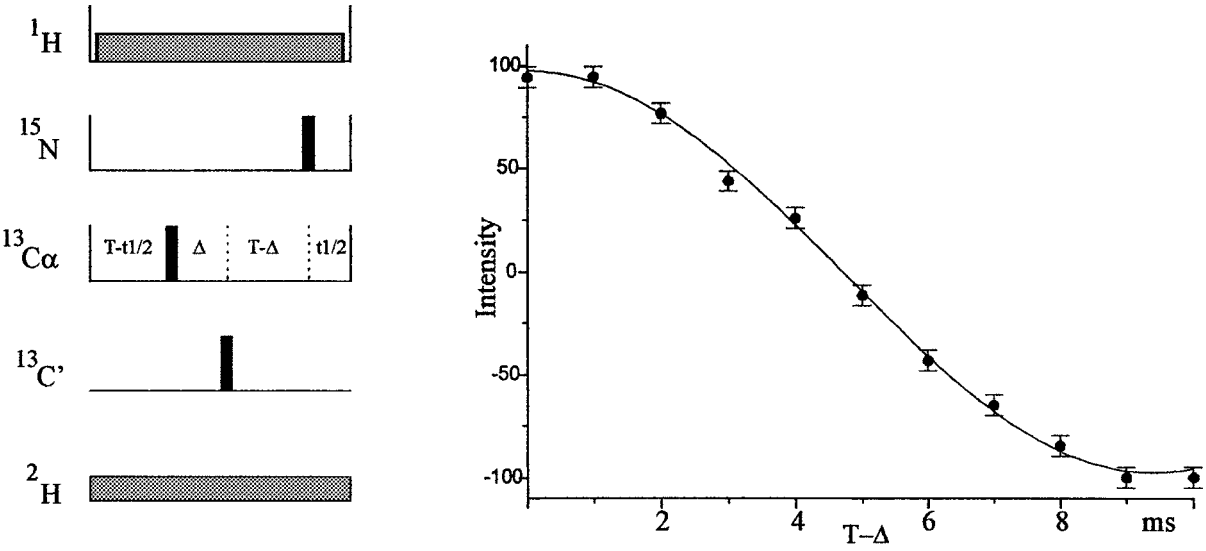

Figure 5. Left: Pulse sequence block for simultaneous Cα chemical shift labelling and constant time intensity modulation of $^{1}J_{C\alpha,C'}$ coupling. The carbonyl 180 °C pulse was applied using a SEDUCE-1 profile with a pulse length of 160 ms. This building block was implemented into a ^{1}H-filtered HNCA sequence (Coughlin et al., 1999). Right: Intensity modulation for I44 in V,E,K,N,D,Q,A,I backbone labelled ubiquitin. The modulation of the $H_i^N C\alpha_i$ resonance intensity is a function of the dephasing delay ($T - \Delta$).

Conclusions

The use of backbone $^{13}C,^{15}N$ enriched proteins results in the abolition of the undesirable one bond $^{1}J_{C\alpha,C\beta}$ coupling. Whereas NMR spectroscopic techniques for removing this coupling like constant time acquisition or selective decoupling suffer from considerable sensitivity losses or incomplete decoupling, the approach presented here offers optimal resolution and sensitivity for all residue types. The backbone resonance assignment for [$^{13}C\alpha,^{13}C'$, ^{15}N,50% $^{2}H\alpha$] labelled proteins requires only minor modifications of standard pulse sequences such as HNCA or HN(CO)CA. In order to remove signals from protonated $^{13}C\alpha$, we implemented a ^{1}H-filter into these triple resonance experiments. Our study demonstrates that the HNCA experiment is a useful tool for the measurement of the scalar and dipolar Cα,C$'$ coupling, either from the observation of a signal splitting in a C$'$-coupled experiment or from the detection of an intensity modulation. For uniformly ^{13}C labelled proteins this coupling is frequently measured using a HNCO approach. Based on estimated relaxation times for Cα and C$'$ we conclude, that at medium or high magnetic fields it should be advantageous to measure the one bond $^{1}J_{C\alpha,C'}$ coupling with the approach described here. Future investigations will focus on the assignment of large proteins utilising the improved sensitivity provided by backbone labelling.

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