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Improved pulse sequences for measuring coupling constants in $^{13}C,^{15}N$-labeled proteins

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SUMMARY

NMR pulse sequences for measuring coupling constants in $^{13}C,^{15}N$-labeled proteins are presented. These pulse sequences represent improvements over earlier experiments with respect to resolution and number of radiofrequency pulses. The experiments are useful for measuring $J_{NH\beta}$, J_{NCO}, $J_{NC\alpha}$, J_{H^NCO} and $J_{H^NH\alpha}$. Applications to chymotrypsin inhibitor 2 (CI-2) are shown.

During the past few years ^{13}C and/or ^{15}N labeling of proteins has found widespread applications in NMR spectroscopy. Labeling was originally introduced mainly for resolution purposes in 3D and 4D NMR, but recently much attention has been focused on the use of homo- and heteronuclear coupling constants in the structural analysis of proteins (e.g., Montelione et al., 1989; Kay and Bax, 1990; Sørensen, 1990; Delaglio et al., 1991; Edison et al., 1991; Wagner et al., 1991; Griesinger and Eggenberger, 1992; Vuister and Bax, 1992). In this article improvements of recent NMR techniques suitable for measuring coupling constants are presented.

The chymotrypsin inhibitor CI-2 (Ludvigsen et al., 1991b) was used as a test molecule for the experiments. A truncated version of CI-2 lacking the 19 N-terminal residues of the total 83 amino acid residues was overexpressed in *E. coli* from an inducible bacteriophage T7 RNA promoter (Tabor, 1990; Studier, 1991). $^{13}C,^{15}N$-labeling was carried out by inoculating the expression strain in M9AX medium containing per liter 3 g KH_2PO_4, 6 g $Na_2HPO_a{\cdot}2H_2O$, 5 g NaCl, 250 mg $MgSO_4{\cdot}7H_2O$ and 10 g of $^{13}C,^{15}N$ double-labeled algae extract prepared as described earlier (Sørensen and Poulsen, 1992). Details on the construction and purification of the recombinant CI-2 will be published elsewhere.

All experiments were performed with a 1 mM sample of double-labeled CI-2 dissolved in

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90%/10% $H_2O/^2H_2O$ at pH 4.2. The spectra were recorded at 25°C on a Bruker AMX-600 spectrometer equipped with a triple resonance 5 mm probe. The spectra were processed by using MNMR software (PRONTO Software, Development and Distribution, Copenhagen).

We first present a simple modification of the HNCA experiment of Madsen and Sørensen (1992) well suited for measurement of $^3J_{H^N H^\alpha}$ couplings (Fig. 1). (The sensitivity-enhancing scheme of Palmer et al. (1991) has been added to all the presented pulse sequences for further enhancement). The value of $^3J_{H^N H^\alpha}$ is important for structure determination because it is correlated with the backbone dihedral angle, ϕ (Karplus, 1959; Bystrov, 1976). Our new technique takes advantage of $^{13}C^\alpha$ to create an E. COSY-like multiplet pattern (Griesinger et al., 1986, 1987) with the large $^1J_{C^\alpha H^\alpha}$ coupling allowing convenient measurement of $^3J_{H^N H^\alpha}$. The actual measurement is performed on the intraresidue $\{^{13}C^\alpha, ^1H^N\}$ peak in the ω_2–ω_3 plane defined by the resonance frequency (ω_1) of the ^{15}N of the same residue (Fig. 2). The E. COSY pattern is due to the fact that the combined effect of the last five proton pulses in Fig. 1 leaves protons not bound to ^{15}N (e.g., $^1H^\alpha$) invariant, which ensures that the spin states of these protons do not change between t_2 and t_3. Thus, separation of the peak components by the large $^1J_{C^\alpha H^\alpha}$ coupling in the ω_2 dimension makes it possible accurately to determine the small displacement due to the $^3J_{H^N H^\alpha}$ coupling in ω_3. The ω_1 dimension serves merely to resolve overlapping multiplets, which means that a 2D version (the four simultaneous 180° pulses removed) of the experiment may suffice in cases where overlap is not a problem. That experiment is similar to one proposed by Wagner et al. (1991).

The limited number of points one can acquire in a multidimensional NMR experiment means that special data processing (integration, interpolation, etc.) is necessary before the exact peak position can be extracted. In practice, a number of ω_3 traces are often added around the center frequency for each multiplet component before relative displacements are measured (Griesinger et al., 1986, 1987). However, in this application we chose to take the whole 2D peak volume for a given ω_2–ω_3 plane into consideration, using a procedure to fit a Lorentzian peak shape to the experimental data. Figure 2 shows an ω_2–ω_3 plane from a 3D spectrum recorded with the pulse sequence in Fig. 1. The values for $^3J_{H^N H^\alpha}$ extracted from this plane (2.9 Hz for Ala[35] and 9.5 Hz

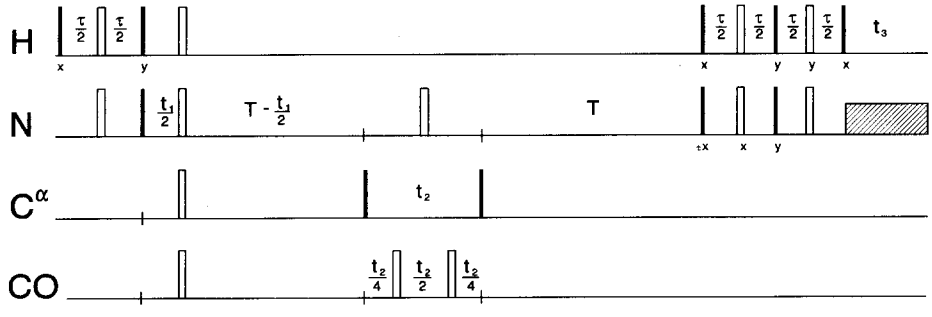

Fig. 1. 3D HNCA experiment for measurement of $^3J_{H^N H^\alpha}$ couplings. For all pulse sequences presented, narrow filled bars represent 90° pulses, broad hollow bars 180° pulses, and hatched frames broadband decoupling. Essential phase relations are indicated below the pulses. Note that α-protons are unperturbed by the element of the last five proton pulses. Quadrature detection in ω_1 and ω_2 is obtained by phase cycling the first 90° ^{15}N pulse and the first 90° ^{13}C pulse, respectively, according to one of the three standard procedures (States et al., 1982; Marion and Wüthrich, 1983; Marion et al., 1989).

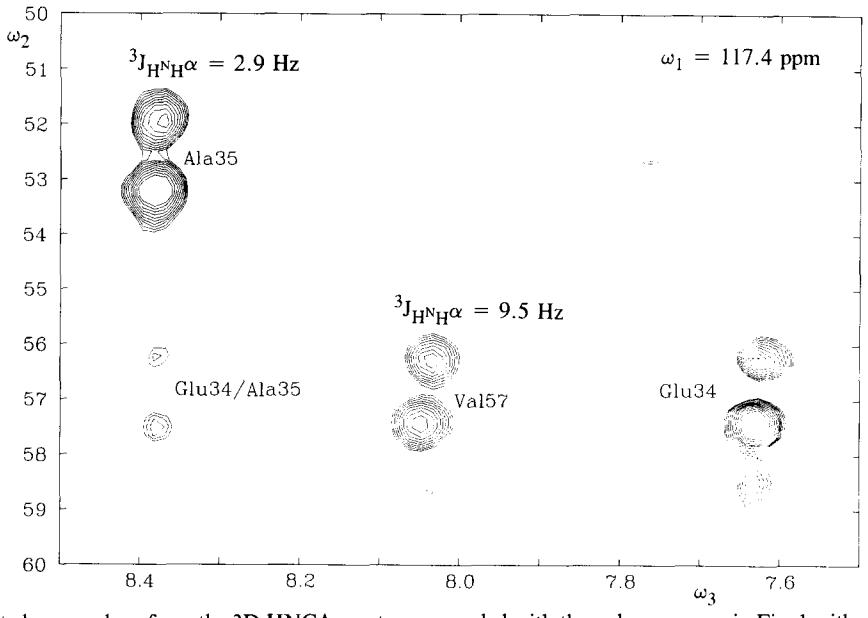

Fig. 2. Selected ω_2–ω_3 plane from the 3D HNCA spectrum recorded with the pulse sequence in Fig. 1 with τ = 5 ms, T = 30 ms, and GARP decoupling of ^{15}N during acquisition. A total of 32 × 128 × 512 complex points were acquired in the t_1, t_2 and t_3 dimensions, with acquisition times of 12.8, 20.5 and 57.3 ms, respectively. Sixteen scans were acquired per real (t_1,t_2) pair distributed between two subexperiments according to the procedure of Palmer et al. (1991). Phase alternation was applied to the first ^{15}N and $^{13}C^\alpha$ 90° pulses independently, and a syncronous phase alternation was applied to the second and third ^{15}N 90° pulse. For visual presentation the data were Lorentz-to-Gauss weighted and zero-filled to twice the size in all three dimensions. The coupling constants were, however, extracted from an unweighted data set, using a fitting procedure. The weak doublet is a sequential correlation caused by coherence transfer through the two-bond coupling $^2J_{NC^\alpha}$ ($^4J_{H^NH^\alpha} \approx 0$). Likewise, the distortion of the peak labeled Glu^{34} is caused by an overlapping peak from such a sequential correlation.

for Val^{57} may be compared with the values reported previously (Ludvigsen et al., 1991a,b) for 3.3 Hz and 9.8 Hz for Ala^{35} and Val^{57}, respectively. Details on the fitting procedure and applications will be described in a forthcoming paper (Rischel, 1993).

A pulse sequence proposed by Delaglio et al. (1991) for measurement of coupling constants between ^{15}N and ^{13}CO or $^{13}C^\alpha$ in protein backbones is shown in Fig. 3A while a simplified version can be found in Fig. 3B. Ideally, the two experiments exhibit identical sensitivity and resolution. An even better version would be to broadband decouple C_i during t_1 and the two surrounding τ delays as indicated by the dashed line in Fig. 3B. Figure 4 shows a spectrum recorded with the pulse sequence in Fig. 3B and some of the coupling constants which can be inferred from the spectrum are indicated.

A final example of pulse economy is a 3D experiment of Archer et al. (1991) (Fig. 5A) to provide qualitative information about the size of $^3J_{NH^\beta}$ couplings in proteins. Figure 5 employs the same nomenclature as Archer et al. (1991) except that an optional spin-lock period has been left out for convenience. In the experiment depicted in Fig. 5A the two simultaneous 180° pulses in the first Δ delay can be moved over a period $\Delta/2$ starting in the middle and ending at the subsequent ^1H 90° pulse in order to label the magnetization with the chemical shift of ^{15}N during t_1. In contrast, the new simpler sequence in Fig. 5B allows the pair of 180° pulses to be moved

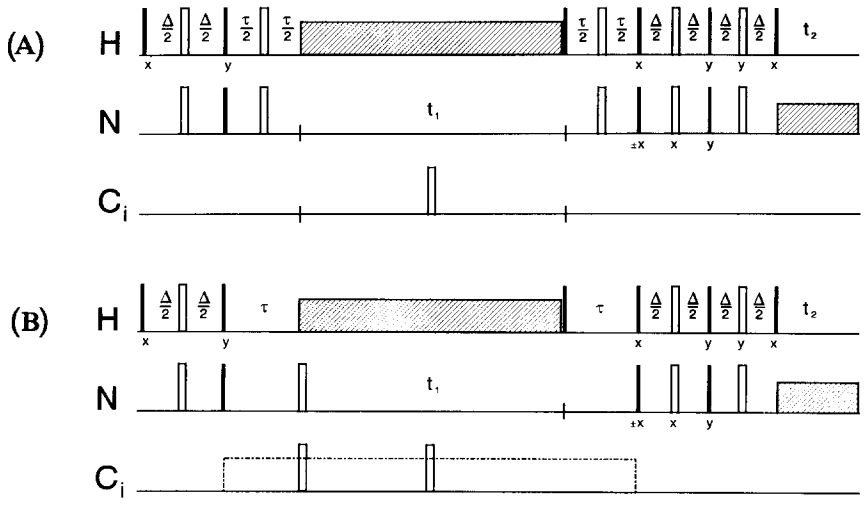

Fig. 3. (A) Pulse sequence for measurement of couplings between ^{13}C and ^{15}N (Delaglio et al., 1991). C_i indicates either C^{α} or CO. (B) The same experiment after reduction of the number of pulses. C_i can be decoupled either by the two 180° pulses or by broadband decoupling.

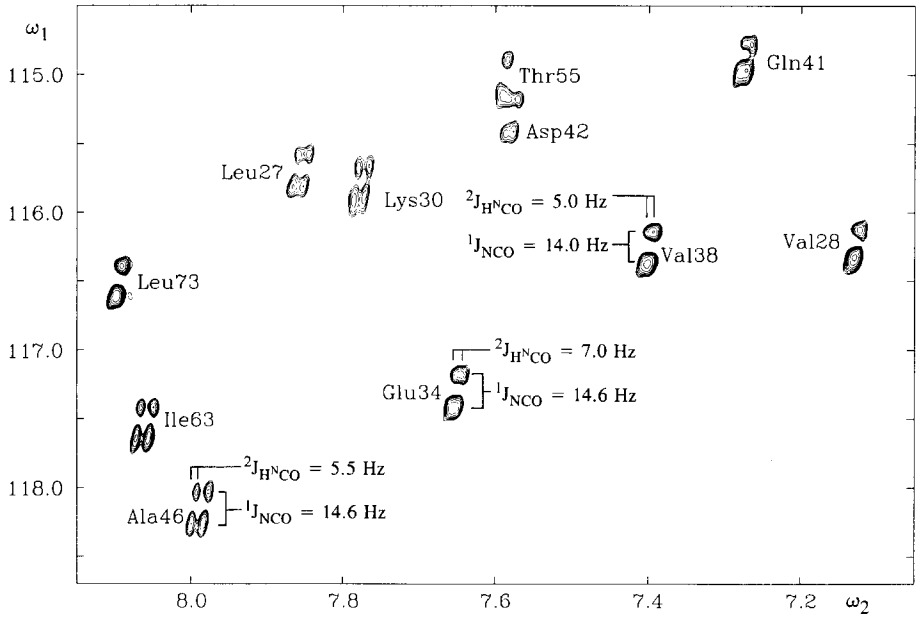

Fig. 4. Spectrum recorded with the pulse sequence in Fig. 3B with $C_i = C^{\alpha}$ decoupled by two 180° pulses (i.e., splittings are due to couplings to ^{13}CO), Δ = 5 ms, τ = 5.4 ms, WALTZ-16 decoupling of the protons, and GARP decoupling of ^{15}N. A total of 2048 × 512 complex points were acquired with acquisition times of 229.4 ms and 204.8 ms in the t_2 and t_1 dimensions, respectively. For each real t_1 value 32 scans were acquired and distributed between two subexperiments (Palmer et al., 1991). Lorentz-to-Gauss weighting was performed in both dimensions. $^3J_{H^NH^{\alpha}}$ couplings account for the multiplet splittings in ω_2. (At present we can not account for the relatively higher intensity in the high-frequency component of the $^1J_{NCO}$ doublet.)

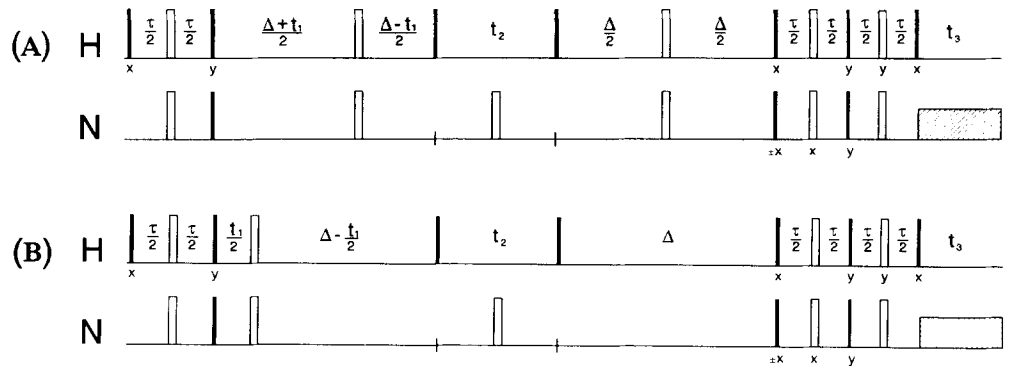

Fig. 5. (A) 3D NMR pulse sequence (Archer et al., 1991) providing qualitative information about $^3J_{NH\beta}$. The maximum t_1 value possible is Δ. (B) Improved and simplified version of the pulse sequence in (A). The maximum possible t_1 value (and hence the resolution in the ω_1 dimension) has been increased by a factor of two compared to (A).

over the entire delay Δ, which results in a two-fold increase in resolution in the ^{15}N dimension without any penalties whatsoever. The same principle has recently been applied to triple resonance 3D NMR experiments (Madsen and Sørensen, 1992).

In conclusion, methods involving ^{13}C,^{15}N-labeling and relying on efficient coherence transfer using optimized pulse sequences are the techniques of choice for large proteins. Furthermore, these methods can easily be extended to more dimensions to overcome problems caused by overlapping peaks in crowded regions of the spectra.

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