Measurements of H^{α}–H^{N} Vicinal Coupling Constants in a Protein with Large Line Widths in a New 3D ^{1}H–^{15}N–^{13}C Quadruple Resonance NMR Experiment

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We present a 3D ^{1}H–^{15}N–^{13}C quadruple resonance NMR experiment that provides accurate measurements of homonuclear H^{α}–H^{N} coupling constants even in proteins with broad lines. The technique follows strategies developed earlier[1,2] but is more sensitive due to a different design of the pulse sequence. This approach can readily be applied to large proteins that are enriched with both ^{15}N and ^{13}C. Here we show experiments on the 164 residue protein T4 lysozyme.

Measurement of coupling constants is crucial for refining protein structures derived from NMR data.[3] Conventional measurements of coupling constants in macromolecules with large line widths have been difficult or even impossible. These measurements have been attempted by analysis of cross peaks in COSY[4] or HMQC[5] spectra, by spectral simulation,[6] or by observation of a null in a series of heteronuclear ^{1}H–^{15}N correlation spectra with different delays.[7] The latter technique requires that the transverse relaxation time be longer than the inverse coupling constant, and delays of up to 200 ms may be required.[7] Previously, we had presented a simple technique for measuring heteronuclear ^{1}H–^{15}N or ^{1}H–^{13}C vicinal coupling constants.[8] An analogous 2D NMR ^{1}H–^{15}N–^{13}C triple resonance experiment was proposed to measure homonuclear H^{N}–H^{α} vicinal coupling constants.[1,2] The technique was successful when applied to small peptides but failed when tried on proteins. The approach presented here is simpler and more sensitive. An earlier version of this experiment was successfully applied to ^{15}N-labeled peptides.[9] In highly ^{13}C enriched proteins, additional complications arise due to carbon–carbon couplings. This is partly alleviated by GARP[13] decoupling of the carbonyl resonances (see Figure 1) and by a different choice of the delay b in Figure 1.

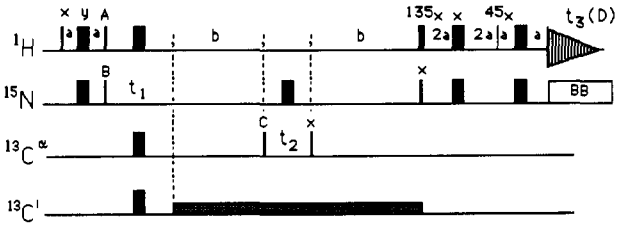

Figure 1. Pulse sequences for the 3D quadruple resonance experiment described in the text. Wide and narrow bars represent 180° and 90° pulses, respectively; 135° and 45° pulses are labeled as such. The delay a is set to $(4J_{HN})^{-1}$, and the delays b are tuned as described in the text. The phase cycles were: A = y, B = $2(x)2(-x)$, C = $x, -x$, D = +, –, –, +. Time-proportional phase incrementation is used on phases B and C. Two carbon channels are used with frequencies in the α-carbon and the carbonyl range. BB stands for broad band GARP[13] nitrogen decoupling during detection. The 180° pulses in both carbon channels are achieved by high-power irradiation at the C^{α} frequency. The long horizontal bar at the carbonyl channel indicates low-power GARP[13] decoupling.

Figure 1 shows the pulse sequence used for the experiment. The sequence consists of a nonrefocused INEPT[10] followed by a ^{15}N evolution period. Decoupling of the antiphase ^{15}N coherence is achieved by a 180° (^{1}H) pulse and a hard 180° (^{13}C) pulse. The delay b leads to evolution of three terms which we write using the operator product formalism of Sørensen et al.:[11]

$$4N^{i}_{y,x}H^{Ni}_{z}C^{\alpha i}_{z}\sin(\pi^{1}J_{N^{i}-C^{\alpha i}}b)\cos(\pi^{2}J_{N^{i}-C^{\alpha i-1}}b) \quad (1)$$

$$4N^{i}_{y,x}H^{Ni}_{z}C^{\alpha i-1}_{z}\sin(\pi^{2}J_{N^{i}-C^{\alpha i-1}}b)\cos(\pi^{1}J_{N^{i}-C^{\alpha i}}b) \quad (2)$$

$$8N^{i}_{x,y}H^{Ni}_{z}C^{\alpha i}_{z}C^{\alpha i-}{}_{1z}\sin(\pi^{1}J_{N^{i}-C^{\alpha i}}b)\sin(\pi^{2}J_{N^{i}-C^{\alpha i-1}}b) \quad (3)$$

N^{i}, H^{i}, and $C^{\alpha i}$ stand for the spin operators of the ^{15}N, ^{1}H, and ^{13}C nuclei, respectively, of residue i. $N_{x,y}$ means N_x or N_y. Terms 1 and 2 will give rise to intraresidue and sequential cross peaks from H^{Ni} along ω_3 to $C^{\alpha i}$ and $C^{\alpha i-1}$, respectively, along ω_2. Term 3 is eliminated by phase cycling. During t_2, we have a ^{15}N–^{13}C heteronuclear multiple quantum evolution, similar as in experiments described by Kay et al.[12] However, the one-bond coupling between the α-proton and the α-carbon is active while the coupling between the nitrogen and the amide proton is refocused due to the nitrogen 180° pulse in the center of the t_2 period. Low-power GARP[13] decoupling of the carbonyls avoids undesirable coupling effects with the α-carbons. Couplings between α- and β-carbons are active and split the α-carbon resonances. The final part of the sequence is a reverse INEPT where the first proton 90° pulse is replaced with a TANGO[14] pulse. This is a 90° pulse selective for protons directly bonded to ^{15}N. It does not mix the two spin states of the α-proton and yields the desired multiplet structure that allows for a convenient measurement of the vicinal coupling constant.

The experiment described yields a 3D spectrum that contains in the ω_3–ω_2 planes (see Figure 2) intraresidue cross peaks between H^{Ni} and $C^{\alpha i}$ originating from term 1. The maximum transfer is at a value of $b \geq (3(^{1}J_{N^{i}-C^{\alpha i}} + {}^{2}J_{N^{i}-C^{\alpha i-1}}))^{-1}$, where the two coupling constants are ca. 11 and 7 Hz, respectively. The factor 3 instead of 2 in the transfer function is to take care of relaxation effects. The experiment also yields cross peaks between H^{Ni} and $C^{\alpha i-1}$ originating from term 2. The maximum transfer is at a value of $b \leq (3(^{1}J_{N^{i}-C^{\alpha i}} + {}^{2}J_{N^{i}-C^{\alpha i-1}}))^{-1}$. In the experiment shown in Figure 2, delay b was set to 33 ms. This is optimal for intraresidue cross peaks, and only a few sequential cross peaks were observed (not shown). This is advantageous for measuring coupling constants because the spectrum is simplified and potential overlap between intraresidue and sequential cross peaks is avoided. All cross peaks are split in doublets along ω_2 with the one-bond H^{α}–C^{α} coupling.

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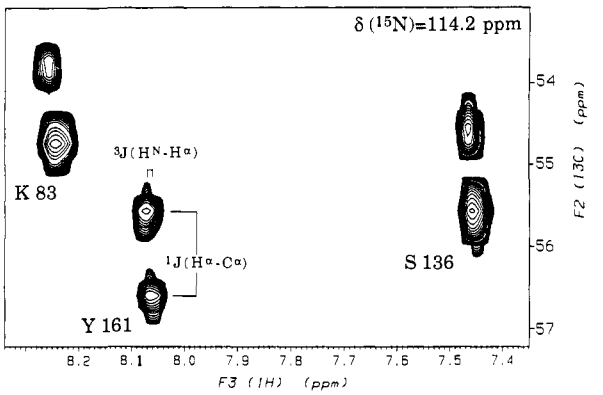

Figure 2. Expansion of an ω_2-ω_3 cross plane of a 3D spectrum recorded with the sequence of Figure 1 on uniformly (>95%) ^{13}C-^{15}N enriched T4 lysozyme at 25 °C. The protein, prepared as described previously,[16] was 4.3 mM in 100 mM KCl, 30 mM potassium phosphate, 5% D_2O, pH 5.5. The cross plane shown is at the ^{15}N frequency of 114.2 ppm containing the C^α-H^N cross peaks of Lys 83, Tyr 161, and Ser 136. The cross peaks are split in doublets along ω_2 with the one-bond H^α-C^α coupling of ~130 Hz. In addition, the two doublet components are displaced along ω_3 with the vicinal H^α-H^N coupling constant. These can readily be measured as 9.0, 5.0, and 4.5 ± 0.5 Hz from the peak positions of the two components. The spectrum was recorded on a Bruker AMX-600 spectrometer equipped with two external synthesizers and amplifiers. The GARP[13] decoupling of the carbonyls was achieved with a GARP box purchased from Tschudin Associates using a 90° pulse length of 400 μs. GARP decoupling of the nitrogens during detection was achieved with the internal nitrogen channel using 90° pulses of 150 μs. The 3D data set consisted of 32, 128, and 512 complex data points in t_1, t_2, and t_3, respectively. The data were zero-filled and truncated after Fourier transformation so that the final digital resolution was 21.7, 30.5, and 2.4 Hz in ω_1, ω_2, and ω_3, respectively. Chemical shifts are quoted relative to sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)propionate (^1H and ^{13}C) and ammonia (^{15}N).

In addition, the two doublet components are displaced along ω_3 with the vicinal H^α-H^N coupling constant. This can readily be measured from the peak positions of the two components. Furthermore, the two components are split along the carbon axis due to the C^α-C^β coupling (30–35 Hz). This is not resolved in Figure 2 due to the low digital resolution along ω_2. Figure 2 shows a small part of an ω_2-ω_3 plane at a nitrogen frequency of 114.2 ppm containing the cross peaks for Lys 83, Tyr 161, and Ser 136 (^1H and ^{15}N assignments of this protein have been published previously,[15] and the assignment of the α-carbon resonances is trivial from this spectrum). The large doublet splitting of ~130 Hz along ω_2 and the displacement of the multiplet components along ω_3 can readily be seen. The H^N-H^α vicinal coupling constants measured are 9.0, 5.0, and 4.5 Hz, respectively. Currently, we are examining the best way to measure the coupling constants, such as separation of peak maxima, centers of mass, fit of line shapes, and the influence of the digital resolution on the precision of the measurements. It is worth mentioning that we can measure all H^N-H^α vicinal coupling constants in T4 lysozyme using this technique. A complete analysis of the coupling constants as well as a comparison with values expected from the X-ray structure is in progress and will be presented elsewhere. The measured values of the coupling constants are significantly smaller than the approximate 15-Hz line widths of the amide resonances and would be difficult to accurately measure using previous approaches.

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Polyether Tethered Oligonucleotide Probes

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Molecules capable of sequence-specific recognition of DNA[1] and RNA[2] have widespread utility in the biotechnology industry and as tools for the study of nucleic acid structure and function. Recognition of RNA is particularly challenging due to its increased potential for structural complexity[3] and the absence of three-dimensional structural data for most molecules.[4] Interestingly, the few well-characterized cases[5] suggest that, in terms of molecular architecture, RNA is related more closely to a globular protein than to duplex DNA.[4]

Recently we described a family of synthetic molecules for the sequence- and structure-specific recognition of RNA.[6] Tethered oligonucleotide probes (TOPs) consist of two deoxyoligonucleotides separated by a flexible, synthetic tether (Figure 1). The two oligonucleotides hybridize to two noncontiguous, single-stranded regions of a target RNA, and the tether traverses the distance between them (Figure 1).[7] When the tether is constructed from a repeating abasic phosphodiester unit that resembles DNA (as in **1** and **2**), TOPs bind cooperatively, monomerically, and with high affinity to two regions (labeled 3′- and 5′-site in Figure 1) of the *Leptomonas collosoma* SL RNA.[8] We questioned whether

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