Communication

A 'just-in-time' HN(CA)CO experiment for the backbone assignment of large proteins with high sensitivity

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Abstract

Among the suite of commonly used backbone experiments, HNCACO presents an unresolved sensitivity limitation due to fast ^{13}CO transverse relaxation and passive $^{13}C_\alpha$–$^{13}C_\beta$ coupling. Here, we present a high-sensitivity 'just-in-time' (JIT) HN(CA)CO pulse sequence that uniformly refocuses $^{13}C_\alpha$–$^{13}C_\beta$ coupling while collecting ^{13}CO shifts in real time. Sensitivity comparisons of the 3-D JIT HN(CA)-CO, a CT-HMQC-based control, and a HSQC-based control with selective $^{13}C_\alpha$ inversion pulses were performed using a $^2H/^{13}C/^{15}N$ labeled sample of the 29 kDa HCA II protein at 15 °C. The JIT experiment shows a 42% signal enhancement over the CT-HMQC-based experiment. Compared to the HSQC-based experiment, the JIT experiment is 16% less sensitive for residues experiencing proper $^{13}C_\alpha$ refocusing and $^{13}C_\alpha$–$^{13}C_\beta$ decoupling. However, for the remaining residues, the JIT spectrum shows a 106% average sensitivity gain over the HSQC-based experiment. The high-sensitivity JIT HNCACO experiment should be particularly beneficial for studies of large proteins to provide ^{13}CO resonance information regardless of residue type.
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The introduction of protein deuteration [1,2] and the recent development of TROSY methodology [3,4] have vastly expanded our capability to study large proteins and protein complexes, with near complete backbone assignment being reported for a single chain protein of 83 kDa [5]. The spectra of these large proteins often contain highly complex and degenerate signals. The unambiguous assignment of these resonances can only be achieved through the use of all three pairs (C_α, C_β, and CO) of connectivities [6]. Among the suite of commonly used backbone triple-resonance experiments, the HNCACO experiment [7] is the least sensitive due to two inherent sensitivity limitations. First, during the $^{13}C_\alpha$–^{13}CO transfer where $^{13}C_\alpha$ magnetization is transverse, passive $^{13}C_\alpha$–$^{13}C_\beta$ coupling degrades the observable signal. Second, when ^{13}CO magnetization is transverse, fast relaxation of carbonyl coherence caused by the large carbonyl chemical shift anisotropy (CSA) leads

to substantial signal loss that becomes more severe with increasing protein size and at higher magnetic field.

Different approaches have been used to improve the sensitivity of the HNCACO experiment. A constant time heteronuclear multiple quantum correlation (CT-HMQC) $^{13}C_\alpha$–^{13}CO transfer places $^{13}C_\alpha$ magnetization on the transverse plane for 28 ms to refocus the undesired $^{13}C_\alpha$–$^{13}C_\beta$ coupling [8]. However, this method also includes a constant time carbonyl evolution period of 11 ms, during which ^{13}CO coherence is susceptible to degradation via rapid transverse relaxation. The alternative to the HMQC-based method employs a heteronuclear single quantum correlation (HSQC) $^{13}C_\alpha$–^{13}CO transfer sequence [7]. While this eliminates the need for a constant time ^{13}CO evolution period, $^{13}C_\alpha$–$^{13}C_\beta$ coupling is not refocused and leads to over 60% signal loss. This drawback can be partially resolved by using selective $^{13}C_\alpha$ pulses during the $^{13}C_\alpha$–^{13}CO INEPT transfers for simultaneous $^{13}C_\alpha$ refocusing and $^{13}C_\alpha$–$^{13}C_\beta$ decoupling [9,10]. However, due to the chemical shift degeneracy of $^{13}C_\alpha$ and $^{13}C_\beta$ nuclei, it has not been

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possible to eliminate the passive $^{13}C_\alpha$–$^{13}C_\beta$ couplings uniformly, leading to severe signal attenuation of peaks from certain residue types, particularly Ser and Leu [5,9,10]. Even with pulse sequence modifications to optimize the collection of Ser signals, a large percentage of expected Ser peaks are still lost [5]. Additionally, residues with $^{13}C_\alpha$ chemical shifts outside the refocusing window of the selective $^{13}C_\alpha$ pulse, such as Gly, Val and Pro, also experience significant signal attenuation [9,10].

In this communication, we describe a novel 'just-in-time' (JIT) HN(CA)CO experiment that combines the advantages of the HMQC- and the HSQC-based experiments by removing $^{13}C_\alpha$–$^{13}C_\beta$ coupling uniformly and minimizing ^{13}CO relaxation losses, thus offering high sensitivity for all resonances regardless of residue type. The pulse sequence of the JIT TROSY-HN(CA)CO experiment is shown in Fig. 1. It begins with the coherence transfer from ^1HN to ^{15}N and ^{15}N to $^{13}C_\alpha$. As in the $^{13}C_\alpha$–^{13}CO transfer element of the CT-HMQC experiment, $^{13}C_\alpha$ magnetization is kept transverse for a constant period of $1/^1J_{C\alpha C\beta}$ to refocus $^{13}C_\alpha$–$^{13}C_\beta$ coupling. However, the ^{13}CO chemical shift is recorded in real time by synchronizing the movement of the two 180° ^{13}CO pulses during the $^{13}C_\alpha$–^{13}CO transfer (marked by asterisks in Fig. 1) with respect to the 90° ^{13}CO flip pulses flanking the ^{13}CO evolution period. This keeps the effective $^{13}C_\alpha$–^{13}CO coupling time at $1/2^1J_{C\alpha CO}$ and allows the real time build up of carbonyl coherence before flipping ^{13}CO magnetization into the transverse plane *just in time* for frequency labeling. The JIT $^{13}C_\alpha$–^{13}CO transfer scheme limits the maximum ^{13}CO evolution time to ~11 ms. Although this restricts the digital resolution of the ^{13}CO dimension, the fast ^{13}CO transverse relaxation in large proteins makes longer acquisition time of limited benefit. ^{15}N and ^1HN shifts are collected using a sensitivity-enhanced TROSY element with suppression of anti-TROSY signals [11]. A similar $^{13}C_\alpha$–^{13}CO transfer scheme has been utilized previously as part of a (HCA)CONH experiment [12]. However, the rapid transverse relaxation of $^{13}C_\alpha$ nuclei in protonated samples has restricted the use of the (HCA)CONH experiment to small- to medium-sized proteins. Because the ^{13}CO CSA relaxation is more severe in large proteins, the signal enhancement of the JIT $^{13}C_\alpha$–^{13}CO transfer is most pronounced for large deuterated proteins.

The JIT $^{13}C_\alpha$–^{13}CO transfer provides significant sensitivity enhancement over the CT-HMQC-HNCACO experiment. At the maximum ^{13}CO resolution attainable in the JIT experiment, the average time for ^{13}CO magnetization in the transverse plane is reduced by 58% compared to the HMQC-based sequence, therefore avoiding substantial signal loss from fast carbonyl relaxation. This sensitivity gain increases for large proteins at high magnetic fields, where the rate of transverse ^{13}CO relaxation is particularly detrimental. Compared to the HSQC-based experiment with selective $^{13}C_\alpha$ refocusing pulses, the JIT experiment is slightly less sensitive for residues experiencing complete $^{13}C_\alpha$ inversion and proper $^{13}C_\alpha$–$^{13}C_\beta$ decoupling, due to an additional delay of 11 ms experienced by transverse $^{13}C_\alpha$ magnetization. The signal loss should be minimal because of the relatively slow relaxation rate of transverse $^{13}C_\alpha$ magnetization in deuterated proteins [2]. However, a significant signal gain is achieved for the remaining residues as the JIT experiment does not rely on selective $^{13}C_\alpha$ pulses to refocus $^{13}C_\alpha$ magnetization or to remove $^{13}C_\alpha$–$^{13}C_\beta$ coupling.

To demonstrate the advantages of our JIT TROSY-HNCACO experiment, we collected 3-D spectra of a 0.9 mM $^2H/^{13}C/^{15}N$ labeled sample of the 29 kDa protein
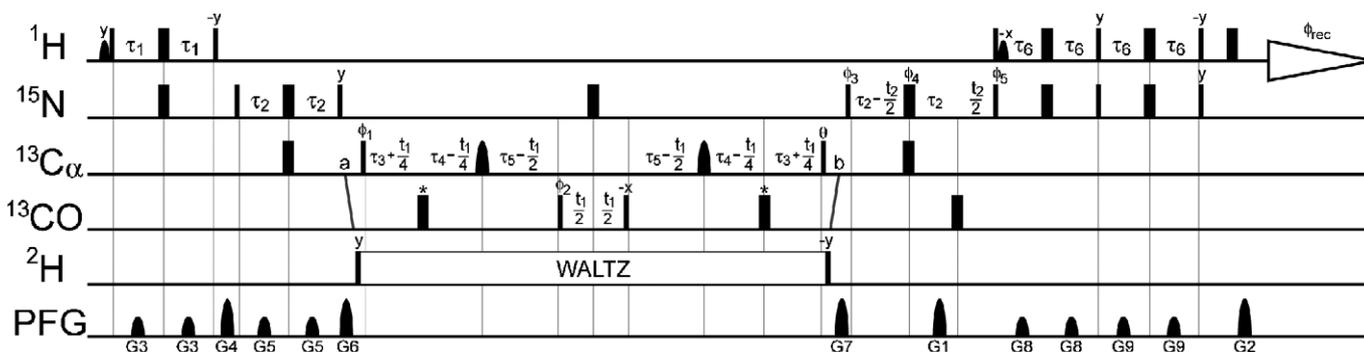

Fig. 1. JIT TROSY-HN(CA)CO experiment. Narrow and wide bars represent 90° and 180° pulses, respectively. All pulses are applied along the *x*-axis unless noted otherwise. 90° selective water pulses are indicated by shaped bars. The ^{13}C carrier frequency is initially set to 57 ppm and shifted to 175 ppm between points *a* and *b*. Shaped aliphatic pulses represent 375 μs off-resonance REBURP pulses centered at 44 ppm. The remaining $^{13}C_\alpha$ pulses are selective rectangular pulses with maximum excitation at 57 ppm and null excitation at 175 ppm. Selective sinc-shaped ^{13}CO pulses are applied at 175 ppm with null excitation at 57 ppm. An empirically optimized phase shift of $\theta = 28°$ is applied to the last 90° $^{13}C_\alpha$ pulse to correct for Bloch–Siegert effects. The positioning of the two asterisked ^{13}CO 180° pulses is synchronized with the carbonyl evolution. The delays are $\tau_1 = 2.4$ ms, $\tau_2 = 12.5$ ms, $\tau_3 = 1/4J_{C\alpha CO} = 4.5$ ms, $\tau_4 = 1/4J_{C\alpha C\beta} - 1/4J_{C\alpha CO} = 2.6$ ms, $\tau_5 = 1/4J_{C\alpha C\beta} = 7.1$ ms, and $\tau_6 = 2.8$ ms. The phase cycling is $\phi_1 = [2x, 2(-x)]$, $\phi_2 = [x, -x]$, $\phi_3 = [4y, 4(-y)]$, $\phi_4 = [8x, 8(-x)]$, $\phi_5 = [x]$, and $\phi_{rec} = [x, -x, -x, x, -x, x, x, -x]$. States-TPPI in F1 is achieved by incrementing ϕ_2 and ϕ_{rec}. The sensitivity-enhanced gradient TROSY scheme requires the inversion of ϕ_5 and G2 for hypercomplex data collection in F2. WALTZ-16 is used for ^2H decoupling. Gradient durations and field strengths are G1 = (2 ms, 20.42 G/cm), G2 = (0.2 ms, 20.36 G/cm), G3 = (0.5 ms, 11.64 G/cm), G4 = (0.9 ms, 21.03 G/cm), G5 = (0.5 ms, 9.60 G/cm), G6 = (0.9 ms, 18.99 G/cm), G7 = (0.9 ms, 19.81 G/cm), G8 = (0.7 ms, 12.05 G/cm), and G9 = (0.7 ms, 12.86 G/cm).

human carbonic anhydrase (HCA) II on an 800 MHz Varian spectrometer at 15 °C. The correlation time of HCA II was estimated to be 23 ns, corresponding approximately to

a 39 kDa protein at room temperature. Two control spectra employing the CT-HMQC-HN(CA)CO and the HSQC-HN(CA)CO with selective $^{13}C_\alpha$ refocusing were
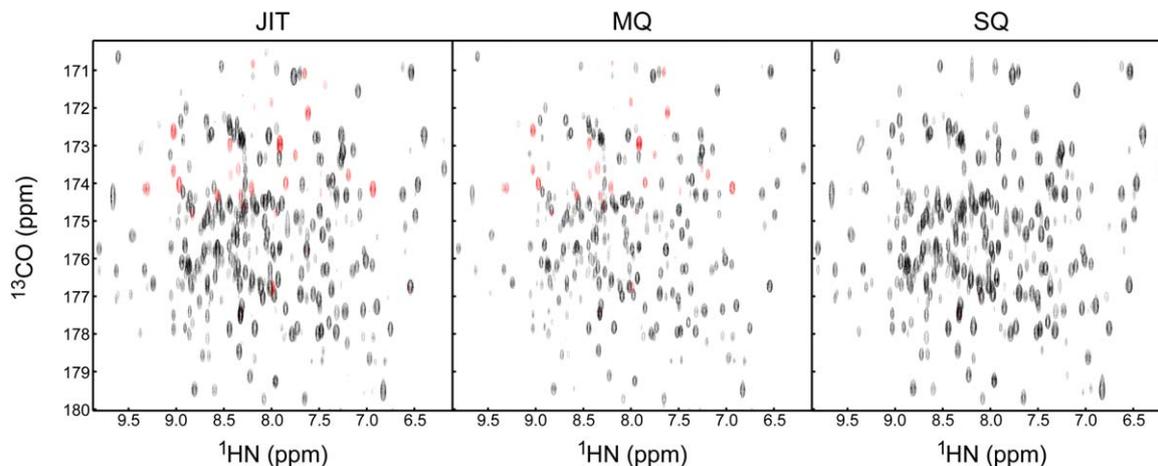

Fig. 2. 2-D ^{1}HN–^{13}CO projections from the JIT HN(CA)CO experiment and CT-HMQC-based [8] and HSQC-based [9,10] controls. Due to the lack of $^{13}C_\alpha$–$^{13}C_\beta$ coupling, glycine peaks appear with negative intensity (in red) in the JIT and CT-HMQC-based experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
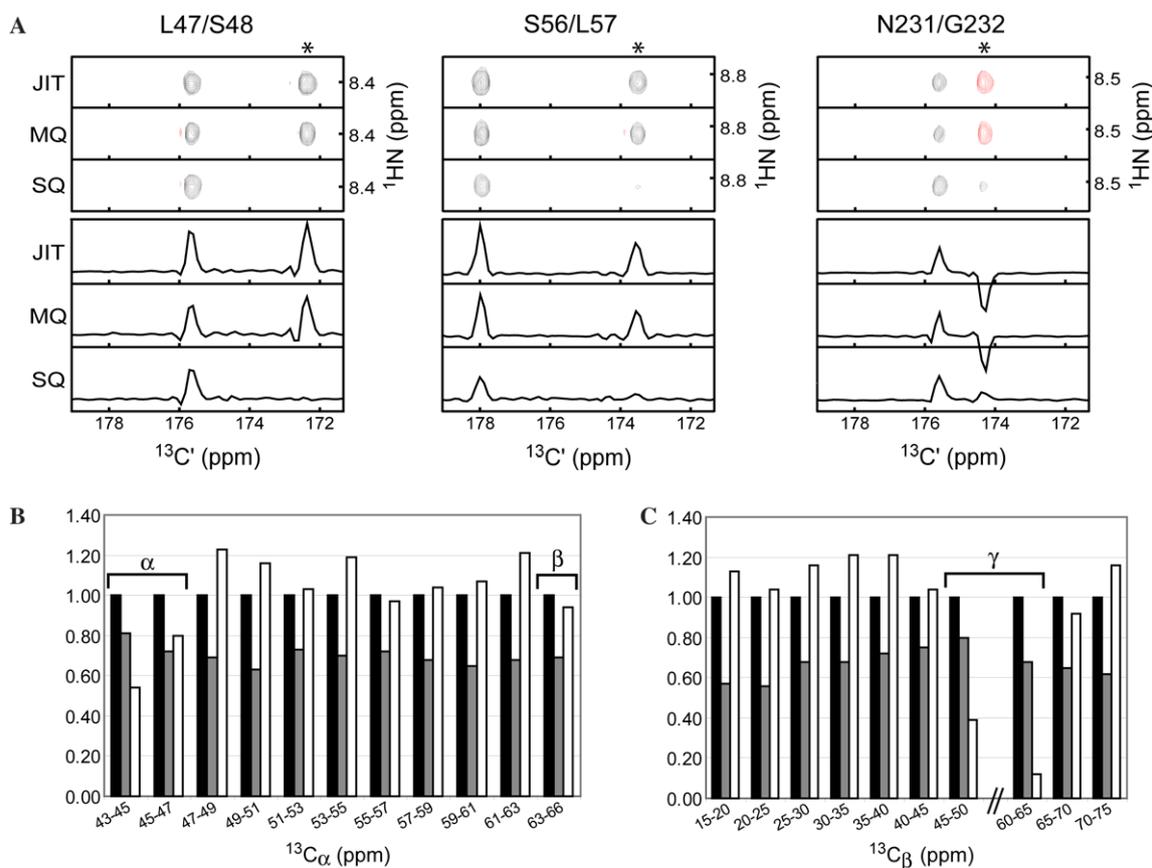

Fig. 3. (A) Slices from the 3-D JIT, CT-HMQC-based [8], and HSQC-based [9,10] HNCACO spectra. Asterisks indicate peaks from residues not properly refocused by the selective $^{13}C_\alpha$ pulses in the HSQC-based control: intraresidue correlations from Ser 48 and Gly 232 and an interresidue correlation from Ser 56/Leu 57. 1-D traces are taken at the centers of peak intensity in the ^{1}HN dimension. The negative intensity of peaks from glycine residues in the JIT and CT-HMQC-based experiments is caused by the absence of $^{13}C_\alpha$–$^{13}C_\beta$ coupling. (B,C) Relative sensitivities of the JIT (black), CT-HMQC-based (gray), and HSQC-based (white) experiments calculated with peak heights versus $^{13}C_\alpha$ and $^{13}C_\beta$ chemical shifts. The sensitivity profiles of the HSQC-based experiment are related to the excitation profile of its selective $^{13}C_\alpha$ inversion pulses. Signal loss is observed for residues at the edges of the $^{13}C_\alpha$ distribution (regions α and β) due to the incomplete refocusing of $^{13}C_\alpha$ magnetization and for residues with $^{13}C_\beta$ shifts between 45 and 65 ppm (region γ) due to improper $^{13}C_\alpha$–$^{13}C_\beta$ decoupling.

also collected for sensitivity comparisons. For each experiment, 26, 64, and 512 complex points were collected in the ^{13}CO, ^{15}N, and ^1HN dimensions, respectively, with a total acquisition time of 32 h. 2-D ^1HN–^{13}CO projections from each experiment are shown in Fig. 2. Two sets of sensitivity comparisons were calculated using peak heights and 3-D integrated peak volumes (reported in parenthesis). On average, the JIT experiment provides a 42% (59%) sensitivity gain over the CT-HMQC-based control. In the HSQC-based experiment, the nonuniform excitation profile of the selective $^{13}C_\alpha$ inversion pulses leads to a significant variation of sensitivity that depends on the $^{13}C_\alpha$ and $^{13}C_\beta$ chemical shifts of each residue (Fig. 3). Effective inversion of $^{13}C_\alpha$ magnetization occurs from 45 to 65 ppm; residues with $^{13}C_\alpha$ shifts inside and $^{13}C_\beta$ shifts outside this window experience effective $^{13}C_\alpha$ refocusing and $^{13}C_\alpha$–$^{13}C_\beta$ decoupling during the $^{13}C_\alpha$–^{13}CO INEPT transfers. For these residues, the JIT experiment averages 16% (19%) less sensitivity than the HSQC-based sequence. For residues with incomplete $^{13}C_\alpha$ refocusing (Fig. 3B, regions α and β) or improper $^{13}C_\beta$ decoupling (Fig. 3C, region γ), the JIT spectrum shows, on average, a 106% (104%) sensitivity gain for intra- and interresidue peaks over the HSQC-based experiment. For HCA II, the 70 peaks that fall into this group include mainly Ser and Gly residues and a smaller number of Leu, Val, and Pro residues. While the JIT spectrum shows a consistent sensitivity advantage for these residues, the degree of sensitivity enhancement varies substantially. For the resonances shown in Fig. 3A (S48, S56, and G232), the selective $^{13}C_\alpha$ inversion pulses in the HSQC-based experiment lead to a nearly complete loss of signal intensity.

In conclusion, we have introduced the JIT TROSY-HNCACO pulse sequence and demonstrated its high and uniform sensitivity compared to HMQC- and HSQC-based controls. While previous HNCACO experiments have not succeeded in both uniformly refocusing detrimental $^{13}C_\alpha$–$^{13}C_\beta$ coupling and minimizing ^{13}CO relaxation losses, the JIT $^{13}C_\alpha$–^{13}CO transfer scheme overcomes both of these limitations. In particular, the real-time ^{13}CO chemical shift evolution should make the JIT experiment especially beneficial for NMR studies of large proteins at high magnetic fields, where the reduction of adverse ^{13}CO CSA relaxation is critical. Given the importance of carbonyl data in obtaining unambiguous sequential assignment for backbone resonances, we believe that our high-sensitivity JIT-HNCACO experiment will provide a powerful new tool for extending NMR methodology to large proteins and protein complexes.

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