^{13}C Line Narrowing by ^{2}H Decoupling in $^{2}H/^{13}C/^{15}N$-Enriched Proteins. Application to Triple Resonance 4D J Connectivity of Sequential Amides

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Short transverse ^{13}C relaxation times, T_2, constitute the principal barrier for the application of heteronuclear J correlation NMR techniques to larger proteins uniformly enriched with ^{13}C and ^{15}N.[1-6] The ^{13}C T_2 is dominated by the strong dipolar interaction with its attached protons.[7] As the magnetogyric ratio of ^{2}H is ~6.5 times lower than that of ^{1}H, the heteronuclear dipolar interaction is greatly reduced by deuteration. Because of the large ^{2}H quadrupolar interaction (~170 kHz), the ^{2}H spin lattice relaxation time, T_1, in proteins is in the millisecond range at a magnetic field strength of 14 T. Therefore, the $^{2}H-^{13}C$ J coupling (~22 Hz) does not result in the triplet shape, expected for a ^{13}C nucleus coupled to a spin-1 nucleus, but gives rise to a collapsed singlet resonance that is broadened by scalar relaxation of the second kind.[8,9] High-power (~2.5 W) ^{2}H decoupling with an RF field strength much stronger than the inverse ^{2}H T_1 effectively removes this broadening and results in a ^{13}C line width that is much narrower than for the protonated ^{13}C.

One of the triple resonance J correlation experiments affected most by the ^{13}C line width is the H(CA)NH experiment[10,11] which relies on magnetization transfer from C^{α} to the backbone ^{15}N nucleus via the relatively small $^{1}J_{NC\alpha}$ (~11 Hz) and $^{2}J_{NC\alpha}$ (~5–8 Hz)[12] couplings. Although experiments have been proposed to alleviate this difficult J correlation step,[13,14] the sequential assignment procedure which is based on J correlation between the intraresidue $^{1}H/^{15}N$ and $^{1}H^{\alpha}/^{13}C^{\alpha}$ resonances and between the $^{1}H^{\alpha}/^{13}C^{\alpha}$ of residue i and $^{1}H/^{15}N$ of residue $i+1$ is complicated by the high degree of overlap among $^{1}H^{\alpha}/^{13}C^{\alpha}$ correlations. Here we describe a procedure which allows J correlation between the much better resolved $^{1}H/^{15}N$ resonances of sequential residues, thereby bypassing the overlapping $^{1}H^{\alpha}/^{13}C^{\alpha}$ pairs. Efficient transfer of magnetization from $^{13}C^{\alpha}$ to ^{15}N is possible in the present case because of the $^{13}C^{\alpha}$ line narrowing afforded by deuteration and ^{2}H decoupling.

Briefly the scheme of Figure 1 functions as follows. After H^{N}_{i+1} evolution during t_1, magnetization is transferred to its attached nucleus N_{i+1} (time a). Following a constant time evolution period, t_2, during which $^{13}C^{\alpha}$ is decoupled from ^{15}N, the $^{15}N_{i+1}$ magnetization is relayed via $^{13}CO_i$ (time b) and $^{13}C^{\alpha}_i$ magnetization (time c) to the $^{15}N_i$ of residue i (time d). The effect of dephasing caused by the homonuclear $^{13}C^{\alpha}-^{13}C^{\beta}$ J coupling during the period where C^{α} magnetization is transverse ($\eta = \eta_1 + \eta_2 + \eta_3$) is effectively eliminated by setting η to $\sim 1/J_{CC}$ (27.6 ms). At time d, a fraction $\sin[\pi\eta(^{1}J_{NC\alpha})]\cos[\pi\eta(^{2}J_{NC\alpha})]$ of the C^{α} magnetization is transferred to the intraresidue ^{15}N, and a smaller fraction, $\sin[\pi\eta(^{2}J_{NC\alpha})]\cos[\pi\eta(^{1}J_{NC\alpha})]$, is transferred back to the amide from which magnetization originated. After the second ^{15}N constant time evolution period, t_3, (time e), magnetization is transferred by means of a reverse INEPT sequence to H^{N} for observation. In the 4D spectrum, the frequency coordinates of J correlations in the F_1, F_2, F_3, and F_4 dimensions then correspond to the chemical shifts of H^{N}_{i+1}, N_{i+1}, N_i, and H^{N}_i, respectively. The "diagonal peaks" at H^{N}_{i+1}, N_{i+1}, H^{N}_{i+1}, and N_{i+1}, due to the above mentioned two-bond $J_{NC\alpha}$ transfer process, are 2–4 times weaker.

Experiments are conducted on a Bruker AMX-600 spectrometer, modified such that the ^{2}H lock receiver is disabled during ^{2}H decoupling. Details regarding this hardware modification will be published elsewhere. The method is demonstrated for a sample containing ~1.4 mM of the protein calcineurin B (19.7 kD), uniformly enriched with ^{2}H, ^{15}N, and ^{13}C to levels of 50%, 98%, and 88%, respectively. A second sample, with higher deuteration (~85%), was also prepared to illustrate directly the ^{13}C line narrowing obtainable. The fact that the deuteration level of the sample used for the 4D experiment is only 50% lowers the sensitivity of this particular experiment, but it permits this sample to be used also for a large range of other experiments that require partial side-chain protonation. Both samples also contain 20 mM $CaCl_2$ and 20 mM CHAPS, a zwitterionic detergent which was shown not to significantly affect the structure or binding affinity of calcineurin B.[15] Experiments were conducted at 37 °C, pH 5.6.

Figure 2 illustrates the ^{13}C resolution enhancement obtained by deuteration and ^{2}H decoupling for a small region of the 2D H(N)CA correlation spectrum,[16] displaying connectivities between amide protons and their intraresidue C^{α}. In the absence of deuteration and ^{2}H decoupling, the $^{13}C^{\alpha}$ resonance is a poorly resolved doublet, split by $^{1}J_{CC}$ coupling with $^{13}C^{\beta}$, with a line width for the doublet components of *ca.* 25 Hz (Figure 2A). In the case of deuteration and ^{2}H decoupling, the C^{α} doublet is well-resolved with line widths of ~10 Hz (Figure 2B). The $^{13}C^{\alpha}$ resonances in the deuterated protein are shifted upfield by ~0.35 ppm, caused primarily by the one-bond ^{2}H isotope effect. The 10-Hz $^{13}C^{\alpha}$ line width is determined primarily by the limited acquisition time in the t_1 dimension of the 2D H(N)CA experiment, and by incomplete deuteration of the amino acid side chains which results in a distribution of two- and three-bond isotope effects.

Figure 3 shows four cross sections through the 4D HN(COCA)-NH spectrum, illustrating J connectivities between the amides of residues F72–F75. Of all the sequential J connectivities expected on the basis of the backbone assignments,[17] 98% were observed, except for a stretch of residues close to the C154K mutation, which causes line broadening of the resonances.[17]

Previous attempts to demonstrate the ^{13}C line narrowing

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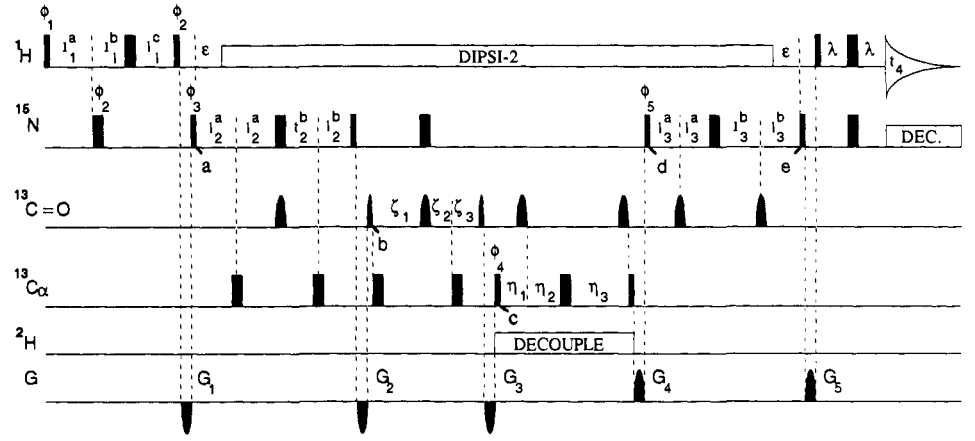

Figure 1. Pulse scheme of the HN(COCA)NH experiment. Narrow pulses correspond to a flip angle of 90°, wide pulses to 180°. Pulses for which the RF phase is not marked are applied along the x axis. Carbonyl pulses have a sin x/x-center-lobe amplitude profile. Carrier frequencies are set to 4.66, 116.5, 177, 56, and 4 ppm for the ^{1}H, ^{15}N, ^{13}C=O, ^{13}C$^\alpha$, and ^{2}H nuclei, respectively. ^{1}H and ^{15}N pulses are applied at 25 and 6 kHz, whereas the ^{13}C$^\alpha$ (^{13}C=O) 90° and 180° pulses have durations of 53 and 47.4 μs (219 and 300 μs), respectively. The ^{1}H decoupling (DIPSI-2)[19], ^{15}N (WALTZ-16), and ^{2}H (cw) decoupling are applied at field strengths of 5, 1.5, and 1.6 kHz, respectively. Phase cycling is as follows: $\phi_1 = x$; $\phi_2 = y,-y$; $\phi_3 = x$; $\phi_4 = 2(x), 2(-x)$; $\phi_5 = x$; acq $= x, 2(-x), x$. Pulsed field gradients are used to suppress artifacts, not to select a coherence transfer pathway.[20] Gradients have a sine bell amplitude profile with a strength of 10 G/cm at their center. Durations are $G_{1,2,3,4,5} = 0.85, 0.25, 0.35, 1.50, 4.00$ ms. Quadrature in the t_1, t_2, and t_3 domains is obtained by changing the phases ϕ_1, ϕ_3, and ϕ_5, respectively, in the usual states–TPPI manner.[21] Delay durations are as follows: $\epsilon = 5.4$ ms; $\lambda = 2.25$ ms; $\zeta_{1,2,3} = 11.1, 6.5, 4.6$ ms; $\eta_{1,2,3} = 4.8, 9.0, 13.8$ ms. The initial delays for the semi-constant-time[22] evolution period (t_1) and constant time evolution periods (t_2 and t_3) are set to $t_1^{a,b,c} = 2.25, 0, 2.25$ ms; $t_2^{a,b} = 5.6$ ms; $t_3^{a,b} = 6.8$ ms. Increments for those delays are set to $\Delta t_1^{a,b,c} = 354, 204, -102$ μs; $\Delta t_2^{a,b} = 275, -275$ μs; $\Delta t_3^{a,b} = 275, -275$ μs.

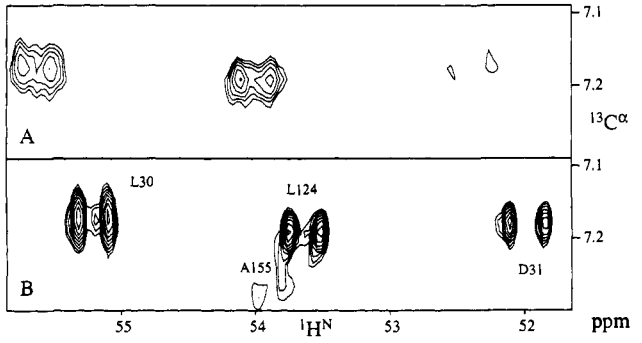

Figure 2. Small regions of the 2D H(N)CA spectrum of (A) fully protonated calcineurin B and (B) randomly 85%-deuterated calcineurin B, with ^{2}H decoupling. Both spectra were recorded and processed identically. The t_1 and t_2 acquisition times used are 73 and 55 ms, respectively, and data are zero filled to yield a digital resolution of 5 Hz (F_1) and 9 Hz (F_2), with no digital filtering in the t_1 dimension.

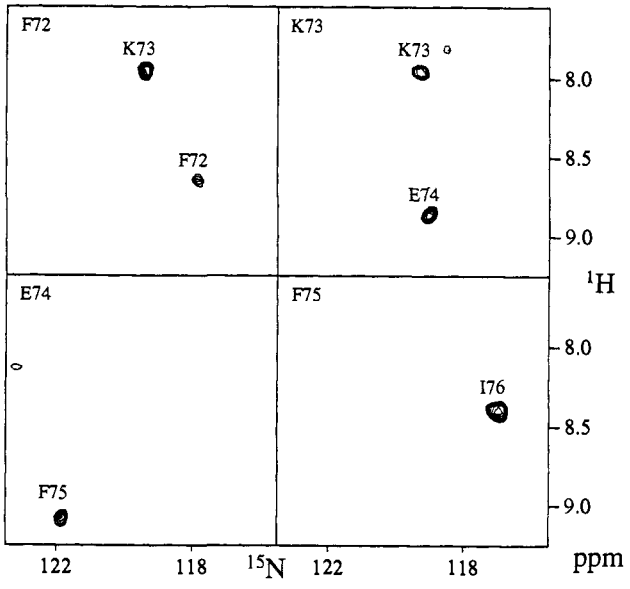

Figure 3. Four (F_1,F_2) cross sections through the 4D HN(COCA)NH spectrum of calcineurin B (50% ^{2}H), taken at the (F_3,F_4) frequencies of the amides of residues F72–F75. Each cross section shows the connection to amide ^{15}N and ^{1}H frequencies of the next residue; panels for F72 and K73 also show the weaker (4D) diagonal peaks to the same residue. The 4D spectrum results from a 22* × 20* × 24* × 512* data matrix, where n* refers to n complex data points. Total accumulation time was 6 days with 32 scans per hypercomplex t_1,t_2,t_3-increment. Acquisition times were 14.5 ms (t_1), 22.0 ms (t_2), 26.4 ms (t_3), and 55.3 ms (t_4). The t_2 and t_3 time domains were extended by means of mirror image linear prediction[23] prior to zero filling and Fourier transformation. The size of the absorptive part of the final 4D spectrum was 64 × 128 × 128 × 1024.

obtained by deuteration and ^{2}H decoupling were only partially successful because the rapid ^{2}H spin lattice relaxation at the low magnetic field strength used (1.4 T) required a stronger ^{2}H decoupling field than could be generated experimentally.[7] At the high magnetic field strength used in our present work (14 T), ^{2}H T_1 relaxation is much longer, and in addition, the ^{2}H decoupling field used in our study is nearly 7 times stronger.

Random fractional deuteration, previously explored in homonuclear ^{1}H NMR,[18] presents a powerful approach for overcoming the natural line width problem in heteronuclear NMR studies of larger ^{13}C/^{15}N-enriched proteins. The present experiment is only a single example of the utility of this approach, but a large range of experiments that can benefit from deuteration is presently under investigation.

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