# $^{13}C$ 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 $C^{\alpha}$ 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 $C^{\alpha}$ magnetization is transverse ($\eta = \eta_1 + \eta_2 + \eta_3$) is effectively eliminated by setting $\eta$ 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 $C^{\alpha}$ 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 $C^{\alpha}$. 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 $C^{\alpha}$ 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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(1) Oh, B. H.; Westler, W. M.; Derba, P.; Markley, J. L. *Science* **1988**, *240*, 908–911.
(2) Ikura, M.; Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659–4667.
(3) Boucher, W.; Laue, E. D.; Campbell-Burk, S.; Domaille, P. J. *J. Am. Chem. Soc.* **1992**, *114*, 2262–2264.
(4) Olejniczak, E. T.; Xu, R. X.; Fesik, S. W. *J. Biomol. NMR* **1992**, *2*, 655–659.
(5) Palmer, A. G., III; Fairbrother, W. J.; Cavanagh, J.; Wright, P. E.; Rance, M. *J. Biomol. NMR* **1992**, *2*, 103–108.
(6) Grzesiek, S.; Döbeli, H.; Gentz, R.; Garotta, G.; Labhardt, A. M.; Bax, A. *Biochemistry* **1992**, *31*, 8180–8190.
(7) Browne, D. T.; Kenyon, G. L.; Packer, E. L.; Sternlicht, H.; Wilson, D. M. *J. Am. Chem. Soc.* **1973**, *95*, 1316–1323.
(8) Abragam, A. *The Principles of Nuclear Magnetism*; Clarendon Press: Oxford, 1961; p 309.
(9) London, R. E. *J. Magn. Reson.* **1990**, *86*, 410–415.
(10) Montelione, G. T.; Wagner, G. *J. Magn. Reson.* **1990**, *87*, 183–188.
(11) Kay, L. E.; Ikura, M.; Bax, A. *J. Magn. Reson.* **1991**, *91*, 84–92.
(12) Delaglio, F.; Torchia, D. A.; Bax, A. *J. Biomol. NMR* **1991**, *1*, 439–446.
(13) Clubb, R. T.; Thanabal, V.; Wagner, G. *J. Biomol. NMR* **1992**, *2*, 203–210.
(14) Clubb, R. T.; Thanabal, V.; Wagner, G. *J. Magn. Reson.* **1992**, *97*, 213–217.
(15) Anglister, J.; Grzesiek, S.; Ren, H.; Klee, C. B.; Bax, A. *J. Biomol. NMR* **1993**, *3*, 121–126.
(16) Grzesiek, S.; Bax, A. *J. Magn. Reson.* **1992**, *96*, 432–440.
(17) Anglister, J.; Grzesiek, S.; Ren, H.; Klee, C. B.; Bax, A. Unpublished results.
(18) LeMaster, D. M.; Richards, F. M. *Biochemistry* **1988**, *27*, 142–150.
(19) Shaka, A. J.; Lee, C. J.; Pines, A. *J. Magn. Reson.* **1988**, *77*, 274–293.
(20) Bax, A.; Pochapsky, S. S. *J. Magn. Reson.* **1992**, *99*, 638–643.

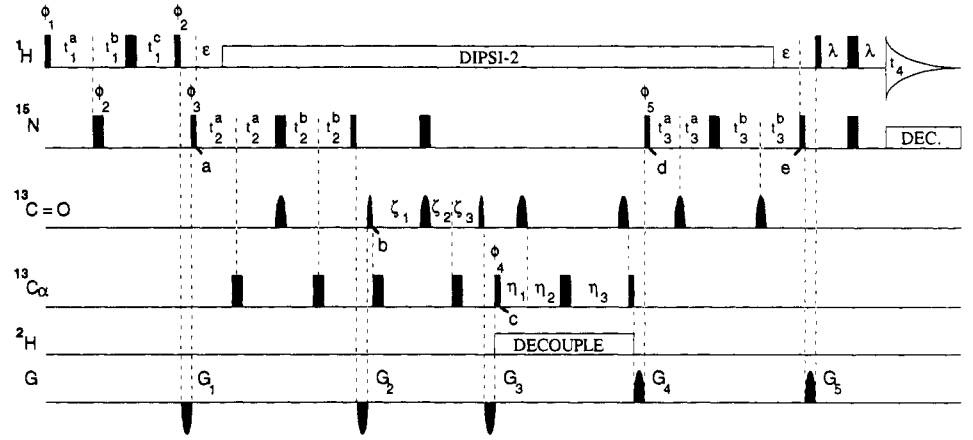


**Figure 1.** Pulse scheme of the HN(COCA)NH experiment. Narrow pulses correspond to a flip angle of 90°, wide pulses to 180°. Pulses for which the RF phase is not marked are applied along the $x$ axis. Carbonyl pulses have a sin $x/x$-center-lobe amplitude profile. Carrier frequencies are set to 4.66, 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 $\mu$s (219 and 300 $\mu$s), 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 $\phi_1$, $\phi_3$, and $\phi_5$, 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 $\Delta t_1^{a,b,c} = 354, 204, -102$ $\mu$s; $\Delta t_2^{a,b} = 275, -275$ $\mu$s; $\Delta t_3^{a,b} = 275, -275$ $\mu$s.

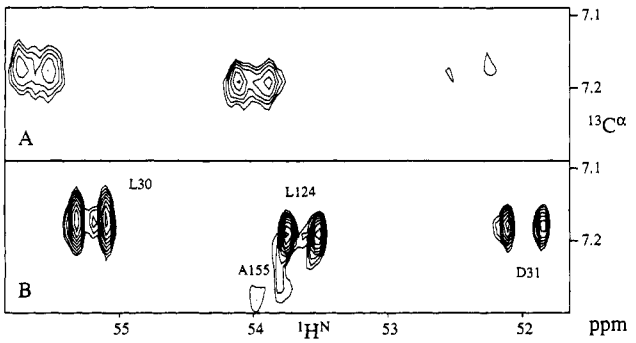


**Figure 2.** Small regions of the 2D H(N)CA spectrum of (A) fully protonated calcineurin B and (B) randomly 85%-deuterated calcineurin B, with $^2$H decoupling. Both spectra were recorded and processed identically. The $t_1$ and $t_2$ acquisition times used are 73 and 55 ms, respectively, and data are 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.

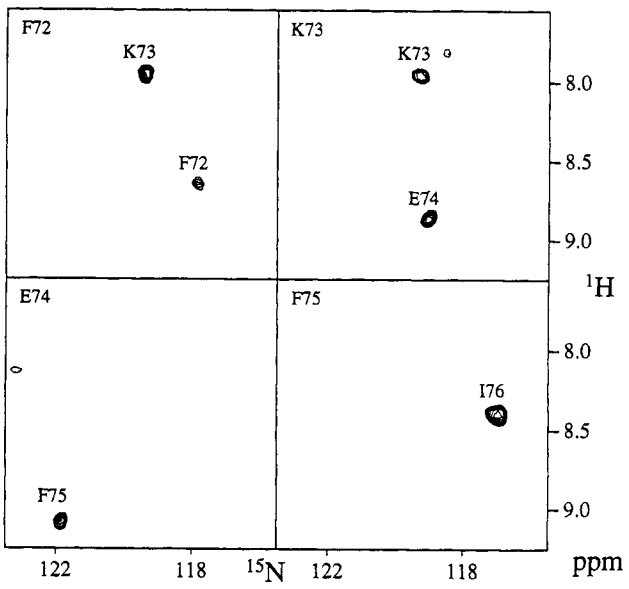


**Figure 3.** Four ($F_1$,$F_2$) cross sections through the 4D HN(COCA)NH spectrum of calcineurin B (50% $^2$H), taken at the ($F_3$,$F_4$) frequencies of the amides of residues F72–F75. Each cross section shows the connection to amide $^{15}$N and $^1$H frequencies of the next residue; panels for F72 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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(21) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393–399.
(22) Grzesiek, S.; Bax, A. *J. Biomol. NMR* **1993**, *3*, 185–204.
(23) Zhu, G.; Bax, A. *J. Magn. Reson.* **1990**, *90*, 405–410.