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The use of heteronuclear cross-polarization for backbone assignment of ^{2}H-, ^{15}N- and ^{13}C-labeled proteins: A pulse scheme for triple-resonance 4D correlation of sequential amide protons and ^{15}N

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Summary

A new four-dimensional pulse scheme is described for the main-chain assignment of proteins by means of the J connectivity of the amide proton and nitrogen resonances of adjacent residues. Since the new experiment, 4D CP-HN(COCA)NH, involves heteronuclear cross-polarization for magnetization transfer from $^{13}C{=}O$ to ^{15}N via $^{13}C^{\alpha}$, a relatively strong WALTZ-16 decoupling rf field is applied to $^{13}C^{\alpha}$ during magnetization transfer. Consequently, $^{13}C^{\alpha}$ is effectively decoupled from its attached ^{2}H in the case of deuterated proteins, in the absence of a decoupling rf field for ^{2}H. This efficiently improves the sensitivity of the experiment through ^{13}C line narrowing. The experiment was performed on a randomly 60% deuterated protein, and the sensitivity of the final 4D spectrum was found to be excellent.

The development of multidimensional triple-resonance NMR techniques has enabled studies of the solution structures of 'medium-size' proteins with molecular masses smaller than 25 kDa (Ikura et al., 1990; Kay et al., 1990; Bax and Grzesiek, 1993). The highest barrier for their application to larger proteins is the short transverse relaxation time, T_2, of $^{13}C^{\alpha}$, since magnetization transfer passes $^{13}C^{\alpha}$ in most multidimensional triple-resonance NMR experiments. The $^{13}C^{\alpha}$ transverse relaxation is dominated by a strong dipolar interaction with $^{1}H^{\alpha}$. Since the gyromagnetic ratio of ^{2}H is 6.5 times smaller than that of ^{1}H, the transverse relaxation of $^{13}C^{\alpha}$ due to the dipole interaction is reduced by deuteration at the H^{α} site. However, due to scalar relaxation of the second kind (Abragam, 1961), the $^{13}C^{\alpha}$ line width is still broadened by deuteration at magnetic fields of 10–15 T. Several groups have shown that ^{2}H decoupling during the periods of transverse ^{13}C magnetization efficiently eliminates the effect of scalar relaxation of the second kind, and thereby results in ^{13}C line narrowing (Grzesiek et al., 1993; Kushlan and LeMaster, 1993; Yamazaki et al., 1994).

However, high-power deuterium decoupling (more than several hundred Hz) leads to additional hardware demands. Another radiofrequency channel is required, in addition to the three rf channels for ^{1}H, ^{15}N and ^{13}C for triple-resonance NMR experiments. Moreover, the ^{2}H channel causes instabilities of the ^{2}H lock, unless ^{2}H lock-holding and -blanking of the ^{2}H channel for decoupling are used. A ^{2}H lock coil in the NMR probe, sufficiently stable for high-power ^{2}H decoupling, is also required.

Recently, several groups have shown that heteronuclear cross-polarization (CP) in solution is as practical for magnetization transfer as pulsed sequences, such as INEPT or DEPT (Zuiderweg, 1990; Ernst et al., 1991; Majumdar et al., 1993; Richardson et al., 1993; Schleucher et al., 1994). It has also been shown to be beneficial under some circumstances, in comparison with corresponding pulsed transfers, such as INEPT (Majumdar et al., 1993; Richardson et al., 1993).

In this communication, we present a new four-dimensional triple-resonance experiment which directly correlates the amide proton and nitrogen resonances of adjacent residues. In contrast to a related experiment introduced by Grzesiek et al. (1993), our experiment involves

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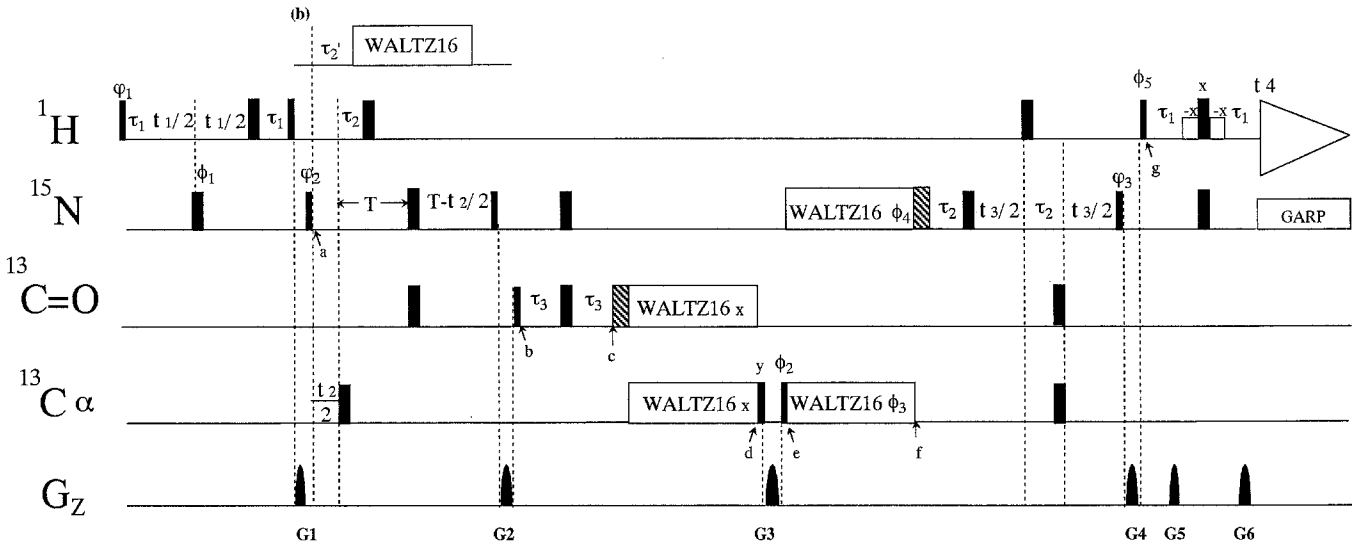

Fig. 1. Pulse scheme for the CP-HN(COCA)NH experiment with heteronuclear cross-polarization. All narrow pulses correspond to a flip angle of 90°, and wide pulses to 180°. Unless indicated otherwise, pulses were applied along the x-axis. All ^{13}C pulses were applied with an rf field strength of 2.87 kHz, except for the $^{13}C{=}O \rightarrow {}^{13}C^{\alpha}$ CP period (from time c to time d), where the rf field strength was 1.95 kHz. The ^{15}N pulses were applied with an rf field strength of 9.47 kHz, except for WALTZ-16 and the subsequent 180° and 90° (φ_3) pulses, where an rf field strength of 2.87 kHz was used. For ^{1}H the field strength was 28 kHz, except for water-selective 90°_{-x} pulses (open rectangles), where the rf field strength was 166 Hz. Typical durations for the cross-polarization periods were 24.65 ms for $^{13}C{=}O \rightarrow {}^{13}C^{\alpha}$ cross-polarization, and 66.8 ms for $^{13}C^{\alpha} \rightarrow {}^{15}$N cross-polarization. The shaded rectangles correspond to 1 ms trim pulses along the x-axis. The ^{13}C carrier frequency was set at 177.3 ppm until the $^{13}C^{\alpha}$ 90° y pulse, and was then switched to 57.5 ppm. The ^{1}H carrier was placed at 8 ppm for the first three pulses, and then changed to 4.6 ppm. The ^{15}N carrier was set at 119.7 ppm. Typical values for the delays were T = 11.2 ms, $\tau_1 = 2.7$ ms, $\tau_2 = 2.7$ ms and $\tau_3 = 11.2$ ms. Instead of a 180° ^{1}H decoupling pulse, WALTZ-16 ^{1}H decoupling can be applied with $\tau_2' = 5.4$ ms (Scheme b). The following phase cycling was employed: ϕ_1 = y,–y; ϕ_2 = 8(y),8(–y); ϕ_3 = 2(x),2(–x); ϕ_4 = 4(x),4(–x); ϕ_5 = x; φ_1 = x; φ_2 = x; φ_3 = x; and Receiver = (x,–x,–x,x), 2(–x,x,x,–x), (x,–x,–x,x). Quadrature detection in F_1, F_2 and F_3 was achieved by altering φ_1, φ_2 and φ_3 in a States-TPPI manner. Gradients have a sine-bell amplitude profile. The durations and strengths of the gradients were as follows: G1 = (2 ms, –12 G/cm); G2 = (2 ms, 7.5 G/cm); G3 = (2 ms, –15 G/cm); G4 = (2 ms, –6 G/cm); G5 = (0.75 ms, 18 G/cm); and G6 = (0.75 ms, 18 G/cm). The gradients G5 and G6 and the two water-selective 90°_{-x} pulses are for the WATERGATE scheme (Piotto et al., 1992).

heteronuclear cross-polarization in solution (Ernst et al., 1991) instead of INEPT for the magnetization transfer from $^{13}C{=}O$ to ^{15}N via $^{13}C^{\alpha}$. Since a strong WALTZ-16 decoupling rf field (more than 1.9 kHz) is applied to $^{13}C^{\alpha}$ during the magnetization transfer, $^{13}C^{\alpha}$ is effectively decoupled from its attached ^{2}H. Thus, applying this pulse sequence with heteronuclear CP to deuterated proteins, we can expect a longer transverse relaxation time for $^{13}C^{\alpha}$ attached to ^{2}H, which results in more efficient magnetization transfer from $^{13}C{=}O$ to ^{15}N through $^{13}C^{\alpha}$ in the absence of ^{2}H decoupling.

Figure 1 illustrates the pulse sequence employed in the experiment. According to the convention introduced by Kay and co-workers (1990), we name the experiment CP-HN(COCA)NH. The flow of magnetization is the same as in the HN(COCA)NH experiment introduced by Grzesiek et al. (1993). After $^1H^N$(i+1) chemical shift evolution during t_1, magnetization is transferred to the attached amide nitrogen ^{15}N(i+1) through INEPT transfer at time point a. In the constant-time evolution period, 2T, and during the subsequent ^{15}N and $^{13}C{=}O$ 90° pulses, the magnetization is relayed to $^{13}C{=}O$(i) at time b, and in-phase $^{13}C{=}O$(i) magnetization appears at time c, after rephasing caused by $^1J(^{15}N(i+1)\text{-}^{13}C{=}O(i))$. From time c to time d, a WALTZ-16 rf field is applied to $^{13}C{=}O$ and $^{13}C^{\alpha}$ at the same field strength, using double selective rectangular pulses, generated by a single rf channel (Vincent et al., 1993; Ito, Y. and Laue, E.D., personal communication). During this period, transverse in-phase $^{13}C{=}O$(i) magnetization is transferred to in-phase $^{13}C^{\alpha}$(i) magnetization by heteronuclear CP (Ernst et al., 1991). Just after the field gradient pulse z-filter, $^{13}C^{\alpha}$-^{15}N CP takes place by the application of a WALTZ-16 decoupling field from time e to time f. In-phase $^{13}C^{\alpha}$(i) magnetization is transferred either to ^{15}N(i), which relies on the intraresidue $^1J_{C\alpha N}$ (~11 Hz) coupling and gives a larger signal in the final spectrum, or to ^{15}N(i+1), which relies on the interresidue $^2J_{C\alpha N}$ (~7 Hz) coupling and gives a smaller signal. Since the WALTZ-16 decoupling rf field, which is applied to $^{13}C^{\alpha}$ during both CP periods, is sufficiently high (more than 1.9 kHz, which is several times larger than the $T_1(^2H)$ relaxation rate) (Grzesiek et al., 1993), the scalar relaxation of the second kind of $^{13}C^{\alpha}$ by its attached ^{2}H is efficiently removed, without an additional ^{2}H decoupling field. The ^{15}N(i) and ^{15}N(i+1) magnetization is dephased relative to their attached protons, and relayed to $^1H^N$(i) and $^1H^N$(i+1), respectively, at time g. Finally, the $^1H^N$(i) and $^1H^N$(i+1) transverse magnetization is refocused and

detected during t_4. In the final 4D spectrum, the frequency coordinates obtained for F_1, F_2, F_3 and F_4 are $^1H^N(i+1)$, $^{15}N(i+1)$, $^{15}N(i)$ and $^1H^N(i)$ as interresidue peaks, and $H^N(i)$, N(i), N(i) and $H^N(i)$ as diagonal peaks, respectively. The diagonal peaks are weaker than the interresidue ones.

The 4D CP-HN(COCA)NH experiment was performed on a sample containing ~ 1.6 mM of the yeast Pho4 DNA binding domain (14.2 kDa as a dimer), pH 6, uniformly labeled with ^{15}N, ^{13}C and ^2H (Shimizu, M. et al., manuscript in preparation). The 60% random ^2H labeling was achieved by culturing *E. coli* cells harboring over-expression plasmids in a 60% D_2O / 40% H_2O solution, containing M9 medium with ^{13}C-glucose (0.12%) and $^{15}NH_4Cl$ (0.1%). NMR measurements were conducted on a Bruker AMX-500 spectrometer with three rf channels at 40 °C. In a series of 1D experiments, we found that the optimum lengths of the CP mixing sequences for $^{13}C{=}O \rightarrow {}^{13}C^{\alpha}$ and $^{13}C^{\alpha} \rightarrow {}^{15}N$ were 24.7 ms (two cycles of the WALTZ-16 scheme for $\gamma B_1 = 1.95$ kHz), and 66.8 ms (eight cycles of

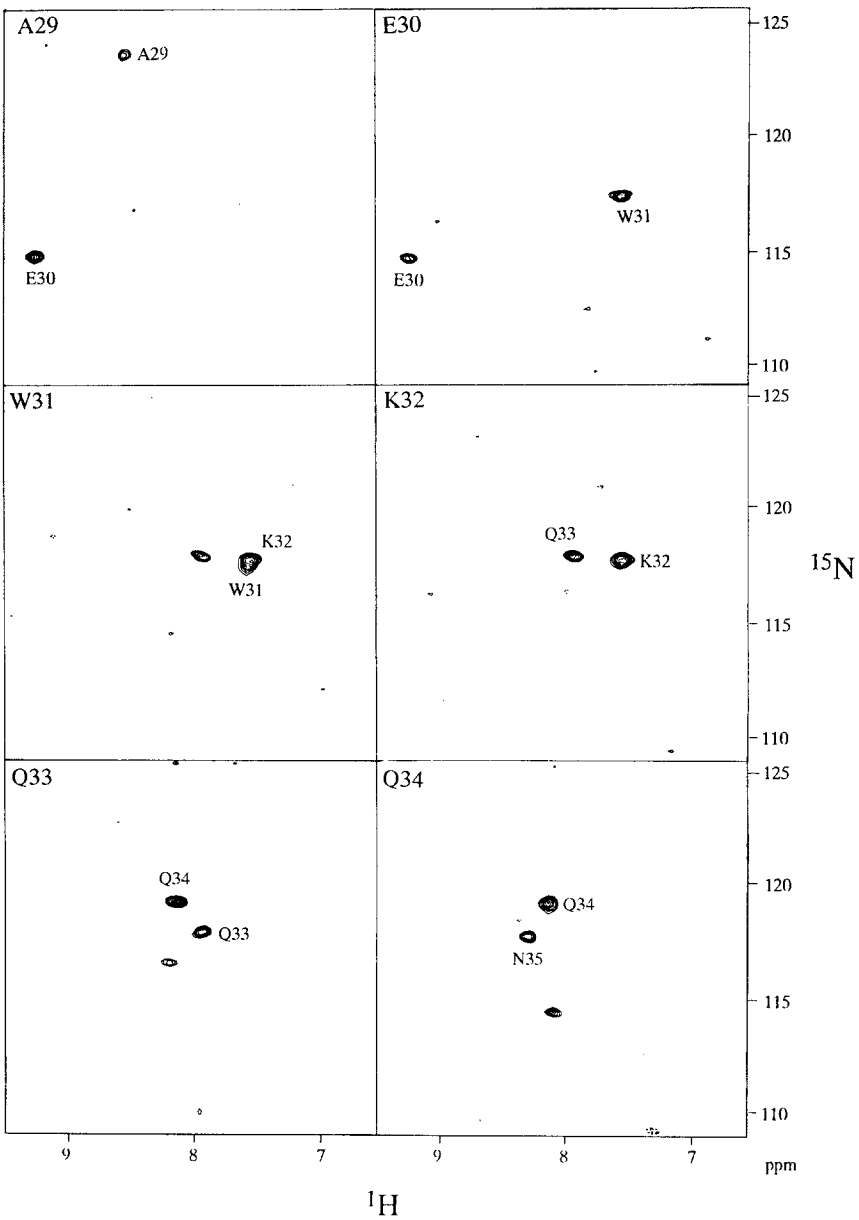

Fig. 2. Six [^1H (F_1), ^{15}N (F_2)] cross sections, at the ^{15}N (F_3) and ^1H (F_4) chemical shifts from Ala^{29} to Asn^{35}, through the 4D CP-HN(COCA)NH spectrum of the Pho4 DNA binding domain (60% ^2H). 12 (F_1)×18 (F_2)×12 (F_3)×1K (F_4) complex points were acquired in 4.6 days, giving maximum acquisition times of 7.99 ms (t_1), 21.5 ms (t_2), 14.3 ms (t_3) and 81.9 ms (t_4). For each FID, 16 transients were accumulated. The spectrum was processed using Fourier transformation in t_3 and t_4 after removal of the H_2O signal by convolution of FIDs (Marion et al., 1989). Only a quarter of the spectrum (in F_4), which covered the whole $^1H^N$ region, was used for further 2D maximum entropy reconstruction in F_1 and F_2 (Laue et al., 1986; Boucher et al., 1991), to give a final spectrum of 64 (F_1)×128 (F_2)×32 (F_3)×512 (F_4) points. The data set was processed using the AZARA suite of programs, provided by Wayne Boucher, Department of Biochemistry, University of Cambridge.

the WALTZ-16 scheme for $\gamma B_1 = 2.87$ kHz), respectively. The rf field strengths for the CP mixing sequences for $^{13}C{=}O \rightarrow {}^{13}C^{\alpha}$ and $^{13}C^{\alpha} \rightarrow {}^{15}N$ were chosen so as to give effective cross-polarization over just the range of $^{13}C^{\alpha}$ chemical shifts, in order to minimize homonuclear magnetization transfer between $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ (Richardson et al., 1993).

Figure 2 shows six (F_1,F_2) cross sections through the 4D HN(COCA)NH spectrum at the ^{15}N (F_3) and ^{1}H (F_4) chemical shifts from Ala[29] to Asn[35], illustrating the sequential (^{1}H,^{15}N) $\rightarrow$ (^{1}H,^{15}N) J connectivities. Stronger cross peaks correspond to interresidue correlations, $^{1}H^{N}$(i+1), ^{15}N(i+1), ^{15}N(i) and $^{1}H^{N}$(i), and weaker cross peaks to diagonal peaks, $^{1}H^{N}$(i), ^{15}N(i), ^{15}N(i) and $^{1}H^{N}$(i). Since the sensitivity of the experiment was excellent, it often gave both interresidue cross peaks and diagonal peaks, which makes sequential main-chain assignment more straightforward.

An additional deuterium WALTZ-16 decoupling field at a strength of 720 Hz, applied from another rf channel during both the $^{13}C{=}O \rightarrow {}^{13}C^{\alpha}$ and $^{13}C^{\alpha} \rightarrow {}^{15}N$ CP periods, did not improve the sensitivity of the experiment, as judged on the first FID of the 4D experiments. This demonstrates that the $^{13}C^{\alpha}$ CP field is sufficient to remove the $^{13}C^{\alpha}$-$^{2}H^{\alpha}$ scalar coupling.

In this communication we have presented a pulse sequence for a CP version of the HN(COCA)NH experiment, applied to a deuterated protein. Since a strong WALTZ-16 rf field is applied to $^{13}C^{\alpha}$ during the heteronuclear CP period, ^{13}C line narrowing can be achieved without ^{2}H decoupling. Consequently, the experiment can be conducted with a commercial three-channel NMR spectrometer without further modifications. To the best of our knowledge, it is the first application of heteronuclear CP for ^{13}C line narrowing by decoupling the ^{13}C-^{2}H scalar coupling in triple-resonance experiments of deuterated proteins. The experiment was proven to be quite sensitive and gave a high-quality four-dimensional NMR spectrum for a 14 kDa protein with a recording time of 4.6 days. Since the chemical shifts of amide protons and amide nitrogens often give the best dispersion among the nuclei in the main chain of proteins, the CP-HN(COCA)NH pulse scheme can be utilized quite effectively for the main-chain assignments of ^{15}N-, ^{13}C- and ^{2}H-labeled proteins, in the absence of ^{2}H decoupling.

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