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A 4D HCC(CO)NNH experiment for the correlation of aliphatic side-chain and backbone resonances in $^{13}C/^{15}N$-labelled proteins

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SUMMARY

We recently proposed a novel four-dimensional (4D) NMR strategy for the assignment of backbone nuclei in spectra of $^{13}C/^{15}N$-labelled proteins (Boucher et al. (1992) *J. Am. Chem. Soc.*, **114**, 2262–2264 and *J. Biomol. NMR*, **2**, 631–637). In this paper we extend this approach with a new constant time 4D HCC(CO)NNH experiment that also correlates the chemical shifts of the aliphatic sidechain (^1H and ^{13}C) and backbone (^1H, $^{13}C_\alpha$ and ^{15}N) nuclei. It separates the sidechain resonances, which may heavily overlap in spectra of proteins with large numbers of similar residues, according to the backbone nitrogen and amide proton chemical shifts. When used in conjunction with a 4D HCANNH or HNCAHA experiment it allows, in principle, complete assignment of aliphatic sidechain and backbone resonances with just two 4D NMR experiments.

The assignment of sidechain protons in NMR spectra of small proteins (< 10 kDa) has traditionally relied on the use of homonuclear two-dimensional (2D) ^1H experiments (Wüthrich, 1986). However, for larger proteins (> 15 kDa), magnetisation transfer in these experiments becomes increasingly inefficient as the ^1H linewidths exceed the value of the $^3J_{HH}$ coupling constants. This has motivated the development of experiments that instead transfer magnetisation via the much larger one bond ^1H–^{13}C and ^{13}C–^{13}C couplings. In addition to providing greatly improved sensitivity, these HCCH experiments also allow separation of the 2D ^1H–^1H correlation spectrum into three or four dimensions according to the ^{13}C chemical shifts of the attached ^{13}C nuclei (Bax et al., 1990a,b; Fesik et al., 1990; Ikura et al., 1991a).

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Nevertheless, despite the improvements in sensitivity and resolution that these experiments provide, overlap does still occur, particularly in the spectra of proteins that have a large number of similar residues or a high helical content. The low chemical shift dispersion of $^{1}H_{\alpha}$ resonances in α-helices is well known, and has recently also been observed for $^{13}C_{\alpha}$ and $^{13}C_{\beta}$ resonances (Spera and Bax, 1991). We have found this to be a major problem in 3D HCCH spectra of predominantly α-helical DNA binding proteins. These contain a large number of lysine and arginine residues which are often on the surface of the protein and therefore largely unstructured. The assignment of their resonances is crucial for studying interactions with nucleic acids and other proteins. For these reasons, we were prompted to develop new experiments designed to separate the sidechain resonances according to the nitrogen and amide proton chemical shifts. These experiments are complementary to those recently proposed by Kay et al., (1992a), where the sidechain correlations are separated by the carbonyl chemical shifts. An additional motivation for developing such experiments is that they often allow straightforward identification of the sidechain, and therefore residue type, during the course of a backbone assignment. When used in conjunction with a 4D HCANNH or HNCAHA experiment (Boucher et al., 1992a,b; Kay et al., 1992b), the new 4D HCC(CO)NNH experiment allows, in principle, complete assignment of sidechain (^{1}H and ^{13}C) and backbone (^{1}H, $^{13}C_{\alpha}$ and ^{15}N) resonances with just two 4D NMR experiments recorded on the same sample in an H_2O solution.

The 4D HCC(CO)NNH experiment is based on our 4D HCA(CO)NNH experiment (Boucher et al., 1992a,b) and is also similar to the 3D CBCA(CO)NH experiment developed by Grzesiek and Bax (1992). Thus only a brief description is given here. The experiment (see Fig. 1) works by transferring ^{1}H magnetisation to ^{13}C, using an INEPT sequence (Morris and Freeman, 1979; Burum and Ernst, 1980); the ^{1}H chemical shift is monitored during t_1. After the simultaneous 90° pulses to ^{1}H and ^{13}C, the antiphase carbon magnetisation (at point a) refocuses with respect to its attached proton during $2\tau_1$ and evolves according to the ^{13}C chemical shift during t_2. Dephasing due to the J_{CC} couplings during t_2, which results in splittings in f_2, is minimised by the short acquisition time in the ^{13}C dimension (see below). The refocused ^{13}C magnetisation (at point b) is then transferred along the sidechain to the $^{13}C_{\alpha}$ nuclei using a z-filtered TOCSY step (Rance et al., 1987). At the end of this step, both in phase and antiphase $^{13}C_{\alpha}$ magnetisation dephases with respect to the attached $^{13}C'$ spin during $2\tau_2$ according to

$$C_x^{\alpha} \rightarrow 2\, C_y^{\alpha}\, C'_z \left[(\sin 2\pi J_{C_{\alpha}C'}\, \tau_2)(\cos 2\pi J_{C_{\alpha}C_{\beta}}\, \tau_2)(\exp(-\, 2\tau_2/T_2^{C_{\alpha}})\right]$$

and

$$-\, 2C_y^{\alpha}\, C_z^{\beta} \rightarrow 2C_y^{\alpha}\, C'_z \left[(\sin 2\pi J_{C_{\alpha}C'}\, \tau_2)(\sin 2\pi J_{C_{\alpha}C_{\beta}}\, \tau_2)(\exp(-\, 2\tau_2/T_2^{C_{\alpha}})\right]$$

If one assumes a value of 21 ms for the $^{13}C_{\alpha}$ T_2 values, a compromise value of 3.6 ms for τ_2 maximises the resulting signal intensity. The magnetisation is then transferred to the $^{13}C'$, ^{15}N and $^{1}H_N$ in an identical manner to the HCA(CO)NNH experiment (Boucher et al., 1992b). For the first half of the pulse sequence, until the end of the TOCSY transfer step, the ^{13}C carrier frequency was set to the centre of the aliphatic region (40.95 ppm) to monitor the ^{13}C chemical shift and to optimise efficiency of the TOCSY transfer. It was then switched to the centre of the $^{13}C_{\alpha}$ region (59.8 ppm) for the remainder of the pulse sequence. We shifted the ^{13}C carrier frequency and adjusted the power level after the TOCSY transfer step, whilst the magnetisation was along the

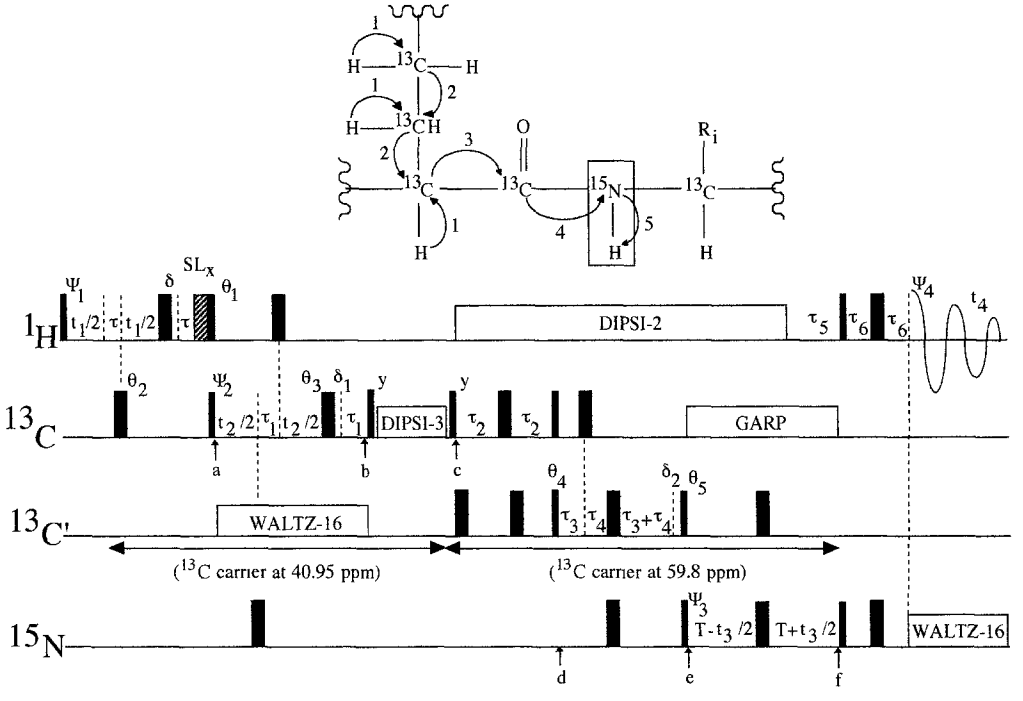

Fig. 1. Pulse sequence and the pathway of magnetisation transfer (inset) for the 4D HCC(CO)NNH experiment. Narrow boxes represent 90° pulses, wider boxes represent 180° pulses and the cross-hatched box represents a spin-lock pulse (1 ms) used for water suppression (Messerle et al., 1989). The ^{13}C carrier was placed at 40.95 ppm until the end of the DIPSI-3 sequence; prior to the 90°$_y$ pulse the frequency was switched to 59.8 ppm and the RF field strength was reduced from 6.25 kHz to 4.43 kHz to minimise excitation of the ^{13}C′ resonances (Kay et al., 1990b). The ^{13}C′ pulses were applied with an RF field strength of 4.43 kHz, using off resonance DANTE sequences (Kay et al., 1990b). For ^{13}C and ^{13}C′ decoupling the RF field strength was 625 Hz. The ^{1}H and ^{15}N pulses were applied with RF field strengths of 11.1 kHz and 3.12 kHz, respectively, except during the decoupling sequences, where the RF field strength was reduced to 5.0 kHz and 1.0 kHz, respectively. The first ^{13}C′ pulse compensates for phase errors due to Block Siegert effects caused by the second ^{13}C′ pulse (Vuister and Bax, 1992). Typical values for the delays are: τ = 1.5 ms, τ_1 = 1.1 ms, τ_2 = 3.6 ms, τ_3 = 4.5 ms, τ_4 = 7.9 ms, τ_5 = 5.4 ms and τ_6 = 2.25 ms. The constant time, T, was set to 12.0 ms. The delay, δ, was set to the initial value of t_1 + the length of the ^{13}C (180°) whilst δ_1 was set to the initial value of t_2 + the length of the ^{1}H (180°) and δ_2 was equal to the length of the ^{13}C (180°). The following phase cycling was employed: θ_1 = 4(y), 4(−y); θ_2 = x, −x; θ_3 = 2(x), 2(y), 2(−x), 2(−y); θ_4 = 2(x), 2(−x); θ_5 = 20°; ψ_1 = x; ψ_2 = 8(x), 8(−x); ψ_3 = x, −x and ψ_4 = (x, −x, x, −x), 2(−x, x, −x, x), (x, −x, x, −x). Unless indicated otherwise, all other pulses were of phase x. Quadrature detection in f_1, f_2 and f_3 was achieved by altering ψ_1, ψ_2 and ψ_3 in a States, States-TPPI and States-TPPI manner, respectively (Marion et al., 1989b), so that the axial peaks are at the edges of the spectrum in all three dimensions after data processing (see legend to Fig. 2).

z axis, so as to avoid problems of phase shifts. As with the HCA(CO)NNH experiment, the ^{15}N chemical shifts are monitored during a constant time period 2T and the $^{1}H_N$ chemical shifts are detected during t_4.

The resulting 4D NMR spectrum is most easily analysed by looking at 2D ^{1}H–^{13}C planes at particular $^{1}H_N$ and ^{15}N chemical shifts; the sidechain of the residue preceding the amide is detected specifically (see inset to Fig. 1). We therefore recorded the spectrum with the best possible digital resolution in the ^{15}N dimension. Good resolution in this dimension does not

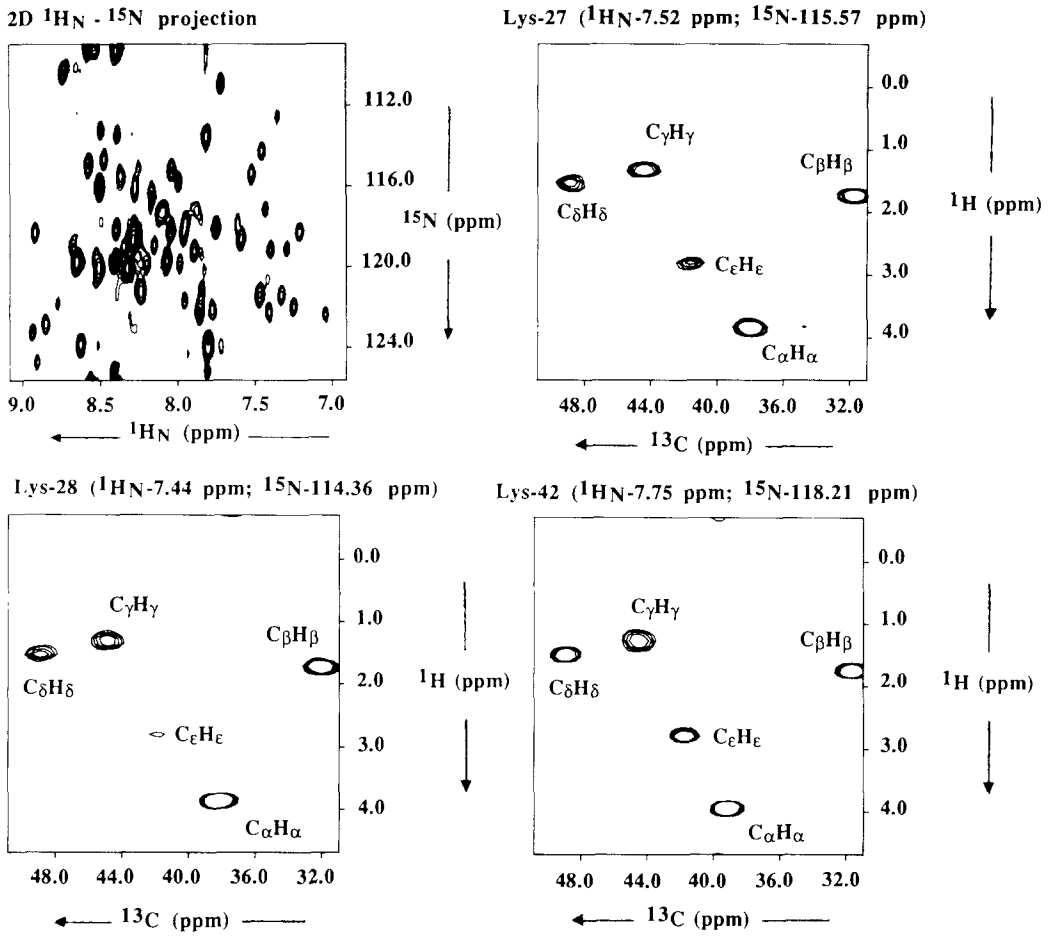

Fig. 2. A 2D $^{1}H_N$–^{15}N projection and the 2D ^{1}H–^{13}C planes for residues Lys^{27}, Lys^{28} and Lys^{42} from the 4D HCC(CO)NNH spectrum of a 3.8 mM sample of the HMG1-A domain, enriched (> 95%) in ^{13}C and ^{15}N, in 10 mM sodium phosphate pH 5.0, 150 mM sodium chloride and 0.2 mM dithiothreitol at 293 K. (Note that the $^{1}H_N$ and ^{15}N chemical shifts are of the residues immediately C-terminal to those labelled). The ^{1}H carrier was placed on the H_2O signal and the spectral width in f_1 was approximately half that in f_4; after data processing the low field half of the spectrum (in f_1) was shifted to the high field end. The ^{13}C spectrum was aliased as discussed in the text. In addition, limited aliasing in the ^{15}N dimension was also used. 20 (t_1) × 8 (t_2) × 24 (t_3) × 512 (t_4) complex points were acquired in 4.5 days to give maximum acquisition times of 6.20 ms (t_1), 2.67 ms (t_2), 23.61 ms (t_3) and 63.5 ms (t_4). The spectrum was first processed by using conventional Fourier transforms in t_3 and t_4, after removal of the H_2O signal by baseline correction of the FIDs (Marion et al., 1989a). The high field half of the spectrum (in f_4) was discarded prior to a 2D maximum entropy reconstruction in f_1 and f_2 (Laue et al., 1986) to give a final spectrum (real part) of 64 (f_1) × 64 (f_2) × 64 (f_3) × 512 (f_4) points. These spectra take ~ 14 h to process on a Silicon Graphics Iris Indigo (R3000) or ~ 9 h on sixteen transputers in a Meiko Computing Surface (Boucher et al., 1991).

compromise the sensitivity of the experiment because the ^{15}N chemical shifts are monitored during the constant time period. The acquisition times in t_1 (^{1}H) and t_2 (^{13}C), however, should be kept as short as possible to minimise losses in sensitivity resulting from relaxation. The ^{13}C NMR spectrum was aliased so that each plane corresponded to three different chemical shifts in a

similar manner to that used in the 4D $^{13}C/^{15}N$- or $^{13}C/^{13}C$-edited NOESY spectra (Kay et al., 1990a; Clore et al., 1991). (If required, the initial t_2 value could be set so that aliased cross peaks have opposite signs to those that are not aliased (Bax et al., 1991). This facilitates analysis of the spectrum, but if cross peaks overlap it could lead to cancellation). For the A domain from HMG1 (Johns, 1982) we were also able to take advantage of the lack of aliphatic proton resonances downfield of ~ 5.0 ppm, allowing us to use a small spectral width in the ^1H dimension without introducing ambiguities (see legend to Fig. 2). In situations where this was not possible, the phase cycling could be reduced and therefore the digital resolution in f_1 increased, by recording the spectrum using pulsed field gradients (Hurd, 1990).

Figure 2 shows a projection of the 4D NMR spectrum of the HMG1 A-domain (83 residues, 9.8 kDa), on to the 2D ^1H_N–^{15}N plane. The good resolution allows us to resolve most of the amide proton–nitrogen cross peaks. In the 4D NMR spectrum this means that we can look at 2D ^1H–^{13}C planes at ^1H_N and ^{15}N chemical shifts that correspond to a single residue. Three such ^1H–^{13}C planes, containing the sidechain resonances of Lys^{27}, Lys^{28} and Lys^{42}, are shown (see Fig. 2). They identify the residue type and determine the ^1H and ^{13}C chemical shifts of the sidechain resonances. Note that the $^{13}C_\gamma$ and $^{13}C_\delta$ cross peaks are aliased from upfield whilst the $^{13}C_\alpha$ crosspeak is aliased from downfield. The cross peaks are easily assigned on the basis of their expected ^1H and ^{13}C chemical shifts (Ikura et al., 1991b). All three residues have very similar chemical shifts, which explains why it had proved impossible to assign the sidechain resonances of these residues using the 3D HCCH-COSY and TOCSY spectra.

In conclusion, the 4D HCC(CO)NNH experiment provides a valuable alternative to the HCA(CO)NNH experiment. It facilitates identification of amino acid residues during the assignment of the backbone nuclei and provides, in principle, complete assignments of aliphatic sidechain resonances as well. Sometimes cross peaks may be missing or weak whatever the choice of mixing time for the TOCSY transfer step. It is likely, therefore, that either a 3D HCCH-COSY experiment or a 4D HCC(CO)NNH experiment with a different mixing time will still be required to complete the assignments. Because it separates the sidechain resonances according to the nitrogen and amide proton chemical shifts, the HCC(CO)NNH experiment alleviates problems of overlap in 3D HCCH spectra. It has similar sensitivity to the 4D HCANNH experiment (Boucher et al., 1992a,b), suggesting that it could be useful for proteins of up to ~ 20 kDa. A further virtue of this 4D NMR experiment is that the spectrum can be recorded with an identical experimental set-up and digital resolution to the 4D $^{13}C/^{13}C$-edited and $^{13}C/^{15}N$-edited NOESY spectra, facilitating automated identification of cross peaks in the latter following the assignment stage.

Note added in proof

After this paper was submitted, similar experiments were published by Montelione et al. (1992) and Logan et al. (1992).

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