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Improved 4D NMR experiments for the assignment of backbone nuclei in $^{13}C/^{15}N$ labelled proteins

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SUMMARY

We recently proposed a novel 4D NMR strategy for the assignment of backbone nuclei in $^{13}C/^{15}N$-labelled proteins (Boucher et al., 1992). Intra-residue (and many sequential) assignments are obtained from a HCANNH experiment, whereas sequential assignments are based on a complementary HCA(CO)NNH experiment. We present here new constant time 4D HCANNH, HCA(CO)NNH and HNCAHA experiments that are more sensitive. Some of the data were presented at the 33rd ENC held at Asilomar, California, U.S.A., in April 1992.

We recently reported two new four-dimensional (4D) heteronuclear triple resonance NMR experiments whose resulting spectra form the basis of a particularly simple sequential assignment strategy (Boucher et al., 1992). Intra-residue (and many sequential) assignments are obtained from a 4D version of a previously published 3D H(CA)NNH experiment (Montelione and Wagner, 1990; Kay et al., 1991). Sequential assignments are based on a complementary 4D HCA(CO)NNH experiment where the magnetisation is specifically transferred via the ^{13}CO group whose chemical shift is not measured. Using this method we have obtained a virtually complete assignment of the backbone nuclei in c-H-*ras* p21 (166 residues, 18.9 kD) (Campbell-Burk et al., 1992). In this paper we present new pulse sequences which give spectra with improved sensitivity.

In our original experiments, the signal evolves as a result of scalar couplings, in addition to the $^{13}C_\alpha$ chemical shift, during t_2. This leads to splitting in f_2 and to modulation of the efficiency of magnetisation transfer. These problems can be avoided by modification of constant time experiments, where the evolution time remains constant and the 180° pulse to $^{13}C_\alpha$ is stepped through this period, allowing evolution solely as a result of the $^{13}C_\alpha$ chemical shift during t_2 (Powers et al.,

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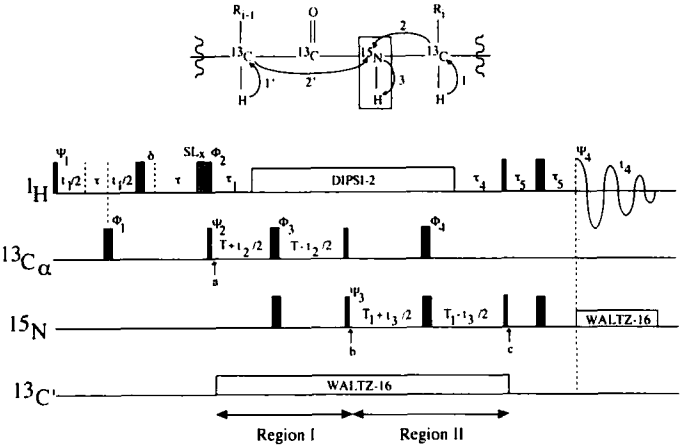
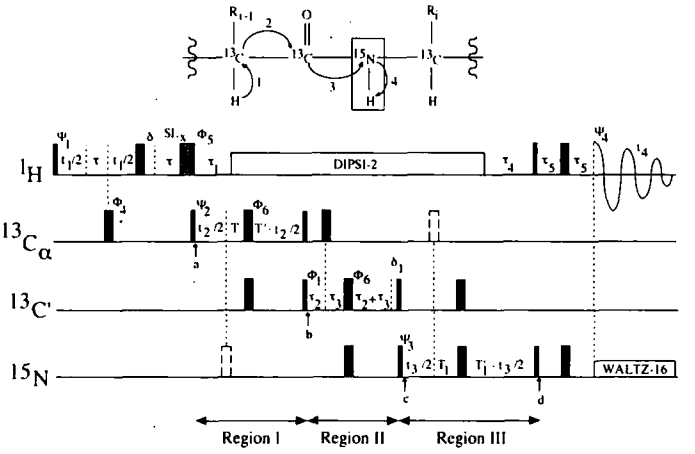
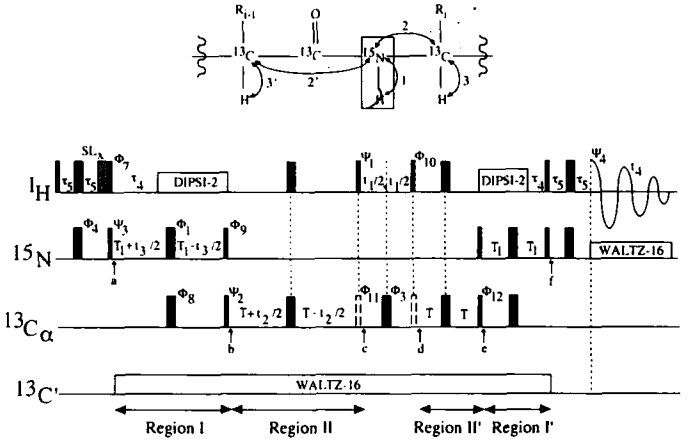
(a) Constant time 4D HCANNH



(b) Constant time 4D HCA(CO)NNH



(c) Constant time 4D HNCAHA



1991). Further improvements can be made by ensuring that relaxation during the pulse sequence is governed by the rate of decay of ^1H-*decoupled* rather than ^1H-*coupled* $^{13}C_\alpha$ magnetisation; the latter relaxes more quickly (Bax et al., 1990; Norwood et al., 1990). In the new sequences (see Fig. 1), evolution as a result of $^{13}C_\alpha$ and ^{15}N chemical shifts takes place in the constant time periods T and T_1, respectively, and magnetisation is maintained in a ^1H-decoupled state by the DIPSI-2 decoupling sequence (chosen for its efficiency in the presence of homonuclear coupling; Shaka et al., 1988).

Figure 1 depicts the magnetisation transfer pathways and the new pulse sequences. The HCANNH experiment (Fig. 1a) can be concisely described using product-operator formalism (Sørensen et al., 1983), where I^α, C^α, N and I^N represent the $^1H_\alpha$-, $^{13}C_\alpha$-, ^{15}N- and ^1H_N-spin operators, respectively. For clarity, terms that do not result in observable magnetisation and all coefficients have been omitted.

$$I_z^\alpha \longrightarrow 2I_z^\alpha C_y^\alpha \text{ (at point a)} \xrightarrow{\text{Region I}} 2C_z^\alpha N_y \text{ (at point b)} \xrightarrow{\text{Region II}} 2I_y^N N_z \text{ (at point c)} \longrightarrow I_x^N \quad (1)$$

The HCA(CO)NNH experiment (Fig. 1b) is very similar. It can be described, where C′ represents the $^{13}C'$-spin operator, as:

$$I_z^\alpha \longrightarrow 2I_z^\alpha C_y^\alpha \text{ (at point a)} \xrightarrow{\text{Region I}} 2C_z^\alpha C'_y \text{ (at point b)} \xrightarrow{\text{Region II}} 2C'_z N_y \text{ (at point c)} \xrightarrow{\text{Region III}} 2I_y^N N_z \text{ (at point d)} \longrightarrow I_x^N \quad (2)$$

To evaluate their relative sensitivity we compared the transfer functions for the two experiments. In the HCANNH experiment, the transfer functions for the intra-residue cross peaks are:

←

Fig. 1. Schematic representation of the magnetisation transfer pathways (insets) and the pulse sequences for the improved HCANNH (a), HCA(CO)NNH (b) and HNCAHA (c) 4D NMR experiments; narrow boxes represent $\pi/2$ pulses, wide boxes represent π pulses and cross-hatched boxes represent spin-lock pulses (1.5 ms), used for water suppression (Messerle et al., 1989). In the HCA(CO)NNH experiment, the 180° pulses in dotted outline are optional. In the HNCAHA experiment, the 90° pulses in dotted outline are required for the HSQC version only. Typical values for the delays are: $\tau = 1.5$ ms, $\tau_1 = 3.4$ ms, $\tau_2 = 4.5$ ms, $\tau_3 = 7.9$ ms, $\tau_4 = 5.4$ ms and $\tau_5 = 2.25$ ms. The constant times T and T_1 were set to 12.4 and 11.5 ms in the HCANNH experiment, 3.8 and 12.0 ms in the HCA(CO)NNH experiment and 1.5 and 12.0 ms in the HNCAHA experiment. In the HCA(CO)NNH experiment, T′ and T′$_1$ were set to T + the length of the $^{15}N(180°)$ and T_1 + the length of the $^{13}C(180°)$, respectively. The delay, δ, was set to the initial value of t_1 + the length of the $^{13}C(180°)$, whilst δ_1 was equal to the length of the $^{13}C(180°)$. The following phase cycling was employed: $\varphi_1 = 2(x),2(-x)$; $\varphi_2 = 2(y),2(-y)$; $\varphi_3 = 4(x),4(y),4(-x),4(-y)$; $\varphi_4 = x,-x$; $\varphi_5 = 4(y),4(-y)$; $\varphi_6 = 2(x),2(y),2(-x),2(-y)$; $\varphi_7 = y,-y$; $\varphi_8 = 16(x),16(-x)$; $\varphi_9 = 8(x),8(-x)$; $\varphi_{10} = 4(x),8(-x),4(x)$; $\varphi_{11} = y$; φ_{12} = either y or x in the HSQC or HMQC versions of the HNCAHA experiment, respectively. For the HCANNH and HCA(CO)NNH experiments, $\psi_1 = x$; $\psi_2 = 8(x),8(-x)$; $\psi_3 = x,-x$ and $\psi_4 = (x,-x,-x,x),2(-x,x,x,-x),(x,-x,-x,x)$. For the HNCAHA experiments, $\psi_1 = 16(x),16(-x)$; $\psi_2 = 2(x),2(-x)$; $\psi_3 = x$ and $\psi_4 = 4(x,-x,-x,x),4(-x,x,x,-x)$ for the HMQC version and $2(x,-x,-x,x,-x,x,x,-x)2(-x,x,x,-x,x,-x,-x,x)$ for the HSQC version. The phase of all other pulses was x. Quadrature detection during t_1, t_2 and t_3 was achieved by independently incrementing the phase of ψ_1, ψ_2 and ψ_3 in a States-TPPI manner (Marion et al., 1989).

for Region I $$\sin(2\pi {}^1J_{C\alpha N}T)\cos(2\pi {}^2J_{C\alpha N}T)\cos(2\pi J_{C\alpha C\beta}T)\exp(-2T/T_{2C\alpha}) \quad (3)$$

for Region II $$\sin(2\pi {}^1J_{C\alpha N}T_1)\cos(2\pi {}^2J_{C\alpha N}T_1)\exp(-2T_1/T_{2N}) \quad (4)$$

In the HCA(CO)NNH experiment they are:

for Region I $$\sin(2\pi J_{C\alpha C'}T)\cos(2\pi J_{C\alpha C\beta}T)\exp(-2T/T_{2C\alpha}), \quad (5)$$

for Region II $$\sin(2\pi J_{NC'}(\tau_2+\tau_3))\sin(2\pi J_{C\alpha C'}\tau_2)\exp(-2(\tau_2+\tau_3)/T_{2C'}) \quad (6)$$

for Region III $$\sin(2\pi J_{NC'}T_1)\exp(-2T_1/T_{2N}) \quad (7)$$

These expressions were evaluated assuming values of 11, 7, 37, 55 and 15 Hz for the ${}^1J_{C\alpha N}$, ${}^2J_{C\alpha N}$, $J_{C\alpha C\beta}$, $J_{C\alpha C'}$ and $J_{NC'}$ scalar couplings, respectively. We assumed ${}^{13}C_\alpha$, ${}^{13}C'$, and ${}^{15}N$ T_2 values for calmodulin (148 residues, 16.7 kD) of 21, 50 and 45 ms, respectively (Kay et al., 1991; Grzesiek et al., 1992). In the HCANNH experiment, the overall transfer function evaluated to 0.07 (0.19×0.37) whilst in the HCA(CO)NNH experiment it evaluated to between 0.15 ($0.5\times0.56\times0.53$) and 0.20 ($0.67\times0.56\times0.53$), depending on whether the 180° pulse to ${}^{13}C_\alpha$ (φ_6, see Fig. 1b) also inverted the attached ${}^{13}C_\beta$ spin or not (allowing refocussing of evolution caused by the $J_{C\alpha C\beta}$ coupling). Because the ${}^1H_\alpha \rightarrow {}^{13}C_\alpha$ and ${}^{15}N \rightarrow {}^1H_N$ steps were common to both experiments, the relative sensitivity, neglecting instrumental factors, was 0.47 (0.07/0.15) to 0.35 (0.07/0.20), making the HCA(CO)NNH experiment two to three times more sensitive than the HCANNH experiment.

In the HCANNH experiment, the small value of the ${}^1J_{C\alpha N}$ coupling relative to the ${}^{13}C_\alpha$ linewidth limited the sensitivity. We therefore designed a different pulse sequence where the rapid relaxation of the ${}^{13}C_\alpha$ spin was not expected to be so deleterious. This HNCAHA sequence (Fig. 1c) transferred magnetisation from ${}^1H_N$ to ${}^1H_\alpha$, via ${}^{15}N$ and ${}^{13}C_\alpha$, and back to ${}^1H_N$, which was detected during t_4. In the correlation with the ${}^1H_\alpha$ spin, the magnetisation evolved during t_1 either as heteronuclear multiple quantum coherence (HMQC) (Müller, 1979) or as heteronuclear single quantum coherence (HSQC) (Bodenhausen and Ruben, 1980). This pulse sequence is similar to the HN(CO)HB experiment (Grzesiek et al., 1992) and HNCAHA sequences developed independently by Wittekind et al., (1992) and Clubb et al., (1992). The HMQC version can be described as:

$$\begin{aligned} I_z^N &\longrightarrow 2I_z^N N_y \text{ (at point a)} \xrightarrow{\text{Region I}} 2N_z C_y^\alpha \text{ (at point b)} \\ &\xrightarrow{\text{Region II}} 4N_z I_y^\alpha C_x^\alpha \text{ (at point c)} \longrightarrow 4N_z I_z^\alpha C_x^\alpha \text{ (at point d)} \\ &\xrightarrow{\text{Region II'}} 2C_z^\alpha N_y \text{ (at point e)} \xrightarrow{\text{Region I'}} 2N_z I_y^N \text{ (at point f)} \longrightarrow I_x^N \end{aligned} \quad (8)$$

In the HCANNH experiment, the ${}^{13}C_\alpha$ magnetisation was transverse during region I ($\sim$ 24.8 ms) (see 1, 3 and Fig. 1a). In contrast, in the HNCAHA experiment (see 8, 10 and Fig. 1c), the magnetisation resided on the ${}^{13}C_\alpha$ spin only during regions II and II' ($\sim$ 6.0 ms) in the HSQC

version of the pulse sequence, although it was also transverse during t_1 in the HMQC version. It should also be noted that homonuclear ^{13}C–^{13}C couplings lead to splitting (in f_1) in the HMQC version of the pulse sequence, but not in the HSQC version. In the HNCAHA experiment, the transfer functions for the intra-residue cross peaks (for all residues except glycine) are:

$$\text{for Regions I and I}' \qquad \sin(2\pi^1 J_{C\alpha N} T_1)\cos(2\pi^2 J_{C\alpha N} T_1)\exp(-2T_1/T_{2N}) \tag{9}$$

$$\text{for Regions II and II}' \qquad \sin(2\pi J_{HC} T)\cos(2\pi J_{C\alpha C\beta} T)\exp(-2T/T_{2C\alpha}) \tag{10}$$

The overall transfer function evaluated to 0.09 ($0.37 \times 0.8 \times 0.8 \times 0.37$). Noting that the HNCAHA experiment involved two ^1H_N–^{15}N INEPT transfers, whilst the HCANNH experiment involved one ^1H_N–^{15}N and one $^1H_\alpha$–$^{13}C_\alpha$ transfer, their relative sensitivity (HNCAHA/HCANNH) is 1.4 (0.07/0.05) after taking this into account. However, compared to the HCANNH experiment, the HNCAHA pulse sequence contains ~3 additional simultaneous $^1H_\alpha$ and $^{13}C_\alpha$ 180° pulses. To evaluate the expected loss in sensitivity, we recorded 1D ^1H–^{13}C HSQC experiments with variable numbers of simultaneous $^1H_\alpha$ and $^{13}C_\alpha$ 180° pulses inserted into a fixed-length evolution period. We found that with each extra pair of pulses, the sensitivity was reduced by ~15% (data not shown). Thus when instrumental factors are taken into account, the two experiments are expected to have similar sensitivity for a protein the size of calmodulin. Further evaluations showed that as the T_2s get shorter, the HNCAHA experiment should become relatively more sensitive and vice versa (data not shown).

The relative sensitivity of the HCANNH and the HNCAHA experiments was compared experimentally by recording spectra for both ubiquitin (76 residues, 8.6 kD) and a truncated form of c-H-*ras* p21 (166 residues, 18.9 kD) uniformly labelled (> 90%) with ^{13}C and ^{15}N. The 2D ^{15}N (f_1)–^1H_N (f_2) NMR spectra provide a comparison of the basic sensitivity of the experiment whilst the 2D $^1H_\alpha$ (f_1)–^1H_N (f_2) NMR spectra illustrate the influence of $^1H_\alpha$ decay rates and homonuclear ^{13}C couplings in the t_1 dimension. We initially compared spectra of ubiquitin where the $^{13}C_\alpha$ T_2s were longer than in calmodulin. The HCANNH experiment was some 5–10% more sensitive than the HSQC version of the HNCAHA experiment, which was in turn 5–10% more sensitive than the HMQC version (data not shown). We then compared spectra of c-H-*ras* p21 where the $^{13}C_\alpha$ T_2s were shorter than in calmodulin. For the 2D ^{15}N (f_1)–^1H_N (f_2) NMR spectra, the HCANNH experiment (Fig. 2a) was ~20% less sensitive than either of the HNCAHA experiments (Figs. 2b and 2c). A similar comparison of the 2D $^1H_\alpha$ (f_1)–^1H_N (f_2) NMR spectra showed that the HCANNH experiment (Fig. 2d) was ~30% less sensitive than the HSQC version of the HNCAHA experiment (Fig. 2e), but both were approximately twice as sensitive as the HMQC version (Fig. 2f). The apparent insensitivity of the HMQC version is at least in part due to splitting as a result of the $J_{C\alpha C\beta}$ coupling. Comparison of the relative sensitivity of the HCANNH and HCA(CO)NNH experiments was more definitive; the latter was approximately twice as sensitive (data not shown). In addition, the new constant time experiments were all found to be significantly more sensitive than those we published originally (Boucher et al., 1992).

In conclusion, evaluation of transfer functions provides a useful tool for the analysis of new pulse sequences. As a result we would advocate the use of either the HCANNH experiment (for smaller proteins) or the HSQC version of the HNCAHA experiment (for larger proteins) in combination with the HCA(CO)NNH experiment. In the HNCAHA experiment, intra-residue cross

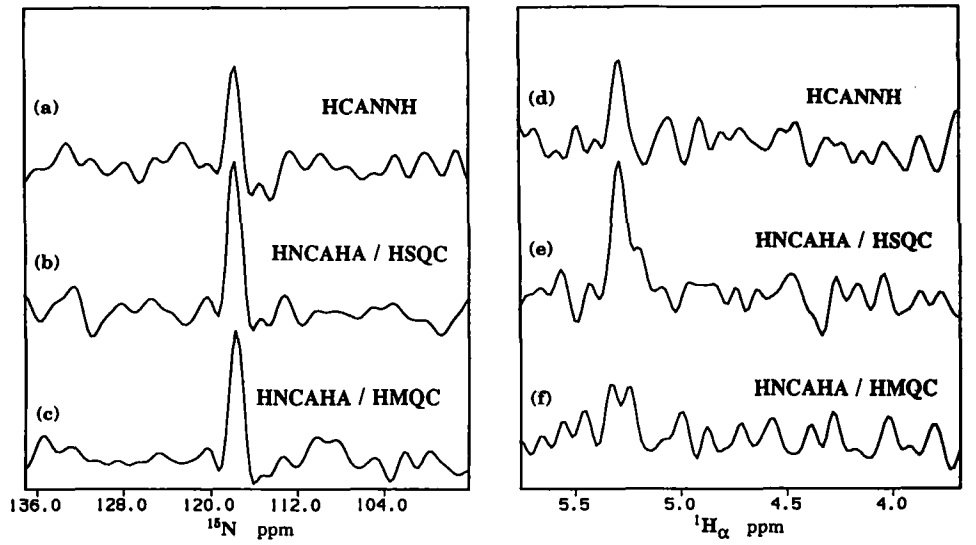


Fig. 2. Comparison of 2D NMR spectra, recorded using the HCANNH and HNCAHA 4D NMR experiments, of a 1.5-mM sample of c-H-*ras* p21 uniformly labelled (>90%) with ^{13}C and ^{15}N. In (a), (b) and (c) f_1-cross sections for Val^{152} from 2D ^{15}N (f_1) – ^1H_N (f_2) NMR spectra recorded using the HCANNH, HNCAHA (HSQC version) and HNCAHA (HMQC version) experiments are shown. In (d), (e) and (f) a similar comparison of f_1-cross sections from 2D $^1H_\alpha$ (f_1) – ^1H_N (f_2) NMR spectra is shown. For the 2D ^{15}N (f_1) – ^1H_N (f_2) and $^1H_\alpha$ (f_1) – ^1H_N (f_2) NMR spectra, the maximum acquisition times in t_1 were 10.4 and 25.6 ms, respectively. All spectra were recorded and processed under identical experimental conditions with $^1H_\alpha$, $^{13}C_\alpha$, ^{15}N and $^{13}C'$ RF field strengths of 11.9 kHz (reduced to 5.0 kHz for the DIPSI-2 decoupling steps), 19.7 kHz, 7.1 kHz and 625 Hz, respectively.

peaks for all residues, including glycine (at low intensity), were present (data not shown) whereas in the HCANNH experiment τ_1 had to be changed to 1.7 ms to optimise detection of glycines, with concomitant advantages and disadvantages (Campbell-Burk et al., 1992). A disadvantage of the HSQC version of the HNCAHA experiment is that one is limited to a maximum acquisition time in the $^{13}C_\alpha$ (t_2) dimension of 3.0 ms, but with maximum entropy data processing this should be adequate. Although the recording time for these 4D NMR experiments is currently determined by the phase cycling, the ability to record phase-sensitive spectra using pulsed field gradients will remove this limitation (Davis et al., 1992).

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Note added in proof

After this work was completed, Clubb et al., (1992) published a similar comparison of the HCANNH and the HNCAHA experiments for T4 lysozyme. Their results suggest that for larger proteins, where the $^{13}C_\alpha$ T_2s may be shorter than in c-H-*ras* p21, the advantage of the HSQC ver-

sion of the HNCAHA experiment may be even greater. After submission, the work described in a poster by Wittekind et al. (1992) was also published (Kay et al., 1992).

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