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Rapid acquisition of three-dimensional triple-resonance experiments using pulsed field gradient techniques

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SUMMARY

A rapid method for recording three-dimensional triple-resonance experiments utilising pulsed field gradient techniques is proposed, and applied to the HNCO experiment. In order to optimise the sensitivity of the method, a short phase cycle is used in conjunction with the pulsed field gradients to select the desired coherence transfer pathway. The method is demonstrated for the HU protein.

A number of 3D triple-resonance experiments which allow the sequential assignments of backbone resonance in uniformly ^{13}C, ^{15}N-labeled proteins have recently been proposed (Kay et al., 1990). One such experiment is the HNCO experiment, which correlates the amide ^{1}H and ^{15}N resonances of an amino acid with the carbonyl resonance of the preceding residue. Such sequential connectivities are combined with the intra-residue and sequential information obtained from HCACO and HCA(CO)N experiments (Ikura et al., 1990) or from HN(CA)CO (Clubb et al., 1992), and in conjunction with other experiments lead to a complete characterisation of the backbone resonances. The HNCO experiment is very sensitive, as the magnetisation transfers are mediated by the large $^{1}J_{NH}$ and $^{1}J_{NC'}$ scalar couplings, and hence it is desirable to record the 3D spectrum with the minimum number of transients per (t_1,t_2) increment.

The use of pulsed field gradient techniques (Maudsley et al., 1978; Bax et al., 1980; Barker and Freeman, 1985) to select the desired coherence transfer pathways has undergone a recent resurgence of interest (Hurd, 1990; Davis et al., 1991; Vuister et al., 1991) as a result of improvements in the design of gradient coils, and offers the tantalising prospect of recording 3D experiments

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using only a single transient per increment (Hurd and John, 1991; Vuister et al., 1992). In this communication we report the results of incorporating pulsed field gradients into a pulse sequence for recording HNCO spectra, and show that this approach can give high quality spectra very rapidly. For optimum sensitivity in a fixed recording time, we show that it is beneficial to combine pulsed field gradients with a short phase cycle.

A pulse sequence for recording HNCO spectra, based on a similar sequence proposed recently (Bax and Ikura, 1991), is shown in Fig. 1A. Only a brief description of the magnetisation transfer steps will be given here. An INEPT transfer (Morris and Freeman, 1979) from NH protons generates anti-phase ^{15}N magnetisation which evolves during t_1, and then refocuses with respect to the $^1J_{NH}$ coupling, and simultaneously dephases due to the one-bond coupling between ^{15}N and C′ during the delays τ_2 and τ_3. The concurrent 90°(^{15}N, C′) pulses effect an INEPT-like transfer into carbonyl antiphase magnetisation (Westler et al., 1988) which evolves during t_2, before being reconverted into ^{15}N transverse magnetisation. Further spin-echo periods for coupling evolution,

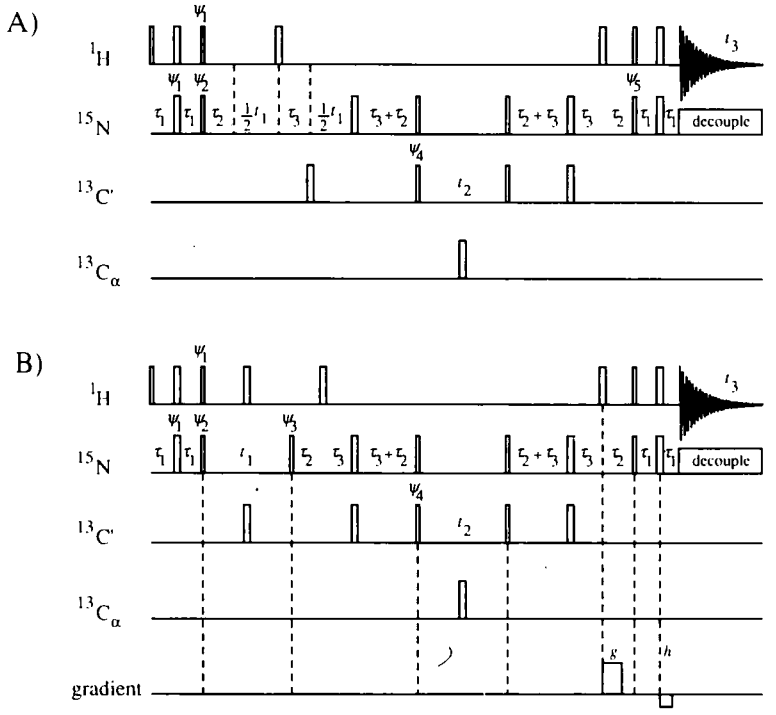

Fig. 1. Pulse sequences for recording HNCO experiments. 90° pulses are indicated by thin boxes; 180° by thick boxes. All pulse phases are x unless otherwise noted. In A, a sequence using only phase cycling procedures to define the experiment is shown. The phase cycling is as follows: $\psi_1 = y, -y$; $\psi_2 = 2(x), 2(-x)$; $\psi_4 = 4(x), 4(-x)$; $\psi_5 = 8(x), 8(-x)$; receiver = x, 2(−x), x, −x, 2(x), 2(−x), 2(x), −x, x, 2(−x), x. In addition, pre-irradiation to suppress the water signal would be employed. In B, a pulse sequence combining pulsed field gradients and phase cycling is shown. The field gradient pulses at points g and h have areas in the ratio 9.88: −1. The phase cycling used is as follows: $\psi_1 = y$, $\psi_2 = x$, $\psi_3 = y$, $\psi_4 = x, -x$; receiver = x, −x. In both pulse sequences, frequency discrimination in t_1 and t_2 is obtained using TPPI (Marion and Wüthrich, 1983) of ψ_2 and ψ_4. The ^{13}C transmitter is placed in the carbonyl region of the spectrum, and the $^{13}C_\alpha$ pulse is generated by a phase-modulated DANTE pulse (Kay et al., 1990).

followed by reverse-INEPT transfer of magnetisation from ^{15}N to ^{1}H, allow detection of the amide protons, amplitude-modulated by the (t_1, t_2) evolution.

When incorporating field gradient pulses into multi-dimensional experiments, two main considerations must be addressed. Firstly, the evolution of chemical shifts and couplings during the gradient pulses must be accounted for (Davis et al., 1991). In the case of triple-resonance sequences, the gradient pulses can be applied during the spin-echo periods, achieving the spatial phase encoding concurrently with the desired coupling evolution. Secondly, special attention must be paid to whether the modulation of the detected signal is phase modulation or amplitude modulation (Keeler and Neuhaus, 1985). Amplitude modulation is a necessity for high resolution experiments, as pure phase lineshapes can then be obtained. Phase-modulated data will normally result in the unacceptably broad phase-twist lineshape, and can only give pure phase lineshapes if pseudo-echo weighting functions are used, which will seriously degrade the signal:noise ratio of the spectrum. Amplitude modulation is obtained when symmetrical coherence transfer pathways in the evolution period t_n contribute equally to the signal detected during t_m, conventionally assumed to be of coherence order -1 (Bodenhausen et al., 1984):

$$\pm p(t_n) \rightarrow -1(t_m)$$

Phase modulation is obtained when only a single coherence order present during the evolution delay contributes to the detected signal:

$$+p(t_n) \rightarrow -1(t_m)$$

Straightforward inclusion of gradient pulses during the incremented evolution periods of multi-dimensional experiments leads to spectra in which phase modulation is obtained (Brereton et al., 1991). It has previously been shown that the detection of amplitude-modulated data is possible when using pulsed field gradients to select the desired coherence transfer pathway (Davis et al., 1992). A simple way of achieving this is to insert a phase purging between the incremented evolution period and the field gradient pulse.

Figure 1B shows a pulse sequence employing a pair of pulsed field gradients for selecting $^{15}N \rightarrow {}^{1}H$ coherence transfer at the end of the sequence. The field gradients are not used to select a coherence transfer pathway passing through carbonyl magnetisation for reasons of sensitivity to be discussed below. The dephasing gradient of strength G is applied at point g in the sequence: transverse ^{15}N magnetisation acquires a spatially dependent phase $\pm\gamma_N G\Delta$ where Δ is the duration of the gradient pulse and γ_N is the gyromagnetic ratio of ^{15}N. This phase is refocused by the second gradient pulse, applied at point h, during which the observable ^{1}H magnetisation acquires a spatially dependent phase of $+\gamma_H G'\Delta'$. Thus, if the relative ratio of the gradient areas is

$$\frac{G\Delta}{G'\Delta'} = \pm\frac{\gamma_H}{\gamma_N}$$

i.e. ± 9.88, then only magnetisation which passed through transverse ^{15}N magnetisation will be detected. Note that the gradient pulses are applied during essential free precession periods and do

not lead to an increase in the overall duration of the pulse sequence. To select carbonyl magnetisation excited by the first C′ 90° pulse, a two-step phase cycle of this pulse is used, which selects the coherence transfer pathway:

$$p_{C'} = 0 \rightarrow p_{C'} = \pm 1$$

The inclusion of the $90°_y$ pulse on ^{15}N immediately after the t_1 period generates some magnetisation which is zz-order with respect to the one-bond ^1H-^{15}N coupling. This does not evolve during

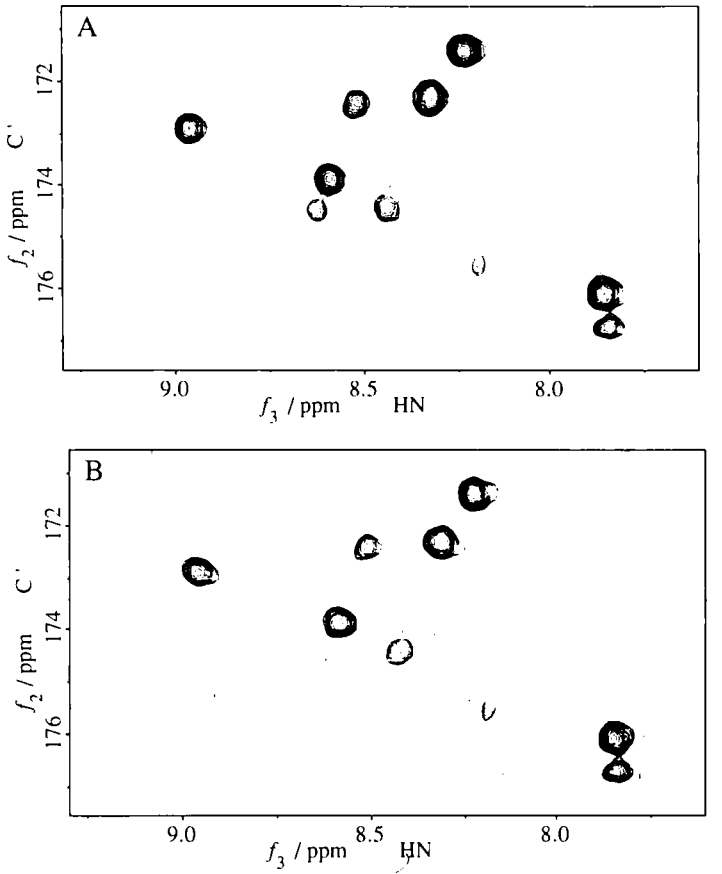

Fig. 2. (f_2,f_3) slices through HNCO spectra of HU protein. (A) Spectrum recorded in 18 h with the pulse sequence of Fig. 1A, using a 16-step phase cycle. (B) Spectrum recorded in only 2.25 h using the pulse sequence of Fig. 1B incorporating pulsed field gradients, and a 2-step phase cycle. The slices are taken at a ^{15}N frequency of 122.2 ppm. Both spectra were acquired with 64 real (t_1) × 64 real (t_2) × 512 complex (t_3) points, with acquisition times of 19.2 ms (t_1) and 11.5 ms (t_2). The ^1H transmitter was placed in the centre of the amide region for spectrum B, but placed at the H_2O frequency for spectrum A to enable pre-irradiation during the relaxation delay. The fixed delays were $\tau_1 = 2.5$ ms, $\tau_2 = 2.75$ ms, $\tau_3 = 12$ ms. The spectra were recorded using a Bruker AMX spectrometer operating at 500 MHz for protons, using a probe-head fitted with actively shielded gradient coils. The pulsed field gradients were implemented as square pulses of 500 μs and 250 μs, with field gradient strengths of approximately 20 Gcm $^{-1}$ and -4 Gcm $^{-1}$. The precise ratio was adjusted to give maximum signal. The spectra were processed using the TRITON software developed in this laboratory; after linear prediction and zero-filling, the final processed data size was 512 (f_3) × 256 (f_2) × 64 (f_1).

the delays τ_2 and τ_3 and is eliminated by the phase cycling of the first $^{13}C'$ 90° pulse, ensuring that the detected signals are amplitude modulated due to ^{15}N chemical shift evolution during t_1.

It is appropriate at this point to discuss why a third field gradient pulse was not employed to select carbonyl magnetisation. If such a gradient pulse were used, the sensitivity of the new sequence per unit of recording time would be one quarter of that obtainable from a phase-cycled experiment recorded with the same number of transient acquisitions. This signal loss occurs because a pair of gradient pulses can only refocus one of the two symmetrical pathways that are present during an evolution delay. Thus, for a 3D experiment, in which three gradient pulses are used (Hurd and John, 1991), only $\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$ of the possible coherence transfer pathways can be refocused:

$$\text{field gradients: } +p(t_1) \rightarrow +p'(t_2) \rightarrow -1(t_3)$$
$$\text{phase cycling: } \pm p(t_1) \rightarrow \pm p'(t_2) \rightarrow -1(t_3)$$

We decided that a pragmatic approach to the use of field gradients was the most appropriate way to exploit their advantages. Using the field gradients to select ^{15}N magnetisation ensures that good suppression of unwanted signals is obtained (Vuister et al., 1991), and that the sensitivity loss relative to a phase-cycled experiment is only a factor of two. This intensity loss will be partly compensated by the increased intensity of labile amide protons which arises from the absence of pre-irradiation of the water resonance. Further benefits may accrue from the reduction of the dynamic range of the detected signals.

Figure 2 compares planes taken from HNCO spectra recorded on a 2-mM, ^{13}C,^{15}N-labeled sample of HU protein from *B. stearothermophilus* (Tanaka et al., 1984) in 95% H_2O with 5% D_2O. Figure 2A shows the $f_1 = 122.2$ ppm plane from a conventionally phase-cycled HNCO spectrum recorded in 18 h, and Fig. 2B shows the identical plane taken from a HNCO spectrum recorded in 2.25 h using a combination of field gradients and phase cycling. It is clear that the latter spectrum is of high quality, suitable for interpretation during the sequential assignment of HU.

In this communication we have demonstrated the application of pulsed field gradient techniques to the triple-resonance HNCO experiment. This experiment is of high sensitivity and so the use of field gradients to reduce the minimum recording time is warranted. We have combined pulsed field gradients and phase cycling methods to optimise the sensitivity of the experiment. The method chosen is equally appropriate for many of the other triple-resonance techniques, though we wish to emphasise that pulsed field gradient methods may not always be the most appropriate for achieving coherence transfer pathway selection, particularly when sensitivity is an over-riding concern. Nevertheless, in many situations the rapid acquisition of triple-resonance spectra without the need for pre-irradiation of the water resonance will be a valuable enhancement of existing methods.

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REFERENCES

Barker, P. and Freeman, R. (1985) *J.Magn. Reson.*, **64**, 334–338.
Bax, A. and Ikura, M. (1991) *J. Biomol. NMR*, **1**, 99–104.
Bax, A., de Jong, P.G., Mehlkopf, A.F. and Smidt, J. (1980) *Chem. Phys. Lett.*, **69**, 567–570.
Bodenhausen, G., Kogler, H. and Ernst, R.R. (1984) *J.Magn. Reson.*, **58**, 370–388.
Brereton, I.M., Crozier, S., Field, J. and Doddrell, D.M. (1991) *J.Magn. Reson.*, **93**, 54–62.
Clubb, R.B., Thanabal, V. and Wagner, G. (1992) *J.Magn. Reson.*, **97**, 213–217.
Davis, A.L., Laue, E.D., Keeler, J., Moskau, D. and Lohman, J. (1991) *J.Magn. Reson.*, **94**, 637–644.
Davis, A.L., Keeler, J., Laue, E.D. and Moskau, D. (1992) *J.Magn. Reson.*, **98**, 207–216.
Hurd, R.E. (1990) *J.Magn. Reson.*, **87**, 422–428.
Hurd, R.E. and John, B.K. (1991) *J.Magn. Reson.*, **92**, 658–668.
Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.
Kay, L.E., Ikura, M. and Bax, A. (1990) *J.Magn. Reson.*, **89**, 496–514.
Keeler, J. and Neuhaus, D. (1985) *J.Magn. Reson.*, **63**, 454–472.
Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **96**, 967–974.
Maudsley, A.A., Wokaun, A. and Ernst, R.R. (1978) *Chem. Phys. Lett.*, **55**, 9–14.
Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
Tanaka, I., Appelt, K., Dijk, J., White, S.W. and Wilson, K.S. (1984) *Nature*, **310**, 376–381.
Vuister, G.W., Boelens, R., Kaptein, R., Hurd, R.E., John, B. and van Zijl, P.C.M. (1991) *J. Am. Chem. Soc.*, **113**, 9688–9690.
Vuister, G.W., Boelens, R., Kaptein, R., Burgering, M. and van Zijl, P.C.M. (1992) *J. Biomol. NMR*, **2**, 301–305.
Westler, W.M., Stockman, B.J. and Markley, J.L. (1988) *J. Am. Chem. Soc.*, **110**, 6256–6258.