Journal of Biomolecular NMR, **15:** 309–313, 1999.
KLUWER/ESCOM

A 4D TROSY-based pulse scheme for correlating $^{1}HN_{i}$,$^{15}N_{i}$,$^{13}C^{\alpha}_{i}$,$^{13}C'_{i-1}$ chemical shifts in high molecular weight, ^{15}N,^{13}C, ^{2}H labeled proteins

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Received 25 August 1999; Accepted 21 October 1999

Key words: 4D NMR, deuteration, high molecular weight proteins, protein assignment, triple resonance NMR, TROSY

Abstract

A 4D TROSY-based triple resonance experiment, 4D-HNCO$_{i-1}$CA$_{i}$, is presented which correlates intra-residue ^{1}HN, ^{15}N, $^{13}C^{\alpha}$ chemical shifts with the carbonyl ($^{13}C'$) shift of the preceding residue. The experiment is best used in concert with recently described 4D TROSY-HNCOCA and -HNCACO experiments [Yang, D. and Kay, L.E. (1999) *J. Am. Chem. Soc.*, **121**, 2571–2575]. In cases where degeneracy of (^{1}HN,^{15}N) spin pairs precludes assignment using the HNCOCA and HNCACO, the HNCO$_{i-1}$CA$_{i}$ often allows resolution of the ambiguity by linking the $^{13}C^{\alpha}$ and $^{13}C'$ spins surrounding the (^{1}HN,^{15}N) pair. The experiment is demonstrated on a sample of ^{15}N, ^{13}C, ^{2}H labeled maltose binding protein in complex with β-cyclodextrin that tumbles with a correlation time of 46 ns.

Introduction

Chemical shift assignment is the first step in any detailed analysis of molecular structure or dynamics by solution NMR. Over the past several years, therefore, considerable effort has been directed towards the development of robust methods for the assignment of resonances in proteins (Bax, 1994) and nucleic acids (Pardi, 1995; Sklenar et al., 1996). In this regard, triple resonance ^{15}N, ^{13}C spectroscopy has emerged as a powerful methodology and complete assignments of proteins in the 15–20 kDa molecular weight range are now routine. Applications to larger proteins generally involve the use of deuteration in addition to ^{15}N, ^{13}C labeling and have extended analysis to proteins of 300–400 amino acids (Gardner and Kay, 1998). TROSY spectroscopy (Pervushin et al., 1997, 1998) promises to be a particularly valuable tool for studies of such high molecular weight systems (Yang and Kay, 1999b).

Our laboratory has developed a number of strategies for the chemical shift assignment of backbone resonances in large, ^{15}N, ^{13}C, ^{2}H labeled proteins. The first approach is based on recording a suite of 3D experiments, including CT-HNCA, CT-HN(CO)CA, HN(CA)CB and HN(COCA)CB, with chemical shift assignment achieved via matching ($^{13}C^{\alpha}$,$^{13}C^{\beta}$) and (^{1}HN,^{15}N) spin pairs (Shan et al., 1996; Yamazaki et al., 1994a,b). The latter two experiments in this list can be recorded in constant-time (CT) mode as well. An important feature of these experiments is that the $^{13}C^{\alpha}$ shifts (and, sensitivity permitting, $^{13}C^{\beta}$) are recorded using CT-spectroscopy for delays on the order of 28 ms. This significantly minimizes the degree of overlap in the carbon dimensions. In addition, in cases of degeneracy of (^{1}HN,^{15}N) spin pairs it is often still possible to assign the overlapping fragments, $\cdots$(N-C^{α}/C^{β}-C′)$_{i}$-(N-HN)$_{i+1}$ and -(HN-N-C^{α}/C^{β}-C′)$_{i+1}\cdots$, by eliminating possibilities on the basis of the $^{13}C^{\beta}$ chemical shift. The CT-$^{13}C^{\beta}$ experiments are particularly powerful in this regard, since the signs of cross peaks are a function of whether an odd or even number of aliphatic carbons are directly coupled to the $^{13}C^{\beta}$ spin (Shan et al., 1996).

In many cases the utility of the above mentioned set of experiments is limited to proteins with correlation times less than approximately 30 ns, even when TROSY is employed. For larger molecules the use of CT-^{13}C spectroscopy results in considerable sensitivity losses, especially when $^{13}C^{\beta}$ chemical shifts are recorded. This is particularly unfortunate since it is precisely in these cases that both the high resolution in ^{13}C dimensions and the resolving power of the $^{13}C^{\beta}$ chemical shift is needed for assignment. With these problems in mind we have recently described a pair of TROSY-based 4D experiments, 4D-HNCACO ($^{1}HN_i$,$^{15}N_i$,$^{13}C^{\alpha}{}_{i/i-1}$,$^{13}C'_{i/i-1}$) and 4D-HNCOCA ($^{1}HN_i$,$^{15}N_i$,$^{13}C'_{i-1}$,$^{13}C^{\alpha}_{i-1}$) for assignment of proteins or protein complexes which are not limited to correlation times in the 30 ns range (Yang and Kay, 1999b). Near complete (>95%) intra- and inter-residue correlations were observed in HNCACO and HNCOCA spectra, respectively, recorded on a 46 ns tumbling complex of ^{15}N, ^{13}C, ^{2}H [Leu, Val, Ile (δ1 only)]-methyl protonated maltose binding protein (MBP, 370 residues, 5 °C) in complex with the cyclic heptasaccharide β-cyclodextrin (Yang and Kay, 1999b).

Although overlap is essentially non-existent in both 4D data sets in the case of MBP, problems frequently arise in chemical shift assignment due to degeneracies of (^{1}HN,^{15}N) and ($^{13}C^{\alpha}$, $^{13}C'$) spin pairs. In this paper we present a third 4D TROSY experiment, 4D-HNCO$_{i-1}$CA$_i$, which significantly reduces the assignment problem in cases of overlap in (^{1}HN,^{15}N) dimensions. The experiment correlates ^{1}HN, ^{15}N and $^{13}C^{\alpha}$ chemical shifts of residue i with the CO shift of the preceding residue. Frequently, it is also possible to observe correlations linking $^{1}HN_i$,$^{15}N_i$,$^{13}C^{\alpha}_{i-1}$,$^{13}C'_{i-1}$ chemical shifts. The utility of the 4D-HNCO$_{i-1}$CA$_i$ is demonstrated on a 1.4 mM sample of ^{15}N, ^{13}C, ^{2}H, methyl protonated MBP at 5 °C.

Results and discussion

Figure 1 provides a schematic illustrating a potential source of ambiguity in assignment using the HNCACO and HNCOCA experiments. In step 1 the $^{13}C^{\alpha}$ and $^{13}C'$ chemical shifts of residue i, obtained from the HNCACO, are used to extend the assignment to the ^{1}HN and ^{15}N of the successive residue using the HNCOCA. The newly identified (^{1}HN,^{15}N) spin pair is, in turn, used to assign the $^{13}C^{\alpha}$ and $^{13}C'$ shifts of residue

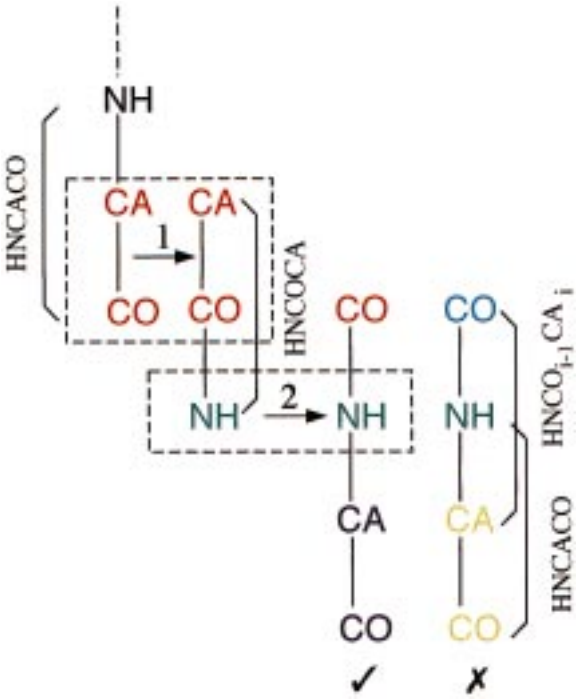

Figure 1. Schematic illustrating the utility of the 4D-HNCO$_{i-1}$CA$_i$ experiment in resolving ambiguities in chemical shift assignment due to degeneracies in ^{1}HN/^{15}N shifts. Like spins (i.e., $^{13}C^{\alpha}$, $^{13}C'$, ^{15}N, etc.) with the same chemical shift have the same color. In step 1 the $^{13}C^{\alpha}$ and $^{13}C'$ chemical shifts of residue i are matched in the HNCACO and HNCOCA experiments (Yang and Kay, 1999b), resulting in the assignment of the (^{1}HN,^{15}N) shifts of the following residue. Because this (^{1}HN,^{15}N) spin pair is not unique it is not possible to continue the assignment if the HNCACO alone is used. The HNCO$_{i-1}$CA$_i$ removes the ambiguity since the $^{13}C'$ shifts of the two possible partners in step 2 are distinct.

($i+1$) from the HNCACO. This procedure works well, assuming that both the ($^{13}C^{\alpha}$,$^{13}C'$) and the (^{1}HN,^{15}N) shifts are unique. In cases where (^{1}HN,^{15}N) spin pairs are degenerate, for example (denoted by the green colored ^{1}HN, ^{15}N spins in Figure 1), several possibilities arise for extending the assignment to the $^{13}C^{\alpha}$ and $^{13}C'$ shifts of residue ($i+1$) (i.e., step 2). The 4D-HNCO$_{i-1}$CA$_i$ removes the degeneracy by linking the $^{13}C^{\alpha}$ and $^{13}C'$ chemical shifts that are directly coupled to the degenerate (^{1}HN,^{15}N) spin pair, as illustrated in Figure 1.

Figure 2 shows the TROSY-based experiment that has been used to provide ($^{1}HN_i$,$^{15}N_i$,$^{13}C^{\alpha}_i$,$^{13}C'_{i-1}$) correlations. The pulse scheme follows our previously published 4D experiments very closely. Briefly, the transfer steps are:

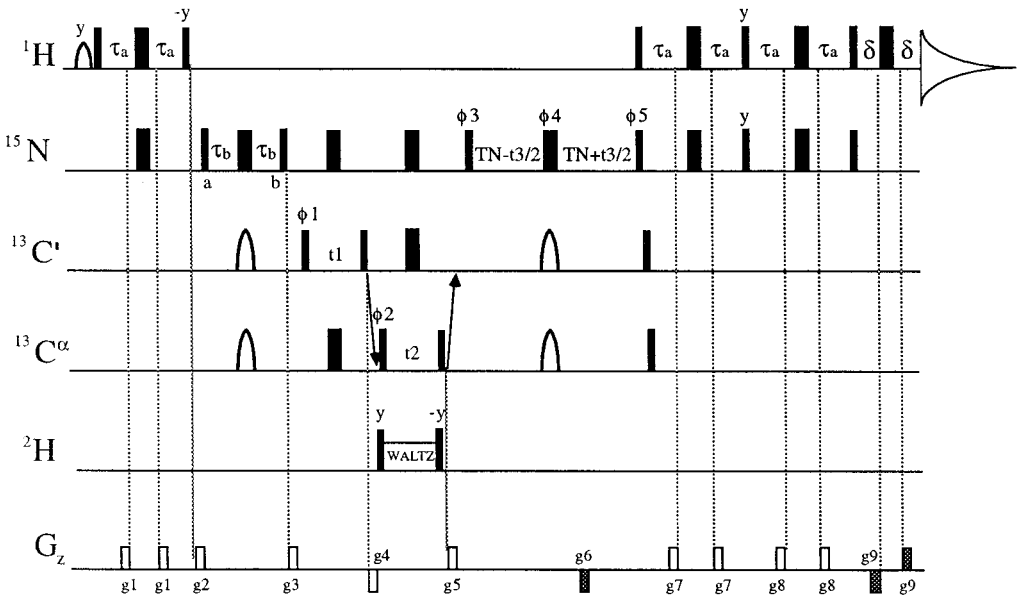

Figure 2. Pulse scheme of the 4D-HNCO$_{i-1}$CA$_i$. All narrow (wide) pulses are applied with a flip angle of 90° (180°) along the x-axis, unless indicated otherwise. The ^{1}H, ^{15}N and ^{2}H carriers are positioned at water, 119 ppm and 4.7 ppm, respectively, while the ^{13}C carrier is initially set to 176 ppm, jumped to 55 ppm prior to the ^{13}C$^\alpha$ pulse of phase ϕ2 and subsequently returned to 176 ppm immediately before gradient g5. All ^{1}H pulses are applied with a 32 kHz field, with the exception of the 7.3 ms 90° water selective EBURP pulse (shaped pulse) (Geen and Freeman, 1991) which uses a 1.0 kHz field at maximum amplitude. ^{15}N pulses employ a 6.2 kHz field, while all rectangular ^{13}C 90° (180°) pulses are applied at a field (Hz) of $\Delta/\sqrt{15}$ ($\Delta/\sqrt{3}$), where Δ is the difference (in Hz) between the centers of the ^{13}C$^\alpha$ and ^{13}C$'$ chemical shift regions (Kay et al., 1990). The ^{13}C$'$ and ^{13}C$^\alpha$ spins are inverted during the transfers from ^{15}N to ^{13}C and back using a 300 μs, 15 kHz CHIRP pulse (Bohlen and Bodenhausen, 1993; Kupce and Freeman, 1995) applied with an 80 kHz sweep centered midway between ^{13}C$^\alpha$ and ^{13}C$'$ spins (∼117 ppm). ^{2}H decoupling is achieved with an 800 Hz WALTZ$_{16}$ field (phases of all pulses applied along the ±x axes) (Shaka et al., 1983), surrounded by 2 kHz field pulses. The delays used are: $\tau_a = 2.2$ ms, $\tau_b = 13.0$ ms, TN = 13.0 ms, $\delta = 0.25$ ms. The phase cycle is: $\phi1$ = (x,−x), $\phi2$ = 2(x),2(−x), $\phi3$ = (x,−x), $\phi4$ = 2(x),2(−x), $\phi5$ = x, rec = 2(x),2(−x). Quadrature detection in F_1 and F_2 is achieved via States-TPPI (Mrion et al., 1989) of $\phi1$ and $\phi2$, respectively, while quadrature detection in F_3 employs the enhanced sensitivity pulsed field gradient approach, where for each value of t_3 separate data sets are recorded for (g6,$\phi5$) and (−g6,$\phi5$+180°) (Kay et al., 1992; Schleucher et al., 1993) . For each successive t_3 value $\phi3$ is incremented by 180° in concert with the receiver. The durations and strengths of the gradients are: g1 = (0.4 ms, 5 G/cm), g2 = (1.0 ms, 10 G/cm), g3 = (0.7 ms, 10 G/cm), g4 = (1.0 ms, −10 G/cm), g5 = (1.5 ms, 15 G/cm), g6 = (1.25 ms, −30 G/cm), g7 = (0.4 ms, 16 G/cm), g8 = (0.4 ms, 14 G/cm), g9 = (62.5 μs, 28.75 G/cm).

$$^1\mathrm{HN} \xrightarrow{^1J_{HN}} {}^{15}\mathrm{N} \xrightarrow{^1J_{N,C'},J_{N,C\alpha}} {}^{13}\mathrm{C}'(t_1),$$
$$^{13}\mathrm{C}^\alpha(t_2) \longrightarrow {}^{15}\mathrm{N}(\mathrm{CT}-t_3) \xrightarrow{^1J_{N,C'},J_{N,C\alpha}} \xrightarrow{^1J_{HN}} {}^1\mathrm{HN}(t_4), \quad (1)$$

where t_i ($i = 1-4$) is an acquisition time and the active couplings involved in each transfer step are indicated above the appropriate arrows. ^{15}N transverse y-magnetization, N_y, at point a in the sequence is transferred to ^{13}C$'$ and ^{13}C$^\alpha$ through the generation of a coherence of the form $N_yC'_zC^\alpha_z$ at point b. The latter coherence can be readily created by allowing transverse ^{15}N magnetization to evolve simultaneously under both $^1J_{N,C'}$, $^1J_{N,C\alpha}$ couplings. Inversion of ^{13}C$'$ and ^{13}C$^\alpha$ magnetization during the INEPT transfer (Morris and Freeman, 1979) is achieved by the application of a single CHIRP pulse (Bohlen and Bodenhausen, 1993; Kupce and Freeman, 1995) (300 μs, 80 kHz sweep; denoted by the shaped ^{13}C$'$, ^{13}C$^\alpha$ pulses in Figure 2). A similar transfer involving simultaneous evolution due to $^1J_{N,C'}$, $^1J_{N,C\alpha}$ occurs during the CT-^{15}N period. Note that because both $^1J_{N,C\alpha}$ and $^2J_{N,C\alpha}$ couplings are operative during the ^{15}N → ^{13}C transfer periods, cross peaks correlating A = (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha_i$,^{13}C$'_{i-1}$) and B = (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha_{i-1}$,^{13}C$'_{i-1}$) can be observed. Because $^1J_{N,C\alpha}$ is most often considerably larger than $^2J_{N,C\alpha}$ (Delaglio et al., 1991), near complete correlations of type A are observed in MBP (> 95%), while approximately 70% of type B correlations have been obtained in this case. A final point of interest concerns the ^{13}C$'$ and ^{13}C$^\alpha$ 90° purge pulses applied following the ^{15}N 90$_{\phi5}$ pulse. These pulses insure that pure absorptive lineshapes are generated in the F_3/F_4 dimensions, as described in detail previously (Yang and Kay, 1999a).

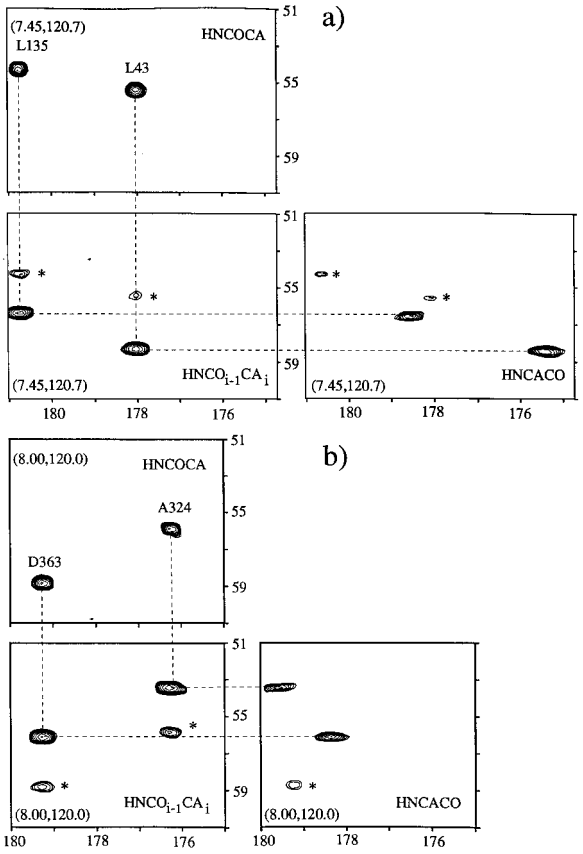

Figure 3. Selected slices of the 4D-HNCOCA, 4D-HNCO$_{i-1}$CA$_i$ and 4D-HNCACO data sets at (^{1}HN,^{15}N) shifts of (7.45 ppm,120.7 ppm) (a) and (8.00 ppm,120.0 ppm) (b), illustrating how the 4D-HNCO$_{i-1}$CA$_i$ can resolve ambiguities in chemical shift assignment created by overlapping (^{1}HN,^{15}N) spin pairs. The 4D-HNCO$_{i-1}$CA$_i$ was acquired as a data set consisting of (14,21,34,576) complex points corresponding to acquisition times of (8.6, 7.4, 20.6, 64 ms) in (t_1, t_2, t_3, t_4). A relaxation delay of 1.4 s and 4 scans/FID were obtained corresponding to a total measuring time of 5.2 days. A sample of 1.4 mM ^{15}N, ^{13}C, ^{2}H [Leu, Val, Ile (δ1 only)]-methyl-protonated MBP/β-cyclodextrin, 20 mM sodium phosphate buffer (pH 7.2), 3 mM NaN_3, 100 mM EDTA, 0.1 mg/mL Pefabloc, 1 μg/μL pepstatin, 90% H_2O/10% D_2O was used, prepared as described previously (Gardner et al., 1998). The data set was recorded on a Varian INOVA 600 MHz spectrometer. The data was processed using NMRPipe (Delaglio et al., 1995) in an identical fashion to what has previously been described for the 4D-HNCOCA and 4D-HNCACO experiments and analyzed using NMRView (Johnson and Blevins, 1994).

Figure 3 illustrates how the HNCO$_{i-1}$CA$_i$ can be used in conjunction with the HNCOCA and HNCACO data sets to help resolve ambiguities in chemical shift assignments presented by degenerate ^{1}HN/^{15}N pairs of chemical shifts. By means of example, Leu 43 and Leu 135 in MBP have nearly equivalent ^{1}HN,^{15}N chemical shifts (Figure 3a). Hence, in the assignment

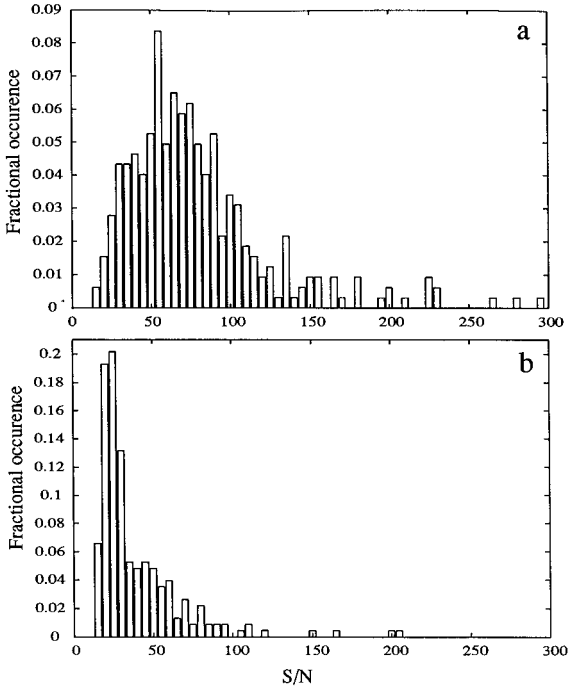

Figure 4. Fractional occurrence vs. signal-to-noise (S/N) of (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha$ $_i$,^{13}C′$_{i-1}$) (a) and (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha$ $_{i-1}$,^{13}C′$_{i-1}$) (b) correlations in the HNCO$_{i-1}$CA$_i$ data set of MBP. It is noteworthy that the sensitivity of the HNCO$_{i-1}$CA$_i$ is similar to the HNCOCA published previously (Yang and Kay, 1999b).

of the ^{13}C$^\alpha$ and ^{13}C′ shift of Leu 43 it would be impossible to choose from the two possibilities presented in the HNCACO. However, because the ^{13}C′ shifts of Lys 42 and Ala 134 are distinct (from each other) this ambiguity can be resolved by the HNCO$_{i-1}$CA$_i$ data set. A second example illustrates a similar problem in the case of Ala 324 and Asp 363, as shown in Figure 3b. Again the HNCO$_{i-1}$CA$_i$ resolves the ambiguity. The cross peaks marked with an asterisk in the spectra derive from the $^2J_{N,C\alpha}$ coupling, and their intensities are considerably weaker than the corresponding peaks originating from the $^1J_{N,C\alpha}$ transfer.

The signal-to-noise ratios of A = (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha$ $_i$,^{13}C′$_{i-1}$) and B = (^{1}HN$_i$,^{15}N$_i$,^{13}C$^\alpha$ $_{i-1}$,^{13}C′$_{i-1}$) correlations are indicated in Figures 4a and b, respectively. Excellent sensitivity, in particular, is noted for the $^1J_{N,C\alpha}$ correlations (a), with cross peaks for over 95% of the expected residues observed.

Conclusions

In summary, a 4D TROSY-based pulse scheme has been described which significantly reduces ambigui-

ties in the assignment of backbone ^{1}HN, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C'$ chemical shifts in cases where (^{1}HN,^{15}N) spin pairs overlap. The high sensitivity and resolution of the 4D $HNCO_{i-1}CA_i$ and the previously described 4D-HNCOCA and 4D-HNCACO makes these experiments particularly well suited for chemical shift assignments of high molecular weight proteins.

Acknowledgements

This research was supported by a grant from the Medical Research Council of Canada (L.E.K.). L.E.K. is a foreign investigator of the Howard Hughes Medical Research Institute.

References

Bax, A. (1994) *Curr. Opin. Struct. Biol.*, **4**, 738–744.
Bohlen, J.M. and Bodenhausen, G. (1993) *J. Magn. Reson.*, **A102**, 293–301.
Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.
Delaglio, F., Torchia, D.A. and Bax, A. (1991) *J. Biomol. NMR*, **1**, 439–446.
Gardner, K.H. and Kay, L.E. (1998) *Annu. Rev. Biophys. Biomol. Struct.*, **27**, 357–406.
Gardner, K.H., Zhang, X., Gehring, K. and Kay, L.E. (1998) *J. Am. Chem. Soc.*, **120**, 11738–11748.
Geen, H. and Freeman, R. (1991) *J. Magn. Reson.*, **93**, 93–141.
Johnson, B.A. and Blevins, R.A. (1994) *J. Biomol. NMR*, **4**, 603–614.
Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.
Kay, L.E., Keifer, P. and Saarinen, T. (1992) *J. Am. Chem. Soc.*, **114**, 10663–10665.
Kupce, E. and Freeman, R. (1995) *J. Magn. Reson.*, **A115**, 273–276.
Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
Pardi, A. (1995) *Methods Enzymol.*, **261**, 350–380.
Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 12366–12371.
Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1998) *J. Am. Chem. Soc.*, **120**, 6394–6400.
Schleucher, J., Sattler, M. and Griesinger, C. (1993) *Angew. Chem. Int. Ed. Engl.*, **32**, 1489–1491.
Shaka, A.J., Keeler, J., Frenkiel, T. and Freeman, R. (1983) *J. Magn. Reson.*, **52**, 335–338.
Shan, X., Gardner, K.H., Muhandiram, D.R., Rao, N.S., Arrowsmith, C.H. and Kay, L.E. (1996) *J. Am. Chem. Soc.*, **118**, 6570–6579.
Sklenar, V., Dieckmann, T., Butcher, S.E. and Feigon, J. (1996) *J. Biomol. NMR*, **7**, 83–87.
Yamazaki, T., Lee, W., Arrowsmith, C.H., Muhandiram, D.R. and Kay, L.E. (1994a) *J. Am. Chem. Soc.*, **116**, 11655–11666.
Yamazaki, T., Lee, W., Revington, M., Mattiello, D.L., Dahlquist, F.W., Arrowsmith, C.H. and Kay, L.E. (1994b) *J. Am. Chem. Soc.*, **116**, 6464–6465.
Yang, D. and Kay, L.E. (1999a) *J. Biomol. NMR*, **14**, 273–276.
Yang, D. and Kay, L.E. (1999b) *J. Am. Chem. Soc.*, **121**, 2571–2575.