Journal of Biomolecular NMR, 2 (1992) 655–659
ESCOM

J-Bio NMR 096

A 4D HCCH-TOCSY experiment for assigning the side chain ^{1}H and ^{13}C resonances of proteins

Edward T. Olejniczak, Robert X. Xu and Stephen W. Fesik*
Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, IL 60064, U.S.A.

Received 2 October 1992
Accepted 7 October 1992

Keywords: 4D NMR; HCCH-TOCSY; Side-chain resonance assignments; Proteins

SUMMARY

A 4D HCCH-TOCSY experiment is described for correlating and assigning the ^{1}H and ^{13}C resonances of protein amino acid side chains that has several advantages over 3D versions of the experiment. In many cases, *both* the ^{1}H *and* ^{13}C chemical shifts can be obtained in the 4D experiment from a simple inspection of the $^{13}C(\omega 3)$, $^{1}H(\omega 4)$ planes extracted at the $^{1}H^{\alpha}(\omega 1)/^{13}C^{\alpha}(\omega 2)$ chemical shifts. Together with the 3D and 4D triple resonance experiments, this allows sequence-specific assignments to be obtained. In addition, the increased resolution of the 4D experiment compared to its 3D counterpart allows automation of resonance assignments.

A prerequisite for determining high-resolution protein structures by NMR is the assignments of side-chain resonances. For larger proteins (> 10 kDa) that are uniformly ^{13}C-labeled, these assignments are obtained from recently developed 3D NMR experiments that correlate the ^{1}H and ^{13}C side-chain resonances by the transfer of magnetization through the large one-bond ^{1}H–^{13}C and ^{13}C–^{13}C J-couplings (Bax et al., 1990; Fesik et al., 1990; Kay et al., 1990a).

In this paper, we describe a 4D HCCH-TOCSY experiment for correlating and assigning the ^{1}H and ^{13}C resonances of protein amino acid side chains. The 4D NMR experiment makes it easier to identify the ^{1}H and ^{13}C signals of the individual amino acid spin systems and facilitates the assignment of these spin systems by amino acid type. In addition, the increased resolution of the 4D experiment compared to its 3D counterpart allows automation of resonance assignments.

Figure 1 depicts the 4D HCCH-TOCSY pulse sequence. After frequency labeling of the protons during the t_1 period, the magnetization is transferred by an INEPT experiment to the attached carbon, which is indirectly detected during t_2. A FLOPSY mixing scheme (Mohebbi and Shaka, 1991) is used to transfer magnetization between carbons, followed by a second ^{13}C evolu-

* To whom correspondence should be addressed.

0925-2738/$ 5.00 © 1992 ESCOM Science Publishers B.V.

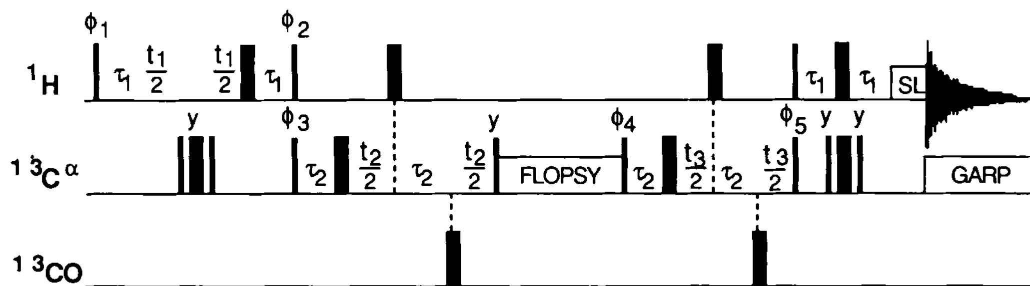


Fig. 1. Pulse sequence for the 4D HCCH-TOCSY experiment. Wide bars correspond to 180° pulses and narrow bars denote 90° pulses. The phase cycling for the pulses consists of: φ1 = x,x,−x,−x; φ2 = 8y,8(−y); φ3 = x,−x; φ4 = x,y,−x, −y,−x,−y,x,y; φ5 = y,x,−y,−x; with the receiver cycled x,−x,−x,x,2(−x,x,x,−x),x,−x,−x,x. Quadrature detection was obtained using States-TPPI (Marion et al., 1989) by incrementing φ1 for t_1, φ3 for t_2 and φ4 for t_3. The carbon and proton carrier frequency was set at 37.8 and 3.68 ppm, respectively.

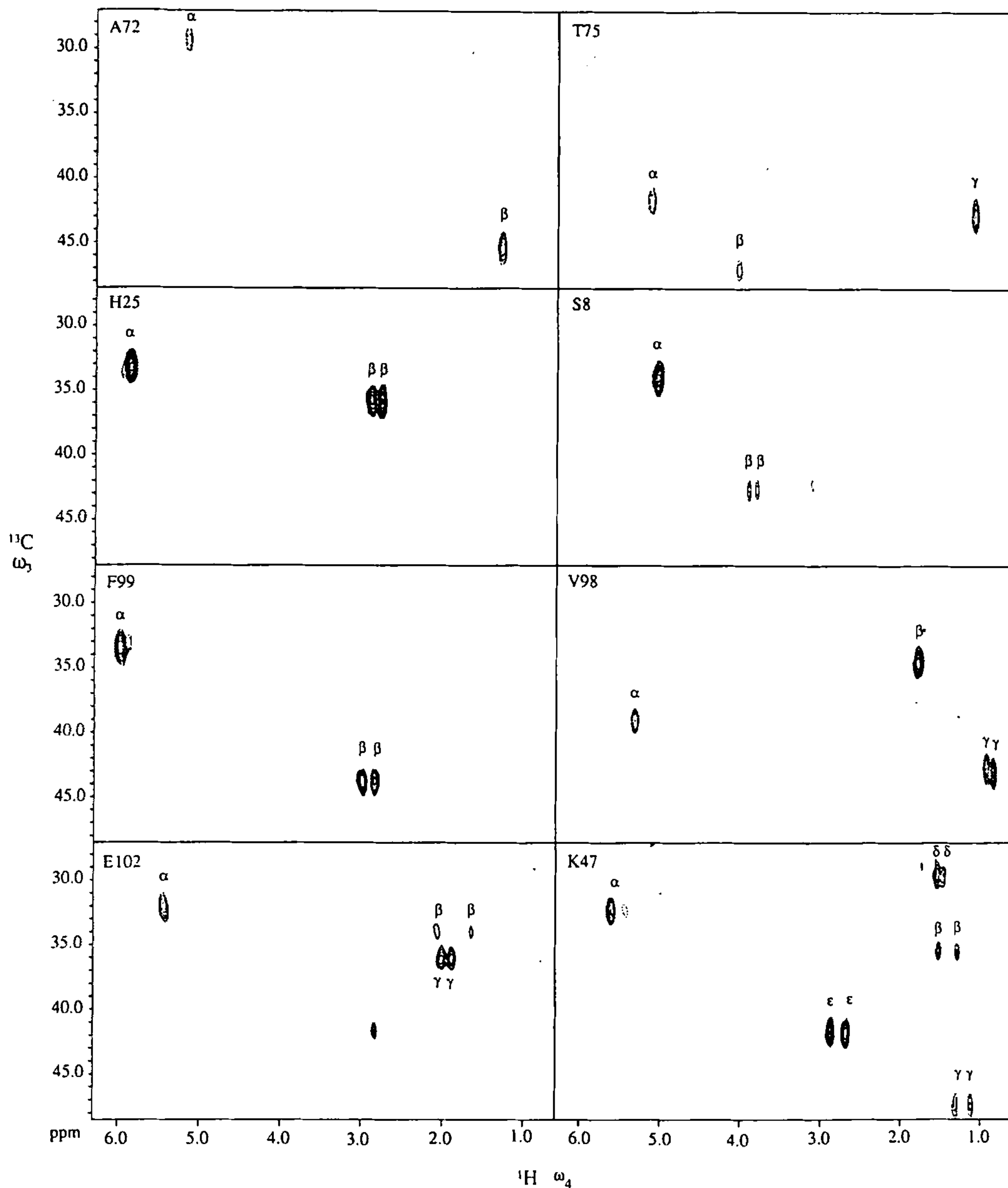
tion period (t_3). The next step involves a reverse INEPT sequence and the detection of the protons during the acquisition (t_4) period. The experiment was optimized by concatenating some of the pulses (Kay et al., 1991), resulting in the elimination of one ^{13}C and two ^{1}H 180° pulses.

Figure 2 depicts several $^{13}C(\omega3)$,$^{1}H(\omega4)$ planes from the 4D HCCH-TOCSY spectrum of [U-^{15}N,^{13}C]FKBP (FK506 binding protein; 11.8 kDa) (Harding et al., 1989; Sekierka et al., 1989) bound to the immunosuppressant, ascomycin (Hatanka et al., 1988). The planes were extracted at the $^{1}H^{\alpha}(\omega1)$ and $^{13}C^{\alpha}(\omega2)$ chemical shifts. As illustrated in Fig. 2, identification of the complete ^{1}H and ^{13}C resonances of the individual amino acid side chains is easily accomplished in many cases by simple inspection of only one plane of the 4D data set. Indeed, under these experimental conditions, nearly all of the expected side-chain resonances were observed in the $^{13}C(\omega3)$,$^{1}H(\omega4)$ planes at the $^{1}H^{\alpha}(\omega1)/^{13}C^{\alpha}(\omega2)$ chemical shifts. Data interpretation was only complicated for those amino acids in which both the α-proton and α-carbon signals overlapped, producing two or more sets of signals on the plane extracted at the $^{1}H^{\alpha}/^{13}C^{\alpha}$ frequencies. However, in most of these cases, the ambiguities could be resolved by carefully locating peak maxima or by analyzing additional planes from the 4D data set.

The 4D HCCH-TOCSY experiment complements 3D and 4D triple resonance NMR experiments (Kay et al., 1990b; Bax and Ikura, 1991; Kay et al., 1991, 1992; Boucher et al., 1992; Olejniczak et al., 1992). From the H^{α}/C^{α} shifts of adjacent amino acids identified in the triple resonance experiments, the ^{1}H and ^{13}C chemical shifts of the side chain are obtained from the 4D HCCH-TOCSY spectrum. As shown in Table 1, many of the amino acids have either unique ^{13}C chemical

→

Fig. 2. $^{13}C(\omega3)$,$^{1}H(\omega4)$ planes from the 4D HCCH-TOCSY spectrum extracted at the $^{1}H^{\alpha}/^{13}C^{\alpha}$ chemical shifts of the indicated residue. The phase ramps in ω2 (180°) and ω4 (360°) were chosen so that all folded resonances had negative contours (dashed lines) relative to resonances within the carbon sweep width. Folded resonances with proton chemical shifts of less than 3.0 ppm can generally be unfolded by subtracting the carbon sweep width (21.8 ppm) from the peak position, while all other folded resonances are unfolded by adding the carbon sweep width to their peak position. FKBP was purified from cells grown on ^{15}N ammonium chloride and [U-^{13}C]acetate as previously described (Edalji et al., 1992). The NMR sample (3 mM) of [U-^{15}N,^{13}C]FKBP/ascomycin (1/1) was prepared in $^{2}H_2O$ buffer (pH = 6.5), containing 50 mM potassium phosphate, 100 mM sodium chloride and 5 mM dithiothreitol-d_{10}. The 4D HCCH-TOCSY experiment was done



with a Bruker AMX600 (600MHz) NMR spectrometer at 30°C, using the pulse sequence shown in Fig. 1. The data set contained $40(t_1) \times 9(t_2) \times 10(t_3) \times 1024(t_4)$ complex points, which were acquired using spectral widths of 5208 Hz ($\omega 1$,^{1}H), 3289 Hz ($\omega 2$,^{13}C), 3289 Hz ($\omega 3$,^{13}C) and 10000 Hz ($\omega 4$,^{1}H). A FLOPSY-8 (Mohebbi and Shaka, 1991) spin lock ($\gamma B_2/2\pi = 7.35$ kHz) mixing period of 22.4 ms, and τ_1 and τ_2 values of 1.5 and 1.2 ms were employed. Power switching of the carbon pulses was implemented before and after the FLOPSY mixing sequence. Sixteen transients were accumulated per increment with a 1.2-s relaxation delay between scans. The total accumulation time was 6.4 days. The NMR data were processed using in-house written software on Silicon Graphics computers. Linear prediction was used to improve windowing and resolution in the indirect detected dimensions. The time-domain data in the indirect detected dimensions were extended by one-quarter of the number of experimental points. Data sets were extensively zero-filled to aid interpolation of the peak positions. The final data set size after zero-filling was $256(\omega 1) \times 32(\omega 2) \times 64(\omega 3) \times 1024(\omega 4)$ points.

TABLE 1
THE RANGE OF ^{13}C CHEMICAL SHIFTS OBSERVED FOR EIGHT DIFFERENT PROTEINS[a]

Res	α	β	γ	δ	ε
Gly	42 – 48				
Ala	49 – 56	18 – 24			
Ser	55 – 62	61 – 67			
Thr	58 – 68	66 – 73	19 – 26		
Val	57 – 67	30 – 37	16 – 25		
Leu	51 – 60	39 – 48	22 – 29	21 – 28	
Ile	55 – 66	34 – 47	25 – 31 14 – 22	9 – 16	
Lys	52 – 61	29 – 37	21 – 26	27 – 34	40 – 43
Arg	50 – 60	28 – 35	25 – 30	41 – 45	
Pro	60 – 67	27 – 35	24 – 29	49 – 53	
Glu	52 – 62	27 – 34	32 – 38		
Gln	52 – 60	24 – 33	32 – 36		
Met	51 – 59	30 – 38	31 – 35		
Phe	52 – 64	36 – 44			
Tyr	54 – 63	37 – 45			
His	53 – 60	27 – 36			
Trp	55 – 63	28 – 29			
Cys	53 – 59	29 – 33			
Cys(s-s)	54 – 61	40 – 48			
Asp	50 – 58	38 – 45			
Asn	49 – 57	33 – 41			

[a] The data were compiled from the ^{13}C chemical-shift assignments of BPTI (Wagner and Brühwiler, 1986), IL-1β (Clore et al., 1990), calmodulin (Ikura et al., 1991), III^{Glc} (Pelton et al., 1991), staphylococcal nuclease H124L (Wang et al., 1992), IL-4 (Powers et al., 1992), *Bacillus subtilis* glucose permease IIA domain (Fairbrother et al., 1992) and FKBP/ascomycin complex (Xu et al., 1992). The ^{13}C chemical shifts are relative to 3-(trimethylsilyl)propionic-$d_4$acid.

shifts (Gly, Ala, Ser, Thr, Val, Leu, Ile, Lys, Arg, Pro) or shifts shared by only a few other residues (Oh et al., 1988; Wagner and Brühwiler, 1986; Clore et al., 1990; Ikura et al., 1991; Pelton et al., 1991; Fairbrother et al., 1992; Powers et al., 1992; Wang et al., 1992; Xu et al., 1992). Thus, the ^{13}C chemical shifts can be used to identify the individual spin systems by amino acid type (Oh et al., 1988) (see Table 1). This information, together with the amino acid sequence, yields the sequence-specific assignments which are necessary for determining the 3D structure.

Compared to the 3D HC(C)H-TOCSY experiment (Bax et al., 1990), the 4D version offers several important advantages. In the 4D experiment, *both* the ^{1}H *and* ^{13}C chemical shifts can be obtained by simple inspection of the $^{13}C(\omega3)$,$^{1}H(\omega4)$ planes extracted at the $^{1}H^{\alpha}(\omega1)/^{13}C^{\alpha}(\omega2)$ chemical shifts (Fig. 1). In the 3D HC(C)H-TOCSY experiment (Bax et al., 1990), only the ^{1}H chemical shifts are found on the $^{1}H^{\alpha}(\omega1)/^{1}H(\omega3)$ planes at the $^{13}C(\omega2)$ frequencies. The proton chemical shifts may overlap (e.g., H^{β} and H^{δ} of K47; H^{β} and H^{γ} of E102) and cannot be readily assigned. In the 4D experiment, the proton chemical shifts are separated by the chemical shifts of the attached ^{13}C nuclei (e.g., K47 and E102, Fig. 1) and are easily assigned on the basis of the characteristic ^{13}C chemical shifts (Table 1). In order to determine the ^{13}C chemical shifts of the amino acid side chains in the 3D HC(C)H-TOCSY experiment, the 3D data set must be searched for similar

patterns of $^1H(\omega 1)/^1H(\omega 3)$ chemical shifts. This process is time-consuming and is hampered by peak overlap. In addition, it is difficult to automate the analysis of the 3D data. In contrast, the increased resolution and symmetry of the peaks in the 4D experiment facilitate both the manual and automatic assignment of the ^1H and ^{13}C chemical shifts.

In summary, a 4D HCCH-TOCSY experiment is described for assigning the ^1H and ^{13}C resonances of amino acid side chains. It has several advantages over 3D versions of the experiment (Bax et al., 1990; Fesik et al., 1990) and, together with the recently described 4D triple resonance experiments (Boucher et al., 1992; Kay et al., 1991, 1992; Olejniczak et al., 1992), allows sequence-specific assignments to be obtained in a relatively short time with only a few experiments.

ACKNOWLEDGEMENTS

We thank Harriet Smith, Earl Gubbins, Jean Severin, and Tom Holzman for the preparation of isotopically labeled FKBP. This research was supported in part by the National Institute of General Medical Sciences (GM45351, awarded to S.W.F.).

REFERENCES

Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Magn. Reson.*, **88**, 425–431.

Bax, A. and Ikura, M. (1991) *J. Biomol. NMR*, **1**, 99–104.

Boucher, W., Laue, E.D., Campbell-Burk, S. and Domaille, P.J. (1992) *J. Am. Chem. Soc.*, **114**, 2262–2264.

Clore, G.M., Bax, A., Driscoll, P.C., Wingfield, P.T. and Gronenborn, A.M. (1990) *Biochemistry*, **29**, 8172–8184.

Edalji, R., Pilot-Matias, T.J., Pratt, S.D., Egan, D.A., Severin, J.M., Gubbins, E.G., Smith, H., Park, C.H., Petros, A.M., Fesik, S.W., Luly, J., Burres, N.S. and Holzman, T.F. (1992) *J. Prot. Chem.*, **11**, 213–223.

Fairbrother, W.J., Palmer, A.G.I., Rance, M., Reizer, J., Saier, M.H., Jr. and Wright, P.E. (1992) *Biochemistry*, **31**, 4413–4425.

Fesik, S.W., Eaton, H.L., Olejniczak, E.T., Zuiderweg, E.R.P., McIntosh, L.P. and Dahlquist, F.W. (1990) *J. Am. Chem. Soc.*, **112**, 886–888.

Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) *Nature*, **341**, 758–760.

Hatanka, H., Kino, T., Miyata, S., Inamura, N., Kuroka, A., Goto, T., Tanaka, H. and Okuhara, M. (1988) *J. Antibiotics*, **41**, 1592–1601.

Ikura, M., Spera, S., Barbato, G., Kay, L.E. and Krinks, M.B., A. (1991) *Biochemistry*, **30**, 9216–9228.

Kay, L.E., Ikura, M. and Bax, A. (1990a) *J. Am. Chem. Soc.*, **112**, 888–889.

Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990b) *J. Magn. Reson.*, **89**, 496–514.

Kay, L.E., Ikura, M. and Bax, A. (1991) *J. Magn. Reson.*, **91**, 84–92.

Kay, L.E., Wittekind, M., McCoy, M.A., Friedrichs, M.S. and Mueller, L. (1992) *J. Magn. Reson.*, **98**, 443–450.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.

Mohebbi, A. and Shaka, A.J. (1991) *Chem. Phys. Letters*, **178**, 374–378.

Oh, B.H., Westler, W.M., Darba, P. and Markley, J.L. (1988) *Science*, **240**, 908–911.

Olejniczak, E.T., Xu, R.X., Petros, A.M. and Fesik, S.W. (1992) *J. Magn. Reson.*, (in press).

Pelton, J.G., Torchia, D.A., Meadow, N.D., Wong, C.-Y. and Roseman, S. (1991) *Biochemistry*, **30**, 10043–10057.

Powers, R., Garrett, D.S., March, C.J., Frieden, E.A., Gronenborn, A.M. and Clore, G.M. (1992) *Biochemistry*, **31**, 4334–4346.

Sekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. and Sigal, N.H. (1989) *Nature*, **341**, 755–757.

Wagner, G. and Brühwiler, D. (1986) *Biochemistry*, **25**, 5839–5843.

Wang, J., Hinck, A.P., Loh, S.N., LeMaster, D.M. and Markley, J.L. (1992) *Biochemistry*, **31**, 921–936.

Xu, R.X., Nettesheim, D., Olejniczak, E.T., Meadows, R., Gemmecker, G. and Fesik, S.W. (1992) *Biopolymers*, (in press).