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

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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($\omega_1$)/$^{13}$C($\omega_2$) 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-
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: \( \phi_1 = x, x, -x, -x \); \( \phi_2 = 8y, 8(-y) \); \( \phi_3 = x, -x \); \( \phi_4 = x, y, -x, -y, -x, -y, y \); \( \phi_5 = y, x, -y, -x \); 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 \( \phi_1 \) for \( \tau_1 \), \( \phi_3 \) for \( \tau_2 \), and \( \phi_4 \) for \( \tau_3 \). The carbon and proton carrier frequency was set at 37.8 and 3.68 ppm, respectively.

The next step involves a reverse INEPT sequence and the detection of the protons during the acquisition (\( \tau_4 \)) 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 \( ^1H \) 180° pulses.

Figure 2 depicts several \( ^{13}C(\omega_3), ^1H(\omega_4) \) 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 \( ^1H(\omega_1) \) and \( ^{13}C(\omega_2) \) chemical shifts. As illustrated in Fig. 2, identification of the complete \( ^1H \) 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 \( ^{13}C(\omega_3), ^1H(\omega_4) \) planes at the \( ^1H(\omega_1)/^{13}C(\omega_2) \) chemical shifts. Data interpretation was only complicated for those amino acids in which both the \( \alpha \)-proton and \( \alpha \)-carbon signals overlapped, producing two or more sets of signals on the plane extracted at the \( ^1H, ^{13}C \) 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 \( H, C \) shifts of adjacent amino acids identified in the triple resonance experiments, the \( ^1H \) 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. 1. Pulse sequence for the 4D HCCH-TOCSY experiment.