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HCCH-TOCSY spectroscopy of ^{13}C-labeled proteins in H_2O using heteronuclear cross-polarization and pulsed-field gradients

Hong Wang and Erik R.P. Zuiderweg*

Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, U.S.A.

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Summary

A pulsed-field gradient-enhanced, heteronuclear cross-polarization-driven, 3D HCCH-TOCSY experiment is described, which in a single scan can achieve nearly ideal solvent suppression for protein samples in H_2O solution. The 3D experiment can be transformed without additional pre- or post-processing, thus leaving solute resonances at the solvent resonance position undisturbed and easily identifiable. As the gradients are used in combination with a ^{13}C z-filter, only minimal relaxation losses are encountered as compared to non-gradient versions.

Assignment of the NMR resonances of larger biomolecules is generally carried out by analyzing a combination of several multidimensional multinuclear experiments. A widely used protocol is to obtain main-chain resonance assignments from three-dimensional (3D) triple- and quadruple-resonance experiments and to compile side-chain resonance assignments from 3D HCCH-COSY and HCCH-TOCSY experiments (see Bax and Grzesiek (1993) and Clore and Gronenborn (1993) for recent reviews). The two assignment subsets are linked through the H^{α} and C^{α} resonance positions. While the original HCCH experiments are based on INEPT (RINEPT) sequences (Bax et al., 1990; Fesik et al., 1990), we have later introduced a sensitive heteronuclear cross-polarization (CP) based 3D HCCH experiment we now refer to as CP-driven HCCH-TOCSY (Majumdar et al., 1993). In most cases, the HCCH-type experiments are acquired using labeled proteins dissolved in D_2O, because of complications with solvent suppression in H_2O-containing samples. It is very desirable, however, to use the same sample of $^{13}C/^{15}N$-labeled protein dissolved in 95% H_2O for both the triple-resonance experiments and the HCCH experiments, in order to avoid chemical shift changes between the experiments associated with isotope effects and with sample handling per se. Kay and co-workers, recognizing this, have recently modified the INEPT-based HCCH-TOCSY experiment with pulsed-field gradients (PFG) to allow 3D HCCH-TOCSY spectra to be recorded in H_2O (Kay et al., 1993). In this communication, we show that pulsed-field gradients can also be successfully incorporated into the 3D CP-driven HCCH-TOCSY experiment, to produce an extremely high degree of solvent suppression in this sensitive experiment. Good solvent-specific suppression is especially important for the HCCH-TOCSY experiment because of the necessity to observe in the direct dimension H^{α} signals, many of which resonate close to or coincident with the water resonance. For ideal solvent suppression, the signals under the water resonance should not be disturbed at all and a flat baseline near water must be obtained. We show that the gradient version of the CP-driven HCCH-TOCSY experiment approaches this ideal situation. The suppression method used should also be applicable to other net-magnetization transfer CP NMR experiments.

The pulse scheme for the PFG-enhanced CP-driven HCCH-TOCSY experiment is shown in Fig. 1. The sequence is very similar to the CP-driven HCCH-TOCSY experiment described previously (Majumdar et al., 1993).

*To whom correspondence should be addressed.

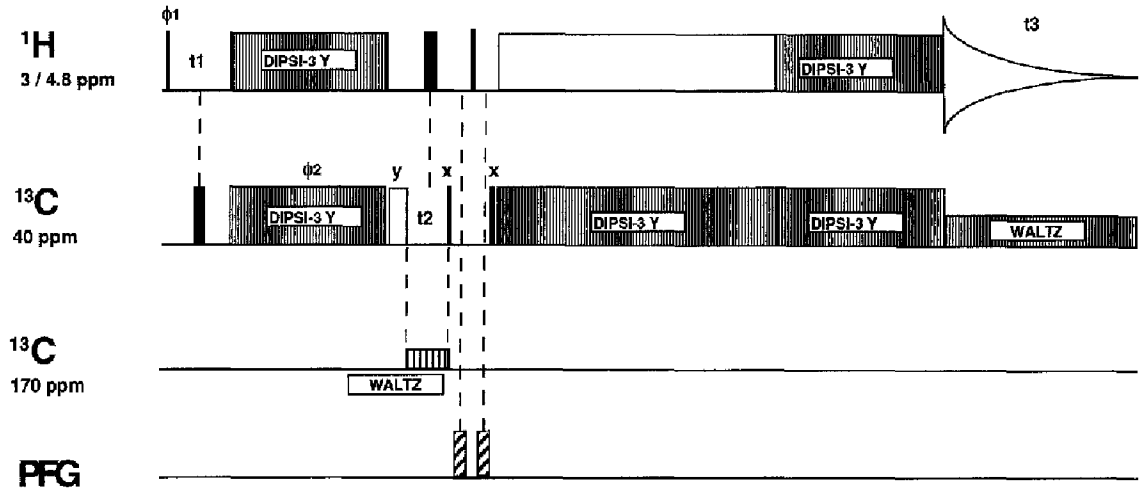

Fig. 1. Pulse sequence for the PFG-enhanced CP-driven HCCH-TOCSY experiment. Narrow lines correspond to 90° pulses, wider lines to 180° pulses. An applicable phase cycling is: ϕ1, x,–x; ϕ2, y,y, –y,–y; receiver, +, –, –, + (see text). The heteronuclear and homonuclear cross-polarizations (vertically hatched boxes) were carried out using synchronous DIPSI-3 sequences (Shaka et al., 1988; Majumdar et al., 1993) with rf field strengths of 9 kHz (generated by a Bruker BLAX-300 linear amplifier for the ^{13}C channel). The heteronuclear CP sequences were 6.0 ms each; the homonuclear TOCSY sequence was 12.1 ms. Carbonyl decoupling was carried out using a Waltz-16 sequence (Shaka et al., 1983) with a 278 Hz rf field strength. Open boxes indicate a ^{13}C trim pulse (1 ms) and a ^{1}H dephasing pulse, both with 9 kHz rf field strength. Quadrature detection (States-TPPI (Marion et al., 1989)) in the indirect ^{1}H dimension was obtained by incrementing the phase of the first ^{1}H pulse; quadrature detection in the ^{13}C dimension (States-TPPI) was achieved by phase-shifting the ^{13}C pulses in the first heteronuclear CP sequence together with the optional trim pulse. The field gradient pulses had a square shape and were 3 and 1 ms in length, and corresponded to 48 G/cm each.

Source proton magnetization, frequency labeled during t_1, is transferred to the attached ^{13}C nucleus over the one-bond scalar coupling via heteronuclear cross-polarization (Bertrand et al., 1978; Bearden and Brown, 1989; Zuiderweg, 1990). The resulting ^{13}C magnetization is frequency labeled in t_2 and subsequently transferred to other carbons in the same residue through a homonuclear TOCSY transfer (Braunschweiler and Ernst, 1983; Bax and Davis, 1985); finally the ^{13}C magnetization is transferred via another heteronuclear cross-polarization sequence to the destination proton and detected. For a more detailed description of these (and unwanted) pathways the reader is referred to the original paper on this experiment (Majumdar et al., 1993). Water suppression in this sequence is achieved by the use of B_0 field gradients as strong homospoil pulses. With the first proton pulse, the water resonance is tipped into the transverse plane. The subsequent heteronuclear cross-polarization, carried out as a complete DIPSI-3 R(–R)(–R)R sequence (Shaka et al., 1988), serves as an efficient isotropic spin-lock for the water resonance, keeping it in the transverse plane. This magnetization is subsequently dephased by a strong pulsed-field gradient. In the meantime, the coherence of interest has been transferred to the attached ^{13}C nucleus and is stored along the z-axis during the PFG block. Water magnetization, relaxed back to the z-axis by T_1 processes during t_1 and t_2, is unaffected by the first gradient pulse. This component is tipped into the transverse plane by a 90° ^{1}H pulse and subsequently dephased by the second field gradient pulse. The PFG-90-PFG block therefore efficiently dephases all water magnetization (Kay et al., 1993). Once all water magnetization has been destroyed, continuous wave irradiation (i.e. a radio-frequency field gradient) is used to dephase any solvent magnetization reemerging by T_1 processes during the ^{13}C TOCSY period. This c.w. irradiation will not affect signals associated with H^α magnetization, because there is no proton magnetization of interest at this time.

The sequence accomplishes ^{13}C pathway selection through z-filtration. In contrast to coherence pathway selection methods based on gradient phase encoding of transverse magnetization (Hurd, 1990), the method presented here avoids the inherent loss of $\sqrt{2}$ in sensitivity associated with echo selection. Gradients occur in the sequence while the magnetization of interest is aligned along the +/– z-axis, thus only incurring sensitivity losses associated with the relatively slow ^{13}C T_1 relaxation. The sequence does contain two additional 90° ^{13}C pulses as compared to the original CP-driven HCCH-TOCSY experiment. However, we found that, even though ^{13}C rf pulses of only limited rf strength were issued, the sensitivity losses caused by these pulses were minimal in the microcell NMR tubes used. In principle, the sequence accomplishes in-phase HCCH pathway selection in a single transient and therefore allows for very high three-dimensional resolution. The HCCH pathway selection is accomplished by the following events: (i) ^{1}H x,y-magnetization associated with non-^{13}C bound protons present during t_1 is spin-locked by the ^{1}H component of the first CP period and dephased by the gradients, as described above; (ii) equilibrium ^{13}C magnetization (S_z) is returned to the z-axis at the end of the first CP period. It is con-

verted into S_x by the ^{13}C z-filter pulse and subsequently dephased by the gradient. The optional ^{13}C trim pulse prior to t_2 accomplishes the same selection. Consequently, only ^{13}C magnetization originating from cross-polarization passes through the gradient z-filter; and (iii) the ^1H magnetization detected during t_3 must originate from ^{13}C via the last CP sequence, since all other ^1H magnetization is retained in a dephased state by the isotropic ^1H component of the CP sequence.

Selection for three-dimensional absorption phase is accomplished by the following: (i) heteronuclear CP sequences only transfer magnetization components that are parallel to the phase of the rf pulses; dispersive ^1H magnetization present during t_1 (I_x) is therefore not transferred; (ii) anti-phase dispersion ^{13}C magnetization ($2I_zS_x$) originating from an imperfectly tuned first CP sequence and present during t_2 is retained in the ^{13}C x,y-plane by the z-filter pulse and dephased by the gradient or destroyed by the trim pulse prior to t_2; and (iii) anti-phase dispersion ^1H magnetization ($2I_xS_z$) present during t_3 and originating from an imperfectly tuned last CP sequence is suppressed by the heteronuclear decoupling.

Accordingly, a single scan per complex t_1,t_2 increment accomplishes complete pathway selection and produces spectra in three-dimensional absorption. In practice, we used a four-step phase cycle, as described in Fig. 1, since experimental time allowed this. Note, however, that this phase cycle does not include any steps to suppress possible dispersion components. The efficiency of single-scan pathway selection can be appreciated from Fig. 2 (see below).

The 3D PFG-enhanced CP-driven HCCH-TOCSY experiment was applied to the 18 kDa peptide binding domain of chaperone protein Hsc70, uniformly labeled with ^{13}C and ^{15}N, dissolved in 95% H_2O / 5% D_2O solution at 25 °C. The 3D spectrum was recorded on a Bruker AMX-500 spectrometer equipped with a self-shielded triple-resonance z-gradient probe. The pulsed-field gradients (PFG) were generated with a Bruker Grasp unit. Figure 2 shows that the solvent suppression produced by this sequence is truly excellent: the water resonance is fully suppressed in *a single scan*. It is important to note that the quality of the single-scan suppression remains equally good during the course of the experiment, due to the precautions taken to dephase solvent magnetization relaxed back during the t_1, t_2 and TOCSY periods (see above). Figure 2 suggests that this HCCH-TOCSY experiment should allow assignment of resonances coincident with that of water. This is indeed the case, as illustrated in Fig. 3 which shows part of the ^1H-^1H plane at F2 = 18.8 ppm (^{13}C) of the completely transformed 3D data set. We want to emphasize that no convolution or any other data massaging technique was used to obtain this spectrum. The results are as good as or better than can be obtained from protein samples dissolved in D_2O using conventional techniques, and also appear to compare favorably with results obtained for the gradient-enhanced INEPT-driven HCCH-TOCSY described by

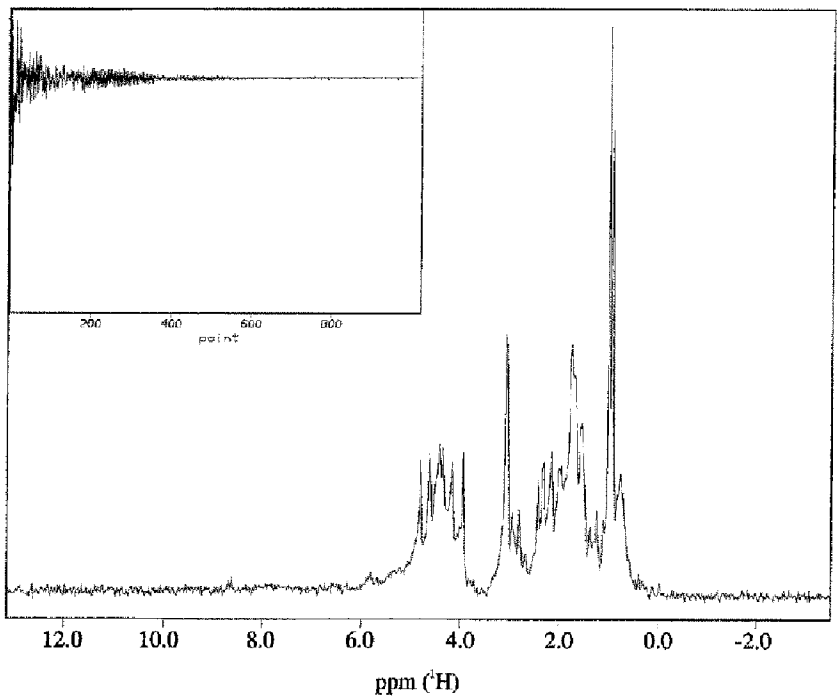

Fig. 2. FID and spectrum obtained with the PFG-enhanced CP-driven HCCH-TOCSY experiment with only one scan following eight dummy scans. The sample is 1.4 mM U-L $^{13}C/^{15}N$ Hsc70 peptide binding domain (18 kDa) in 95% H_2O, 25 °C in a 5 mm microcell (Shigemi Inc., Allison Park, PA). No window function, convolution or other pre- or post-data massaging was used.

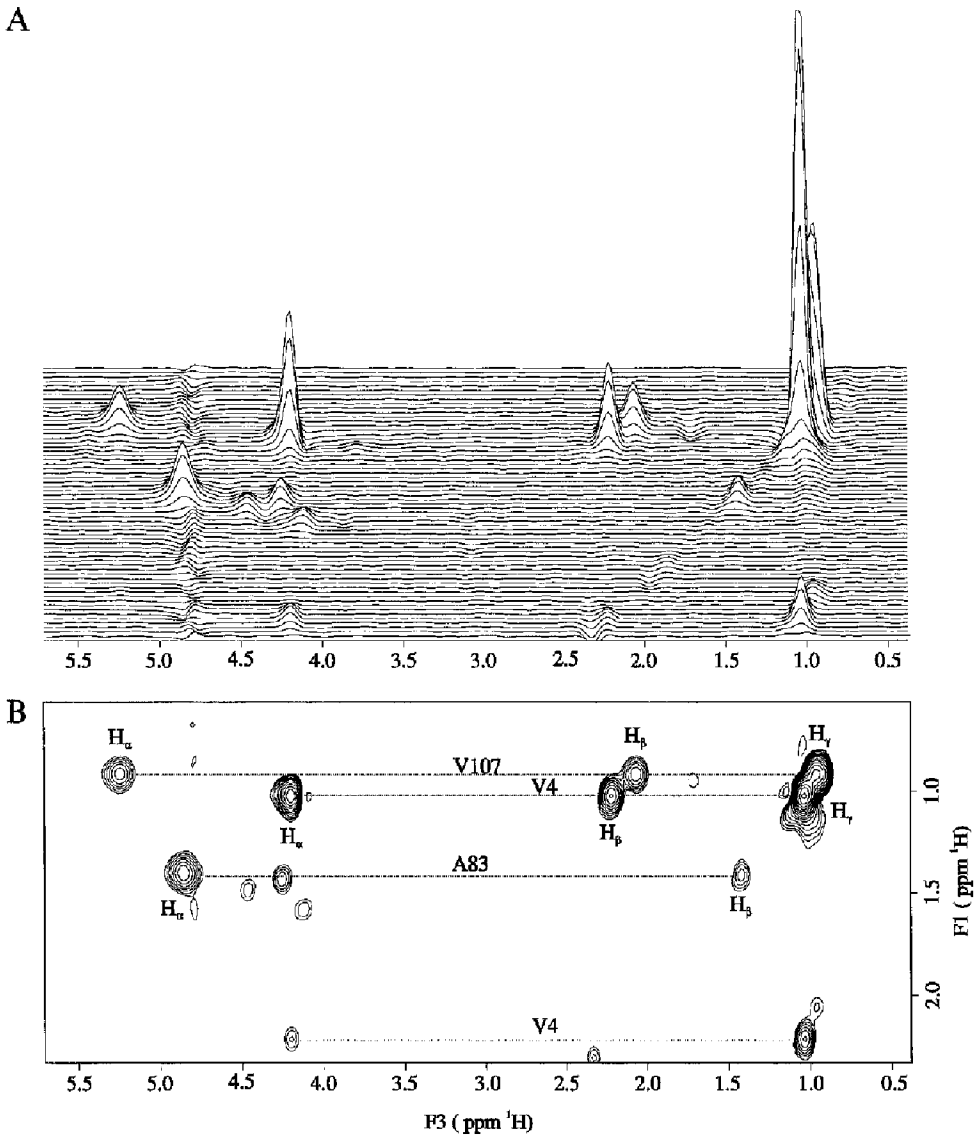

Fig. 3. 3D PFG-enhanced CP-driven HCCH-TOCSY spectrum obtained at 500 MHz with 1.4 mM U-L $^{13}C/^{15}N$ Hsc70 peptide binding domain (18 kDa) in 95% H_2O, 25 °C. (A) A stacked plot and (B) a contour plot of a region of the ^1H-^1H plane through the 3D spectrum at the $^{13}C^{\gamma}$ frequencies of Val^{107} and Val^4 and the $^{13}C^{\beta}$ frequency of Ala^{83} (18.8 ppm) showing the direct HCCH traces (solid lines) and 'ghost' traces (dashed line) for these residues. The vertical disturbance at 4.83 ppm in the stacked plot marks the location and intensity of the residual H_2O resonance. A data matrix of 128 × 60 × 1024 complex points (t_1,t_2,t_3) was acquired with 4 scans per increment and a relaxation delay of 0.8 s. Spectral widths of 3704, 8197 and 8333 Hz were used in F1, F2 (^{13}C) and F3, respectively. ^1H and ^{13}C carriers were placed in the center of the respective aliphatic spectra. The ^1H carrier was placed at 4.83 ppm during and after the z-filter. The spectrum was processed on a Silicon Graphics Personal Iris workstation using FELIX 2.0 and 2.05 software (formerly Hare Research, Inc.). No convolution or other pre- or post-data massaging was used, leaving protein signals coincident with the solvent resonance undisturbed. Lorentzian–Gaussian transformation and squared shifted sine-bell window functions were used to apodize the direct and indirect dimensions, respectively. The final frequency domain matrix was 256 × 128 × 2048 real points (t_1,t_2,t_3).

Kay et al. (1993). In Fig. 3B, the HCCH-TOCSY traces for Val^{107}, Val^4 and Ala^{83} are identified. The H^{α} resonance of Ala^{83}, exactly degenerate with the H_2O resonance, is totally undisturbed and easily identified, as can be seen from the stacked plot of this spectral region (Fig. 3A). The stacked plot also shows that the CP-driven HCCH-TOCSY data is virtually free of artifacts. A ghost trace (Majumdar et al., 1993) at the F1 frequency, corresponding to the H^{β} resonance for Val^4, can be seen on its C^{γ} plane. Ghost traces are caused by the transfer of ^1H source magnetization to remote ^{13}C signals (here H^{β} to C^{γ}) during the first heteronuclear CP transfer, and are in most cases helpful to confirm the assignments.

The use of pulsed-field gradients as strong homospoil pulses for water suppression is not new, although it has not been much used in conjunction with z-filters (Kay et al., 1993). A very common application is to use PFGs to spoil unwanted coherences when all desired coherence is converted to zz-order in INEPT-based experiments (Bax and Pochapski, 1992). This approach works well when no

frequency labeling is required for the first proton period, as in 'out-and-back'-type experiments such as HNCA and its derivatives (Bax and Grzesiek, 1993). A solvent suppression problem occurs with the *zz*-filter gradient dephasing approach in INEPT-based net-transfer experiments such as HCCH or HBHA(CBCACO)NH (Grzesiek and Bax, 1992) when frequency labeling is required for the indirect proton dimension: the solvent resonance aligns with the z-direction during the zz-period of the sine-quadrature component of the indirect ^{1}H dimension. As a result, the solvent suppression is less than satisfactory for that quadrature component, giving rise to poor baselines near the water in the completely transformed spectrum. In the current PFG-enhanced CP-driven experiment, this difference in solvent suppression in quadrature pairs does not occur. The water signal remains locked in the transverse plane by the (for the solvent) isotropic DIPSI-3 sequence, irrespective of the phase of the first pulse that created it. Thus, in CP-driven experiments this can be exploited for PFG dephasing of equal effectiveness for both quadrature components. Another important benefit is that the (indirect) proton carrier frequency can be set to the center of the aliphatic proton region without complications, to reduce the spectral width in the t_1 dimension and to optimize the cross-polarization between aliphatic protons and carbons.

In conclusion, we have shown that pulsed-field gradients can be incorporated into the 3D CP-driven HCCH-TOCSY experiment to produce truly excellent spectra of ^{13}C-labeled proteins dissolved in H_2O solution. As the solvent suppression / coherence selection in the proposed scheme occurs while the ^{13}C magnetization is aligned along the z-axis, only minimal sensitivity losses due to T_1 relaxation occur. The character of the DIPSI-3 sequence allows solvent suppression to be equally good for all quadrature components, and the sequence is optimized for excellent solvent suppression in the presence of solvent T_1 relaxation.

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