Sensitivity improvement in 2D and 3D HCCH spectroscopy using heteronuclear cross-polarization

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SUMMARY

A new method, which employs a sequence of heteronuclear-homonuclear-heteronuclear Hartmann-Hahn (HEHOHEHAHA) cross-polarization steps for obtaining through-bond H-C-C-H correlations in larger proteins ($M_r > 15$ kDa), is presented. The method has significantly higher sensitivity compared to INEPT-HOHAHA-INEPT-based techniques. An additional feature of this experiment is that well-phaseable spectra may be obtained with a minimal (4-step) phase cycle and, consequently, experimental time can be utilized towards obtaining high resolution in indirect dimensions. Results from 2D and 3D HEHOHEHAHA experiments on T4-lysozyme are presented.

INTRODUCTION

High-resolution three-dimensional (3D) solution structures of proteins can only be determined when NMR assignments are available for both back-bone and side-chain ^1H resonances. For smaller systems, these assignments are obtained from the concerted analysis of 2D COSY/DQFCOSY/RELAY/HOHAHA spectra with NOE data (Wüthrich, 1986). For medium-size proteins the analysis of 3D ^{15}N-resolved NOESY-HSQC and HOHAHA-HSQC data might be sufficient for full spectral analysis (Fesik and Zuiderweg, 1990; Clore and Gronenborn, 1991; Stockmann et al., 1992). For larger proteins ($M_r > 15$ kDa), assignments for backbone resonances can be obtained from 3D and 4D triple and quadruple resonance experiments (Driscoll et al., 1990; Grzesiek et al., 1992). However, side-chain assignments cannot be readily made for these larger systems. The sensitivity of the ^1H-^1H HOHAHA-HSQC experiment, from which these assignments should be extracted, is too low for larger systems since the line widths of the ^1H resonances become much larger than the ^1H-^1H scalar couplings.

A potential route for side-chain assignment is found in the so-called HCCH experiments, where

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for uniformly ^{13}C labeled molecules, ^1H magnetization is transferred to the ^{13}C nucleus of the bound carbon atom over a large one-bond heteronuclear scalar coupling ($^1J_{^1H^{13}C}$ = 130–160 Hz), followed by a Hartmann-Hahn (Bax et al., 1990; Fesik et al., 1990; Olejniczak et al., 1992) or pulsed (Kay et al., 1990) ^{13}C-^{13}C transfer over the one-bond ^{13}C-^{13}C coupling ($^1J_{^1H^{13}C}$ = 35–55 Hz). The magnetization is subsequently transferred to protons for detection. Thus, indirect ^1H-^1H correlations are obtained using exclusively large one-bond scalar interactions. In addition, ^{13}C assignments are obtained that are important for the analysis of ^{13}C-resolved 3D and 4D NOESY spectra (Clore et al., 1991; Zuiderweg et al., 1991). The HCCH experiments are in principle suitable for NMR of proteins up to 30 kDa.

Three-dimensional HCCH experiments are generally carried out using refocused-INEPT/^{13}C-^{13}C HOHAHA/reverse-refocused-INEPT (RINEPT) sequences (Bax et al., 1990). These sequences contain a multitude of ^{13}C INEPT pulses that are performed at a power level suitable for the ^{13}C-^{13}C HOHAHA because of r.f. coherence requirements (typically at $\gamma B_1/2\pi$ = 8.5 kHz). These longer pulses have pronounced offset effects over the spectral width area of interest (the aliphatic ^{13}C region is 10 kHz at 14 T) and efficiency of transfer and phase behavior may become problematic at the spectral edges. Equally critical for the sensitivity of the HCCH experiment is minimization of the residence time of the ^{13}C magnetization in the xy plane since ^{13}C T_2 and $T_{1\rho}$ relaxation is relatively fast. The pulse-based experiments contain two ^{13}C free precession periods that serve to refocus and defocus ^{13}C-^1H couplings in addition to the ^{13}C-^{13}C HOHAHA period. Especially for HOHAHA experiments with short mixing times, these do-nothing free precession periods become a disproportionately large sensitivity sacrifice.

Here we propose (Zuiderweg, 1992) to use heteronuclear cross-polarization in HCCH experiments to address some of the shortcomings of the pulse-based approach. Instead of using refocused INEPT and RINEPT to effectuate the $^1H \rightarrow ^{13}C$ and $^{13}C \rightarrow ^1H$ transfers, we use heteronuclear cross-polarization (CP) sequences consisting of bursts of amplitude-modulated r.f. pulses delivered simultaneously from two r.f. channels. We and others have demonstrated that heteronuclear CP is more efficient than refocused INEPT for simple ^1H-^{13}C transfer (Bearden and Brown, 1989; Zuiderweg, 1990; Ernst et al., 1991), and similar benefits are expected for the double-transfer case. We refer to the CP-based experiment as Heteronuclear-Homonuclear-Heteronuclear-Hartmann-Hahn spectroscopy (HEHOHEHAHA). In this paper, we present 2D and 3D versions of this experiment on T4-lysozyme (M_r = 19 kDa), demonstrating its higher sensitivity. A discussion of the various coherence-transfer pathways that are involved shows that a minimal 4-step phase cycle is sufficient to obtain well-phaseable spectra. This provides the additional advantage that higher resolution is possible in indirect dimensions.

MATERIALS AND METHODS

NMR spectroscopy

All spectra were recorded with a ^{13}C-labeled sample of T4-lysozyme in 90% H_2O/10% ^2H_2O, at a temperature of 30 °C. The sample concentrations used were 2 mM for the spectra in Figs. 2, 3 and 4A, and 3 mM for the spectrum in Fig. 4B. The DIPSI-3-based HEHOHEHAHA sequence shown in Fig. 1 was used. Since the solvent was H_2O, a 1-s presaturation was applied.

For the 1D and 2D spectra shown in Figs. 1 and 2, respectively, the heteronuclear CP mixing time (τ_1, τ_3 in Fig. 1) as well as the ^{13}C-^{13}C HOHAHA period (τ_2 in Fig. 1) was 6.7 ms. The 2D data
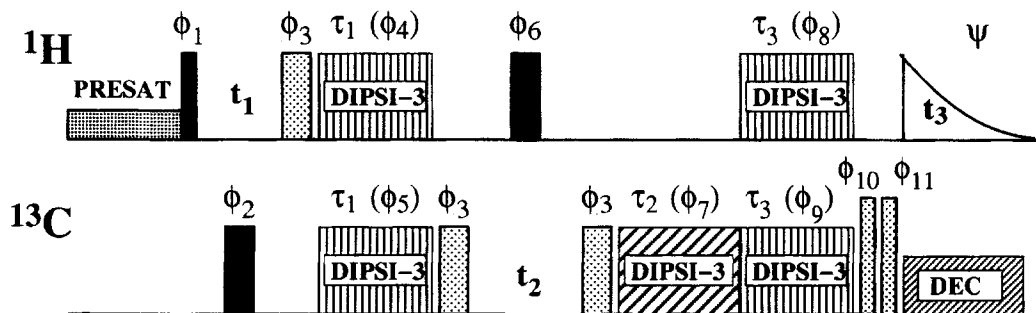
Fig. 1. Pulse scheme of the 3D HEHOHEHAHA experiment proposed here. Pulses with phases ϕ_1, ϕ_{10} and ϕ_{11} correspond to 90° rotations and those with ϕ_2 and ϕ_6 are 180° rotations. Pulses with phase ϕ_3 correspond to optional trim pulses. Phase cycles ϕ_1–ϕ_{11} are: ϕ_1 = x,x,−x,−x, ϕ_2 = x, ϕ_3 = y, ϕ_4 = y, ϕ_5 = y,−y,y,−y, ϕ_6 = x, ϕ_7 = y, ϕ_8 = y, ϕ_9 = y, ψ = x,−x,−x,x. The two 90° purge pulses prior to data acquisition are also optional and serve to suppress side-bands originating from imperfect decoupling of anti-phase magnetization (20) (by inverting the S_z magnetization on alternate scans with a 180° pulse), especially when GARP decoupling is used. These are not essential in the case of WALTZ-16 decoupling which produces fewer side-bands. In case they are used, the phase cycling is ϕ_{10} = 8(x), ϕ_{11} = 4(x), 4(−x) and ϕ_6 = 4(x), 4(−x). All other phase cycles remain unchanged. τ_1 and τ_3 are the heteronuclear CP mixing times and τ_2 is the ^{13}C-^{13}C HOHAHA mixing time.

was acquired with 64 t_2 scans and 160 complex t_1 data points. For the CP experiments, the r.f. field strength was 8.1 kHz for both nuclei; the pulsed experiments used 8.1 kHz throughout for the ^{13}C fields and full power (23 kHz) for the INEPT ^{1}H fields.

For the 3D experiment in Fig. 4A, acquisition parameters (t_1, t_2, t_3) were: carrier (ppm): 2.1, 41.6, 2.1; acquisition time (ms): 48.5, 13.5, 143; spectral widths (Hz): 3571, 9260, 3571; data points (complex): 174, 125, 512; CP mixing times τ_1, τ_2, τ_3 were all set to 6.5 ms; r.f. field strength (both nuclei): 8.1 kHz; 8 scans were acquired per (t_1, t_2) increment; WALTZ-16 decoupling was used during acquisition. The net data accumulation time was approximately 260 h on a Bruker AMX-500 (11.7 T) spectrometer. All trim pulses shown in Fig. 1 were applied (1 ms), but the purge pulses were not. For the spectrum in Fig. 4B, the acquisition parameters (t_1, t_2, t_3) were: carrier (ppm): 4.73, 41.30, 4.73; acquisition time (ms): 8.52, 11.26, 145; spectral widths (Hz): 7042, 11363, 7042; data points (complex): 60, 128, 1024; τ_1, τ_3 = 6.52 ms, τ_2 = 19.56 ms; r.f. field strength (both nuclei): 8.3 kHz; scans: 8 per (t_1, t_2) increment; decoupling: GARP; accumulation: 123 h on a Bruker AMX-600 (14 T) spectrometer. Only the first trim pulse in Fig. 1 was applied, along with the purge pulses. In both experiments, a commercial triple resonance probe (^{1}H/^{2}H inside, ^{13}C/^{15}N outside) was used. ^{1}H fields were delivered from standard E.coupler hardware while ^{13}C fields were delivered from a Bruker BSV-10 amplifier for the spectrum in Fig. 4A and a Bruker BLAX-300 linear amplifier for the spectrum in Fig. 4B since the maximum CW power available from the standard BSV-10 amplifier on the 600 MHz spectrometer was insufficient for the r.f. requirements ($\gamma B_1/2\pi \geq$ 8.3 kHz). No ^{13}C-O decoupling was performed. Quadrature detection in t_1 was achieved by shifting the phase of the first ^{1}H pulse; in t_2 by shifting the phase of the entire ^{13}C CP train and the first ^{13}C trim pulse.

Data processing was carried out on Silicon Graphics workstations using FELIX 2.0 (Hare Research Inc.) augmented with several routines. For the spectra in Fig. 4, the final matrix dimensions and digital resolution (Hz/pt) in F_3, F_2, F_1 were (4A): 1024(3.48)∗256(36.2)∗256(14.0) and (4B): 512(3.44)∗256(44.0)∗512(13.8). In Fig. 4B, extension of t_1 (^{1}H) from 80 complex data
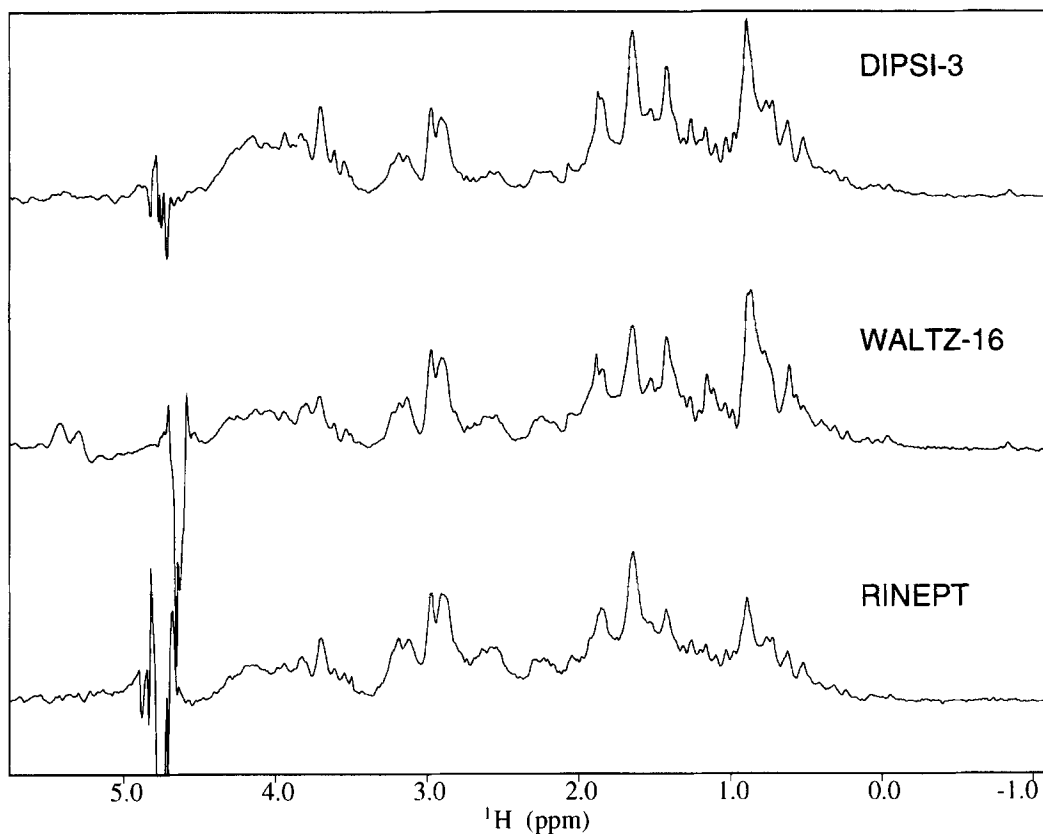
Fig. 2. One-dimensional ^{1}H spectra generated with the pulse sequences INEPT-(DIPSI-3)-RINEPT, HEHOHEHAHA (WALTZ-16)-(DIPSI-3)-(WALTZ-16) and HEHOHEHAHA (DIPSI-3)-(DIPSI-3)-(DIPSI-3). Experimental conditions are described in Materials and Methods. The peaks appearing downfield of the H_2O resonance in the middle panel (WALTZ-16) are artifactual.

points to 120 complex points was carried out by linear prediction followed by apodization (Olejniczak and Eaton, 1990).

RESULTS AND DISCUSSION

Figure 1 shows the pulse sequence for a 3D version of the experiment. Figure 2 shows a comparison of the efficiency of the double-transfer ^{1}H-^{13}C-^{13}C-^{1}H using INEPT-DIPSI-RINEPT and a 2D-HEHOHEHAHA sequence. The figure shows that DIPSI-3 (Shaka et al., 1988) based heteronuclear CP transfers yield additional sensitivity in the resulting ^{1}H spectrum as compared to the INEPT approach. This sensitivity difference is apparent at the edges of the ^{1}H spectral window (also corresponding to the edges of the ^{13}C window). It is clear that DIPSI-3-based CP sequences perform better than WALTZ-16 (Shaka et al., 1983) based sequences. Heteronuclear CP sequences need to be carried out with pulse trains containing 180° phase shifts only; as was demonstrated previously (Zuiderweg, 1990), sequences such as MLEV (Levitt et al., 1982) yield spectra with undesirable phase characteristics. As expected, the newer FLOPSY sequences
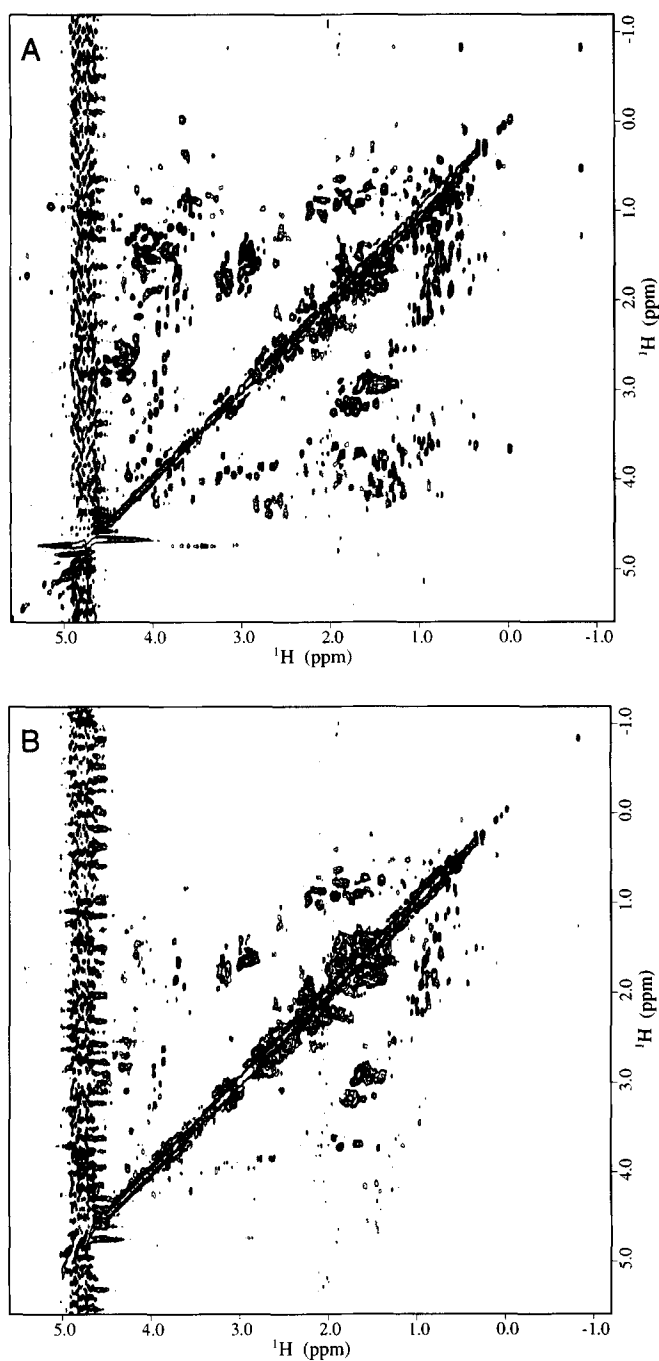
Fig. 3. Two-dimensional HCCH spectra of T4-lysozyme obtained by using HEHOHEHAHA (A) and INEPT-DIPSI-RINEPT (B). The (DIPSI-3)-(DIPSI-3)-(DIPSI-3) sequence of Fig. 1 was used in A. Experimental parameters were interactively optimized for both experiments. The ^{13}C evolution times in INEPT-DIPSI-RINEPT were 1.1 ms for both τ and δ, as recommended (Bax et al., 1990). The ^{1}H pulses were high power for INEPT-DIPSI-RINEPT (23 kHz) and CP power for HEHOHEHAHA (8 kHz). The ^{13}C fields were 8 kHz for both experiments.

(Kadkhodaie et al., 1991) with nonorthogonal phases do not produce good HEHOHEHAHA spectra either (results not shown).

The advantage of HEHOHEHAHA spectroscopy becomes more evident when comparing 2D spectra (Fig. 3). The CP-based sequence produces many cross peaks (Fig. 3A) while homonuclear ^{13}C-^{13}C transfer has been much less effective in the INEPT-based approach (Fig. 3B). The cross-peak sensitivity in the CP data is partially due to ^{13}C-^{13}C HOHAHA transfer that occurs also during the heteronuclear CP sequences (Zuiderweg, 1990) in addition to that taking place in the homonuclear HOHAHA period. Thus, more efficient utilization of the ^{13}C xy magnetization for ^{13}C-^{13}C HOHAHA transfer is made in the CP experiment. This is important because ^{13}C transverse magnetization has very limited lifetime even in well-behaved proteins such as T4-lysozyme.

The 2D HEHOHEHAHA spectrum was recorded with a minimal phase cycle of four steps for coherence pathway selection. The phase of the first ^{13}C-^{13}C CP train was inverted to invert the phase of the incoming ^{13}C magnetization. Concomitant cycling of receiver phase was carried out to select for this path involving ^{13}C. Cycling of the first ^{1}H pulse with receiver inversion was used to select a proton origin of that ^{13}C path. It is clear from the 2D spectrum that this limited cycling is sufficient to obtain good pure absorption-phase data. The INEPT-based experiment (Bax et al., 1990) was carried out with the recommended 16-step cycle, here augmented to 32 steps by using inversion of the first ^{1}H pulse for axial peak suppression.

Figure 4 shows two ^{1}H-^{1}H planes from 3D HEHOHEHAHA experiments carried out on a ^{13}C-labeled T4-lysozyme sample, with ^{13}C-^{13}C HOHAHA mixing times of 6 ms at 11.7 T (Fig. 4A) and 20 ms at 14 T (Fig. 4B). The data is interpreted as a classical ^{1}H-^{1}H HOHAHA correlation edited by the ^{13}C shift (F_2) of the ^{13}C nucleus bound to the proton giving rise to the F_1 chemical shift. Even at these high magnetic fields, 8.3-kHz CP fields were sufficient to bring about virtually complete spin-system correlation. Simple counting yielded 144 nonoverlapping traces with ^{13}C frequencies in the C_α region for this 164 amino acid protein. It may be stressed here that the sample was in H_2O. Nonetheless, good-quality spectra were obtained. Clearly, the quality can be further improved by using D_2O as solvent. Since very limited phase cycling was required, experimental time could be well utilized for obtaining high resolution in the F_1 and F_2 dimensions. In the spectrum in Fig. 4A the resolution along F_1 is comparable to that available in typical 2D spectra. This is highly desirable in the upfield regions of the spectra where especially proton chemical-shift dispersion is poor. The high resolution is demonstrated by the clear separation of the γ protons of E 122. In the spectrum with a longer ^{13}C-^{13}C HOHAHA mixing time (20 ms), full magnetization transfer over the side chain was accomplished in many cases, as can be seen in Fig. 4B. In order to be able to assess the phase behavior of the experiment, we opted to sample the complete aliphatic ^{13}C region without folding. It is clear from the traces that the phasing in the ^{1}H dimensions is excellent in the experiment. However, there are some phase distortions in the ^{13}C dimension (generally less than 20 degrees) of the diagonal peaks, for the chosen experimental set-up. Optionally, the ^{13}C dimension can be folded to achieve the same resolution with fewer data points along this axis. However, the occurrence of ghost traces in the experiment (see below) may complicate the interpretation of the folded data.

In order to describe the phase characteristics of the experiment and why very limited phase cycling is sufficient for absorption-phase cross peaks we consider the equations governing magnetization transfer by cross-polarization in weakly coupled IS-spin systems. In homonuclear systems, assuming an efficient mixing sequence such that $H_{CP} = 2\pi J\mathbf{I}\cdot\mathbf{S}$, the CP Hamiltonian is
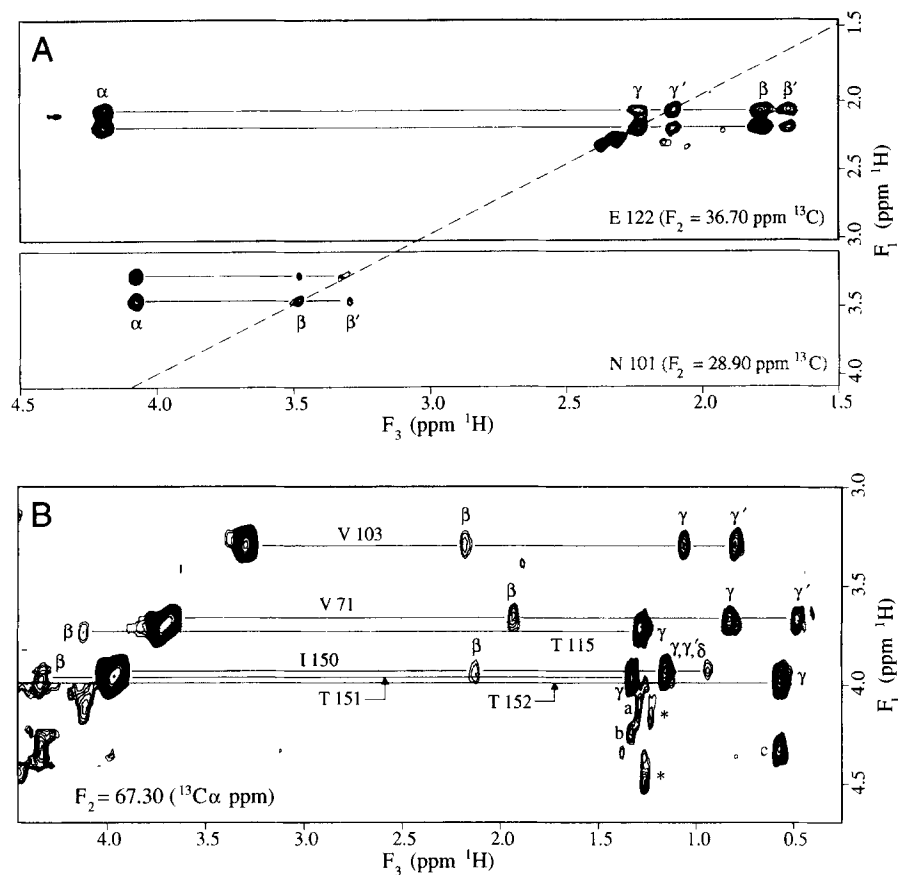
Fig. 4. F_1-F_3 cross sections through a 3D HEHOHEHAHA experiment carried out on ^{13}C labeled T4-lysozyme. Panel (A) is a composite of the $^{13}C_\gamma$ plane of E 122 and the $^{13}C_\beta$ plane of N 101 and shows the resolution available in this experiment. In (B), a $^{13}C_\alpha$ plane is shown, illustrating complete magnetization transfer along the side chain originating from the H_α (diagonal) proton, demonstrating the efficiency of the longer HOHAHA step. The peaks marked (a) and (b) are 'ghosts' of T 115 and T 151, respectively. (c) is cross talk from the $^{13}C_\beta$ plane (67.60 ppm) of T 152, and not a ghost. The peaks marked with a '*' are yet to be assigned.

isotropic and therefore, all magnetization components of spin I will be transferred to spin S for any orientation of the CP field, according to (Braunschweiler and Ernst, 1983):

$$I_\alpha \rightarrow 1/2 \ (1 + \cos2\pi J\tau)I_\alpha + 1/2(1 - \cos2\pi J\tau)S_\alpha + 1/2 \ (2I_\beta \ S_\gamma - 2I_\gamma S_\beta)\sin2\pi J\tau \qquad (1a)$$

and

$$2I_\alpha \ S_\beta \rightarrow 1/2 \ (1 + \cos2\pi J\tau)2I_\alpha S_\beta + 1/2 \ (1 - \cos2\pi J\tau)2S_\alpha I_\beta - 1/2 \ (I_\gamma - S_\gamma)\sin2\pi J\tau \qquad (1b)$$

where (α, β, γ) is a cyclic permutation of (x, y, z). In heteronuclear systems, the CP Hamiltonian is anisotropic (Chingas et al., 1981) and consequently, for spin-lock along an axis α in the doubly rotating frame of spins I and S, only I_α and $I_\beta S_\gamma$ are exclusively transferred to S (Chandrakumar, 1985).

$$I_\alpha \rightarrow 1/2 \ (1 + \cos\pi J\tau)I_\alpha + 1/2 \ (1 - \cos\pi J\tau)S_\alpha + 1/2 \ (2I_\beta S_\gamma - 2I_\gamma S_\beta)\sin\pi J\tau \tag{2a}$$

and

$$2I_\beta S_\gamma \rightarrow 1/2 \ (1 + \cos\pi J\tau)2I_\beta S_\gamma + 1/2 \ (1 - \cos\pi J\tau)2S_\beta I_\gamma - 1/2 \ (I_\alpha - S_\alpha)\sin\pi J\tau \tag{2b}$$

Magnetization transfer is complete at $\tau = 1/(2J)$ for homonuclear transfer and at $\tau = 1/J$ for heteronuclear transfer. Refocused INEPT also requires $\tau = 1/J$ for complete transfer in IS systems. The transfer functions for magnetization transfer in Eqs. 2a,b are altered marginally when cw r.f. fields are replaced by windowless composite pulse sequences (Ernst et al., 1991), but their basic form remains the same.

The above equations indicate that both homonuclear CP (TOCSY/HOHAHA) and heteronuclear CP can give rise to mixed-phase line shapes when timings are not (or cannot be, see below) chosen accurately. For the HEHOHEHAHA experiment undesirable phase characteristics can potentially accumulate at many different steps. Figure 5 shows the possible magnetization-transfer pathways in an I(H)-S(C)-Q(C)-I'(H) fragment. At first glance, it appears that (a) very fine tuning of mixing periods, (b) extensive phase cycling, and (c) an excessive number of trim pulses or z-filters are necessary to obtain pure absorption 3D spectra. Closer inspection reveals, however, that this is not necessary. Many undesirable pathways will be bleached by the anisotropy of the heteronuclear CP. For instance, S_x, generated by path a of arm (A), is not transferred to spin I during τ_3 if the CP field is along the y-axis. In our spectra, this is evidenced by the lack of quadrature images in the ω_2 dimension. Other components such as c will be negligibly small since a two-step transfer $S_xQ_z \rightarrow S_y \rightarrow I_y$ is required during τ_3. Other pathways such as I can, in principle, lead to a $Q_xI_z \rightarrow I_y$ transfer but in practice will be cancelled due to lack of resolvable coupling between Q and I. Finally, any undesirable anti-phase I_yS_z/I_xS_z magnetization during acquisition will be destroyed by the ^{13}C decoupling sequence.

It thus turns out that only a few pathways (c, d, e and f) give rise to detectable magnetization. These are shown in bold lines in Fig. 5. Pathways d and e are absorptive whereas c and f give rise to dispersive ^{13}C components. Paths c and f are minor, since they depend on J_{CC} which is not well developed during t_2; they also have sine-dependent transfer functions during the TOCSY periods. Accordingly, in principle, no trim pulses or extensive phase cycling is necessary for absorption-mode spectra. However, in order to destroy the small dispersive components, a y-trim pulse immediately following τ_1 eliminates the entire pathway B. This also has the effect of reducing the intensity of diagonal peaks since the $S_xI_z \rightarrow I_y$ transfer is blocked. A second y-trim pulse just before τ_2 eliminates the dispersive component S_xQ_z in pathway c as well. For 3D spectra acquired using these two trim pulses, we have found that most peaks can be easily phased to pure absorption. However, some residual phase distortions were observed, especially with intense body diagonals. These distortions may be partially attributed to small relaxation delays between scans and/or incomplete dephasing by the trim pulses. Comparison of this experiment on two different spectrometers showed surprising differences in performance. The difference between the sensitivity of HEHOHEHAHA relative to INEPT-based sequences was much larger on a 600 MHz system (data not shown) than on a 500 MHz system. Also, the effects of ^{13}C trim pulses were vastly different on the two systems. We attribute these differences to different r.f. inhomogeneities of the probes used. It was found that ^{13}C trim pulses resulted in unacceptably reduced sensitivity of the experiment in the 600 MHz system. Thus, we acquired the HEHOHEHAHA spectrum on this
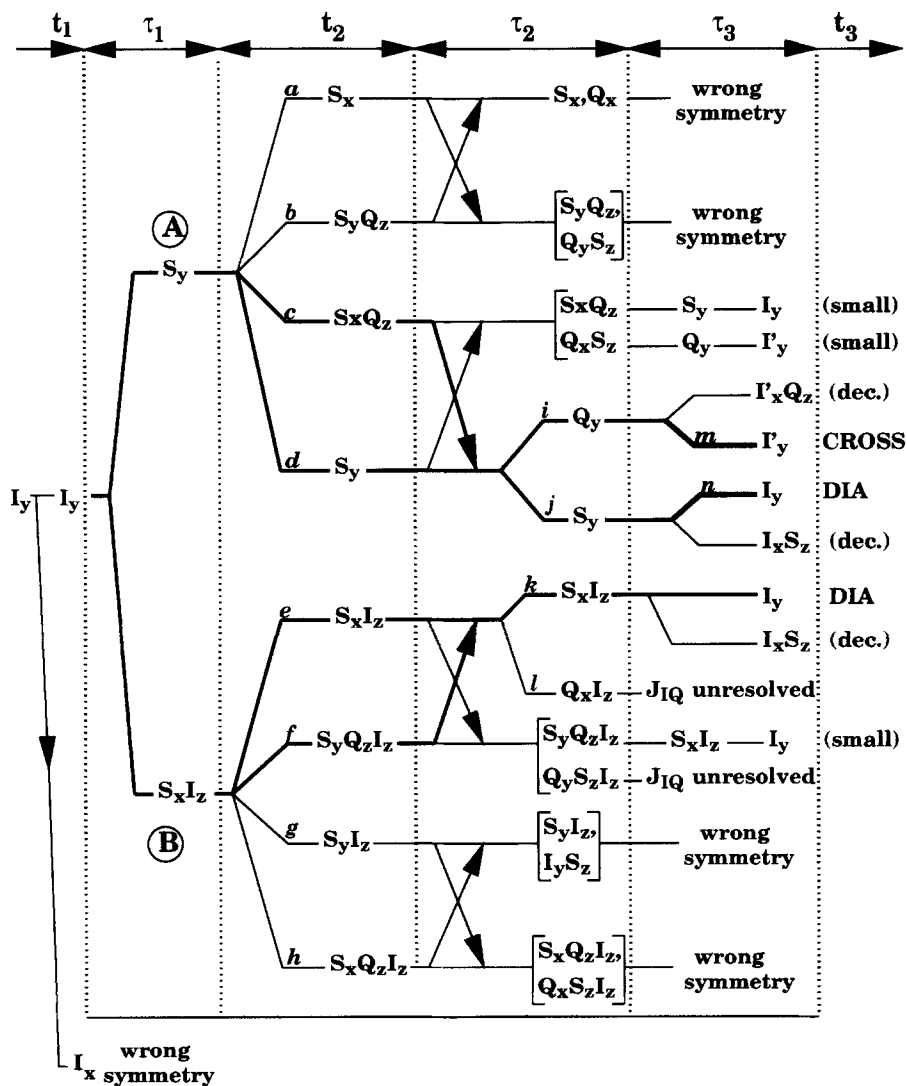
Fig. 5. A 'tree' showing the possible coherence-transfer pathways in a I(H)-S(C)-Q(C)-I'(H) fragment, derived using Eqs. 1 and 2. The most significant pathways contributing to observable magnetization are shown in bold lines. Operator terms labeled '(dec.)' are canceled by decoupling during acquisition. Those marked 'wrong symmetry' are not transferred to the other nucleus during heteronuclear CP.

system without trim pulses (Fig. 4B). The data was fully phaseable for the cross peaks, but more severe distortions were observed on the diagonals. Since the cross peaks were in phase, the spectra without the trim pulses were found to be usable.

The τ dependence of magnetization-transfer functions in refocused INEPT is very different for CH_n (n = 1,2,3) groups (Ernst et al., 1987). Approximately uniform excitation is obtained at $\tau = 0.8/J$ (τ being the total time for an $H_{ny} \rightarrow C_y$ transfer), with transfers between 78% (n = 1) and 93% (n = 3). The τ dependence is more even for these groups in CP experiments, and transfer

functions remain between 50% and 100% in the range 0.5/J–1J (Bertrand et al., 1978) as compared to refocused INEPT, where the transfer function can even become zero in the same range. Most uniform CP transfer is also obtained at $\tau = 0.8/J$, with transfers between 88% (n = 1) and 96% (n = 3). For the current experiments, $\tau_1 \approx 1/J$ was used, which enhances the transfer within CH groups at the expense of methyls and methylenes. This value was chosen because of the limits set upon the mixing time in the DIPSI-3 sequence and the maximum CW power obtainable at the ^{13}C frequency on our spectrometer.

The homonuclear S-Q transfers occurring during the heteronuclear CP pulse trains as evidenced in the 2D spectra are advantageous since additional correlations are obtained. In the 3D experiment as reported here, only the second of these CP periods contributes to such signal enhancement. The first transfer will partially give rise to ^{13}C signals with the 'wrong' ^1H labeling and will thus not add to the integral of the main correlation traces. For instance, magnetization starting on H_β can be transferred via C_β to C_α by the first CP pulse train. One will thus find a 'ghost' trace with H_β ^1H frequency (F_1) on a C_α plane (F_2). We have found that these ghost traces are of low intensity when present (Fig. 4B) and do not interfere with spectral analysis, and can in fact be helpful in this process. Of course, it is possible to keep one of the transfers as an INEPT/RINEPT step to avoid ghosting. However, the intrinsically higher efficiency of cross-polarization transfer as compared to INEPT (Zuiderweg, 1990) would then be sacrificed as well.

CONCLUSIONS

In summary, we have shown that CP-based 2D and 3D HCCH spectroscopy is a good alternative to INEPT-based versions because significant improvements in sensitivity can be obtained with this approach. A further favorable feature of the HEHOHEHAHA experiment is that well-phaseable spectra can be acquired with minimal phase cycles, thus allowing high-resolution sampling in indirect dimensions, and/or the sampling of more dimensions.

ACKNOWLEDGEMENTS

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