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Isotope-Filtered 2D HOHAHA Spectroscopy of a Peptide–Protein Complex Using Heteronuclear Hartmann–Hahn Dephasing

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The NMR spectra of a protein and a tightly bound ligand can be separated by isotope filtering and editing procedures if either the protein or the ligand can be enriched with ^{13}C and ^{15}N (*1–7*). For a cloned protein, overexpressed in *Escherichia coli*, isotopic enrichment is readily accomplished and also greatly facilitates determination of its solution structure by NMR. The ligand frequently is much smaller in size than the protein so that its spectrum can be analyzed by conventional 2D techniques, provided that protein resonances are suppressed by means of isotope filtering. The most commonly used procedures for suppressing the ^1H signal of the isotopically enriched protein involve either spin-echo difference-type methods (*2–5*) or purging of the isotope-attached ^1H signals (*6, 7*). In either of these two approaches, additional delays in the pulse sequence are required to invert the phase of the isotope-coupled ^1H signals relative to the signal of protons not coupled to $^{15}N/^{13}C$ (*2–5*), or to build up antiphase $^1H-\{^{15}N/^{13}C\}$ coherence (*6, 7*). For larger complexes which are subject to rapid transverse relaxation, these additional delays can significantly attenuate the resonance intensity of the unlabeled ligand. Here we present a method for obtaining ^{13}C-filtered HOHAHA spectra of unlabeled ligands which does not require significant purging or filtering delays. The method relies, in part, on heteronuclear dephasing of ^1H magnetization which has an initial phase perpendicular to the effective rotation axis of a homonuclear Hartmann–Hahn-type mixing scheme.

The pulse scheme for the ^{13}C-filtered HOHAHA experiment is sketched in Fig. 1. Aside from the ^{13}C RF pulses, the sequence is virtually identical to the experimental scheme we commonly use for 2D studies of proteins (*8*). After isotropic mixing with a DIPSI-2 scheme for a period T, in-phase transverse y magnetization is flipped back to the z axis at time c. The subsequent delay period, of total duration $T/2$, serves to offset the positive NOE that builds up during the DIPSI mixing scheme (*9*). Provided that indirect NOE effects during the short period $T/2$ are negligible, this method of suppressing NOE contributions in HOHAHA/TOCSY spectra is functionally analogous to the more elegant Clean-TOCSY experiment (*9*). The additional short delay, 2Δ ($\sim$4 ms), between time points b and c was not used in our previous description of the experiment, but as discussed previously (*10*), such a short delay following the isotropic mixing scheme has an effect which, to first order, is similar to lengthening the duration of the mixing period by Δ. Consider, for simplicity, a system of two homonuclear coupled spins, I and K. Isotropic mixing followed by J evolution during the period 2Δ then results in (*10*)

$$2I_y \rightarrow I_y\{\cos(2\pi J_{IK}\Delta) + \cos[2\pi J_{IK}T + \Delta]\} + K_y\{\cos(2\pi J_{IK}\Delta) - \cos[2\pi J_{IK}(T + \Delta)]\} + \text{other terms}, \quad [1]$$

where "other terms" refers to antiphase magnetization at time c. Provided $J_{IK}(T + \Delta) < 1/2$, the amount of in-phase K-spin magnetization increases during the time 2Δ, thereby increasing cross-peak intensity. Hence, the 2Δ period in the pulse scheme of Fig. 1 does not merely serve as a heteronuclear filtering delay but also can be considered as part of the magnetization-transfer period.

Heteronuclear filtering in the scheme in Fig. 1 is obtained by three different filters, two of which involve heteronuclear dephasing during the ^1H isotropic mixing scheme and a third one, during the delay 2Δ, which is of the conventional purging type (*6, 7*). Heteronuclear dephasing during homonuclear isotropic mixing, to the best of our knowledge, has not been previously used in high-resolution NMR and is briefly discussed below. Although, at first sight, the simultaneous irradiation with two synchronized DIPSI-2 schemes on the ^1H (I spin) and ^{13}C (S spin) channels appears similar to heteronuclear cross polarization, the in-phase ^1H magnetization in the present case is aligned *perpendicular* to the axis along which RF irradiation takes place and equations previously derived for heteronuclear cross polarization (*11, 12*) are not applicable. In the present case, there is no net transfer of magnetization from ^1H to ^{13}C; instead, as shown below, in-phase ^1H magnetization (I_y) evolves into antiphase I_xS_z

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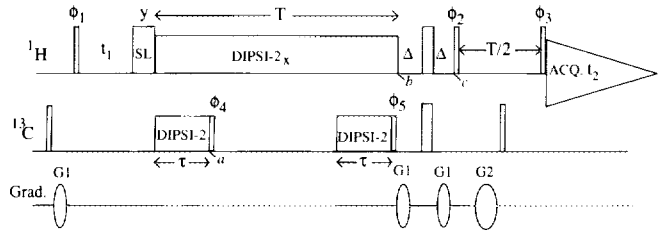


FIG. 1. Pulse scheme of the ^{13}C-filtered 2D HOHAHA experiment. Narrow pulses correspond to 90° flip angles and wide pulses to 180°. Unless indicated, all pulses (including the DIPSI pulses) are applied along the x axis. SL denotes a short (1 ms) spin lock (trim pulse). Gradient pulses are sine-bell shaped with a strength of 10 G/cm at their center. Gradients G1 (1 ms) and G2 (5 ms) have opposite polarity. Phase cycling is as follows: $\phi_1 = x, -x$; $\phi_2 = 4(x), 4(-x)$; $\phi_3 = 8(x), 8(y), 8(-x), 8(-y)$; $\phi_4 = 2(y), 2(-y), \phi_5 = 8(x), 8(y), 8(-x), 8(-y)$; Rec. $= P, -P, Q, -Q, -P, P, -Q, Q$, with $P = x, -x, x, -x$ and $Q = y, -y, y, -y$. Quadrature in the t_1 dimension is obtained by changing ϕ_1 in the regular States–TPPI manner.

magnetization when the DIPSI RF field is applied along the x axis.

The truncated Hamiltonian for a heteronuclear two-spin I–S system during the application of this mixing scheme equals $\mathcal{H} = (1/2)J_{IS}(I_yS_y + I_zS_z)$. Using the commutator relationship $[I_yS_y + I_zS_z, I_y] = -iI_xS_z$, one finds after application of the synchronized DIPSI$_x$ scheme for a time τ

$$I_y \rightarrow I_y\cos(\pi J_{IS}\tau/2) - 2I_xS_z\sin(\pi J_{IS}\tau/2). \quad [2]$$

For an I_2–S spin system, one obtains

$$I_y^1 \rightarrow (1/2)(I_y^1 + 4I_x^1I_y^2S_x)\cos(\pi J_{IS}\tau\sqrt{2}) - \sqrt{2}I_x^1S_z\sin(\pi J_{IS}\tau\sqrt{2}) + (1/2)(I_y^1 - 4I_x^1I_y^2S_x). \quad [3]$$

Calculations for a methyl group (I_3–S) are lengthy, but a simple result is obtained when considering the effect of the mixing scheme on the total y magnetization $(I_y^1 + I_y^2 + I_y^3)$,

$$\begin{aligned}(I_y^1 + I_y^2 + I_y^3) \rightarrow (I_y^1 + I_y^2 + I_y^3)\{&(1/6)\cos(\pi J_{IS}\tau/2) \\ &+ (1/4)\cos(\pi J_{IS}\tau\sqrt{3}/2) \\ &+ (7/12)\cos(\pi J_{IS}\tau)\cos(\pi J_{IS}\tau\sqrt{3}/2) \\ &+ (1/\sqrt{3})\sin(\pi J_{IS}\tau)\sin(\pi J_{IS}\tau\sqrt{3}/2)\} \\ &+ \text{other terms.} \quad [4]\end{aligned}$$

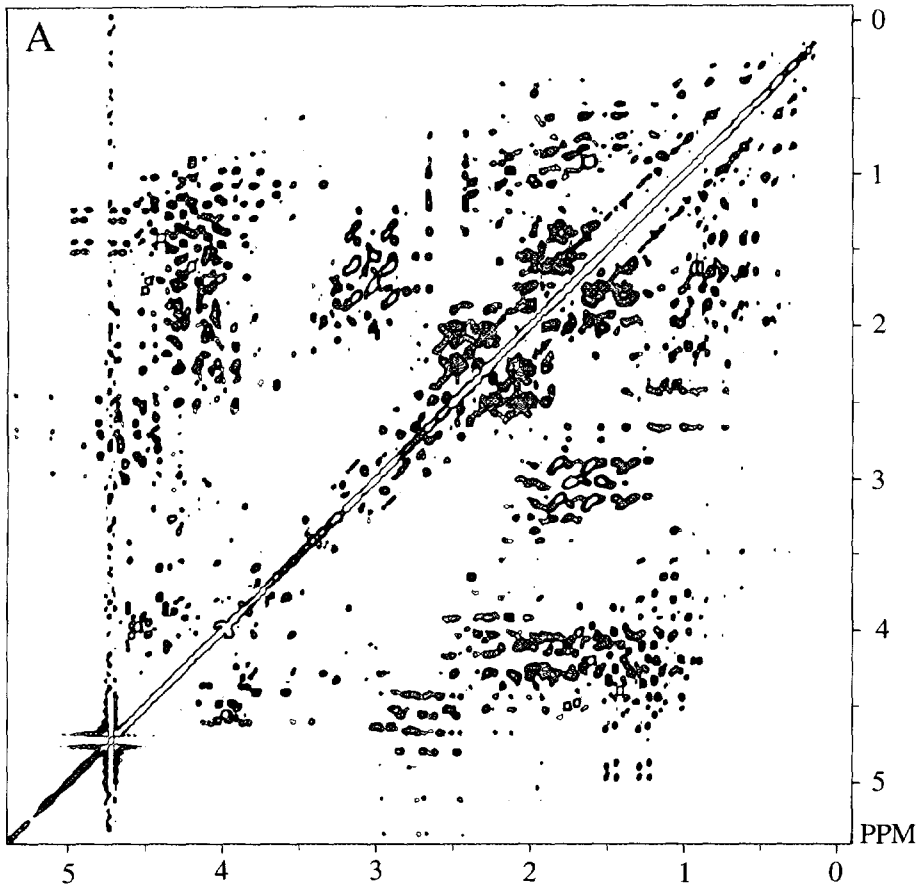
where other terms refer to unobservable operator products, most of which contain an S_z, S_x, or S_y term. The result of Eq. [4] agrees with the time dependence of $I_y^1 + I_y^2 + I_y^3$ obtained from a numerical simulation, using the program ANTIOPE (*13*).

After heteronuclear dephasing has been applied for a period τ (time a), a phase-cycled ^{13}C purge pulse is applied to destroy any operator products that contain an S_z or S_x term. For methyl groups, operator products containing S_y are not eliminated by this purge pulse, but for the short duration of τ used (9 ms), such operator products have very small coefficients and may be ignored. The optimal duration for τ differs for methine ($1/^1J_{CH}$), methylene ($\sqrt{2}/^1J_{CH}$), and methyl groups. As can be seen from Eq. [5], methyl groups have a first local minimum of ~0.2 near $\tau = 1.3/^1J_{CH}$ and a first null near $3.2/^1J_{CH}$. This behavior is quite different from that observed during heteronuclear cross polarization (*11, 12, 14–16*), for which the maximum reduction in CH_2 and CH_3 intensity would be 1/2 and ~1/3, respectively. Heteronuclear cross polarization in the present scheme will occur if the phase of the ^{1}H isotropic mixing period is changed by 90° relative to the preceding trim pulse and the succeeding 90° ^{1}H pulse, applied at time c.

The heteronuclear dephasing filter is applied twice, once at the beginning and once at the end of the HOHAHA mixing period. The first functions primarily on protons which are labeled with the F_1 frequency whereas the second filter acts primarily in the F_2 dimension. In practice, we used three repeats of the DIPSI-2 (*17*) cycle for heteronuclear dephasing and a total of 15 cycles for homonuclear isotropic mixing. Due to RF inhomogeneity and offset effects, heteronuclear dephasing occurs at rates that are somewhat slower than predicted by Eqs. [2]–[4]. Experimentally, a heteronuclear dephasing period of 9 ms was optimal for minimizing the H$^\alpha$ signal in a sample of uniformly ^{13}C-enriched alanine, with the ^{13}C carrier positioned at 43 ppm. Under these conditions, methine carbons are attenuated much more than methylene and methyl groups. Therefore, the delay Δ of the last purge filter was adjusted to optimize suppression of these signals by optimizing for a relatively small $^1J_{CH}$ value of 128 Hz (Δ = 1.95 ms).

The experiment in Fig. 1 is demonstrated for a sample containing 1 mM protein calmodulin (CaM), isotopically uniformly enriched (>97%) in ^{13}C and ^{15}N, complexed with 4 mM Ca^{2+} and 1 mM unlabeled 26-residue peptide fragment of skeletal muscle myosin light chain kinase, commonly

FIG. 2. The 600 MHz 2D HOHAHA spectrum of a 1 mM calmodulin–M13 complex, recorded with the scheme in Fig. 1, (A) omitting the ^{13}C pulses and (B) with the ^{13}C filtering in Fig. 1. The mixing time (T) was 45 ms. Each spectrum results from a 300* × 1024* data matrix, with 2 × 32 scans for each complex t_1 increment and a total measuring time of 7.5 hours per spectrum. The ^{1}H RF field strengths for the high-power pulses and for the DIPSI mixing were 25 and 9.6 kHz, respectively. For ^{13}C all pulses were applied using a 9.6 kHz RF field. Between scans, presaturation with a very weak RF field (~3 Hz) was used to reduce the intensity of the residual HDO resonance.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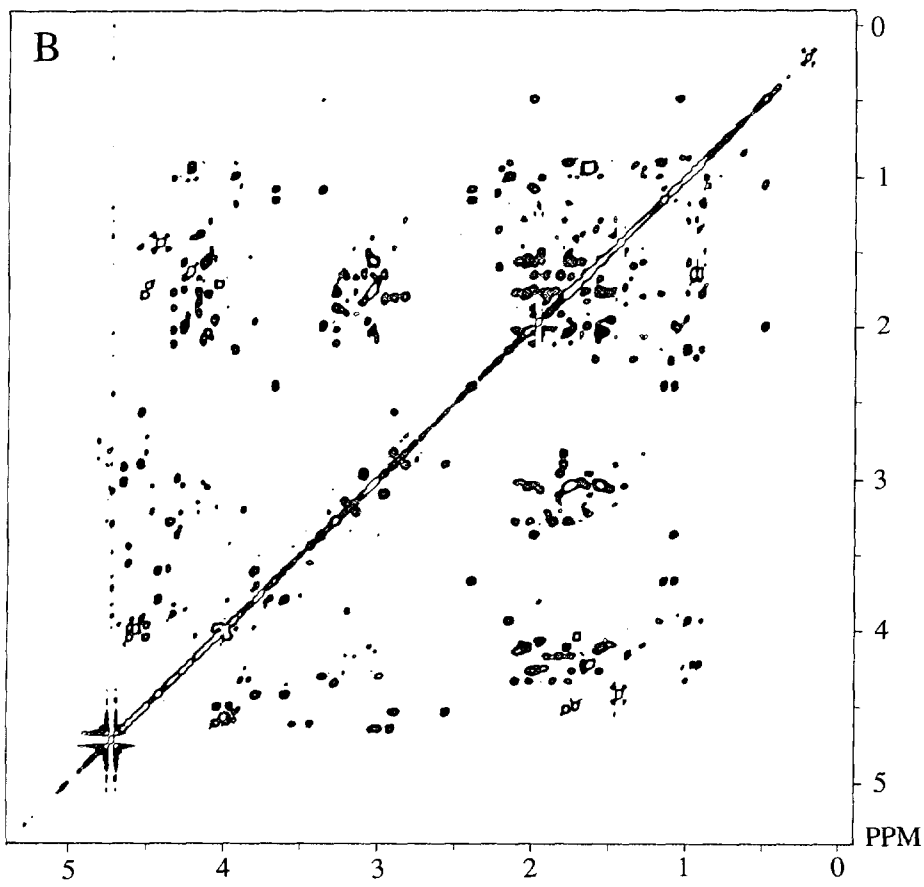
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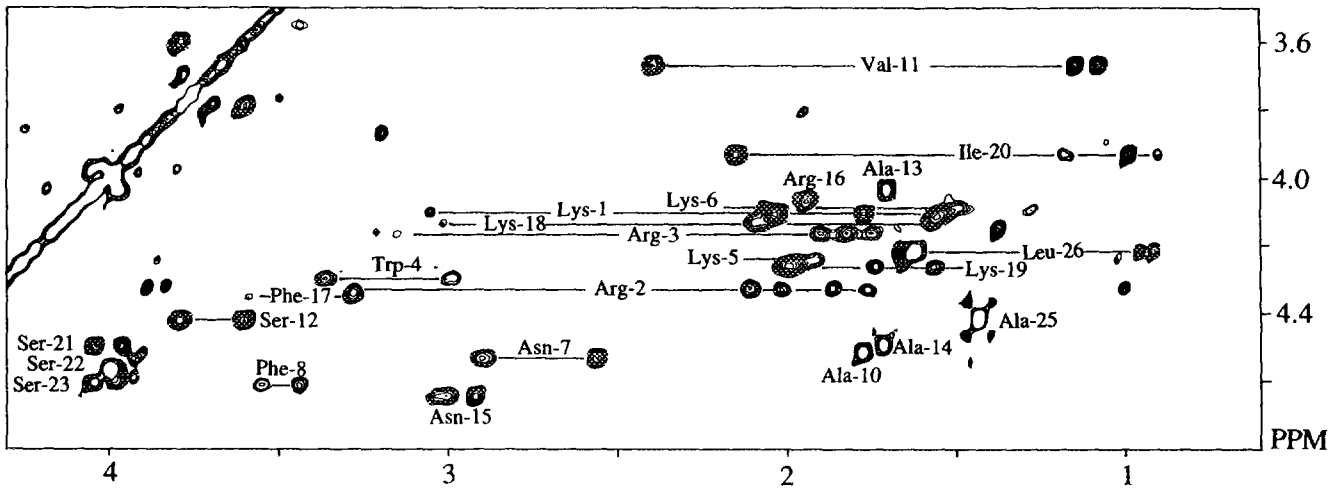

FIG. 3. Expansion of the spectral region of Fig. 2B which shows the connectivities between H^{α} and side-chain resonances.

referred to as M13 (*18*). The three-dimensional structure of the 20 kDa CaM–M13 complex has been solved by NMR (*19*) and, using a peptide homologous to M13, by X-ray crystallography (*20*). The sample was dissolved in D_2O, pH 6.8. Experiments were conducted on a Bruker AMX-600 spectrometer, equipped with a triple-resonance probehead and a pulsed-field-gradient accessory. The temperature of the air regulating the sample temperature was set to 30°C, but due to RF heating the actual sample temperature was higher by about 3°C (*21*).

Figure 2 compares the 2D HOHAHA spectrum of the calmodulin–M13 complex in the absence (Fig. 2A) and in the presence (Fig. 2B) of ^{13}C filtering. Each spectrum was acquired in 7.5 hours. Both spectra were recorded under identical conditions, except that for Fig. 2A the ^{13}C amplifier was turned off; i.e., no ^{13}C pulses were applied. The duration of the isotropic mixing period was 45 ms, using 15 DIPSI-2 cycles with ^{1}H and ^{13}C RF field strengths of 9.6 kHz. Clearly, the abundance of calmodulin cross-peak signals present in Fig. 2A has been effectively suppressed in Fig. 2B by the application of the three independent ^{13}C filters.

Figure 3 shows an expanded region of the connectivities involving M13 H^{α} protons. The high quality of the ^{13}C-filtered HOHAHA spectrum made it possible to obtain nearly complete assignments for the peptide side chains, even for many of the lysine and arginine residues. For Lys^{1} and Lys^{18}, for example, J connectivity between H^{α} and H^{ϵ} protons is observed, and, similarly, correlations between H^{α} and H^{δ} are present for Arg^{2} and Arg^{3}, suggesting that the side-chain resonances of these four residues are considerably narrowed by rapid conformational averaging.

The ^{13}C-filtered HOHAHA pulse scheme described above is an extremely effective experiment for obtaining resonance assignments of unlabeled ligands bound tightly to a ^{13}C-enriched protein. The prerequisite is, of course, that the transverse relaxation rates of the ligand in its complexed state be sufficiently long to permit coherence transfer via homonuclear ^{1}H J couplings. Considering that nearly all possible side-chain connectivities are observed for the 20 kDa calmodulin–peptide complex, we anticipate that the technique is applicable to even larger complexes. Indeed, preliminary experiments indicate that the experiment is useful for significantly larger complexes between DNA and DNA-binding proteins.

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