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A New Multi-quantum Version of the HBHA(CBCACO)NH Experiment with Enhanced Sensitivity for Partially Deuterated Samples

Ruth M. Gschwind,[1] Horst Kessler, and Gerd Gemmecker[2]

Institut für Organische Chemie und Biochemie, TU München, Lichtenbergstrasse 4, D-85747 Garching, Germany

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A new multi-quantum version of the HBHA(CBCACO)NH experiment for partially deuterated protein samples is presented. The method is based on the significant reduction of the proton and carbon relaxation rates due to multi-quantum delays in highly deuterated proteins recently published by our group. The introduction of a multi-quantum period in the coherence transfer pathway of the HBHA(CBCACO)NH experiment yields a dramatic increase of sensitivity—on average 46% with a 75% deuterated sample of the homodimeric 31 kDa *E. coli* IIA^{Man} domain. Additional resolution in the proton dimension can be achieved by a *double time shared* approach keeping the ^{1}H single-quantum period at a minimum.

Key Words: **triple-resonance NMR; partially deuterated proteins; multi-quantum; proton side-chain assignment.**

INTRODUCTION

Recently, the use of partially deuterated samples has become the method of choice for structural investigations of larger proteins (≥20 kDa) by NMR (*1*). Partial deuteration causes a significant reduction of the transverse relaxation rates and signal linewidths, due to the substantially smaller gyromagnetic ratio γ of deuterium relative to protium (*2, 3*). This leads to a drastic improvement of resolution and signal intensity, the major problems when dealing with NMR spectra of large molecules. A wealth of triple-resonance NMR techniques has been developed for the backbone assignment of partially deuterated proteins (*4–6*), as well as for the ^{13}C assignment of side-chains (*7*).

However, the effect of partial deuteration on spectra of carbon-bound (i.e., $^{1}H^{\alpha}$ and side-chain) *protons* is much smaller than on ^{13}C signals. The explanation is that substitution of the carbon-bound ^{1}H by a ^{2}H spin effectively reduces the ^{1}H, ^{13}C dipolar couplings as the main relaxation source for the ^{13}C atom; however, for the relaxation of a ^{13}C-bound *proton* the contribution of the ^{13}C spin (not affected by partial deuteration) is in the same order of magnitude as the contribution of neighboring protons (which is greatly diminished by partial deuteration).

MULTI-QUANTUM TECHNIQUES

It has been known for many years that—in the slow tumbling limit—the dipolar coupling between two nuclei can be rendered ineffectual by creating a two-spin multi-quantum (MQ) coherence of the two spins (*8*). This effect has been exploited in the case of ^{1}H–^{15}N spin pairs in proteins (*9*); also, for RNA with its relatively low proton density, a significant intensity gain in ^{1}H–^{13}C MQ vs SQ experiments has recently been demonstrated (*10*). However, ^{1}H–^{13}C MQ versions have rarely been applied to proteins, because the inevitable evolution of ^{1}H, ^{1}H J couplings during MQ periods often tends to neutralize the relaxation gains (*11*); double ^{1}H, ^{13}C constant time MQ versions have recently been shown to retain at least a small (10%) intensity gain for $^{13}C^{\alpha}$ over the corresponding single-quantum (SQ) techniques (*12*). An exception are the numerous $^{1}H^{\alpha}$–$^{13}C^{\alpha}$ MQ techniques recently published for otherwise completely deuterated protein samples (*13*).

Recently, we showed in the example of the ed-H(CCO)NH-TOCSY experiment and a HMQC/HSQC comparison that the use of MQ periods instead of SQ periods can yield an increase of sensitivity in the case of the *partially deuterated* samples now widely used in protein NMR (*14*). In this Communication, we demonstrate with a new version of the HBHA(CBCACO)NH experiment that dramatic sensitivity gains from MQ periods are not limited to pulse sequences containing long editing delays, but can also be realized by implementation in the coherence transfer pathway of common triple resonance experiments.

In the case of a sample with a considerable level of random deuteration, ^{1}H–^{13}C spin pairs can be interpreted in reasonable approximation as two-spin systems, since the effects of other nuclei are now becoming negligible relative to the ^{1}H–^{13}C dipolar coupling (other neighboring protons being mostly substituted by ^{2}H). Thus the transformation of a ^{1}H SQ period to a ^{1}H, ^{13}C MQ period should now greatly reduce relaxation losses, since it removes the only remaining major relaxation source for the proton (i.e., the directly bound ^{13}C spin).

[1] Present address: Fachbereich Chemie, Philipps–Universität Marburg, Hans Meerwein-Straße, D-35032 Marburg.

[2] To whom correspondence should be addressed. E-mail: Gerd.Gemmecker@ch.tum.de.

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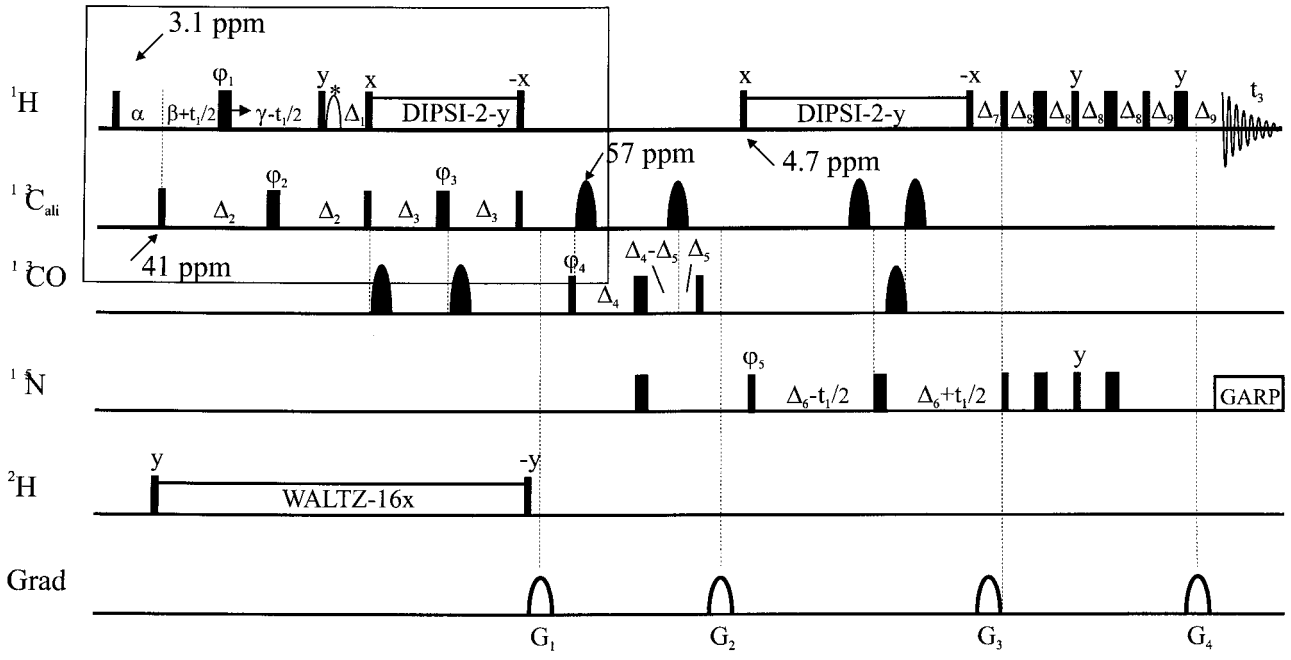

FIG. 1. Pulse sequence for the new MQ-HBHA(CBCACO)NH experiment. The boxed section can be substituted by any of the three versions (a)–(c) shown in Fig. 2, yielding the standard time-shared SQ experiment, the new constant time MQ version (shown here) and the new double time-shared MQ experiment, respectively. All 90° (180°) pulses are represented by narrow (wide) rectangles, HERMITE-shaped selective inversion pulses (*18*) for aliphatic and carbonyl ^{13}C (220 μs) by solid sine bells. The rectangular pulses on aliphatic $^{13}C^{ali}$ and ^{13}CO were calibrated to provide a null in their excitation profiles at the ^{13}CO and $^{13}C^{ali}$ frequencies, respectively. Quadrature detection in F1 was achieved by States-TPPI cycling of the first ^{1}H pulse (*19*), γ is decremented. The delay settings are as follows: $\alpha = 3$ ms, $\beta = 0.6$ ms, $\gamma = 3.6$ ms, $\Delta_1 = 2.0$ ms, $\Delta_2 = 3.1$ ms, $\Delta_3 = 3.7$ ms, $\Delta_4 = 13.5$ ms, $\Delta_5 = 4.55$ ms, $\Delta_6 = 13.5$ ms, $\Delta_7 = 5.6$ ms, $\Delta_8 = 2.65$ ms, $\Delta_9 = 1.5$ ms. Magic angle gradients (*20*) were used for quadrature detection in F2 based on the echo–antiecho scheme (*21*) and artefact suppression. The gradient strengths (z component) in percent of the full strength (ca. 70 G/cm) were as follows: $G_1 = 30\%$, $G_2 = 70\%$, $G_3 = 70\%$ and $G_4 = 7.1\%$; the phase cycle is: $\varphi_1 = 4(y), 4(-y)$; $\varphi_2 = 4(x), 4(y), 4(-x), 4(-y)$; $\varphi_3 = 8(x), 8(-x)$; $\varphi_4 = 2(x), 2(-x)$; $\varphi_5 = (x), (-x)$; $\varphi_{rec} = x, 2(-x), x, -x, 2(x), -x$. The open sine bell marked with an asterisk is an optional selective 90° pulse to change the experiment to a water flip-back version; in this case the ^{1}H carrier frequency must be set to the water frequency.

Indeed, we were able to observe promising intensity gains in 2D ^{1}H, ^{13}C heteronuclear MQ correlation (HMQC) experiments of 75% deuterated protein samples over the SQ version (HSQC) (*14*). However, the most important applications for such MQ techniques will be more complicated 3D NMR spectra that routinely require measuring times of several days. Many of these experiments contain relatively long (i.e., tens of milliseconds) fixed delays for the evolution or refocusing of heteronuclear couplings. Such periods can be advantageously combined with ^{1}H indirect evolution times into ^{1}H, ^{13}C MQ periods with superior relaxation qualities, without compromising the intensity gains by increasing the overall length of the pulse sequence.

THE MQ-HBHA(CBCACO)NH EXPERIMENT

Here the advantages of MQ periods in heteronuclear NMR experiments with partially deuterated protein samples will be demonstrated on a new version of the HBHA(CBCACO)NH technique (Fig. 1). This standard experiment for the assignment of side-chain proton resonances correlates the H^α and H^β shifts of amino acid i with the $^{15}N/^1H^N$ shifts of the following amino acid $i + 1$ (*15*). The pulse sequence starts with an evolution time for the H^α and H^β spins, then the magnetization is transferred onto the directly bound C^α and C^β resonances. During the following ^{13}C–^{13}C COSY transfer step, C^β magnetization is partially transferred onto C^α, while part of the C^α magnetization stays there. The magnetization is then further transferred from the $^{13}C^\alpha$ spin via the carbonyl carbon onto the amide nitrogen and proton of the next amino acid.

This transfer path lends itself easily to the employment of MQ coherences. In the standard version (Fig. 2a) the time-shared proton evolution period (*16*) is followed by the $^1H \rightarrow {}^{13}C$ transfer step and the delay for the evolution of the $^{13}C^\beta$–$^{13}C^\alpha$ coupling (in preparation for the $^{13}C^\beta \rightarrow {}^{13}C^\alpha$ transfer step). These two delays can be combined to a constant time MQ period (Fig. 2b) without adding additional delays to the pulse sequence, which could otherwise partially offset the intensity gain from the favorable MQ relaxation. In fact, for the first ^{1}H increment both pulse sequences *2a* and *2b* are identical in length, but in the standard sequence *2a* the overall length increases due to the time-shared t_1 incrementation, while the length of MQ sequence *2b* stays constant.

For this kind of multi-quantum approach the maximum length of the ^{1}H evolution time (and, hence, the resolution in the ^{1}H dimension) is limited by the duration needed for the evolution of the $^{13}C^{\beta}$–$^{13}C^{\alpha}$ coupling. For the standard setting $2\Delta_2 = (4\ ^1J_{CC})^{-1}$ this amounts to ca. 7.2 ms, corresponding to a ca. 70 Hz resolution in the $^1H^{\alpha}/^1H^{\beta}$ dimension (after zero-filling).

For a better resolution, the ^{1}H evolution period can be converted from *constant time* to a double *time-shared* approach (cf. Fig. 2c). This is an extension of the "shared time" evolution (*16*), which combines the chemical shift evolution with a scalar transfer period (e.g., $^1J_{HC}$). In our version, both the $^1J_{HC}$ and the following $^1J_{CC}$ evolution delay are used for chemical shift evolution. The ^{1}H chemical shifts evolve during a period $(a' + b' + b' - c' - \alpha' + \gamma')$ (cf. Fig. 2c), whereas $^1J_{HC}$ evolves during $(a' + b' - b' + c')$. In subsequent t_1 increments a' is incremented by $a'(initial)/k_H$, α' decremented by $a'(initial)/k_H$, c' decremented by $c'(initial)/k_H$ and γ' incremented by $\gamma'(initial)/k_H$ (with k_H equal to the number of ^{1}H increments plus one). The additional resolution compared to the constant time version (cf. Fig. 2c) is achieved by the incrementation of the remaining b' delays *by (dwelltime/* $2\text{-}(a'(initial)/k_H + \alpha'(initial)/k_H)$). Thus the ^{1}H evolution time can be extended to the desired degree, while the necessary additional ^{1}H SQ periods ($2b'$ in Fig. 2c) are kept at a mini-

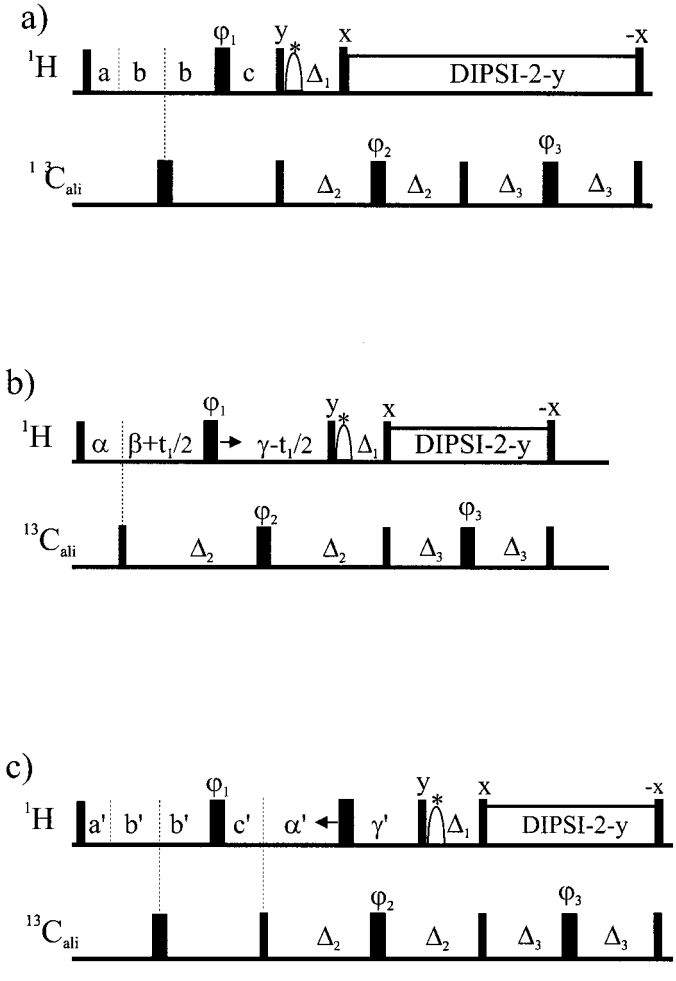

FIG. 2. Modules for the ^{1}H evolution period (boxed part of pulse sequence 1): (a) time-shared standard SQ version used for comparison, with the additional delays set to a = 1.5 ms, b = 2 μs, c = 1.5 ms; (b) new constant time MQ version for optimum signal intensity (cf. Fig. 1); (c) new double time-shared MQ version for enhanced resolution, with the additional parameters: a' = 1.5 ms, b' = 2 μs, c' = 1.5 ms; α' = 2.1 ms, γ' = 2.1 ms (for incrementation scheme see text).

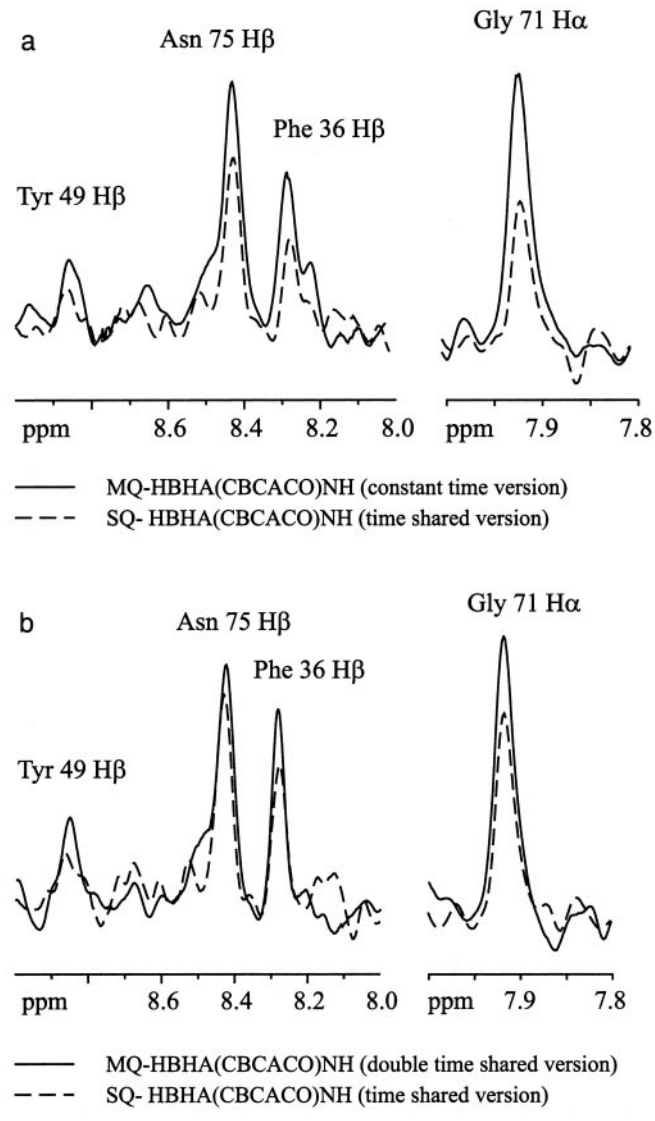

FIG. 3. Representative traces from pseudo-3D MQ-HBHA(CBCA)CONH experiments (solid line), compared to a SQ-HBHA(CBCA)CONH experiment with shared time evolution in the indirect dimension (dashed line). The spectra were recorded on a [U-^{15}N/^{13}C/75%/^{2}H]-labeled sample of the IIA^{Man} dimer of *E. coli* (*17*) (31 kDa, dimer concentration 0.9 mM, 310 K). Each experiment was recorded as 2D ^{1}H, ^{1}H plane with 52 points in the indirect ^{1}H dimension and 512 scans (measuring time ca. 22 h each). (a) Constant time MQ version (sequence *2b*) with 7.2 ms evolution time, average gain in intensity 46%; (b) double time shared MQ version (sequence *2c*) for enhanced resolution, evolution time 16 ms, average gain in intensity 23%.

mum to reduce relaxation losses. The intensity gain of this version will, however, be lower than for the *constant time* version (Fig. 2b), according to the relation between the lengths of the SQ and MQ periods used.

RESULTS

Both versions of the new MQ-HBHA(CBCACO)NH experiment were tested on a 75% deuterated sample of the homodimeric 31 kDa IIA^{Man} domain of *E. coli* (*17*). The *constant time* MQ version (pulse sequence 2b) yielded on average 146% of the signal intensity of the standard SQ version (pulse sequence 2a), while the time-shared MQ version (Fig. 2c), allowing for a higher resolution in the ^1H dimension, still gave on average 123% intensity (for an evolution time of 16 ms, i.e., 31 Hz resolution). (See also Fig. 3.)

In summary, the comparison of the new MQ version to the standard SQ version of the HBHA(CBCACO)NH experiment shows that, for partially deuterated proteins, the introduction of MQ periods in the coherence transfer pathway of triple resonance experiments can indeed yield significant intensity gains in protein NMR spectra. It can be expected that similar results will be achieved for the corresponding MQ versions of several other NMR experiments.

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