

Heteronuclear X-Filter ^1H PFG Double-Quantum Experiments for the Proton Resonance Assignment of a Ligand Bound to a Protein

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A novel X-filter experiment based on ^1H PFG DQ spectroscopy is described. Excellent suppression of proton bound to ^{13}C and ^{15}N is achieved. Successful application of the method to a protein–ligand complex is demonstrated. © 1998 Academic Press

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Isotope-edited NMR experiments have been shown to be very useful for studying the inter- and intramolecular interactions in macromolecular complexes (1–16). In this type of experiments one of the two components forming the complex is labeled with ^{13}C and/or ^{15}N isotopes. The X filter then selects the magnetization which originated solely from one of the two components. The resulting ^1H spectra contain a reduced number of resonances and therefore are amenable to detailed spectral analysis. For protein–ligand complexes it is usually the protein which is labeled with the isotope. This is because the labeling of the ligand can sometimes be chemically very difficult and/or prohibitively expensive. Several methods have been proposed for obtaining X-filter spectra. These can be divided into two classes: experiments which involve spin-echo difference spectroscopy (5) and experiments which use purging of the isotope-attached ^1H magnetization (8, 9). Both methods require additional delays in the pulse sequence in order to create heteronuclear antiphase magnetization. A third method which has been used in the ^{13}C -filter ^1H TOCSY experiment achieves ^{13}C -bound proton suppression via heteronuclear dephasing during the homonuclear isotropic mixing period (14).

The different values of the heteronuclear one-bond J_{CH} coupling constant in proteins represent an intrinsic difficulty in the application of ^{13}C -filter experiments. These scalar couplings range from about 125 Hz for methyl groups to about 170 Hz for aromatic spins (for histidine the coupling is 220 Hz). In order to improve the suppression of protons attached to ^{13}C , pulse sequences containing double filters, tuned to two different $^1J_{\text{CH}}$ values, have been proposed (9, 10, 15, 16).

In this Communication we report pulse sequences based on the purging schemes, homospoil gradients, and coherence

selection gradients which achieve excellent ^{13}C -bound proton suppression. The pulse sequences for different variants of this experiment are shown in Fig. 1. The schemes in Figs. 1a and 1b are the ^{13}C -filter 2D double-quantum (DQ) experiments with and without coherence selection gradients, respectively. The experiment in Fig. 1a is based on the previously reported phase-sensitive ^1H PFG DQ experiment (17). For completeness we report in Fig. 1c the ^{13}C -filter PFG-refocused ^1H DQ experiment. The ^{13}C -filter refocused ^1H DQ experiment without coherence selection gradients is easily derived from the schemes of Fig. 1b and 1c. When triple-axis gradients are available the three coherence selection gradients should be gradient tilted at the magic angle (MAG). The use of MAGs results in the complete suppression of the H_2O signal (17, 18) due to the zeroing of the demagnetizing field effect (19, 20). Proper phase cycling of the first ^1H 90° pulse is used in the version of the experiment with read-out pulse $\beta = 45^\circ$ or $\beta = 60^\circ$ (antiecho selection) and $\beta = 135^\circ$ or $\beta = 120^\circ$ (echo selection) to reduce the effects of radiation damping during the acquisition period (18). In the excitation DQ period two PFGs of equal sign and intensity and one or two 90° ^{13}C pulses are applied. The two gradients have several functions. First they destroy heteronuclear multiple-quantum coherence created by the ^{13}C pulses, second they act like an EXORCYCLE scheme (21, 22) in refocusing only proton coherence inverted by the 180° pulse, and finally they inhibit the creation of H_2O DQ coherence via the radiation-damping mechanism (23, 24). The scheme of Fig. 1a shows also how to perform simultaneous suppression of protons attached to ^{15}N and ^{13}C .

In order to understand the function of these pulse sequences, let us consider the spin system I–K, where I and K represent two protons scalarly coupled with a coupling J^{IK} . We will then consider the behavior of the relevant terms, using the product operator formalism (25–27), in the presence and absence of the heteronuclear spins S and S'. These spins are coupled to spin I and spin K with heteronuclear scalar couplings $^1J_{\text{IS}}$ and $^1J_{\text{KS}'}$, respectively. Furthermore we assume that the heterospins S and S' are scalarly coupled with a coupling $^1J_{\text{SS}'}$.

The amount of DQ coherence between spins I and K

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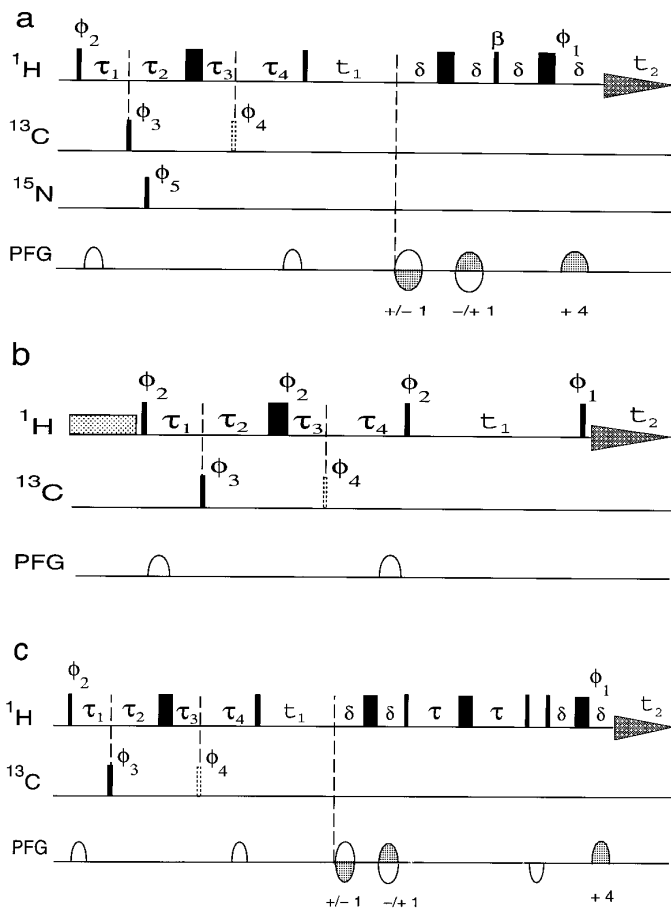


FIG. 1. Pulse sequences for the 2D ^{13}C -filter DQ experiment with coherence selection gradients (a) and with phase cycling (b). Pulse sequence in (c) is the 2D ^{13}C -filter PFG-refocused DQ experiment. Narrow and wide bars correspond to 90° and 180° hard pulses, respectively. The experiment in (a) can be recorded with $\beta = 90^\circ/90^\circ$, with $\beta = 45^\circ/135^\circ$, or with $\beta = 60^\circ/120^\circ$. The phases are $\phi_1 = 8(x), 8(-y), 8(-x), 8(y), \phi_2 = 4(x), 4(-x), \phi_3 = (x, -x), \phi_4 = 2(x), 2(-x), \phi_5 = 32(x), 32(-x), \phi_{\text{rec}} = 4(x), 8(-x), 4(x)$ for the experiment in (a) recorded with $\beta = 90^\circ/90^\circ$ and for the experiment in (c). $\phi_1 = 4(x), 4(-y), 4(-x), 4(y), \phi_3 = (x, -x), \phi_4 = 2(x), 2(-x), \phi_5 = 16(x), 16(-x)$ for the experiment in (a) with $\beta = 45^\circ/135^\circ$ or $(60^\circ/120^\circ)$. The phases of the first ^1H 90° pulse and the receiver are in (a) $\phi_2 = x, \phi_{\text{rec}} = 4(x), 4(-x)$ for $\beta = 45^\circ$ (or 60°) and $\phi_2 = -x, \phi_{\text{rec}} = 4(-x), 4(x)$ for $\beta = 135^\circ$ (or 120°). The phases in (b) are $\phi_1 = (x), \phi_2 = 2(x), 2(y), 2(-x), 2(-y), \phi_3 = (x, -x), \phi_4 = 8(x), 8(-x), \phi_{\text{rec}} = 2(x), 2(-x)$. Furthermore half or an entire cycle of CYCLOPS (33) can be applied in (b) by incrementing all ^1H pulse phases and the receiver in steps of $\pi/2$. The length $|\tau_1 + \tau_2|$ which is equal to $|\tau_3 + \tau_4|$ corresponds to half the DQ excitation period. The delay τ_1 is set to $1/(2J_{\text{CH}})$ and τ_4 is set to $1/(2J'_{\text{CH}})$, where $J_{\text{CH}} \neq J'_{\text{CH}}$. As described in the text, an additional third ^{13}C 90° pulse can be included after a delay τ_5 from the first ^{13}C 90° pulse. The first two gradients, applied at the beginning and end of the DQ excitation period, are short and weak PFGs of equal sign and strength. The last three PFGs in (a) and the third, fourth, and sixth PFGs in (c) are coherence selection gradients. Their strengths must obey the ratio $+1:-1:-4$ or $-1:+1:+4$ for the antiecho pathway selection and $-1:+1:-4$ or $+1:-1:+4$ for the echo pathway selection. For excellent H_2O suppression these PFGs should be MAGs. The delay δ corresponds to the length of the PFG plus $100 \mu\text{s}$ of gradient recovery time. The echo and antiecho data sets in (a) and (c) are then properly added (34), resulting in mostly pure-absorption spectra. Quadrature detection in t_1 in (b) is achieved with the TPPI method (35) where

present at the beginning of the evolution period and originating from spin I is proportional to

$$\{2Q\}_{\text{IK}} \propto (\cos \pi J_{\text{IS}}\tau_1)^2 \sin \pi J_{\text{IK}}(\tau_1 + \tau_2 + \tau_3 + \tau_4) \quad (\text{pulse sequence with one } 90^\circ \text{ }^{13}\text{C} \text{ pulse}) \quad [1]$$

$$\begin{aligned} \{2Q\}_{\text{IK}} \propto & [\cos \pi J_{\text{IS}}\tau_1 \cos \pi J_{\text{IS}}\tau_4 \cos \pi J_{\text{IS}}(\tau_2 - \tau_3) \\ & - \cos \pi J_{\text{SS}}(\tau_2 + \tau_3) \cos(\tau_2 + \tau_3)\omega_{\text{S}} \\ & \times \sin \pi J_{\text{IS}}\tau_1 \sin \pi J_{\text{IS}}\tau_4] \\ & \times \sin \pi J_{\text{IK}}(\tau_1 + \tau_2 + \tau_3 + \tau_4) \quad (\text{pulse sequence with two } 90^\circ \text{ }^{13}\text{C} \text{ pulses}). \quad [2] \end{aligned}$$

In the absence of the spins S and S' the amount of $\{2Q\}_{\text{IK}}$ is simply proportional to

$$\{2Q\}_{\text{IK}} \propto \sin \pi J_{\text{IK}}(\tau_1 + \tau_2 + \tau_3 + \tau_4). \quad [3]$$

In deriving Eq. [2] we have not considered the effect of the phase cycle of the 90° ^{13}C pulses. The second term of Eq. [2] originates from the heteronuclear term $I_y K_2 S_y$ present after the first 90° ^{13}C pulse. This term represents a superposition of antiphase heteronuclear DQ and zero-quantum (ZQ) coherence. The second 90° ^{13}C pulse converts the heteronuclear multiple-quantum term into ^1H antiphase single-quantum coherence. This term can be suppressed by using a two-step phase cycling of the first 90° ^{13}C pulse. Another possibility for its suppression is to locate the first PFG after the first 90° ^{13}C pulse. Equation [2] simplifies then to

$$\begin{aligned} \{2Q\}_{\text{IK}} \propto & [\cos \pi J_{\text{IS}}\tau_1 \cos \pi J_{\text{IS}}\tau_4 \cos \pi J_{\text{IS}}(\tau_2 - \tau_3)] \\ & \times \sin \pi J_{\text{IK}}(\tau_1 + \tau_2 + \tau_3 + \tau_4). \quad [4] \end{aligned}$$

The suppression of unwanted DQ coherence in Eq. [1] is proportional to the square of $(\cos \pi J_{\text{IS}}\tau_1)$. This implies that the use of a τ_1 value which does not perfectly match $1/(2J_{\text{IS}})$ can still provide good ^1H DQ suppression of protons attached to ^{13}C nuclei. Therefore the experiment is recorded with a value for τ_1 which is tuned to a compromise J value.

In the version of the experiment with two 90° ^{13}C pulses, Eq. [4], it is possible to optimize the suppression of unwanted DQ coherence simply by using two different values for τ_1 and τ_4 . For proteins these two values are tuned for the aliphatic and aromatic $^1J_{\text{CH}}$ heteronuclear coupling con-

all the ^1H pulses preceding the evolution period are incremented in step of 45° in concert with t_1 . The simultaneous suppression of ^{15}N - and ^{13}C -bound ^1H magnetization is shown in (a). It can be applied in the same way in (b) and (c). The time between the first 90° ^1H pulse and the 90° ^{15}N pulse is equal to 5.4 ms ($1/(2J_{\text{NH}})$). For suppression of only ^{13}C -bound ^1H magnetization it is sufficient to remove the ^{15}N 90° pulse in (a).

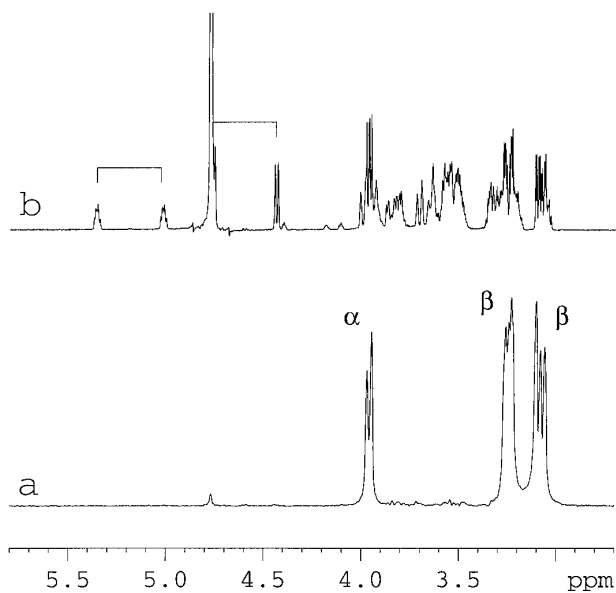


FIG. 2. One-dimensional ^1H reference spectrum of a mixture of ^{13}C -labeled glucose and ^{12}C phenylalanine dissolved in D_2O (b) and the first serial file of a ^{13}C -filter 2D PFG DQ experiment (a) recorded with the pulse sequence of Fig. 1a. The angle β was 135° and only the first ^{13}C 90° pulse was applied. An absolute value display is shown in (b). The spectra have been recorded at $T_e = 297$ K on a Bruker DMX-500 spectrometer. A triple-resonance probe equipped with an actively shielded z -gradient coil connected to a Bruker 10A amplifier was used. The delays τ_1 and $|\tau_1 + \tau_2|$ were 2.9 and 15 ms, respectively. The strength of the five 1.25-ms-long sine-shaped z -PFGs was $+0.35$, $+0.35$, -5 , $+5$, and -20 G/cm, respectively. A total of 16 scans with a repetition time of 1.4 s were recorded. Lines showing the $^1J_{\text{CH}}$ splitting of the anomeric protons are drawn in (b). The resonances of the unlabeled phenylalanine spin system are labeled in (a).

stants, respectively. Furthermore the term $\cos \pi J_{\text{IS}}(\tau_2 - \tau_3)$, where $|\tau_2 - \tau_3| = |\tau_1 - \tau_4|$, is less than 1 ($\tau_1 \neq \tau_4$), resulting in a further suppression of the unwanted magnetization.

In systems with very different $^1J_{\text{CH}}$ heteronuclear couplings it is possible to improve the suppression of the ^{13}C - ^1H signals by inserting in the pulse sequence of Fig. 1 an additional third ^{13}C 90° pulse after a delay τ_5 from the first ^{13}C 90° pulse and at a delay τ_6 before the 180° ^1H pulse. The term $\tau_5 + \tau_6$ corresponds to the τ_2 value of Fig. 1. In this experiment the delay τ_5 can be tuned to a third $^1J_{\text{CH}}$ value. The use of this extra ^{13}C 90° pulse was not required in the examples reported here because the suppression of the ^{13}C - ^1H signals was already excellent with only one and two ^{13}C 90° pulses.

Figure 2b shows the 1D reference spectrum of a mixture of ^{13}C -labeled glucose and unlabeled phenylalanine in D_2O . The values of the $^1J_{\text{CH}}$ couplings for glucose range from 170 to 140 Hz. The spectrum in Fig. 2a represents the first serial file of a 2D ^{13}C -filter DQ experiment recorded with the pulse sequence of Fig. 1a with only one 90° ^{13}C pulse. The angle of the read-out pulse was 135° . An absolute value presenta-

tion is displayed. Only the resonances of the unlabeled amino acid are visible in the ^{13}C -filter DQ spectrum. Note the almost complete suppression of the glucose signals despite the different values for $^1J_{\text{CH}}$ present in this molecule.

Figures 3a and 3b show 2D ^{13}C -filter DQ spectra recorded for the mixture with the pulse sequence of Fig. 1a and Fig. 1b, respectively. The 1D reference spectrum of the mixture is shown in Fig. 3c. The spectrum in Fig. 3a was recorded with read-out pulses $\beta = 45^\circ/135^\circ$, and both spectra were acquired with one ^{13}C 90° pulse. Only the cross peaks of the phenylalanine spin system are visible in these 2D spectra. The remote peaks which are strong in Fig. 3b are significantly attenuated in Fig. 3a and not visible at this contour level.

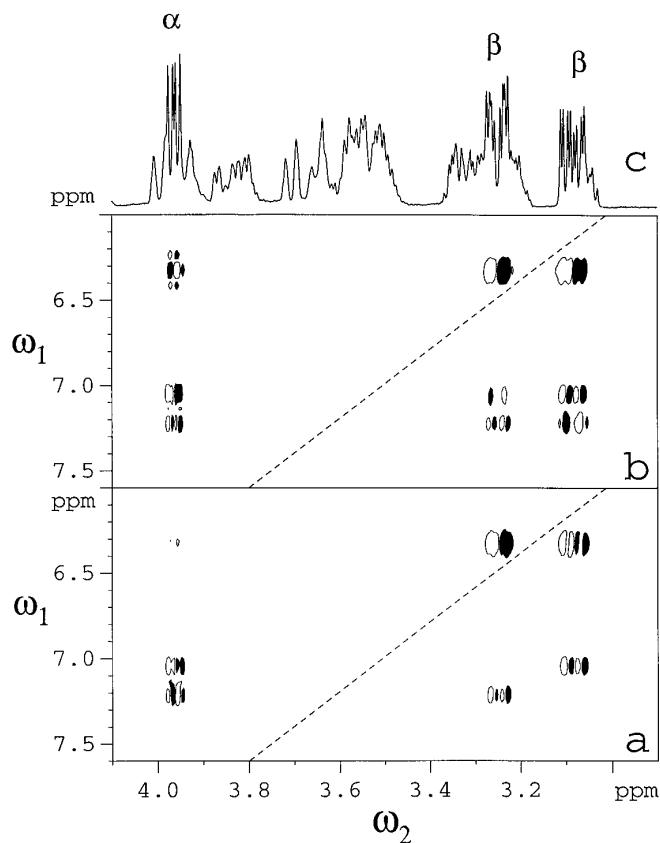


FIG. 3. (a, b) Two-dimensional ^{13}C -filter DQ spectra of the mixture recorded with the pulse sequence of Fig. 1a and Fig. 1b, respectively. The read-out pulse in (a) was $45^\circ/135^\circ$ and the spectra in (a, b) have been recorded with only the first 90° ^{13}C pulse. A total of 16 scans have been acquired for each of the 256 t_1 increments. The ω_1 and ω_2 spectral widths were 13 and 8 ppm, respectively. The data were multiplied with a cosine window function in both dimensions prior to Fourier transformation. The strengths of the gradients were $+0.35$ and $+0.35$ G/cm in (b) and $+0.35$, $+0.35$, -5 , $+5$, -20 (echo selection $\beta = 135^\circ$), $+0.35$, $+0.35$, $+5$, -5 , and -20 (antiecho selection $\beta = 45^\circ$) G/cm in (a). The other experimental parameters are the same as described in the legend to Fig. 2. The reference spectrum of the mixture with the labels for the phenylalanine resonances is shown in (c). The dashed line in (a, b) corresponds to the pseudo DQ diagonal. The strong remote peaks in (b) are effectively attenuated in (a) and are not visible at this contour level.

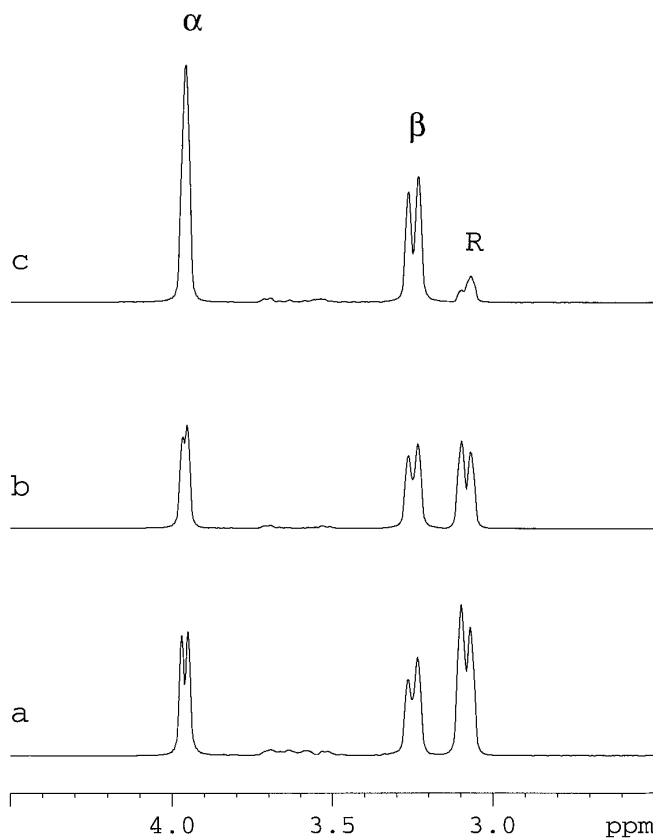


FIG. 4. Row at $\omega_1 = 7.2$ ppm extracted from three different ^{13}C -filter 2D DQ spectra recorded for the mixture. The spectra have been recorded (a) with the pulse sequence of Fig. 1b, (b) with the pulse sequence Fig. 1a and $\beta = 90^\circ/90^\circ$, and (c) with the pulse sequence of Fig. 1a and with $\beta = 45^\circ/135^\circ$. The acquisition and processing parameters are the same as described in the legends to Figs. 3 and 4. All the acquisition and processing parameters were the same for all three experiments. The data are plotted at the same level. The strongest direct peaks and the weakest remote peak, labeled with an R, are observed in (c).

Signal intensity comparisons for the three versions of the ^{13}C -filter DQ experiments are reported in Figs. 4a–4c. A row taken at $\omega_1 = \omega^{\alpha}\text{H} + \omega^{\beta}\text{H}$ was extracted from three ^{13}C -filter 2D DQ spectra recorded under the same experimental conditions with the pulse sequences of Figs. 1a and 1b. For ease of comparison an absolute value presentation is displayed. The strongest direct signals and the weakest remote signals are observed in the $45^\circ/135^\circ$ PFG DQ spectrum (Fig. 4c). Note that the excellent suppression of the ^{13}C -bound ^1H magnetization in the 2D DQ experiment is achieved with only the use of a ω_1 filter. No additional ω_2 filter is necessary to improve the suppression of unwanted magnetization. The weak glucose signals present in these spectra originate mainly from the residual unlabeled glucose molecules (^{13}C labeling was 97%).

A more challenging system for the application of the technique is represented by a protein–ligand complex. The ^{13}C and ^{15}N doubly labeled protein is the inserted-domain (I-domain) of the leukocyte function-associated antigen (LFA-

1) consisting of 188 amino acids and with MW 21,399 (non labeled). The unlabeled ligand is a natural product. Intermolecular NOEs between ligand and protein and the same self-diffusion coefficient observed for the ligand and for the protein indicate that this ligand binds strongly to the protein. Figure 5 shows an expanded region of the 2D ^{13}C -filter DQ spectrum recorded for the complex with the pulse sequence of Fig. 1a with $\beta = 45^\circ/135^\circ$ read-out pulses and with the use of two ^{13}C 90° pulses. The concentration of the sample was $500\ \mu\text{M}$ and the measuring time for the experiment was 7.5 h. Note the complete suppression of ^{13}C -bound ^1H DQ signals of the protein. This experiment has allowed complete unequivocal spectral assignment of the bound ligand. It should be pointed out that the T_2 values of the protein ^1H resonances are shorter than the T_2 values of the ligand resonances due to the presence in the protein of the strong one-bond ^{13}C – ^1H dipolar interaction. Magnetization losses via T_2 during the excitation DQ period will be more pronounced for the protein resonances. This helps further in suppressing the DQ coherences originating from the protein.

The ^{13}C isotope enrichment of a protein is not always

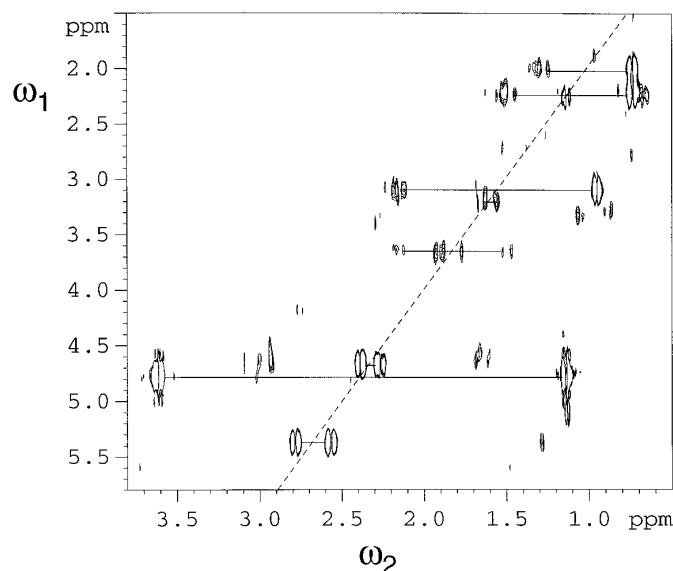


FIG. 5. An expanded region of the ^{13}C -filter DQ spectrum recorded for a $500\ \mu\text{M}$ solution of the ^{13}C and ^{15}N uniformly labeled inserted-domain (I-domain) of the leukocyte function-associated antigen (LFA-1) complexed with an unlabeled ligand. The sample was in D_2O and the experiments were recorded at $T_c = 297\ \text{K}$. The spectrum has been acquired with the pulse sequence of Fig. 1a with $\beta = 45^\circ/135^\circ$ and with two ^{13}C 90° pulses. The length $|\tau_1 + \tau_2|$ was only 10 ms in order to reach a compromise between sufficient DQ creation and signal losses limitation due to rapid T_2 relaxation. A total of 96 scans for each of the 200 t_1 increments were recorded. The repetition time was 1.4 s corresponding to a total measurement time of 7.5 h. The ω_1 and ω_2 spectral widths were 18 and 12 ppm, respectively. The strengths of the gradients were $+0.65, +0.65, -2.5, +2.5, -10$ (echo selection $\beta = 135^\circ$), $+0.65, +0.65, +2.5, -2.5, -10$ (antiecho selection $\beta = 45^\circ$) G/cm. The dashed line corresponds to the pseudo DQ diagonal. Some of the connectivities between direct peaks are drawn.

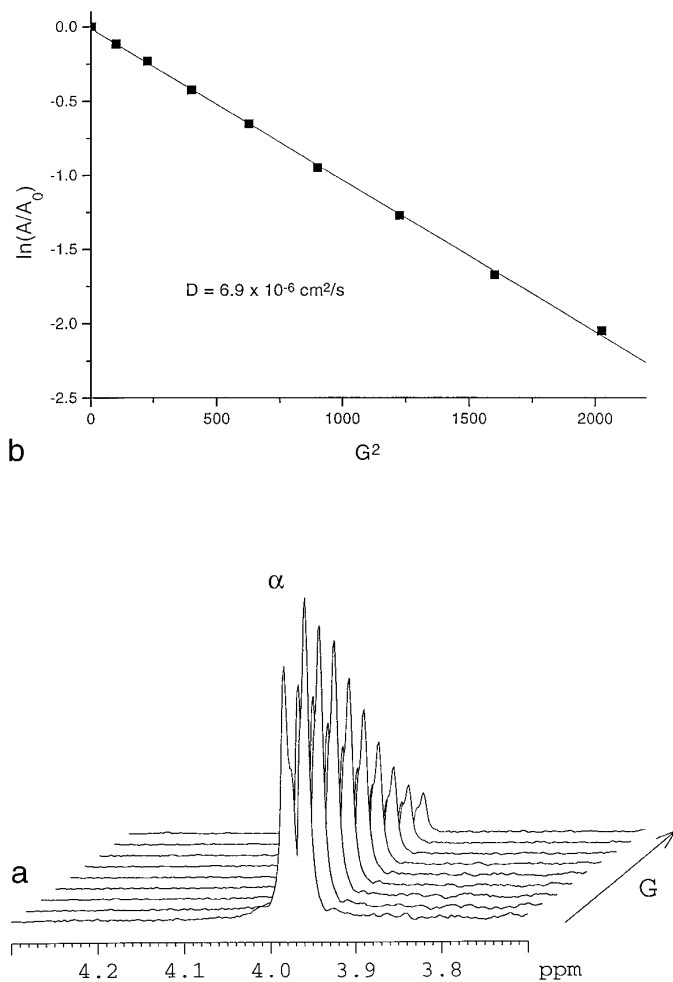


FIG. 6. (a) Behavior of the signal intensity of the $^{\alpha}\text{H}$ resonance of Phe as a function of the strength of the first two z -PFGs of the scheme of Fig. 1a. A total of nine 1D ^{13}C -filter ^1H PFG DQ spectra were recorded for the mixture of glucose and phenylalanine. The scheme with two ^{13}C 90° pulses was employed. The angle of the read-out pulse was 135° , t_1 was $3 \mu\text{s}$ long, and only the echo pathway was selected. An absolute value presentation is displayed. Sixteen scans with a repetition time of 2.5 s were recorded for each spectrum. The τ_1 and τ_2 periods were 3.3 and 2.9 ms, respectively. The delay between the start of the first two z -PFGs was 27.1 ms. The length of the first two rectangular PFGs and of the three sine-shaped coherence selection PFGs was 2.80 and 0.66 ms, respectively. The gradient recovery time was $100 \mu\text{s}$ long. The strength of the first two PFGs in the nine spectra, starting from the lowest value, was 1.5, 10, 15, 20, 25, 30, 35, 40, and 45 G/cm, respectively. The strength of the three coherence selection PFGs was -2.5 , $+2.5$, and -10 G/cm, respectively. The data were multiplied with a cosine window function prior to Fourier transformation. Note the complete suppression of the ^{13}C - ^1H glucose signals at or near the $^{\alpha}\text{H}$ resonance of Phe. (b) Plot of the natural logarithm intensity of the peak in (a) vs G^2 , where G is expressed in G/cm. The line represents the linear fit. The D value of Phe determined from the slope of the line is $6.9 \times 10^{-6} \text{ cm}^2/\text{s}$ with an R value of 0.999.

100%. The residual ^{12}C -bound proton signals give rise in the 2D ^{13}C -filter TOCSY and COSY experiments to a residual diagonal signal, especially in the aliphatic spectral region. The presence of this diagonal can obscure the observation

of cross peaks between two almost degenerate ligand resonances. The absence of diagonal peaks in the ^{13}C -filter DQ experiment overcomes this problem and DQ connectivities between almost degenerate resonances are visible (Fig. 5). The only peaks which lie on the pseudo DQ diagonal are the so-called methyl group forbidden peaks (28, 29). These originate from the differential relaxation rates of degenerate single-quantum transitions of magnetically equivalent nuclei.

An experiment which is usually employed in the spectral assignment of a ligand bound to a protein is the ^{13}C -filter TOCSY experiment. This experiment can be applied to larger systems than the DQ reported here. However, the assignment process based solely on the TOCSY experiment can be fallible, especially when the ligand is a nonpeptidic compound. Therefore the ^{13}C -filter DQ experiments described here represent a useful complement to the ^{13}C -filter TOCSY experiment for obtaining unambiguous spectral assignments.

The two PFGs located at the beginning and end of the DQ excitation period can also be used to determine the self-diffusion coefficient of unlabeled molecules. A series of 1D or 2D X-filter ^1H PFG DQ experiments are recorded with the pulse sequence of Fig. 1 with increasing strength of the first two PFGs, similarly to that previously reported (30, 31). The intensities of the signals are then measured and analyzed according to the Stejskal–Tanner equation (32). The application of the method to the mixture of glucose and phenylalanine is shown in Fig. 6. A total of nine 1D ^{13}C -filter DQ spectra recorded with the pulse sequence of Fig. 1a and with increasing strength for the first two PFGs are reported in Fig. 6a. The excellent suppression of the ^{13}C - ^1H glucose signals which resonate at or near the chemical shift of the $^{\alpha}\text{H}$ resonance of phenylalanine (see Fig. 2a) allows the determination, with high accuracy, of the self-diffusion coefficient of phenylalanine. A plot of the signal intensity against G^2 , the linear regression fit, and the D value for Phe are shown in Fig. 6b. The method can thus be used to identify, in a mixture of unlabeled compounds in solution with a ^{15}N - and/or ^{13}C -labeled protein, those molecules which interact with the protein.

In conclusion it has been shown that the ^{13}C -filter DQ experiments represent sensitive methods for the assignment of the proton resonances of an unlabeled ligand bound to a ^{13}C uniformly labeled protein. The suppression of the ^{13}C -bound proton magnetization is very efficient in these experiments. Successful application of the technique to a macromolecular complex of $\text{MW} \cong 24,000$ has been presented. This method can also be used to measure the self-diffusion coefficients of unlabeled ligands in solution with a labeled macromolecule.

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