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Communication

Observing in-phase single-quantum ^{15}N multiplets for NH_2/NH_3^+ groups with two-dimensional heteronuclear correlation spectroscopy

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ABSTRACT

Two-dimensional (2D) $F1$-^1H-coupled HSQC experiments provide 3:1:1:3 and 1:0:1 multiplets for AX_3 and AX_2 spin systems, respectively. These multiplets occur because, in addition to the $2S_yH_z^a \rightarrow 2S_yH_z^a$ process, the coherence transfers such as $2S_yH_z^a \rightarrow 2S_yH_z^b$ occurring in t_1 period provide detectable magnetization during the t_2 period. Here, we present a 2D $F1$-^1H-coupled ^1H–^{15}N heteronuclear correlation experiment that provides a 1:3:3:1 quartet for AX_3 spin system and a 1:2:1 triplet for AX_2. The experiment is a derivative of 2D HISQC experiment [J. Iwahara, Y.S. Jung, G.M. Clore, Heteronuclear NMR spectroscopy for lysine NH_3 groups in proteins: unique effect of water exchange on ^{15}N transverse relaxation. J. Am. Chem. Soc. 129 (2007) 2971–2980] and contains a scheme that kills anti-phase single-quantum terms generated in the t_1 period. The purge scheme is essential to observe in-phase single-quantum multiplets. Applications to the NH_2 and NH_3^+ groups in proteins are demonstrated.

For heteronuclear AX_3 and AX_2 spin systems, one-dimensional NMR measurement on nucleus A that comprises a single excitation pulse immediately followed by detection without decoupling generally gives an in-phase 1:3:3:1 quartet and a 1:2:1 triplet, respectively, provided that relaxation rates for individual multiplet components are identical. It is because overall modulations of detected magnetizations due to J and chemical shift evolutions are

$$\exp(i\Omega t)\cos^3\pi Jt = \frac{1}{8}\exp\{i(\Omega-3\pi J)t\} + \frac{3}{8}\exp\{i(\Omega-\pi J)t\} + \frac{3}{8}\exp\{i(\Omega+\pi J)t\} + \frac{1}{8}\exp\{i(\Omega+3\pi J)t\} \quad (1)$$

for an AX_3 spin system, and

$$\exp(i\Omega t)\cos^2\pi Jt = \frac{1}{4}\exp\{i(\Omega-2\pi J)t\} + \frac{1}{2}\exp(i\Omega t) + \frac{1}{4}\exp\{i(\Omega+2\pi J)t\} \quad (2)$$

for AX_2. For simplicity sake, we use terms such as '1:3:3:1' and '1:2:1' hereafter, although actual intensity ratios of multiplet components can deviate due to cross-correlations [1].

In the case of a two-dimensional heteronuclear correlation experiment, it is not trivial to obtain the in-phase 1:3:3:1 quartet and 1:2:1 triplet. In an $F1$-^1H-coupled HSQC experiment (such as one shown in Fig. 1A), heteronuclear AX_3 and AX_2 spin systems exhibit 3:1:1:3 quartet and 1:0:1 triplet, respectively [2–4], because not only the $2S_yH_z^a \rightarrow 2S_yH_z^a$ process but also the coherence transfers such as $2S_yH_z^a \rightarrow 2S_yH_z^b$ occurring during the t_1-evolution period generate magnetizations detectable in the t_2-period. With the additional contributions, the real part of the overall modulation due to J and chemical shift evolutions in the t_1-period for AX_3 is given by:

$$(\cos^3\pi Jt_1 - 2\sin^2\pi Jt_1\cos\pi Jt_1)\cos\Omega t_1 = \frac{3}{8}\cos(\Omega-3\pi J)t_1 + \frac{1}{8}\cos(\Omega-\pi J)t_1 + \frac{1}{8}\cos(\Omega+\pi J)t_1 + \frac{3}{8}\cos(\Omega+3\pi J)t_1, \quad (3)$$

resulting a 3:1:1:3 quartet. Likewise, the corresponding modulation for AX_2 is:

$$(\cos^2\pi Jt_1 - \sin\pi Jt_1\cos\pi Jt_1)\cos\Omega t_1 = \frac{1}{2}\cos(\Omega-2\pi J)t_1 + \frac{1}{2}\cos(\Omega+2\pi J)t_1, \quad (4)$$

which gives a 1:0:1 triplet. Since it appears to be a doublet, the multiplet itself does not indicate whether the spin system is of AX_2 or AX unless the true J-coupling is known.

In the present study, we have developed a new 2D ^1H–^{15}N correlation experiment to observe an in-phase 1:3:3:1 quartet for a NH_3^+ group and a 1:2:1 triplet for a NH_2 group along $F1$ axis. Fig. 1B shows the 2D ^1H–^{15}N $F1$-coupled ^1H–^{15}N heteronuclear correlation experiment to observe 1:3:3:1 and 1:2:1 multiplets for NH_3^+ and NH_2, respectively. The experiment was derived from the water-flip-back 2D ^1H–^{15}N HISQC (*h*eteronuclear *i*n-phase *s*ingle

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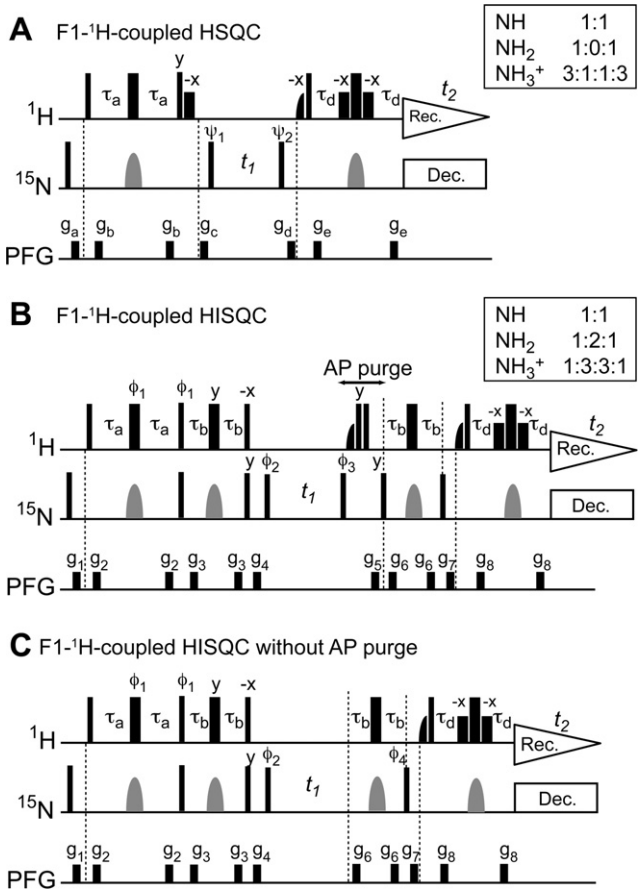

Fig. 1. Two-dimensional ^1H–^{15}N correlation experiments to observe in-phase ^{15}N multiplets. (A) *F*1-^1H-coupled ^1H–^{15}N HSQC (B) *F*1-^1H-coupled ^1H–^{15}N HISQC (C) *F*1-^1H-coupled HISQC without the AP purge scheme. Thin and thick bars represent 90° and 180° pulses, respectively. Unless indicated otherwise, pulse phases are along x. Water-selective half-Gaussian (2.0 ms) and soft-rectangular (1.2 ms) 90° pulses are represented by half-bell and short-bold shapes, respectively. A gray bell-shape represents a ^{15}N 180° pulse (rectangular or shaped; see legends for Figs. 2 and 3). The ^1H carrier position was set at the water resonance. The delay τ_a, for which the optimal value is considerably shorter than $(4^1J_{NH})^{-1}$ because of fast ^1H relaxation caused by rapid water exchange for NH_3^+/NH_2 groups, was set to 2.0–2.7 ms. The other delays: $\tau_b = 1.3$ ms; $\tau_d = \tau_a - 1.2$ ms. Phase cycles: $\psi_1 = \{x, -x\}$, $\psi_2 = \{2x,2(-x),x\}$, and rec. $= \{x,2(-x),x\}$ for A; $\phi_1 = \{y, -y\}$, $\phi_2 = \{2y, 2(-y)\}$, $\phi_3 = \{4y,4(-y)\}$, $\phi_4 = \{4x,4(-x)\}$, and rec. $= \{x,2(-x),x, -x,2x, -x\}$ for B and C. Quadrature detection in the t_1-domain was achieved using States-TPPI, incrementing the phase ψ_1 for A and ϕ_2 for B and C. Field-gradients were optimized to minimize the water signal. For higher sensitivity, water-flip-back principle [13] is implemented in each experiment. The pulse sequence in panel C, which does not include the AP purge scheme, is just for comparison purpose and of no practical use (see Figs. 2C, 3C and 3F).

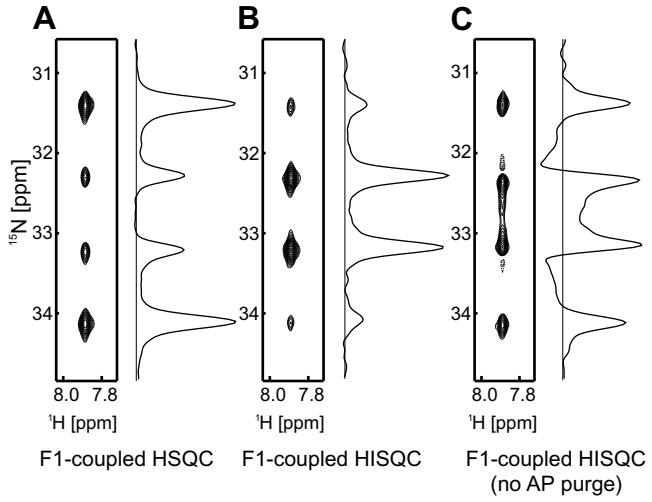

Fig. 2. ^{15}N multiplets observed for the Lys57 NH_3^+ group of $^2H/^{15}N$-labeled homeodomain bound to 24-bp DNA (Solid contours, positive; Dashed, negative). Spectra in panels A, B and C were recorded at 16 °C with the pulse sequences shown in Figs. 1A–C, respectively. The ^{15}N carrier position was at 30 ppm and r-SNOB pulses [14] selective to lysine $^{15}N\zeta$ nuclei were employed for ^{15}N 180° pulses. Acquisition times for ^1H and ^{15}N dimensions were 54 and 79 ms, respectively. For data processing, 60°-shifted sine-bell window functions were applied prior to Fourier transformations. The protein–DNA complex was prepared as described previously [15–18] and dissolved with a buffer of 20 mM sodium phosphate and 20 mM NaCl (pH 5.8, 100% ^1H_2O). The solution was sealed into the inner compartment of the co-axial NMR tube, and D_2O for NMR lock was put in the outer compartment to avoid NH_2D and NHD_2 species [2]. Data were collected at ^1H-frequency of 800 MHz and analyzed with the NMRPipe [19] and NMRView [20] programs. The *J*-coupling was measured to be 74 Hz.

*q*uantum *c*oherence; Fig. 1C) experiment for NH_3^+ groups [2], and therefore we refer to it as *F*1-^1H-coupled HISQC. This pulse sequence starts with the ^1H excitation, and the coherence transfer form H_y to N_x occurs before the t_1 period. The length of delay τ_b (=1.3 ms) is a compromise to simultaneously observe NH_3^+, NH_2, and NH, and overall *J*-modulations for these groups through four τ_b periods are given by $3\cos^4 2\pi J\tau_b \sin^2 2\pi J\tau_b$ (=0.49 with $J = 74$ Hz), $2\cos^2 2\pi J\tau_b \sin^2 2\pi J\tau_b$ (=0.74 with $J = 89$ Hz), and $\sin^2 2\pi J\tau_b$ (=0.55 with $J = 93$ Hz), respectively. Due to these attenuations along with relaxation loss during the additional schemes, the sensitivity of the *F*1-^1H-coupled HISQC experiment is roughly a half of that of the *F*1-^1H-coupled HSQC. A similar experiment that starts with the ^{15}N excitation instead of the ^1H excitation could be more sensitive if the magnetization loss during the coherence transfer from H_y to N_x in the scheme of Fig. 1B is over 90% ($\approx 1-\gamma_N/\gamma_H$), which is not the case in the present study; however, such an experiment that starts on ^{13}C with NOE enhancement via ^1H saturation should be with acceptable sensitivity for ^1H-^{13}C systems [5]. At the beginning of the t_1 period, the observed magnetization is an in-phase single-quantum term N_y or N_x, depending on the phase ϕ_2. Since there is no ^1H-decoupling during the t_1 period, anti-phase single-quantum terms such as $2N^+H_z$, $4N^+H_zH_z$, and $8N^+H_zH_zH_z$ are generated. The scheme right after the t_1-period (hereafter, referred to as the AP purge scheme; indicated with an arrow in Fig. 1B) kills the $2N^+H_z$ and $8N^+H_zH_zH_z$ terms, so only N^+ and $4N^+H_zH_z$ terms can survive. The reason for the survival of $4N^+H_zH_z$ is that $4N_zH_xH_x$ generated by ^1H 90° pulses in the AP purge scheme cannot be killed with the pulse field gradient because it is a homonuclear zero-quantum term [6–8]. However, the following scheme for coherence transfers does not allow such zero-quantum terms to become observable magnetizations in the t_2 acquisition period. Therefore, only the in-phase single quantum term N^+ at the end of the t_1 period is detectable. Since the real part of the overall modulation for the N^+ term in t_1 is given by $\cos^n \pi J t_1 \cos \Omega t_1$ (*n*, number of hydrogens), the spectra obtained with this pulse sequence should show 1:3:3:1, 1:2:1, and 1:1 multiplets for NH_3^+, NH_2, and NH, respectively.

Using the pulse sequences shown in Fig. 1, we recorded 2D ^1H–^{15}N heteronuclear correlation spectra on NH_3^+/NH_2 groups in proteins (Figs. 2 and 3). Data were collected with Varian 800- or 750-MHz NMR systems. Fig. 2 displays spectra recorded on the Lys57 NH_3^+ group of the HoxD9 homeodomain bound to 24-bp DNA. Owing to formation of an ion-pair with a DNA phosphate group, this NH_3^+ group exhibits relatively slow hydrogen-exchange with water molecules and the ^1H–^{15}N cross peak from this group can clearly be observed [2]. Just as expected from considerations above, *F*1-^1H-coupled HSQC (Fig. 2A) and *F*1-^1H-coupled HISQC (Fig. 2B) exhibits in-phase quartets of 3:1:1:3 and 1:3:3:1 types, respectively. Actual intensity ratios deviate from these numbers

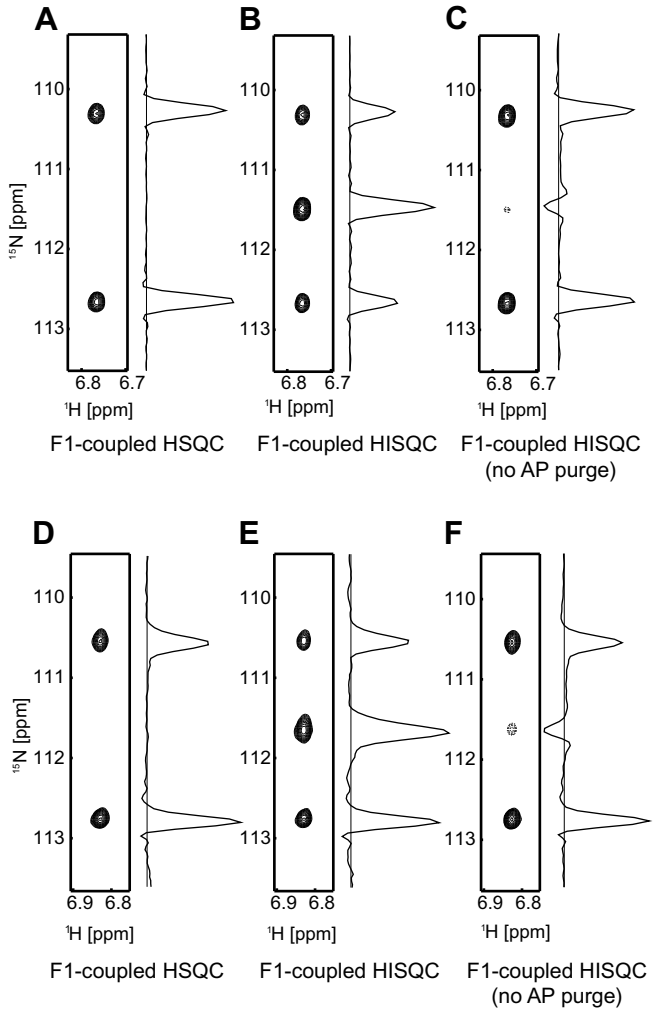

Fig. 3. ^{15}N multiplets observed for NH_2 groups. (A, B and C) Spectra recorded on Gln20 NH_2 group in ^{15}N-labeled HMGB1 A-domain. Data were collected at 25 °C with a 750-MHz spectrometer. Acquisition times for ^{1}H and ^{15}N dimensions were 60 and 66 ms, respectively. The protein was prepared according to previous literature [10,21] and dissolved with a buffer of 50 mM TrisHCl and 100 mM KCl (pH 7.4, 100% $^{1}H_2O$). The protein solution was sealed into the inner compartment of the co-axial tube, and D_2O for NMR lock was put in the outer compartment to avoid NHD species. (D, E and F) Spectra recorded on the Gln12 NH_2 group of ^{2}H/^{15}N-labeled HoxD9 homeodomain bound to 24-bp DNA. The sample is identical to that used for Fig. 2. Spectra were recorded at 16 °C with a 800-MHz spectrometer. Acquisition times for ^{1}H and ^{15}N dimensions were 54 and 72 ms, respectively. All ^{15}N 90° and 180° pulses were rectangular with the rf strength of 6 kHz and the carrier position at 116 ppm. For data processing, 60°-shifted sine-bell window functions were applied prior to Fourier transformations.

because the relaxation rates for inner and outer components of the quartet are different due to cross-correlations [2,5,9].

Fig. 3 shows spectra recorded on side-chain NH_2 groups of glutamine (Gln) residues in proteins. Panels A, B and C display spectra recorded on Gln20 in the ^{15}N-labeled HMGB1 A-domain. The rotational correlation time τ_r for this protein at 25 °C is 9 ns [10]. The NH_2 group exhibited 1:0:1 triplets in the $F1$-^{1}H-coupled HSQC spectrum (Fig. 3A) and 1:2:1 triplets in the $F1$-^{1}H-coupled HISQC spectrum (Fig. 3B). The J-coupling was measured to be 89 Hz. For a system with a long τ_r, the relaxation rates of individual triplet components for a AX_2 spin system can be quite different because of cross-correlations between distinct relaxation mechanisms [11]. Such a case is clearly seen in the spectra measured on the Gln12 NH_2 groups in the ^{2}H/^{15}N-labeled HoxD9 homeodomain bound to 24-bp DNA at 16 °C (Fig. 3D, E and F). The value of τ_r is 15 ns for this system. In this case, the downfield components are substantially shaper than the other components in triplets.

Although one may think that removal of ^{1}H-decoupling from the original HISQC experiment [2] would simply result in 1:3:3:1 and 1:2:1 multiplets, such a pulse sequence (Fig. 1C) does not give the desired multiplets. This occurs because the anti-phase single-quantum terms generated in the t_1-period also become ^{1}H magnetizations detectable in the t_2 acquisition period. In fact, the spectra measured with the simplistic pulse sequence on the same NH_3^+ and NH_2 groups (Figs. 2C and 3C, F) are very different from those measured with the AP purge scheme (Figs. 2B and 3B, E). Intensity ratios are far from 1:3:3:1 for NH_3^+ and 1:2:1 for NH_2; indeed, the multiplets in Fig. 3C and F are more similar to 1:0:1 triplets. In addition, some contributions from the anti-phase terms occur with 90°-shifted phases that cause dispersive distortion of the multiplets, which is evident especially in Fig. 2C. Thus, the AP purge scheme is essential to obtain 1:3:3:1 and 1:2:1 multiplets.

In conclusion, we have demonstrated the 2D $F1$-^{1}H-coupled ^{1}H–^{15}N correlation experiment that permits observation of in-phase 1:3:3:1 quartets for NH_3^+ groups and 1:2:1 triplets for NH_2 groups along the $F1$ axis. This experiment provides a means to distinguish AX, AX_2, and AX_3 spin systems in a straightforward manner. It is particularly useful when ^{1}H chemical shifts are degenerated. For example, the deprotonated state of an alkyl amino group (NH_2) shows a single ^{1}H resonance because of rapid chiral inversion [12]. In such a case, it is hard to distinguish AX and AX_2 spin systems with $F1$-^{1}H-coupled HSQC unless J-coupling is already known, because a 1:0:1 triplet appears to be a doublet. A 1:2:1 triplet is easier to interpret. It should be noted that a rapid hydrogen exchange with a rate greater than $2\pi J$ can cause the self-decoupling effect that results in a ^{15}N singlet even in absence of ^{1}H-decoupling. Considering the range of $^{1}J_{NH}$ coupling constants, however, it is likely that such a rapid hydrogen exchange simply broadens the signal beyond the detection limit in the present case, because the hydrogen exchange also increases ^{1}H transverse relaxation rates. Finally, it should be pointed out that the principle presented here can readily be applied to ^{1}H-^{13}C systems.

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