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COMMUNICATION

# Effective strategy to assign $^{1}H$-$^{15}N$ heteronuclear correlation NMR signals from lysine side-chain $NH_3^+$ groups of proteins at low temperature

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**Abstract** Recent studies have shown that lysine side-chain $NH_3^+$ groups are excellent probes for NMR investigations of dynamics involving hydrogen bonds and ion pairs relevant to protein function. However, due to rapid hydrogen exchange, observation of $^{1}H$-$^{15}N$ NMR cross peaks from lysine $NH_3^+$ groups often requires use of a relatively low temperature, which renders difficulty in resonance assignment. Here we present an effective strategy to assign $^{1}H$ and $^{15}N$ resonances of $NH_3^+$ groups at low temperatures. This strategy involves two new $^{1}H/^{13}C/^{15}N$ triple-resonance experiments for lysine side chains. Application to a protein-DNA complex is demonstrated.

**Keywords** Proteins · Side chains · Lysine · $NH_3^+$ groups · $^{1}H/^{13}C/^{15}N$ resonances

Hydrogen bonds and ion pairs involving protein side chains are of fundamental importance in protein functions such as molecular recognition and catalysis. Recently it was demonstrated that lysine (Lys) side-chain $NH_3^+$ groups are very useful probes for NMR studies of protein dynamics involving hydrogen bonds and ion pairs (Anderson et al. 2013; Esadze et al. 2011; Zandarashvili et al. 2011, 2013). Heteronuclear $^{1}H$-$^{15}N$ cross peaks from Lys $NH_3^+$ groups are also useful for identifying molecular interfaces between protein and ligand (Blaum et al. 2010; Poon et al. 2006). Resonance assignment for Lys $NH_3^+$ groups typically requires not only standard experiments such as (H)C(CO)NH, H(CCO)NH, and HCCH-TOCSY, which correlate side-chain and backbone resonances, but also Lys-selective NMR experiments (Andre et al. 2007; Iwahara et al. 2007). This assignment process is not trivial because rapid hydrogen exchange renders difficulty in $^{1}H$ detection of $NH_3^+$ groups. To mitigate this problem, observation of $^{1}H$ signals from Lys $NH_3^+$ groups often requires the use of relatively low pH and temperature. However, the use of a low temperature can substantially reduce sensitivity of most triple-resonance experiments due to a longer molecular rotational correlation time, and thereby hamper resonance assignment.

Resonance assignment for Lys side-chain $NH_3^+$ groups requires long-range correlation spectra because these amino groups are located at the fifth positions (i.e., $\zeta$ position) from C$\alpha$ atoms. Short-range correlations between $NH_3^+$ and $^{1}H\varepsilon/^{13}C\varepsilon$ resonances are typically insufficient for unambiguous assignment due to poor dispersion of their chemical shifts. While long-range correlation NMR methods such as the 3D (H)CCENH3 (Iwahara et al. 2007) may in principle allow for assignment of Lys $NH_3^+$ resonances, this approach can be impractical at low temperatures due to rapid $^{13}C$ transverse relaxation. In fact, it is well known that rapid $^{13}C$ transverse relaxation can diminish the efficiency of $^{13}C$-$^{13}C$ Hartmann-Hahn cross polarization if a molecular rotational correlation time $\tau_r$ is relatively long (Fischer et al. 1996).

In this paper, we present two distinct approaches to assign Lys side-chain $NH_3^+$ groups at a low temperature. One of the approaches is to obtain long-range $^{15}N$-$^{13}C$ correlations by taking advantage of very slow $^{15}N$ transverse relaxation of $NH_3^+$ groups. Due to bond rotations along the symmetry axis and cross correlation between three $^{15}N$-$^{1}H$ dipole–dipole interactions, NMR relaxation of the in-phase single-quantum term $N_x$ for $NH_3^+$ groups is extremely slow and occurs in a bi-exponential manner

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(Esadze et al. 2011; Zandarashvili et al. 2013). In our previous studies, initial transverse relaxation rates $R_{2,ini}$ for $NH_3^+$ groups were in the range between 0.9 and 4.5 $s^{-1}$ for the systems with $\tau_r = 8.5$ and 10.8 ns (Anderson et al. 2013; Esadze et al. 2011); the average $R_{2,ini}$ for the Lys side-chain $NH_3^+$ groups that directly interact with DNA phosphate groups in the HoxD9 homeodomain-DNA complex was 1.7 $s^{-1}$ (Anderson et al. 2013), which corresponds to a transverse relaxation time of ~580 ms. Therefore, even a time over 100 ms can readily be used for $^{15}N$-$^{13}C$ coherence transfer via *J*-evolution between $N_x$ and $2N_yC_z$ terms for a relatively small scalar coupling. This coherence transfer is substantially more efficient than coherence transfer via *J*-evolution from $C_x$ to $2C_yN_z$, because the $^{15}N$ transverse relaxation of $NH_3^+$ groups is far slower than relevant $^{13}C$ transverse relaxation of $CH_2$ groups.

From this perspective, we have developed the 3D H3NCG experiment (Fig. 1), which provides a long-range correlation between $^1H/^{15}N$ resonances of the $NH_3^+$ groups and $^{13}C\gamma$ resonance for Lys side chains. This 3D experiment relies on relatively small scalar coupling constants $^3J_{NC}$ between the $^{15}N\zeta$ and $^{13}C\gamma$ nuclei. Absolute values of the $^3J_{NC}$ constants are in a range of 1–3 Hz, and depend on Lys $\chi_4$ torsion angles, which can undergo dynamic transitions between gauch and trans conformers (Huang and MacKerell 2013; Zandarashvili et al. 2011). Owing to the slow $^{15}N$ transverse relaxation of $NH_3^+$ groups during the coherence transfers between the $N_x$ and $2N_yC_z$ terms, the magnetizations of interest survive a total of 200 ms for the evolution of the small $^3J_{NC}$ couplings between the $^{15}N\zeta$ and $^{13}C\gamma$ nuclei in the 3D H3NCG experiment. Because $^{15}N$ chemical shifts of lysine side-chain $NH_3^+$ groups are unique (~33 ppm), $^{15}N$ r-SNOB pulses (Kupče et al. 1995) selective to $^{15}N\zeta$ are used to suppress potentially aliased signals from arginine and backbone $^{15}N$ nuclei. In this experiment, undesirable coherence transfer between $^{15}N\zeta$ and $^{13}C\varepsilon$ nuclei is avoided by using $^{13}C$ I-BURP2 pulses (Geen and Freeman 1991) that invert $^{13}C\gamma$ (~25 ppm) but do not affect $^{13}C\varepsilon$ (~42 ppm) nuclei. Although these I-BURP2 pulses also invert $^{13}C\delta$ (~28 ppm), $^2J_{NC\delta}$ coupling constants are undetectably small in lysine side chains (Zandarashvili et al. 2011). Thus, these $^{15}N$ and $^{13}C$ shaped pulses permit selective observation of $^1H\zeta/^{15}N\zeta/^{13}C\gamma$ cross peaks from lysine side chains. Using the 3D H3NCECD (Iwahara et al. 2007) and 3D H3NCG spectra, three resonances of $^{13}C\gamma$, $^{13}C\delta$, and $^{13}C\varepsilon$ nuclei can be obtained for each lysine side-chain $NH_3^+$ group. Because the $^{13}C$-$^{15}N$ coherence transfer in the H3NCG and H3NCECD experiments is not exposed to rapid $^{13}C$ transverse relaxation, these experiments are more sensitive than the (H)CCENH3 experiment that involves coherence transfer via *J*-evolution from $C_x$ to $2C_yN_z$. It should also be noted that the H3NCG and H3NCECD experiments with $^2H$ decoupling can be readily performed for perdeuterated proteins. Although deuteration should substantially slow $^{13}C$ transverse relaxation and therefore improve $^{13}C$-$^{13}C$ coherence transfer (Yamazaki et al. 1994), the lack of side-chain $^1H$ nuclei in perdeuterated proteins makes the (H)CCENH3 experiment impractical.

Using a 750-MHz spectrometer, we recorded 3D H3NCG and 3D H3NCECD spectra for the Egr-1—DNA complex at 10 °C. This complex contains the Egr-1 zinc-finger protein (90 amino acids) and 12-bp DNA with Egr-1's target sequence. Our group uses the complex to study dynamics in DNA scanning and recognition by Egr-1 (Esadze et al. 2014; Zandarashvili et al. 2012). The Egr-1 zinc-finger protein and 12-bp DNA were purified as previously described (Esadze and Iwahara 2014; Takayama et al. 2010). A 280-µl solution of 0.8 mM complex in a buffer of 20 mM potassium succinate (pH 5.8), 2 mM $ZnCl_2$, and 20 mM KCl was sealed into an inner tube of co-axial NMR tube. To avoid deuterated species of $NH_3^+$ groups, $D_2O$ for NMR lock was separately sealed in a thin outer layer of the co-axial NMR tube (Iwahara et al. 2007). The relatively low temperature, 10 °C, was required to observed $^1H$-$^{15}N$ HISQC (Iwahara et al. 2007) cross peaks from all six Lys side-chain $NH_3^+$ groups of the Egr-1—DNA complex under these solution conditions. 3D (H)CCENH3 spectrum (Iwahara et al. 2007) recorded at this temperature did not show signals from any Lys residues other than Lys89, which is located at the disordered C-terminal tail (data not shown). Because the (H)CCENH3 experiment involves the $^{13}C$-$^{15}N$ coherence transfer schemes via *J*-evolution from $C_x$ to $2C_yN_z$ terms and the $^{13}C$-$^{13}C$ cross polarization scheme, rapid $^{13}C$ transverse relaxation can severely reduce sensitivity at low temperatures. In contrast, the 3D H3NCECD and 3D H3NCG spectra clearly show signals from all Lys side-chain $NH_3^+$ groups (Fig. 2). As described above, higher sensitivity of these experiments can be attributed largely to efficient $^{13}C$-$^{15}N$ coherence transfer via *J*-evolution from $N_x$ to $2N_yC_z$ terms.

Our second approach to assign lysine side-chain $NH_3^+$ resonances at low temperatures is to observe $^{15}N\zeta$ resonances through $^1H$ detection of lysine side-chain $CH_2$ groups at higher temperatures. In this approach, we use a new 2D heteronuclear long-range correlation spectrum, (H2C)N(CC)H-TOCSY, which shows cross peaks of lysine side-chain $^{15}N\zeta$ and $CH_2$ proton resonances. Figure 3 illustrates the pulse sequence for this experiment. Owing to the detection of carbon-attached $^1H$ nuclei, this experiment is not hindered by rapid hydrogen exchange of $NH_3^+$ groups, and therefore can be performed using a high temperature at which $^{13}C$-$^{13}C$ Hartmann-Hahn cross polarization can occur efficiently. As described previously (Iwahara et al. 2007;

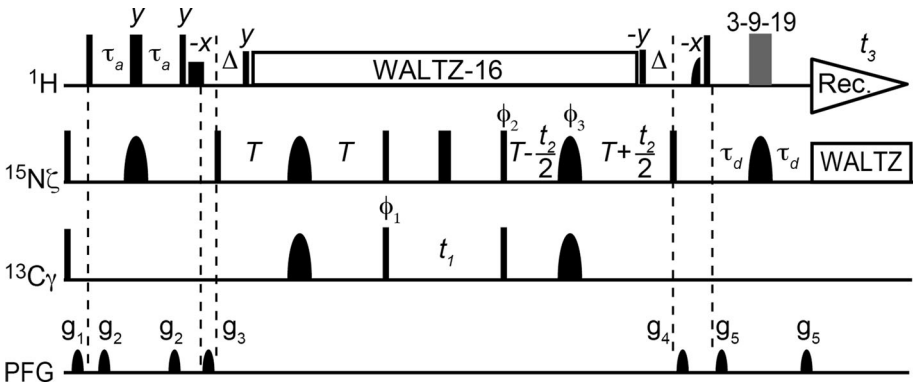


**Fig. 1** Pulse sequences for the 3D H3NCG experiment for Lys side-chain $NH_3^+$ resonance assignment. Thin and bold *bars in black* represent hard rectangular 90° and 180° pulses, respectively. Unless indicated otherwise, pulse phases are along *x*. Carrier positions: $^1$H, the position of the water resonance; $^{15}$N, 33 ppm; and $^{13}$C, 20 ppm. Short-bold bars represent water-selective soft-rectangular $^1$H 90° pulses (1.2 ms). The 3-9-19 pulse train-based WATERGATE scheme (Piotto et al. 1992) was used to suppress the water signal. The $^{13}$Cγ-selective pulses should not affect $^{13}$Cε nuclei (∼42 ppm). RF strengths for $^1$H and $^{15}$N WALTZ-16 composite pulses (Shaka et al. 1983) were 3.3 kHz and 1.0 kHz, respectively. *Shaped pulses*: $^1$H half-Gaussian 90° pulse (2.1 ms); $^{13}$C I-BURP2 180° pulse (1.2 ms); and $^{15}$N r-SNOB 180° pulse (1.03 ms). Delays: $\tau_a = 2.7$ ms; $\delta = 2.6$ ms; and $T = 50$ ms. Phase cycles: $\phi_1 = [2x, 2(-x)]$, $\phi_2 = [x, -x]$, $\phi_3 = [4x, 4y]$, and receiver = $[x, -x, -x, x, -x, x, x, -x]$. Quadrature detections for indirect $^{13}$C and $^{15}$N dimensions were achieved using States-TPPI (Marion et al. 1989) for $\phi_1$ and $\phi_2$, respectively

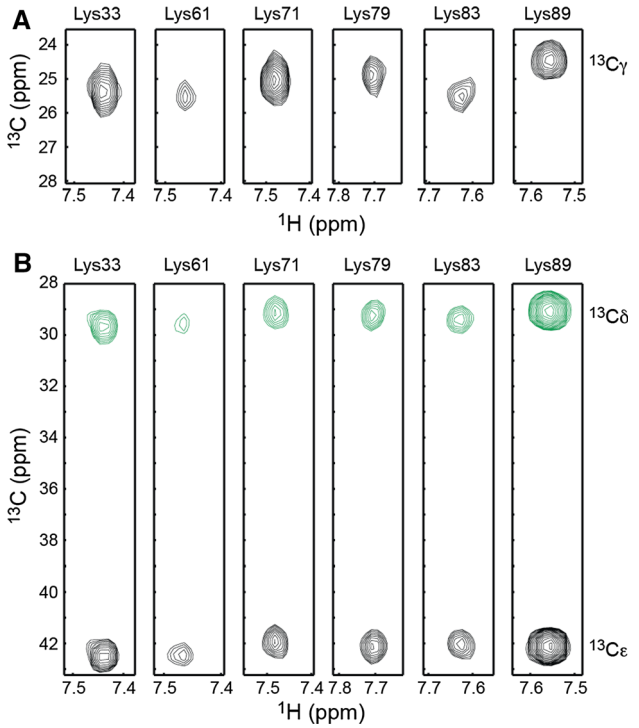


**Fig. 2** Strips of the 3D H3NCG (A) and H3CECD (B) spectra for all Lys side-chain $NH_3^+$ groups of the Egr-1 – DNA complex at 10 °C. Negative contours are shown in *green*. The $^1$H and $^{15}$N spectral widths for were 18.0 ppm and 4.7 ppm, respectively, in both experiments. The $^{13}$C spectral widths were 20.7 ppm in H3NCG and 26.0 ppm in H3NCECD. In each experiment, 32 scans were accumulated per FID, and numbers of complex points for $^1$H, $^{13}$C, and $^{15}$N dimensions were 810, 32, and 32, respectively. The total experimental time was 60 h each. These spectra were recorded at the $^1$H frequency of 750 MHz using a Bruker Avance III spectrometer equipped with a non-cryogenic TXI probe

Andre et al. 2007), $^{15}$N transverse magnetization is maintained to be in-phase with respect to $^1$H via $^1$H-WALTZ-16 decoupling so that scalar relaxation due to hydrogen exchange is avoided. Although this experiment can be implemented as a 3D experiment with a $^{13}$C dimension, we used the 2D version to achieve a higher resolution in the $^{15}$N dimension as well as a higher sensitivity. Figure 4 shows the 2D (H2C)N(CC)H-TOCSY spectrum recorded for the Egr-1—DNA complex. While rapid hydrogen exchange makes it impossible to detect $^1$H signals from the Lys $NH_3^+$ groups of the Egr-1—DNA complex at 35 °C, the (H2C)N(CC)H-TOCSY spectrum for the same sample at this temperature exhibited many $^1$H-$^{15}$N cross peaks from Lys side chains (Fig. 4C). In this spectrum, there was significant variation in signal intensity, presumably because individual Lys side chains exhibit different degrees of relaxation loss, depending on fast internal motions or slow conformational exchange (Foster et al. 1997). Because multiple side-chain $^1$H resonances are observed at high resolution along the $^1$H direct-detection dimension with excellent separation by sharp $^{15}$Nζ resonances, the (H2C)N(CC)H-TOCSY spectrum greatly facilitates $^{15}$Nζ resonances assignment for lysine $NH_3^+$ groups. The temperature dependence of the H2(C)N spectra (Andre et al. 2007) allows us to track the change of $^{15}$Nζ resonances as a function of temperature (Fig. 4B). These data together with the 3D H3NCG and H3NCECD spectra allowed us to unambiguously assign $^1$H-$^{15}$N HISQC signals from the Lys $NH_3^+$ groups of the Egr-1—DNA complex at 10 °C (Fig. 4A).

In conclusion, we have presented an effective strategy for resonance assignment of lysine $NH_3^+$ groups that can

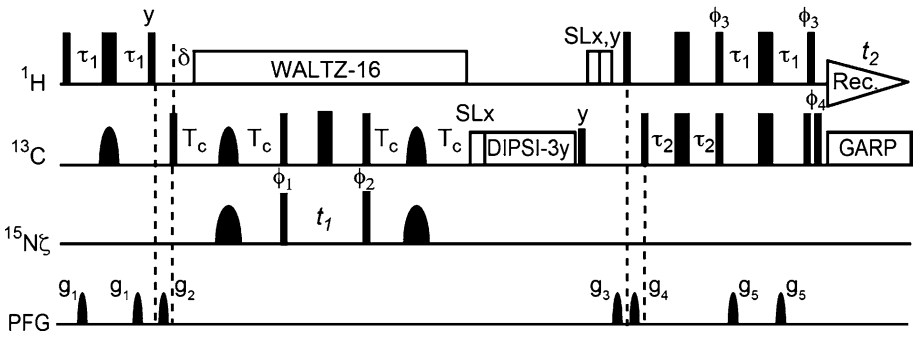


**Fig. 3** The pulse sequence of the 2D (H2C)N(CC)H-TOCSY experiment. This experiment provides correlation between Lys side-chain $^{15}$Nζ and $^{1}$H resonances, and corresponds to a hybrid of the H2CN (Andre et al. 2007) and HCCH-TOCSY (Kay et al. 1993) experiments for $H_2O$ samples. $^{13}$C shaped pulses are Lys $^{13}$Cε-selective r-SNOB pulses (980 us). Length of the $^{13}$C- spin lock (SL) was 1 ms. $^{13}$C carrier position was 43 ppm. The rf strength of the DIPSI-3 scheme (the total length, 16.9 ms) was 9.6 kHz and that of hard $^{13}$C pulses was 20.8 kHz. $^{15}$N *shaped pulses* are Lys $^{15}$Nζ-selective r-SNOB pulses (1.0 ms). $^{15}$N carrier position was 32 ppm. Delays: $\tau_1 = 1.6$ ms; $\tau_2 = 1.1$ ms; $\delta = 1.7$ ms; and $T_c = 20$ ms. *Phase cycles*: $\phi_1 = [x, -x]$; $\phi_2 = [2x, 2(-x)]$, $\phi_3 = [4x, 4y]$; $\phi_4 = [4x, 4(-x)]$; and receiver = [x, −x, −x, x, −y, y, y, −y]. Quadrature detections for indirect $^{15}$N dimensions was achieved using States-TPPI for $\phi_1$

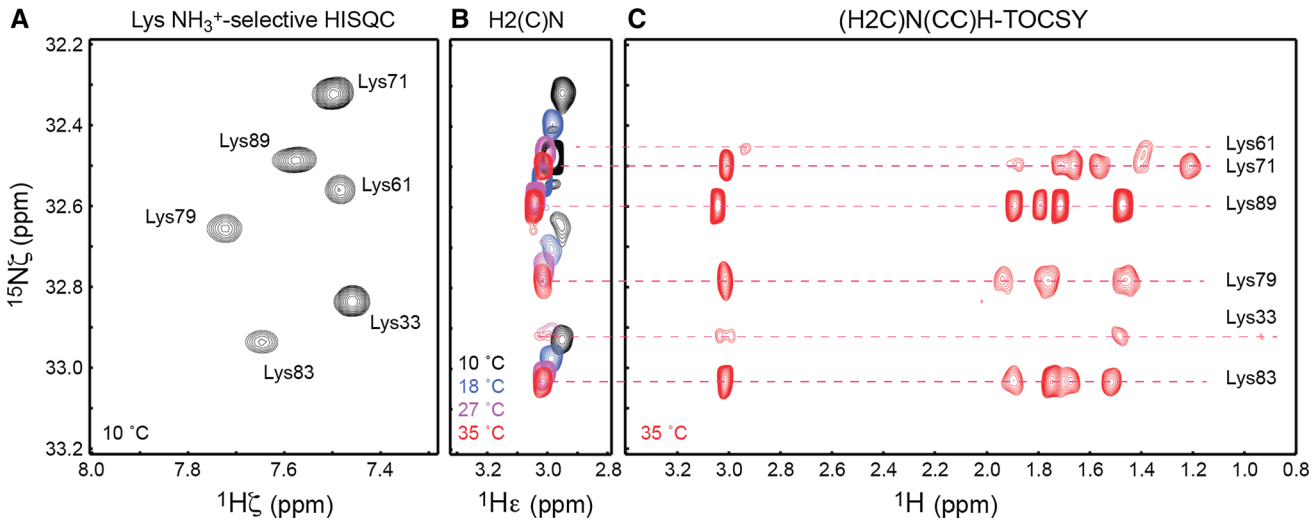


**Fig. 4** Use of 2D (H2C)N(CCH)-TOCSY spectrum and temperature dependence of 2D H2(C)N spectra for assignment of Lys $NH_3^+$ groups. The spectra were recorded for 0.8 mM $^{13}$C/$^{15}$N-labeled Egr-1—DNA complex at pH 5.8. **A** The lysine $NH_3^+$-selective HISQC (Iwahara et al. 2007) spectrum recorded at 10 °C. **B** Lysine side-chain-specific H2(C)N (Andre et al. 2007) spectra recorded at 10 °C (*black*), 18 °C (*blue*), 27 °C (*magenta*), and 35 °C (*red*). **C** (H2C)N(CC)H-TOCSY spectrum recorded at 35 °C. The spectra were recorded with a Bruker Avance III spectrometer equipped with a QCI cryogenic probe operated at the $^{1}$H frequency of 600 MHz

be observed only at relatively low temperatures. Our new approaches can help expand applicability of the NMR methods for characterizing Lys side-chain $NH_3^+$ groups (Anderson et al. 2013; Andre et al. 2007; Esadze et al. 2011; Iwahara et al. 2007; Segawa et al. 2008; Tomlinson et al. 2009; Williamson et al. 2013; Zandarashvili et al. 2011, 2013). As a consequence, dynamics of hydrogen bonds and ion pairs involving Lys side chains may become more assessable for a larger number of proteins and protein complexes.

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