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More line narrowing in TROSY by decoupling of long-range couplings: shift correlation and $^1J_{NC'}$ coupling constant measurements

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Abstract

Since the introduction of RDCs in high-resolution NMR studies of macromolecules, there is a growing interest in the development of accurate, and sensitive methods for determining coupling constants. Most methods for extracting these couplings are based on the measurement of the splitting between multiplet components in *J*-coupled spectra. However, these methods are often unreliable since undesired multiple-bond couplings can considerably broaden the multiplet components and consequently make accurate determination of their position difficult. To demonstrate one approach to this problem, G-BIRD$^{(r)}$ decoupled TROSY sequences are proposed for the measurement of $^1J_{NH}$ and $^1J_{NC'}$ coupling constants. Resolved or unresolved splittings due to remote protons are removed by a G-BIRD$^{(r)}$ module employed during t_1 and as a result, spectra with narrow, well-resolved peaks are obtained from which heteronuclear one-bond couplings can be accurately measured. Moreover, introduction of a spin-state-selective α/β-filter in the TROSY sequence allows the separation of the $^1J_{NC'}$ doublet components into two subspectra which contain the same number of peaks as the regular TROSY spectrum. The $^1J_{NC'}$ couplings are obtained from the displacement between the corresponding peaks in the subspectra.

Keywords: TROSY; G-BIRD$^{(r)}$; Spin-state-selective α/β-filter; Scalar couplings; $^1J_{NH}$; $^1J_{NC'}$; Protein

1. Introduction

There is still an increasing interest in improving the NMR spectral resolution of labeled biomolecules. In addition to the apparent resolving power of TROSY [1–4], the driving force behind such efforts is the bewildering array of available coupling constants which are important structural constraints utilizing torsional angle dependency. Manifestation of through H-bond scalar coupling [5–11] or sensitivity of $^1J_{NC'}$ coupling [12] to hydrogen bonding gives spectroscopic support to appreciate the importance of weak interactions in biomolecular interactions. More recently, introduction the concept of residual dipolar couplings (RDC) [13–17] opened a new avenue for structure refinement. All these techniques require precise and accurate determination of coupling constants which are often comparable to the natural linewidth in the spectra. There are two basic ways of obtaining coupling constants. Either splittings (e.g., E.COSY type methods) [18–20] or intensities (quantitative *J* correlation spectroscopy) [21–25] can yield coupling information. Of course, pros and cons may be mentioned for and against each approaches and many improvements of existing techniques appeared in the literature. Sensitivity is more crucial for the quantitative *J* methods, while resolution is the

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Achilles heel of E.COSY related techniques. In addition to the commonly used one-bond ^{15}N–^{1}H (or ^{13}C–^{1}H) dipolar couplings, backbone one-bond ^{15}N–$^{13}C'$ dipolar couplings can also provide valuable orientational constraints for protein structure determination. A number of quantitative J correlation [26–28] and E.COSY-type frequency splitting [29–33] experiments have been developed for measuring these couplings.

We have recently introduced a simple method [34] to improve the precision of one-bond coupling constant measurements in carbohydrates or peptides, with the obvious purpose of improving the quality of RDC measurements. In that work application of a group selective pulse combination G-BIRD$^{(r)}$, [35,36] successfully decoupled remote ^{1}H couplings in the indirect $F1$ dimension and yielded sharp doublets allowing easy measurement of small changes due to the presence of anisotropic interactions. In addition, we have also shown the use of the same principle to decouple long-range interactions from the most slowly relaxing component of the ^{15}NH pattern in ^{15}N ubiquitin though details were not published. Earlier, we have also demonstrated the line narrowing effect of band-selective SESAM homodecoupling during data acquisition in a TROSY-JM experiment [37,38].

Here, we describe the application of remote proton decoupling [39] in TROSY experiments of ^{15}N or $^{15}N/^{13}C$ labeled proteins. As it is well known, TROSY selects the most slowly relaxing multiplet component from the four lines of an ^{15}NH doublet in the two-dimensions of the experiment. However, the ultimate advantage of the line narrowing effect of opposing CSA and dipolar relaxation mechanism is hampered by a few unresolved long-range scalar couplings such as $^{2}J_{N,H\alpha}$, $^{3}J_{N,H\beta,H\beta'}$, $^{3}J_{N,H\alpha(i-1)}$ in the 1–4 Hz range or $^{1}J_{N,C\alpha}$ $^{2}J_{N,C\alpha}$ in between 1 and 12 Hz. While splittings due to carbon couplings are easily removed by broadband inversion/decoupling methods, this is not the case for long-range proton couplings. In the case of the tested small protein we used group-selective pulses, because these provide 'perfect' inversion profile. However, for bigger proteins other techniques may perform better. Efficient decoupling in the indirect ^{15}N dimension not only improves the signal to noise ratio, but the improved resolution may be beneficial when spectral congestion is serious, e.g., in α-helical proteins. Line narrowed TROSY variants may also be useful for the seed spectra of automatic assignment procedures.

2. Experimental

All experiments were performed on a BRUKER DRX 500 NMR spectrometer equipped with a TXI z-gradient probe and using XWINNMR 2.6 software. The TROSY spectra were recorded in 1.7 mM [U-^{15}N] or [U-^{15}N,^{13}C]ubiquitin (95%:5% = H_2O:D_2O), pH 4.7 (purchased from VLI, Southeastern, PA) at 308 K. All spectra were acquired with high spectral resolution of ca. 0.4–0.6 Hz/point allowing accurate measurement of couplings in the $F1$ dimension. For processing shifted squared cosine window function and double zero filling was applied. Other experimental details are given in the relevant figure captions.

3. Results and discussion

3.1. G-BIRD$^{(r)}$ decoupled TROSY sequences designed for ^{15}N- and $^{15}N/^{13}C$-labeled proteins

The TROSY pulse schemes augmented with the gradient-enhanced BIRD$^{(r)}$ module, G-BIRD$^{(r)}$ are depicted in Fig. 1. The G-BIRD$^{(r)}$ pulse applied at midway of the evolution period t_1, inverting all remote proton magnetization (protons two-, three-bond apart from ^{15}N), will refocus the undesired multiple-bond proton–nitrogen coupling modulation by the end of t_1. In case of $^{15}N/^{13}C$ labeled proteins, additionally both the nitrogen–carbon and proton–carbon coupling evolution should be refocused during the experiment to obtain the maximum resolution and sensitivity improvement. To this end, appropriately positioned composite 180° carbon pulses are incorporated into the pulse scheme as shown in Fig. 1B. The first carbon inversion pulse together with the two nitrogen 180° pulses refocus the J_{NC} evolution during t_1. The last two carbon 180° pulses applied during δ' at time points indicated by the dotted lines in Fig. 1 refocus the nitrogen–carbon and proton–carbon coupling evolution, respectively, during the rest of the experiment. Note that δ' is equal to three times the duration of the gradient pulse δ and a delay of δ is allowed between the corresponding pair of carbon 180° and nitrogen 90° pulses.

The resolution and sensitivity improvement achieved by the proposed G-BIRD$^{(r)}$ decoupled TROSY scheme is illustrated in Fig. 2. $F1$ traces of a crosspeak of [U-^{15}N,^{13}C]ubiquitin obtained with TROSY experiments allowing different coupling evolutions during t_1 are shown for comparison. The top trace (A) obtained with a regular TROSY designed for ^{15}N-labeled protein shows a broad multiplet due to unresolved nitrogen–carbon ($^{1,n}J_{NC}$) and nitrogen–proton couplings ($^{n}J_{NH}$). The pulse scheme of Fig. 1A yields a bit narrower multiplet (B) by decoupling the remote protons with the G-BIRD$^{(r)}$ module. Trace (C) was obtained with a carbon decoupled TROSY experiment including composite 180° carbon inversion pulses for efficient decoupling. It is apparent that the TROSY experiment of Fig. 1B designed and optimized for $^{15}N/^{13}C$ labeled proteins, refocusing all undesired coupling evolution during t_1 yields the sharpest, and most intensive

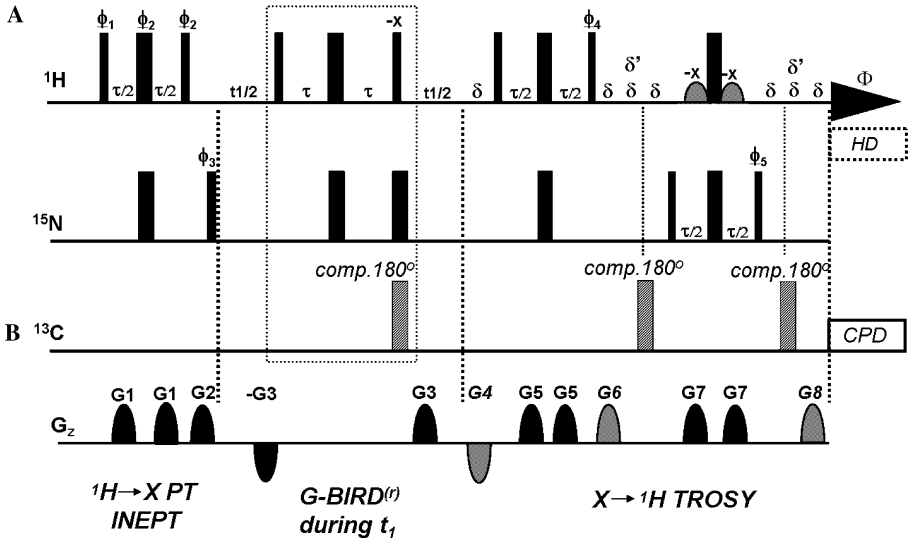

Fig. 1. G-BIRD$^{(r)}$ decoupled TROSY sequences optimized for ^{15}N- (A) and ^{15}N/^{13}C-labeled (B) proteins. Hard 90° and 180° pulses are marked by narrow and wide bars, respectively, with the phase x, unless indicated otherwise. A combination of water flip-back [51] and Watergate scheme [52] is used for solvent suppression. Selective excitation of water magnetization is achieved by 90° Gaussian pulses of 2 ms duration applied on water resonance; they are indicated by shaded half ellipses. Except for acquisition when the ^{1}H carrier frequency is set to the middle of the amide proton region, the ^{1}H transmitter is on-resonance for the water signal. The G-BIRD$^{(r)}$ element during t_1 refocuses all undesired multiple-bond proton–nitrogen coupling modulation. Decoupling of remote protons can be also achieved applying band-selective (H_α, H_β) proton inversion at midway of t_1 instead of the G-BIRD$^{(r)}$ module (see inset). Due to relaxation considerations, this shorter pulse sequence (1 ms $G3$ cascade) might be more suitable for applications on larger proteins. In case of ^{15}N/^{13}C-labeled proteins appropriately positioned composite 180° carbon pulses (indicated by hatched bars and their positions marked by dotted lines in Fig. 1B) refocus all undesired nitrogen–carbon and proton–carbon coupling evolution during the experiment. Carbons are decoupled during acquisition with GARP sequence. The optional band-selective homo-decoupling of H_α,H_β protons during acquisition [37,38] is also indicated. Delay durations: $\tau = 1/(2^1J_{NH})$ and $\delta' = 3\delta$, where $\delta = 1.2$ ms includes the duration of the shaped gradient pulse of 1 ms and a recovery delay of 200 μs. Echo–antiecho selection is achieved by the shaded pulsed field gradients. For ^{1}H and ^{15}N spins the echo is obtained for $\phi_1 = -y$, $\phi_2 = -x$, $\phi_4 = y$, $\phi_5 = y$, $G4 = -35$, $G6 = 15$, and $G8 = 10$ G/cm. In the second experiment the antiecho signal is obtained for $\phi_1 = y$, $\phi_2 = x$, $\phi_4 = -y$, $\phi_5 = -y$, $G4 = -40$, $G6 = 10$, and $G8 = 15$ G/cm. The amplitudes of other gradients are as follows: $G1 = 12$, $G2 = 35$, $G3 = 7.5$ (0.5 ms duration), $G5 = 14$ and $G7 = 28$ G/cm (0.5 ms duration). $\phi_3 = x{-}x$ and the receiver phase $\Phi = x{-}x$. This phase cycling scheme selects the most slowly relaxing component of the ^{15}N-^{1}H multiplet. $\phi_4 = -y$ and $\phi_5 = y$ selects the second sharpest component in $F1$ allowing the measurement of $^1J_{NH}$ from the signal displacement.

signal (D). The signal narrowing achieved is 8.4–6.0 = 2.4 Hz, which is in the range of unresolved couplings. The resolution enhancement is inherent advantage of the fully $F1$-decoupled TROSY scheme, but the practical sensitivity improvement can be lower, depending on the size of the protein, due to the increased total duration of the experiment. Mixing of TROSY and anti-TROSY components can potentially cause cross-talk in the spectra if J-spreading is significant. Under our conditions such artifacts were not observed. Clean TROSY was proposed earlier to reduce similar, relaxation induced artifacts [40]. It should be noted that decoupling of remote protons can also be achieved using band-selective (H_α,H_β) proton inversion applied at midway of t_1 instead of the G-BIRD$^{(r)}$ module, as shown in the inset of Fig. 1. In our hands (using the same ubiquitin sample) this method gave similar results. However, due to relaxation considerations, this shorter pulse sequence might be more suitable for larger proteins. Band-selective homo-decoupling during acquisition is also an option for further sensitivity and resolution improvement, as demonstrated in earlier works [37,38].

The TROSY experiment with different phase cycling allows one to select for the second largest (sharpest) ^{15}N-doublet component in $F1$. The displacement of the relevant peaks measured from the two edited TROSY spectra yields the $^1J_{NH}$ coupling constant with high accuracy.

3.2. Spin-state-selective α/β-filtered, G-BIRD$^{(r)}$ decoupled TROSY for one-bond nitrogen–carbonyl carbon, $^1J_{NC'}$ coupling constant measurement in ^{15}N/^{13}C labeled protein

The modified TROSY scheme of Fig. 3 proposed for determining $^1J_{NC'}$ is a straightforward extension of the carbon and G-BIRD$^{(r)}$ decoupled TROSY experiment discussed above. The two experiments differ in the followings: all composite carbon 180° pulses of Fig. 1B are replaced by C_α/C_β band selective inversion pulses to refocus the undesired $^{1,2}J_{NC\alpha}$, $^{2,3}J_{NC\beta}$ coupling evolution, while retaining the desired $^1J_{NC'}$ modulation during the experiment. So the G-BIRD$^{(r)}$ sequence together with the band selective carbon pulses eliminate

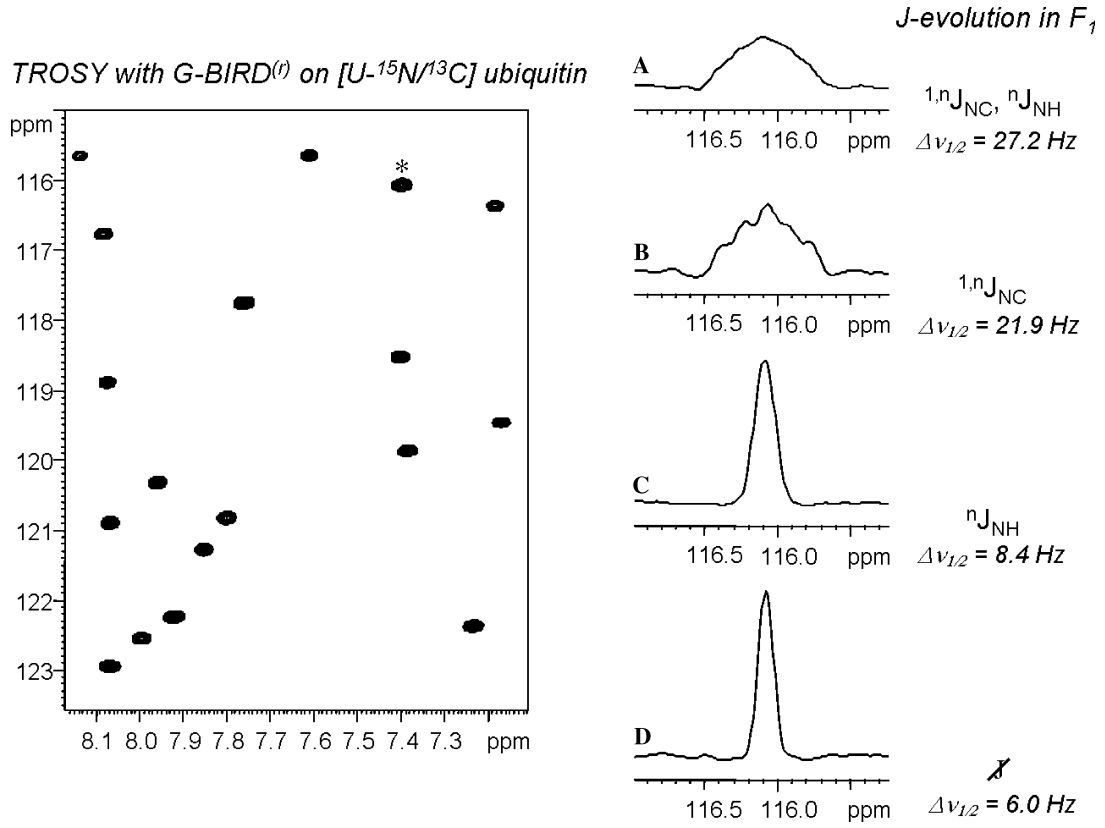

Fig. 2. Expansion of the G-BIRD$^{(r)}$ decoupled TROSY spectrum of [U-^{15}N,^{13}C]ubiquitin recorded with the pulse sequence in Fig. 1B. $F1$-traces extracted at crosspeak labeled by asterisk (*) from different TROSY spectra recorded with TROSY variants allowing and/or suppressing evolution of different couplings in $F1$ are also depicted. Of course, splittings due to carbon couplings can be easily removed by appropriate broadband inversion or decoupling methods, however, to demonstrate the attainable resolution enhancement step-by-step the inclusion of ^{13}C-coupled traces is inevitable. Trace (A) obtained with conventional TROSY sequence designed for ^{15}N-labeled proteins allowing evolution of both $^{1,n}J_{CN}$ and $^{n}J_{NH}$ couplings during t_1. Trace (B) obtained with the pulse sequence in Fig. 1A allowing evolution of $^{1,n}J_{CN}$, but suppressing that of $^{n}J_{NH}$ with the G-BIRD$^{(r)}$ module. Trace (C) obtained with TROSY sequence including a composite 180° pulse at midway of t_1 to refocus evolution of $^{1,n}J_{CN}$ couplings during t_1. Trace (D) obtained with the pulse sequence in Fig. 1B optimized for ^{15}N/^{13}C labeled proteins suppressing all undesired coupling modulation during the course of the experiment. Line width measured at half-height are also depicted beside the corresponding traces to quantify the achieved resolution improvement. Experimental parameters: proton 90° pulse of 11.2 μs, carbon 90° pulse of 16.0 μs, nitrogen 90° pulse of 35.0 μs, and carbon 90° pulse of 80.0 μs for GARP decoupling. Spectral widths in $F1$ ($F2$) dimension = 1300 (2750) Hz, number of t_1 increments = 512, number of data points in $F2$ = 512, number of scans = 8, relaxation delay = 1.5 s. The carrier frequencies in the ^{1}H, ^{15}N, and ^{13}C channels are positioned at 4.7 ppm (water resonance), 8.5 ppm (middle of amide proton region, before acquisition), 119, and 47 ppm, respectively. Selective 90° Gaussian pulse of 2 ms is applied to the water resonance. The duration of the field gradient pulse is 1 ms, unless indicated otherwise in the caption of the pulse scheme.

all coupling modulation except that of $^1J_{NC'}$. As a result sharp and well-resolved $F1$-doublets appear in the TROSY spectrum (Fig. 4), allowing accurate measurement of $^1J_{NC'}$ from the separation of the doublet lines.

To reduce the number of peaks in the spectrum and avoid the overlap of doublets, a spin-state-selective α/β-filter [41–50] can be introduced prior to t_1. The pulse scheme of the α/β-filter is shown as an inset of Fig. 3. In-phase and antiphase doublets are recorded in two experiments according to the followings; applying the ^{15}N and $^{13}CO_{sel}$ inversion pulses simultaneously in the middle of the $4\tau'$ evolution period of $1/(2^1J_{NC'})$ yields antiphase doublets before t_1. In-phase doublets are obtained when the first and third $^{13}CO_{sel}$ inversion pulses are applied together with the ^{15}N 180° pulse. The doublet components can be edited into two separate subspectra by adding and subtracting the corresponding in-phase and antiphase doublets as shown in Fig. 4. The resultant spectra have narrow and well-resolved signals due to the suppression of any undesired coupling modulation during the experiment and the number of crosspeaks remains the same as in the regular TROSY correlation map. The displacement of the corresponding peaks measured from the edited subspectra yields the $^1J_{NC'}$ coupling constant with high accuracy. The chosen filter element is preferred for its favorable editing properties and insensitivity for J-scatter [44]. However, for bigger proteins, shorter filters should probably replace the tested variant.

4. Conclusions

We have shown that the ultimate resolving power of TROSY in ^{15}N and ^{15}N/^{13}C labeled biomolecules can be

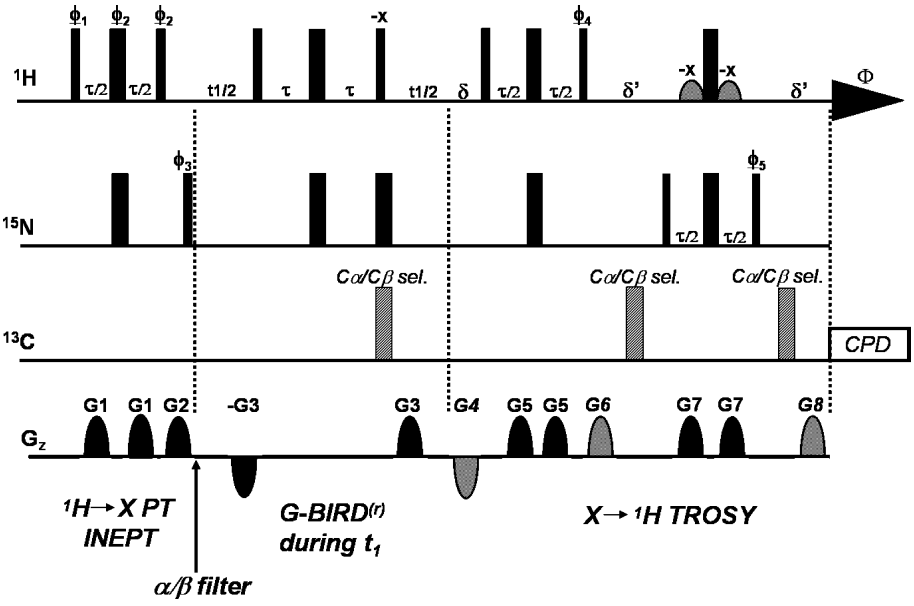

Fig. 3. α/β Filtered, G-BIRD$^{(r)}$ decoupled TROSY sequence for the measurement of $^{1}J_{NC'}$ couplings from [^{15}N, ^{1}H] correlation maps of ^{15}N/^{13}C-labeled proteins. Gaussian Cascade (*G*3) [53] C_α/C_β band selective 180° pulses are applied to refocus the undesired $^{1,2}J_{NC\alpha}$, $^{2,3}J_{NC\beta}$ coupling evolution, while retaining the desired $^{1}J_{NC'}$ modulation during the experiment. Spin-state-selective α/β-filter, shown as inset, can be introduced before t_1 for spectrum editing. Duration of τ' is 8.33 ms (corresponding to $1/(8^{1}J_{NC'})$). Selective excitation of CO carbons is achieved by *G*3 pulse. For other details of the experiment (phase cycling, gradient pulses, delay durations, etc.) see caption of Fig. 1.

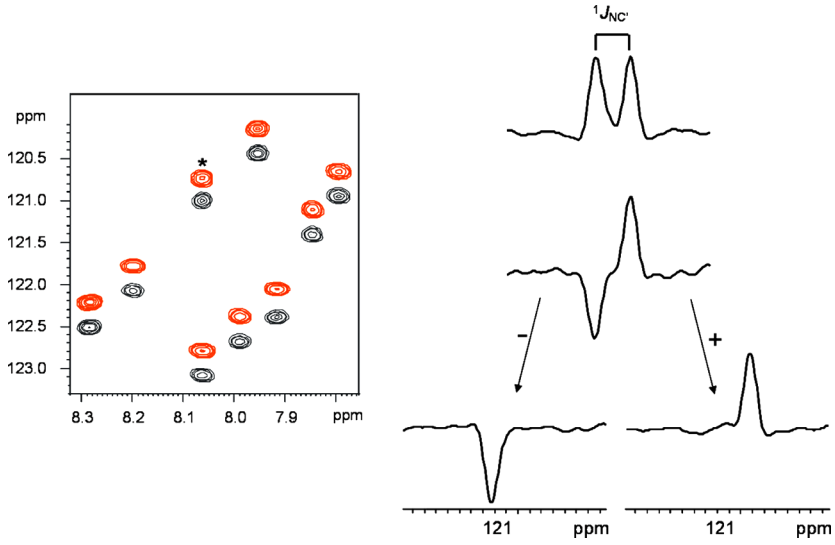

Fig. 4. Overlay of the edited TROSY subspectra shows the $^{1}J_{NC'}$ displacement of the relevant peaks in *F*1. Traces selected at the crosspeak labeled by asterisk (*) from the "in-phase" and "antiphase" spectrum, and the corresponding edited single multiplet components are also depicted. Band selective ^{13}C excitation is achieved by *G*3 180° pulse of 270 μs. For other experimental parameters see caption of Fig. 2.

obtained if the numerous unresolved long-range coupling interactions are decoupled in the *F*1 dimension. The sensitivity gain in the case of ubiquitin can be significant, but, for larger proteins and at higher fields, band-selective proton-decoupled variants of the present method, the traditional TROSY or other concurrent methods may be superior. However, the resolving capability of the G-BIRD$^{(r)}$ decoupled TROSY allows highly accurate determination of $^{1}J_{NH}$ and $^{1}J_{NC'}$ couplings in the *F*1 frequency domain, which is essential for the extraction of residual dipolar couplings.

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