Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Magnetic Resonance 163 (2003) 360–368
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Communication

$^{1}H,^{15}N,^{13}C$-triple resonance NMR of very large systems at 900 MHz

John Chung* and Gerard Kroon

Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Received 10 January 2003; revised 15 April 2003

Abstract

We provide quantitative signal to noise data and feasibility study at 900 MHz for $^{1}H–^{15}N–^{13}C$ triple resonance backbone assignment pulse sequences obtained from a medium sized ^{2}H, ^{13}C, ^{15}N labeled protein slowed down in glycerol–water solution to mimic relaxation and spectroscopic properties of a much larger protein system with macromolecular tumbling correlation time of 52 and 80 ns, respectively, at 296 and 283 K (corresponding to molecular weights of 130 and 250 kDa). Comparisons of several different schemes for transferring magnetization from proton to nitrogen and back to proton confirms Yang and Kay's 1999 prediction that avoiding the unfavorable relaxation properties of $^{1}H–^{15}N$ multiple quantum coherence in the TROSY phase cycle of the final $^{15}N–^{1}H$ transfer before acquisition is crucial for maximal sensitivity from these very large molecular weight systems. We also show results which confirm some predictions regarding the superiority of TROSY at 900 MHz vs. 800 MHz especially as the molecular weights become very large.

1. Introduction

Since Wuthrich and coworkers first showed [1] that relaxation cross-correlation effects between chemical shift and dipolar interaction tensors can be exploited to obtain narrower line-widths even in very large biomacromolecules (MW $\gg$ 50 kDa), there have been a number of publications utilizing the Transverse Relaxation Optimized Spectroscopy (TROSY) method in ever larger molecular weight systems [2].

The present work seeks to examine the utility of existing TROSY-based schemes to obtain $^{1}H–^{15}N–^{13}C$ type triple resonance spectra in as high a molecular weight systems as possible (or as large a macromolecular rotational correlation time, τ_c) and ascertain the upper limits of the feasibility of acquiring spectra from experiments like HNCO or HNCA for sequential assignment purposes.

We would also like to address the question of how much advantage the 900 MHz system has over lower field spectrometers, such as the 800 MHz, when it comes to maximizing the TROSY effect for larger molecular weight systems. Quantitative comparisons between 800 and 900 MHz are shown for medium size protein and very large systems in Appendix A.

2. Methods

2.1. Sample used

We want to model the rotational tumbling behavior of very large molecular weight systems (rotational correlation time $\tau_c \gg 40$ ns, MW $\gg$ 100 kDa) while having sufficiently well characterized spectra of a well-known and well-behaved sample which can be expressed with triple labeling and solubilized at high concentrations and is stable over very long periods of time; thus we have taken a 22 kDa triple-labeled protein, LFA-1 (human Leukocyte Function Associated Antigen-1) [3] and slowed it down in glycerol–water solution in order to be able to access rotational correlation times in excess of 90 ns at near 273 K. The sample was prepared as follows. To a 2.5 mM LFA-1 (uniformly enriched in ^{13}C, ^{15}N, 70% enriched in ^{2}H) in 95/5% H_2O/D_2O solution (pH 7.5, 2 mM NaCl) was added 40% glycerol-d_8(v/v), making the sample about 2 mM in LFA-1 (molar ratio of H_2O:glycerol $\sim$6).

* Corresponding author. Fax: 1-858-784-7454.
E-mail address: chung@scripps.edu (J. Chung).

1090-7807/$ - see front matter © 2003 Published by Elsevier Science (USA).
doi:10.1016/S1090-7807(03)00150-2

It was determined by relaxation measurements (^{15}N T_1/T_2 ratio) on 40 backbone resonances that the sample had a rotational correlation time, τ_c of 52 ± 2 ns at 296 K (or 54 ± 1.6 ns over 33 resonances) corresponding to molecular weight of 130 kDa. At 283 K, ^{15}N T_1 and T_2 measurements are no longer possible, so fitting of 1D-CRIPT [4] experimental intensity build-up and decay as a function of mixing time to Eq. (1) of [4] ($R_C = 200$, $R_I = 430$) was used to determine a correlation time of 80 ns, corresponding to molecular weight of roughly 250 kDa. Using comparisons of viscosity measurements tabulated [5] at 293 and 273 K for 50 wt% glycerol in water, we can estimate a value of $\tau_c > 85$ ns at 283 K and confirm the rotational correlation times of 52 and 80 ns determined at 296 and 283 K, respectively, by the above relaxation time ratios and 1D-CRIPT intensity build-up methods.

2.2. NMR experiments

Data were acquired on Bruker DRX-800 and Avance-900 spectrometers. All spectrometers were equipped with triple resonance (^{1}H, ^{13}C, ^{15}N) probeheads also fitted with triple axis gradients. The following experiments/pulse sequences were designed and/or optimized for evaluation of triple resonance experiments at very large molecular weights:

(1) HNCO-clean-TROSY (Fig. 1a);
(2) ^{15}N,^{13}C-MQ-HNCO-clean-TROSY (Fig. 1b);
(3) ^{15}N,^{13}C-MQ-HNCO (Fig. 1c);
(4) HNCO-Yang/Kay-TROSY (Fig. 1d);
(5) ^{15}N,^{13}C-MQ-HNCO-CRINEPT-TROSY (Fig. 1e).

HNCO-clean-TROSY is of the clean-TROSY variant devised by Sorensen and coworkers [6–8] in which ^{1}H, ^{15}N double and zero-quantum coherences are selected in

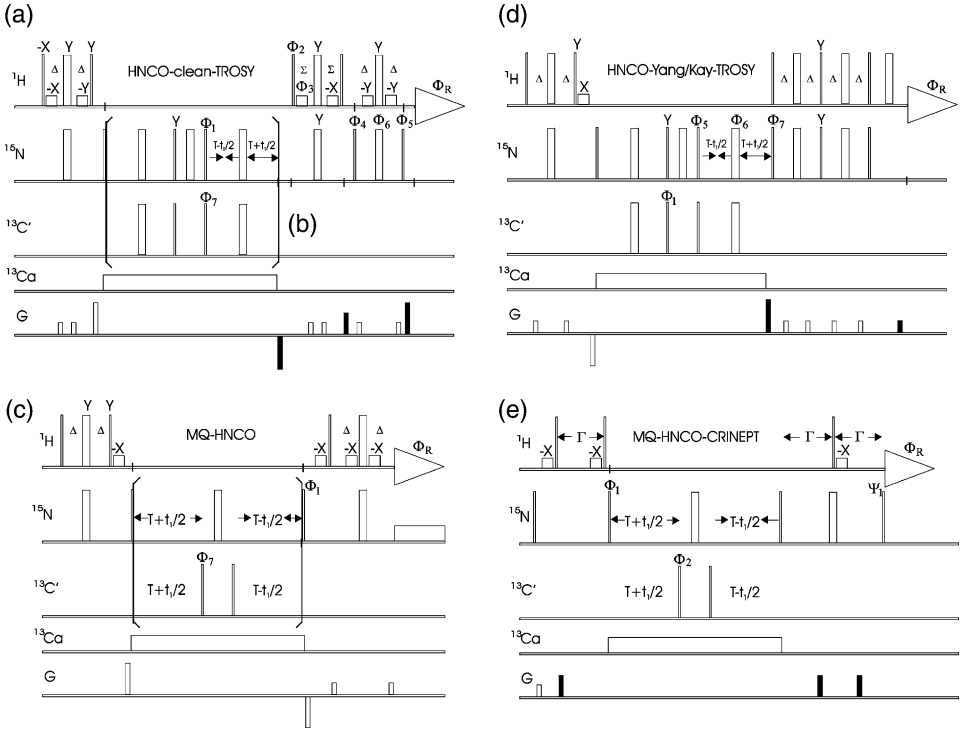

Fig. 1. (a) HNCO clean-TROSY using the scheme of Sorensen and coworkers [5–7] with modifications to ensure water saturation is minimized at 900 MHz. Delays were set as follows: $\Delta = 2.5$ ms; $\Sigma = 2.0$ ms; $T = 10.0$ ms. Phase cycle is $\phi_1 = x, -x$; $\phi_2 = \phi_3 = y$; $\phi_4 = x, x, -x, -x$; $\phi_5 = \phi_6 = y, y, -y, -y$; $\phi_7 = 4(x), 4(-x)$; $\phi_R = x, 2(-x), x, -x, 2(x), x$. Quadrature in 15N is obtained by changing the signs and amplitudes of the three gradients shown in filled bars while inverting ϕ_2, ϕ_3, and ϕ_5, as prescribed in [6]. (b) HNCO clean-TROSY using ^{15}N–^{13}C multiple quantum coherence for ^{13}C evolution, obtained by replacing the bracketed region of (a) with the analogous bracketed region of (c) and setting the first ^{15}N $\pi/2$ to ϕ_1. ^{15}N chemical shift is obtained by incrementing the first interval T while decrementing the second interval T (this procedure causes slight phase twists in the ^{1}H–^{15}N plane due to modulation of ^{15}N chemical shift with ^{13}C–^{15}N scalar coupling). Phase cycle is identical to (a). (c) HNCO using only the INEPT scheme for ^{1}H–^{15}N and ^{15}N–^{1}H transfer but TROSY principle applied for ^{15}N magnetization only; also uses ^{15}N–^{13}C multiple quantum coherence for ^{13}C evolution. Delays were set as follows: $\Delta = 2.5$ ms; $T = 20.0$ ms. Phase cycle is $\phi_1 = x, -x$; $\phi_7 = x, x, -x - x$; $\phi_R = x, -x, -x, x$. Quadrature in ^{15}N is obtained by States-TPPI of ϕ_7 while incrementing and decrementing the first and second T as in (b) above. (d) HNCO TROSY scheme of Yang and Kay [9] with very little modifications. Delays were set as follows: $\Delta = 2.5$ ms; $T = 10.0$ ms. Phase cycle is $\phi_1 = x, x, -x, -x$; $\phi_5 = y, -y$; $\phi_6 = 4(x), 4(-x)$; $\phi_7 = x$; $\phi_R = x, -x, -x, x$. Quadrature in ^{15}N is obtained by the enhanced sensitivity gradient method, where for each value of t_1 separate data sets are recorded for the last gradient (+/−) and ϕ_7 ($x/-x$). (e) HNCO CRINEPT-TROSY using 2D CRINEPT scheme prescribed by Riek et al. [4] and also using ^{15}N–^{13}C multiple quantum coherence as in (b) and (c) above. Delays were set as follows: $\Gamma = 3.0$–3.5 ms; $T = 20.0$ ms. Phase cycle is: $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_R = x, -x, -x, x$. Quadrature in ^{15}N is obtained by States-TPPI of ϕ_1, along with inversion of ψ_1 and storing of data in two different locations and recombining in processing as prescribed in [4]. Due to this procedure, in order to obtain quantitative results in comparison to the other methods (a–d), the number of scans per t_1 increment must be half as many for this scheme.

the refocusing reverse-INEPT portion of the sequence just prior to data acquisition; modifications were made by adding water flip-back pulses to ensure highest sensitivity on the 900 MHz spectrometer which seemed much more sensitive to radiation damping effects than the 800, presumably due to the 900 probe circuit's higher Q factor. The first S^3 CT element [7] in this sequence was empirically set to $2\,\text{ms} \times 2$ by minimizing the well-known TROSY artifacts [9]. HNCO-Yang/Kay-TROSY was used without any modifications based on the published sequence of Yang and Kay [10]. See text for discussions of the other pulse sequence schemes.

3. Results and discussion

In order to examine possible triple resonance schemes for very large molecular weight systems, it is convenient to recognize that the typical ^1H–^{15}N–^{13}C–^{15}N–^1H hetero-nuclear COSY/relay experiments are all built upon the building block of ^1H–^{15}N–^1H 2D experiments such as TROSY [1] and CRINEPT [4], whose sensitivity and resolution characteristics have been analyzed extensively by various groups. Obviously a particular scheme which best preserves the ^{15}N multiplet component (spin state) with the longest transverse relaxation time without mixing it with other shorter-lived components would best succeed as a building block for triple resonance experiments of larger molecular weight systems. Two of the ^1H–^{15}N–^1H schemes considered here are the TROSY modifications of Sorensen's group (clean-TROSY) [6], and CRINEPT. Whereas the TROSY scheme of Pervushin and all others preserves just the up-field ^1H and down-field ^{15}N narrow component, the CRINEPT scheme does nothing to phase cycle out the so-called anti-TROSY (down-field ^1H and up-field ^{15}N) component but relies on the line-width of the anti-TROSY component being so large as to not be of appreciable intensity relative to the TROSY component. There is one other ^1H–^{15}N–^1H scheme which similarly leaves untouched the anti-TROSY component, shown by Yang and Kay [10], which we also consider here.

Following the work of Yang and Kay [10] in which quantitative estimates of TROSY/anti-TROSY intensity ratios were given for triple resonance experiments where there are long constant time delays for ^{15}N–^{13}C coherence transfer and back, we have ascertained that when the rotational correlation time of a system is long, only the TROSY (narrow) component of the two possible ^{15}N multiplets survives even without any attempts to remove or select certain components with appropriate 'TROSY' phase cycling. For the molecular weights we are considering ($\tau_c > 80\,\text{ns}$), it can be seen that the Yang and Kay scheme used for simple double resonance 2D spectrum (obtained by taking Kay, et al.'s 1992 gradient sensitivity enhanced HSQC scheme [11] and adding a ^{15}N $\pi/2$ pulse simultaneously to the last ^1H $\pi/2$ pulse, removing the ^1H π pulse in t_1, and turning off ^{15}N decoupling during acquisition) is already free of the anti-TROSY cross-peak even without a long ^{15}N constant time evolution period (with the exception of the Asn and Gln NH_2 side-chains).

Since a triple resonance pulse sequence based on CRINEPT has not yet been published, we have chosen to create such a sequence utilizing ^{15}N, ^{13}C multiple-quantum coherence transfer, the MQ-HNCO-CRINEPT, shown in Fig. 1e. This sequence is a simplification of motifs used by Salzmann et al. [12] and discussed by Loria et al. [13] and minimizes the number of ^{15}N and ^{13}C π pulses which were shown to be of negligible effect for correlation times of 52 ns but are expected to be unfavorable at the largest molecular weights. In Fig. 1e, the first constant time delay of $\sim$20 ms is incremented while the second 20 ms delay is decremented en bloc in order to achieve chemical shift evolution in ^{15}N. While this may seem unfavorable due to the modulation of the ^{15}N–^{13}C J evolution with chemical shift evolution, causing phase twisted ^{15}N line-shapes, in practice the scheme works well, and the phase twist is not noticeable due to the large line-widths involved; modulation of the ^{15}N–^{13}C transfer function (as $\text{Sin}(\pi * {}^1J_{NC}(T - t_1/2))$) is not unfavorable for the maximum t_1 used (10 ms) when T is 20 ms and assuming $^1J_{NC}$ of 15 Hz. An advantage of the ^{15}N, ^{13}C MQ coherence transfer is its simplicity, and it can be readily incorporated into the complex quadrature and multiplet phase recovery scheme of 2D CRINEPT as published by Riek et al. [4].

We have also chosen to do a simple INEPT/reverse-INEPT based HNCO using the same ^{15}N,^{13}C MQ transfer (since it gave cleaner and better signal intensity than the single quantum version with more ^{15}N and ^{13}C pulses), shown in Fig. 1c. This is no different from a standard HNCO sequence with the proton composite pulse decoupling removed (and can be compared to Yang and Kay's Fig. 2C of [10]).

For TROSY based sequences, there are two considered here. The clean-TROSY method of Sorensen group gives the maximal signal with the least artifacts for most systems we have studied at 900 MHz. However, like the TROSY scheme of Pervushin and others, this method requires ^{15}N–^1H multiple quantum coherence to be present during the first part of the transfer from ^{15}N back to ^1H before acquisition, as pointed out by Yang and Kay [10]. Thus we have done a direct comparison of the Sorensen method and the Yang/Kay method TROSY schemes incorporated into HNCO sequences. The ^{15}N–^{13}C multiple quantum transfers were considered (Fig. 1b) in addition to the traditional single quantum $^{13}C'$ evolution for the Sorensen version of TROSY in Fig. 1a; for Yang/Kay scheme, only the single quantum version, Fig. 1d, was considered, based on its 1D signal intensity being the same as the multiple quantum version.

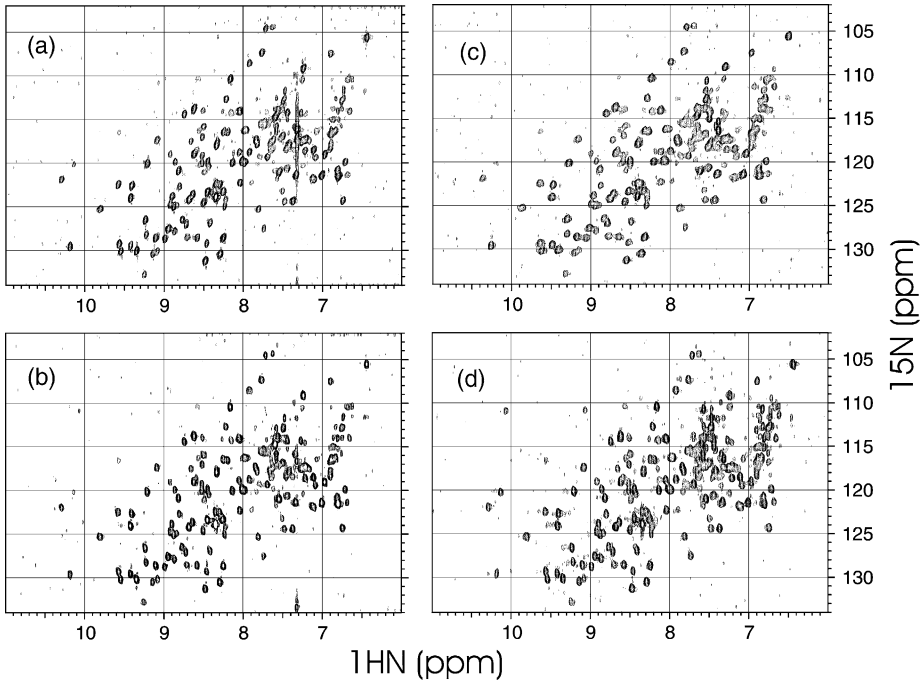

Fig. 2. 2D HNCO ^{1}H–^{15}N spectra of LFA-1 in 40% glycerol-d_8 at 296 K (corresponding to 52 ns τ_c) using various pulse sequence schemes of Fig. 1. In all four, data matrices consisting of (64, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (21.9 and 71.2 ms) in (F1, F2) were recorded with 64 transients/FID with a repetition delay of 2.0 s, and all at the same receiver gain setting and contour plotted at the same levels. (a) Using pulse scheme of Fig. 1b; (b) using pulse scheme of Fig. 1a; (c) using pulse scheme of Fig. 1c; and (d) using pulse scheme of Fig. 1d.

3.1. $T = 296$ K ($\tau_c = 52$ ns)

Fig. 2 shows comparison of 2D ^{15}N–^{1}H correlation spectra of LFA-1 in glycerol at 296 K (corresponding to 52 ns τ_c), acquired using the various HNCO pulse schemes of Fig. 1 (relaying through the ^{13}C but not evolving ^{13}C chemical shift). Quantitative comparison of peak intensities is shown in Table 1 for these 2D data. As is evident from the intensities of some of the weakest peaks, the quantitative results are consistent with visual inspection of the spectra. Signal to noise comparisons between 3D HNCO using Yang/Kay motif and clean-TROSY motif gave results consistent with Table 1 (data not shown); it is clear that the Yang/Kay-TROSY motif is markedly better than regular TROSY schemes which store magnetization as multiple-quantum coherence during part of the transfer from ^{15}N to ^{1}H. The sensitivity of the 3D HNCO is such that >95% of all known and assigned peaks for LFA-1 system in water at 298 K were found.

3.2. $T = 283$ K ($\tau_c = 80$ ns)

To assess the sensitivities of ^{1}H–^{13}C–^{15}N correlation schemes of Fig. 1 at 283 K and below ($\tau_c > 80$ ns), we acquired simple 2D ^{1}H–^{15}N double-resonance spectra (^{1}H–^{15}N–^{1}H only, without relay through ^{13}C). In Fig. 3 are shown clean-TROSY (a), CRINEPT (b), water-flip-

Table 1
Relative signal to noise comparisons for various triple resonance pulse schemes for 52 ns protein

	A. HNCO clean-TROSY	B. MQ-HNCO clean-TROSY	C. MQ-HNCO	D. HNCO Y/K-TROSY
A. HNCO clean-TROSY	—	0.93 ± 0.15 (132)	1.10 ± 0.22 (81)	0.63 ± 0.12 (123)
B. MQ-HNCO clean-TROSY	1.10 ± 0.13 (125)	—	1.28 ± 0.22 (61)	0.74 ± 0.10 (72)
C. MQ-HNCO	0.88 ± 0.16 (78)	0.82 ± 0.25 (62)	—	0.60 ± 0.18 (110)
D. HNCO Y/K-TROSY	1.56 ± 0.24 (110)	1.38 ± 0.23 (72)	1.70 ± 0.30 (87)	—

Quantitative comparison of peak intensities of the various 2D HNCO spectra shown in Fig. 2 using pulse schemes of Fig. 1 reveals the results shown here for LFA-1 in 40% glycerol sample at 296 K. The number of peaks analyzed is shown in parentheses, and the letter in front of each name refers to the name convention in Fig. 1. Relative sensitivity for a particular pulse sequence is viewed by selecting a row and looking across the columns for comparison with other pulse sequences; the cell numbers transposed across the diagonal should be and is roughly reciprocal, confirming the reliability of the automated peak picking and hand editing of the peak tables before averages determined. The results show that HNCO-Yang/Kay-TROSY scheme is superior to the other three.

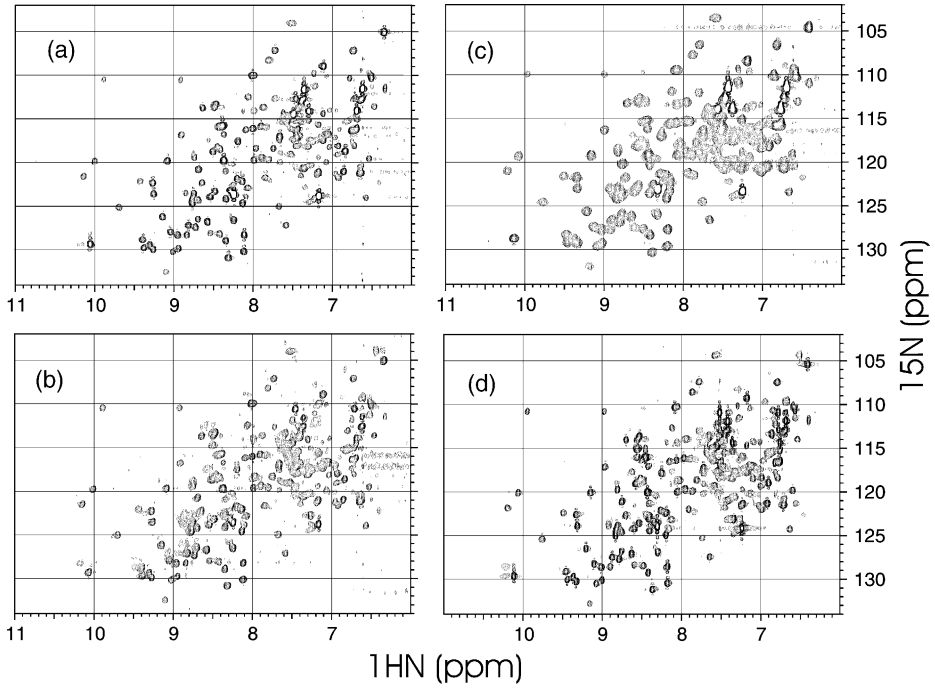

Fig. 3. 2D ^{1}H–^{15}N COSY spectra of LFA-1 in 40% glycerol-d_8 at 283 K (corresponding to 80 ns τ_c) using various pulse sequence schemes, but all at the same receiver gain setting and total experiment time. (a) Clean-TROSY; (b) CRINEPT-TROSY; (c) water flip-back HSQC; and (d) Kay-coupled-HSQC–TROSY. In a, c, and d data matrices consisting of (64, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (17.5 and 71.2 ms) in (F1, F2) were recorded with 64 transients/FID with a repetition delay of 2.0 s. In (b), two separate data matrices with 32 transients/FID was used with inversion of the last ^{15}N $\pi/2$ pulse and shuffled appropriately to obtain pure phase TROSY/anti-TROSY line-shapes as prescribed by Riek et al. [4]. Although difficult to tell from these spectra, quantitative peak height comparisons yielded the Kay-coupled-HSQC–TROSY scheme to have significantly higher sensitivity (40%) over clean-TROSY scheme which in turn had better sensitivity (10 and 25%, respectively) over HSQC and CRINEPT schemes at this temperature.

back HSQC (c), and Kay gradient sensitivity enhanced HSQC without t_1/t_2 decoupling and with extra ^{15}N $\pi/2$ pulse before acquisition (d). At this temperature, the 2D ^{1}H–^{15}N clean-TROSY has sensitivity advantage of about 25% relative to 2D ^{1}H–^{15}N CRINEPT, and so we expect this advantage to transfer to the triple resonance scheme if we simply inserted ^{15}N–^{13}C multiple quantum transfer scheme to either sequence to make the triple resonance schemes of Fig. 1. We do not expect CRINEPT to be useful or better than TROSY schemes at this temperature representing correlation time of a 250 kDa macromolecule. However, Yang/Kay-TROSY was an impressive further 40% gain over clean-TROSY, making it an ideal building block for triple resonance experiments.

To confirm these results, we show, in Fig. 4, 2D ^{1}H–^{15}N spectra from the triple resonance HNCO experiments of Fig. 1 (similar results as Fig. 2), this time at 283 K. Quantitative comparison of peak intensities between two of these spectra, Yang/Kay-TROSY based HNCO (Fig. 4d) and clean-TROSY based HNCO (Fig. 4a) shows 1.49 ± 0.26 for 54 peaks compared, consistent with comparison of just the ^{1}H–^{15}N transfers; superiority of the Yang/Kay-TROSY is convincingly demonstrated. The sensitivity of the 3D HNCO at this temperature is such that >95% of all the peaks present for 3D HNCO at 296 K were also present.

Comparisons of 2D ^{13}C HNCO spectra for pulse schemes 1a, b, c, and d show similar results as the 2D N–H correlation (data not shown). Fig. 5 shows a 2D ^{13}C Yang/Kay-TROSY-HNCA spectrum (same as Fig. 1d but without the band selective decoupling of the $^{13}C'$—replaced with shifted π pulses during $^{13}C\alpha$ evolution—but with ^{2}H decoupling instead), also at 283 K, clearly demonstrating the feasibility of backbone assignments at MW > 250 kDa, especially if higher deuteration levels are utilized. However, a further relay through the carbon for sequence such as HN(CO)CA or HN(CA)CO is likely to fail at these high fields with unfavorable carbonyl relaxation properties due to its large CSA.

3.3. $T = 277K$ ($\tau_c > 100$ ns)

Going further down in temperature to 277 K reduces the signal intensity in all of these experiments by at least 60% compared to the intensities at 283 K. Since even a 2D ^{1}H–^{15}N HNCO spectrum does not seem feasible at this temperature, we have acquired 2D ^{1}H–^{15}N correlation spectra using clean-TROSY (Fig. 6a), Kay-HSQC (Fig. 6d, with no decoupling in t_1/t_2 but added ^{15}N $\pi/2$ at

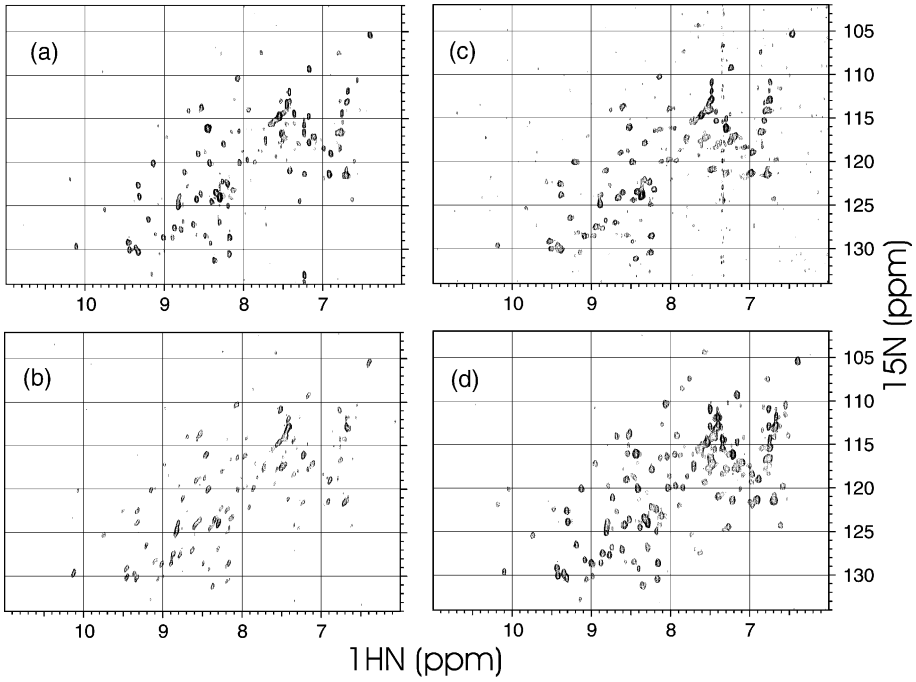

Fig. 4. 2D HNCO ^{1}H–^{15}N spectra of LFA-1 in 40% glycerol-d_8 at 283 K (corresponding to 80 ns τ_c) using various pulse sequence schemes of Fig. 1. In all but (b), data matrices consisting of (64, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (21.9 and 71.2 ms) in (F1, F2) were recorded with 192 transients/FID with a repetition delay of 2.0 s. In (b), two separate data matrices consisting of (64, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (21.9 and 71.2 ms) in (F1, F2) were recorded with 96 transients/FID with a repetition delay of 2.0 s, so the total experiment time are the same in all four. (a) Using pulse scheme of Fig. 1a; (b) using pulse scheme of Fig. 1e; (c) using pulse scheme of Fig. 1c; and (d) using pulse scheme of Fig. 1d. In (a) and (c), receiver gain setting was 0.1 and 0.25, respectively, of the spectra (b) and (d) and so are plotted accordingly at lower levels.

the end), and ^{15}N CRINEPT-TROSY schemes (Figs. 6b and c). Based on factor of 2.4 increase in viscosity going from 293 to 273 K (5), $\tau_c > 100$ ns is expected at temperature of 277 K, and as expected CRINEPT-TROSY is superior to the other schemes for ^{1}H–^{15}N correlation. At 277 K, however, the ^{15}N T_2 is too short to effect significant transfer to the ^{13}C ('out and back'). Thus the advantages of the CRINEPT scheme is too little (over other schemes) and too late to be of use as a module in triple resonance experiments.

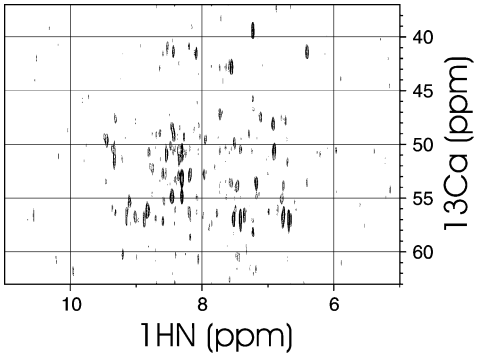

Fig. 5. 2D HNCA ^{1}H–^{13}C spectrum of LFA-1 in 40% glycerol-d_8 at 283 K using pulse sequence scheme similar to that of Fig. 1d. The carrier was moved from carbonyl to alpha carbon, and alpha carbon band-selective decoupling was replaced with phase-modulated π pulses set to decouple carbonyl carbons and also deuterium decoupling. Data matrix consisting of (32, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (10.8 and 71.2 ms) in (F1, F2) were recorded with 256 transients/FID with a repetition delay of 2.0 s.

4. Conclusion

Triple resonance backbone experiments for assignment of proteins of molecular weights in excess of 250 kDa and rotational correlation time of greater than 80 ns should be quite readily achievable with the highest field instrumentation available currently. This work shows experimental verification of the prediction of Yang and Kay who first correctly pointed out that the TROSY element which carefully avoids the short transverse relaxation time of ^{1}H–^{15}N multiple quantum coherence in the transfer from ^{15}N back to ^{1}H before acquisition is indeed superior for all molecular weight species which have ^{15}N transverse relaxation times long enough to effect anti-phase transfer to the direct bound ^{13}C. What is even better, this pulse sequence is almost exactly the same as the existing sequences for triple resonance spectroscopy which were published by the Toronto group in the early/mid 1990s; minimal modification of these pulse sequences will be sufficient to effect the necessary changes for observing triple resonance spectra of very large molecular weight species.

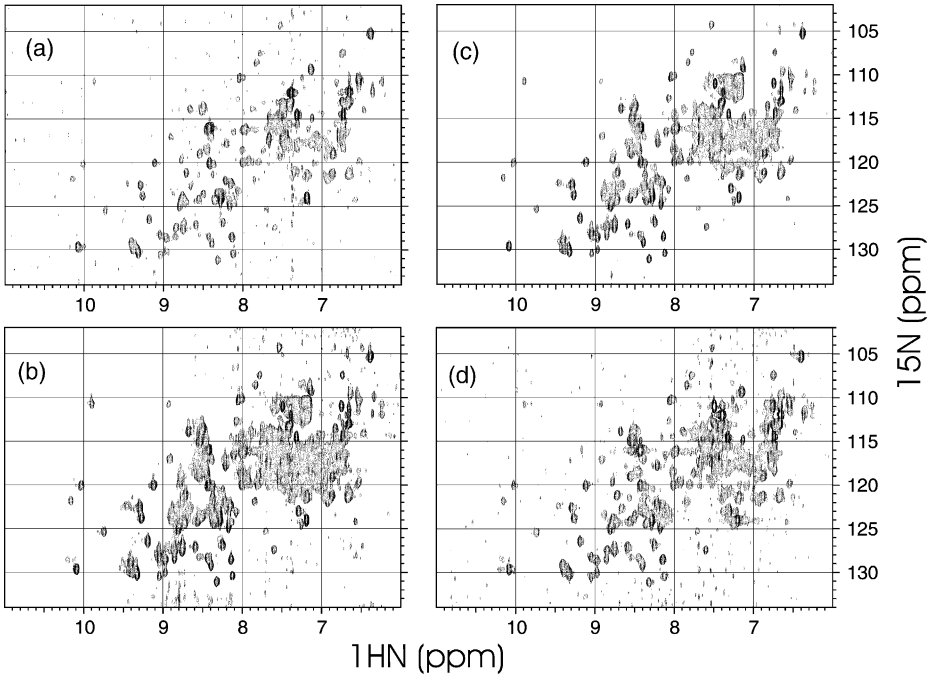

Fig. 6. 2D ^{1}H–^{15}N correlation spectra of LFA-1 in 40% glycerol-d_8 obtained at 277 K using various pulse sequence schemes but all at the same receiver gain setting and total experiment time. (a) clean-TROSY; (d) Kay coupled-HSQC–TROSY; (b) and (c) CRINEPT-TROSY plotted at 1.41× and 2× the contour level of (a) and (d). In (a) and (d), data matrices consisting of (64, 1024) complex points in (t_1, t_2) corresponding to acquisition times of (17.5 and 71.2 ms) in (F1, F2) were recorded with 64 transients/FID with a repetition delay of 2.0 s. In (b) and (c), two separate data matrices with 32 transients/FID was used with inversion of the last ^{15}N $\pi/2$ pulse and shuffled appropriately to obtain pure phase TROSY/anti-TROSY lineshapes as prescribed by Riek et al. [4]. Analysis shows CRINEPT to be superior for most non-side-chain peaks at this temperature.

Acknowledgments

We thank Drs. Ad Bax, Roland Riek, Kwaku Dayie, and Mirko Hennig for helpful discussions. We also gratefully acknowledge Drs. Peter Wright and H. Jane Dyson for continued support and encouragement.

Appendix A

Here we give a summary of sensitivity comparison between the new Avance-900 (energized in July, 2001) and a DRX-800 (energized in August, 1998). Comparison of the newer 900's 0.1% ethyl-benzene sensitivity standard relative to the 800 yields as much as 50% gain (probably due to improvements in probe technology in the three years, since all things being ideal, the sensitivity should scale as $H_0^{1.5}$, or a mere 19% for 900 vs. 800). However for an aqueous protein sample such as LFA-1 (without glycerol) in 95% H_2O/5% D_2O at temperature of 277 K, the sensitivity improvement for ^{15}N clean-TROSY experiments on two slightly different samples and on two different 800 MHz probes and time-span >3 months (determined by peak heights, not volumes, normalized by noise level for each spectrum) was 1.36 ± 0.16 over 118 peaks. For simple water flip-back HSQC experiment also, the gain was 1.36.

Various groups have predicted the advantage of the 900 MHz spectrometer over lower field machines when doing TROSY type experiments on large systems. For systems as large as MW $\sim 10^6$ Da ($\tau_c \sim 350$ ns), we have seen predictions of 900:800 sensitivity gain of 7 (K. Dayie, personal communication); while such gains might be possible, a particularly useful analysis was given by Brutscher [14] who compared HSQC vs. TROSY intensity ratios as a function of the CSA-DD cross-correlation rate constant factored with the time during which ^{15}N magnetization is in the transverse plane in a given experiment. In this way he was able to provide a useful guideline for which types of experiments the TROSY method has advantages over non-TROSY method, given a particular molecular weight and the experimental magnetic field strength. The general conclusion was that TROSY has substantial gains over non-TROSY and has greater utility, especially for larger molecular weight systems in experiments such as triple resonance HNCO, HNCA or hydrogen-bond or long-range coupling constant measurements where the ^{15}N magnetization is in the transverse plane for long periods of time (40–100 ms). In order to quantify the TROSY effect at 900 vs. 800, we have acquired constant-time TROSY experiments with ^{15}N constant time delays of 40 and 80 ms and at both 800 and 900 MHz for the LFA-1 sample in 40% glycerol-d_8 at 296 and 283 K (as

above); we have also acquired the same data on LFA-1 (22 kDa) without glycerol at 277 K as a reference. The relative sensitivity gains are shown in Table 2. We hoped to be able to account for the expected sensitivity gain from 800 to 900 MHz from TROSY as being due to these three factors: (A) peak intensity increase from reduced ^{15}N transverse relaxation of the narrow component at the two fields (the ^{15}N 'line-narrowing effect', $(R_{2N\ 900}/R_{2N\ 800})^{-1}$); (B) peak intensity gain in the directly detected dimension from reduced ^{1}H transverse relaxation of the narrow component at the two fields (the ^{1}H 'line-narrowing effect,' $(R_{2H\ 900}/R_{2H\ 800})^{-1}$); and (C) the reduced signal attenuation during the ^{15}N constant time, T, of the form $\exp[(R_{2N\ 800} - R_{2N\ 900}) \times T]$. For the 40% glycerol system, calculations based on equations of Pervushin et al. [1], assuming high levels of deuteration, gives ^{15}N R_2 value of 27 and 30 s^{-1} at 900 and 800 MHz, respectively, at 296 K (52 ns τ_c) for the narrow component; in Table 2, intensity ratio of 2.49 and 2.34 for rows 3 and 4 at 900 and 800 MHz is a measure of $\exp[R_{2N}\ (T_{80\,ms} - T_{40\,ms})]$, consistent with R_{2N} values of 22.8 and 21.3 s^{-1}, respectively. For this range of R_2 values we expect the factor (C) above to be 1.10–1.22 going from 40 to 80 ms, and the slight increase in sensitivity gain as data shown in rows 1 and 2 are in agreement within experimental error limits. Taking 900 vs. 800 gain in intensity of 2.6 from row 1 of Table 2, factoring out 1.36 due to normal probe sensitivity differences between 800 and 900, factor of 1.1 for (A) and 1.1 for (C) above, we are still left with almost 1.6 in sensitivity gain; assuming that sensitivity gain due to proton line-narrowing effect, factor (B), is also 1.1 (this is difficult to estimate for all the resonances due to the large variation in remote proton density and proton CSA values which contribute to the proton line-width differential substantially [2]), we are left with about 1.4 in sensitivity gain going from 800 to 900 MHz which is still to be accounted for.

Table 2
Constant-time (CT) TROSY sensitivity comparison at 900 MHz vs. 800 MHz for LFA-1

	95% H_2O at 277 K	40% glycerol-d_8 at 296 K
A. 900:800 (CT 40 ms)	1.65 ± 0.15 (127)	2.60 ± 0.78 (66)
B. 900:800 (CT 80 ms)	1.64 ± 0.19 (108)	2.75 ± 0.54 (32)
C. 40:80 ms (900 MHz)	1.45 ± 0.23 (176)	2.49 ± 0.53 (102)
D. 40:80 ms (800 MHz)	1.41 ± 0.16 (152)	2.34 ± 0.70 (34)

Quantitative comparison of peak intensities of LFA-1 samples in water and water/glycerol between 800 and 900 MHz using clean-TROSY method of Sorensen [5–7] with 40 and 80 ms constant time delays to attenuate ^{15}N transverse magnetization. The number of peaks analyzed is shown in parentheses. Results shown in rows 1 and 2 are obtained by comparing 900 vs. 800 MHz while holding the constant time fixed respectively at 40 and 80 ms. Rows 3 and 4 are obtained by taking the same datasets and comparing 40 and 80 ms data at fixed frequency of 900 and 800 MHz, respectively.

When the glycerol-d_8 sample was cooled to 283 K, the 800 MHz data did not yield sufficient number of peaks at the 80 ms constant time to do proper comparisons; at 40 ms constant time, we see gain of about 3.2 of 900 over 800. Attempt to account for this rather large gain by factoring out 1.36 (due to inherent and probe sensitivity differences from 800 to 900), 1.17, 1.1, and 1.1 (due to factors C, A and B, respectively) leaves over 1.6 in unaccounted-for difference in sensitivity between 800 and 900 MHz. Possibly this is due to the per-scan signal-to-noise ratio at lower field being poor enough that the assumed gain of square root of the number of scans rule in Fourier signal averaging can not be applied here. Nevertheless we can see clearly that *for increasingly larger systems the gain is larger on the 900 relative to the 800*; when we take the gain of 1.65 (from row 1 and 2 of Table 2) for LFA-1 at 277 K above (900 vs. 800) and factor out 1.36, then we can say that for this size protein the gain due to TROSY on going from the 800 to the 900 is a modest 1.2. For the larger system, assuming the same 1.36 to be the same factor accounting for non-TROSY sensitivity difference between the 800 and the 900, the TROSY gain factor would be about 1.9 and 2.3 for glycerol sample at 296 and 283 K, respectively. For systems with correlation times of 320 ns (MW$\sim 10^6$ Da), factors (A) and (C) are expected to be 1.1 and 1.9 going from 800 to 900 MHz for 40 ms constant time. While it is not clear how one can extrapolate the unaccounted-for gains for larger systems, the large increase due to factor (C) alone would put a lower limit gain of about 3.7 for the shortest constant time experiments feasible; when multiplied by the 1.2 probe sensitivity factor, gain of 4.5 is expected upon going from 800 to 900 MHz with conventional probe-heads. However these very hopeful numbers for sensitivity gain may not be realizable for actual systems approaching 1 MDa molecular weight for which this work has shown that triple resonance type experiments requiring long ^{15}N transverse magnetization delays are likely to fail. The sensitivity gains observed at 900 MHz in the current experiments will be further increased at still higher magnetic fields, up to a proton Larmor frequency of 1.1 GHz where TROSY effects are expected to be maximal for NH spin systems [1].

References

[1] K. Pervushin, R. Riek, G. Wider, K. Wüthrich, Attenuated T-2 relaxation by mutual cancellation of dipole–dipole coupling and chemical shift anisotropy indicates an avenue to NMR structure of very large biological macromolecules in solution, Proc. Natl. Acad. Sci. USA 94 (1997) 12366–12371.

[2] K. Pervushin, Impact of transverse relaxation optimized spectroscopy (TROSY) on NMR as technique in structural biology, Q. Rev. Biophys. 33 (2000) 161–197.

[3] R.W. Kriwacki, G.B. Legge, U. Hommel, P. Ramage, J. Chung, L.L. Tennant, P.E. Wright, H.J. Dyson, Assignment of ^{1}H, ^{13}C and ^{15}N resonances of the I-domain of human leukocyte function associated antigen-1, J. Biomol. NMR 16 (2000) 271–272.
[4] R. Riek, G. Wider, K. Pervushin, K. Wüthrich, Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules, Proc. Natl. Acad. Sci. USA 96 (1999) 4918–4923.
[5] Handbook of Tables for Applied Engineering Science, second ed., CRC Press, Inc., Boca Raton, FL, 2000, p. 96.
[6] T. Schulte-Herbruggen, O.W. Sorensen, Clean TROSY: Compensation for relaxation-induced artifacts, J. Magn. Reson. 144 (2000) 123–128.
[7] M.H. Lerche, A. Meissner, F.M. Poulsen, O.W. Sorensen, Pulse sequences for measurement of one-bond ^{15}N–^{1}H coupling constants in the protein backbone, J. Magn. Reson. 140 (1999) 259–263.
[8] A. Meissner, T. Schulte-Herbruggen, J. Briand, O.W. Sorensen, Double spin-state-selective coherence transfer. Application for two-dimensional selection of multiplet components with long transverse relaxation times, Mol. Phys. 95 (1998) 1137–1142.
[9] M. Rance, J.P. Loria, A.G. Palmer III, Sensitivity improvement of transverse relaxation-optimized spectroscopy, J. Magn. Reson. 136 (1999) 92–101.
[10] D. Yang, L.E. Kay, Improved (HN)-1H-detected triple resonance TROSY-based experiments, J. Biomol. NMR 13 (1999) 3–10.
[11] L.E. Kay, D. Keifer, T. Saarinen, Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, J. Am. Chem. Soc. 114 (1992) 10663–10665.
[12] M. Salzmann, K. Pervushin, G. Wider, H. Senn, K. Wuthrich, Trosy in triple resonance experiments: New perspectives for sequential NMR assignment of large proteins, Proc. Natl. Acad. Sci. USA 95 (1998) 13585–13590.
[13] J.P. Loria, M. Rance, A.G. Palmer III, Transverse-relaxation-optimized (TROSY) gradient-enhanced triple-resonance NMR spectroscopy, J. Magn. Reson. 141 (1999) 180–184.
[14] B. Brutscher, Principles and applications of cross-correlated relaxation in biomolecules, Concept. Magn. Reson. 12 (2000) 207–229.