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Very simple combination of TROSY, CRINEPT and multiple quantum coherence for signal enhancement in an HN(CO)CA experiment for large proteins

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ABSTRACT

Sensitivity enhancement in liquid state nuclear magnetic resonance (NMR) triple resonance experiments for the sequential assignment of proteins is important for the investigation of large proteins or protein complexes. We present here the 3D TROSY-MQ/CRINEPT-HN(CO)CA which makes use of a ^{15}N–^{1}H-TROSY element and a ^{13}C′–^{13}CA CRINEPT step combined with a multiple quantum coherence during the ^{13}CA evolution period. Because of the introduction of these relaxation-optimized elements and 10 less pulses required, when compared with the conventional TROSY-HN(CO)CA experiment an average signal enhancement of a factor of 1.8 was observed for the membrane protein-detergent complex KcsA with a rotational correlation time τ_c of around 60 ns.

1. Introduction

In order to study the structure or the dynamical behavior of a protein, the NMR resonances have to be assigned to specific nuclei of that protein. The standard assignment strategy starts with the protein backbone assignment. This is achieved by through-bond scalar coupling connectivities, which connect ^{1}HN and ^{15}N with ^{13}CA, ^{13}CB or ^{13}C′ (^{13}CO) of neighboring amino acid residues [1]. In this strategy the magnetization starts on ^{1}HN, is then transferred to ^{15}N and from there further transferred to either ^{13}CA or ^{13}C′ and in an optional next step further to ^{13}CB, ^{13}C′ or ^{13}CA, respectively. The carbon of choice is then frequency labeled and the magnetization is transferred back to the amide proton, via a nitrogen frequency-labeling step. While this approach works excellent for smaller proteins it can meet its limitations for larger systems. Hence, sensitivity enhancement in liquid state nuclear magnetic resonance (NMR) spectroscopy is especially important for the investigation of large proteins, such as protein complexes or membrane proteins solubilized in detergents or bicelles. In addition to deuteration, mainly two pulse sequence strategies have been employed to increase sensitivity. On the one hand, instead of single quantum magnetization transfers, zero and double quantum coherences (multiple quantum, MQ) have been used [2–4]. They are advantageous because they are, to the first order, not affected by the dipolar relaxation mechanism between the two nuclei of the coherence [3,5–7]. On the other hand, transverse relaxation optimized spectroscopy (TROSY) lead to a breakthrough in the investigation of large proteins [8]. In TROSY only the one component of the amide proton and amide nitrogen doublet of doublets is selected, for which the two main relaxation mechanisms, the dipole–dipole and the chemical shift anisotropy (CSA) relaxation mechanisms, almost cancel each other [8]. In addition, also the cross correlation-induced polarization transfer [9] (CRIPT) can be used to study very large proteins with rotational correlation times $\tau_c > 300$ ns, that are not accessible to standard NMR methods. This method uses cross-correlated relaxation between dipole–dipole coupling and CSA for polarization transfer, instead of the fast relaxing polarization transfers mediated by scalar couplings. A combination of both polarization transfers, CRIPT and the scalar coupling mediated transfer termed insensitive nuclei enhanced polarization transfer (INEPT) [10], is called CRINEPT [11]. In theory CRINEPT always yields a higher polarization transfer than the INEPT or CRIPT alone. In addition, the CRINEPT element requires also less radio-frequency pulses reducing signal loss due to pulse inhomogeneities.

As we shall see in the following by using a combination of all methods mentioned the signal-to-noise in a TROSY-HN(CO)-CA experiment can yield significant enhancement. This experiment is important for the sequential assignment since only the combination of the HNCA spectrum with the HN(CO)CA spectrum connects the ^{13}CA atoms of neighboring amino acids unambiguously. In the case of large proteins, where many CA_{i-1} resonances in the HNCA spectrum can be missing, a reasonably good HN(CO)CA spectrum, even with low resolution, can be of vital importance for a successful backbone assignment.

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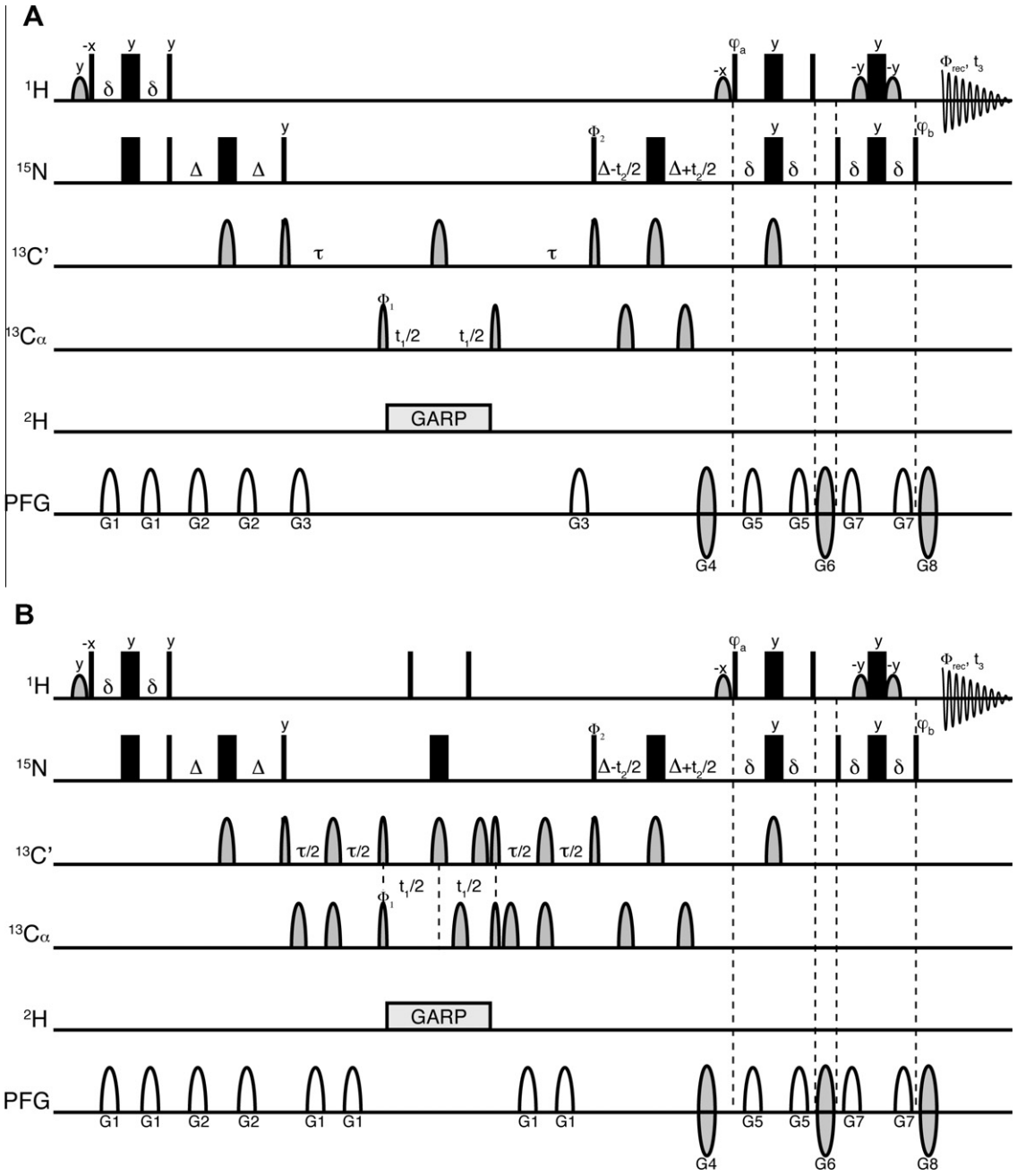

Fig. 1. Experimental scheme for the (A) TROSY-MQ/CRINEPT-HN(CO)CA and (B) TROSY-HN(CO)CA. The narrow and wide black bars indicate nonselective 90° and 180° pulses, respectively. Narrow and wide sine bell shapes represent selective 90° and 180° pulses, respectively. For ^{13}C Gaussian Cascade shapes have been used. The small sine bell shapes on the line labeled with ^{1}H denote soft, water-selective 90° pulses with a duration of 1 ms. These pulses keep the water magnetization stored along the +*z*-axis throughout the whole experiment. The gray box labeled with GARP indicates a GARP decoupling scheme [15,16] for ^{2}H. The radio-frequency pulses on ^{1}H, ^{15}N, ^{13}C′, ^{13}CA and ^{2}H were applied at 4.7, 116.5, 172, 54 and 4.7 ppm, respectively. The line labeled PFG shows the position of sine shaped pulse magnetic field gradients applied along *z*-axis. G1: 1 ms, 1 G/cm; G2: 1 ms, 1 G/cm; G3: 0.7 ms, 22.5 G/cm; G4: 1 ms, −35/−40 G/cm; G5: 1 ms, 1 G/cm; G6: 1 ms, 15/10 G/cm; G7: 1 ms, 22.5 G/cm; G8: 1 ms, 9.935/15.065 G/cm. The delays δ, Δ and τ are 2.3 ms, 12 ms and 8 ms, respectively. The phase cycle is $\Phi_1 = x, x, -x, -x$, $\Phi_2 = x, -x$ and $\Phi_{rec} = x, -x, -x, x$. All other radio-frequency pulses are applied either with phase *x* or as indicated above the pulse. For ^{15}N–^{1}H correlation phases $\phi_a = -y$ and $\phi_b = -y$ and gradients G4, G6 and G8 set to −35, 15 and 9.935 G/cm, respectively select the echo, whereas $\phi_a = y$ and $\phi_b = y$ and gradients G4, G6 and G8 set to −40, 10 and 15.065 G/cm, respectively select the antiecho coherences. Quadrature detection in the ^{13}C dimension is achieved by the States-TPPI method [17] applied to the phase Φ_1.

2. Materials and methods

2.1. NMR sample preparation

The KcsA sample was prepared as described in Baker et al. [12]. The KcsA was ^{15}N, ^{13}C, ~70% ^{2}H labeled, the concentration was 700 μM in sodium acetate buffer, pH 4, with ~60 mM *n*-dodecylphosphocholin (DPC). In addition, the small model protein GB3 was used as well. The deuterated (non-perdeuterated) ^{2}H, ^{13}C, ^{15}N-labeled NMR samples contained 350 μL of 4 mM protein solution, respectively, in 95% H_2O, 5% D_2O, 50 mM potassium phosphate buffer, pH 6.5, and 0.5 mg/mL sodium azide.

2.2. NMR spectroscopy

All spectra were measured either on a Bruker 700 Avance or a 600 Avance spectrometer equipped with a cryogenic probehead at 37°C. The 2D-^{1}H/^{13}C planes of either the TROSY-MQ/

CRINEPT-HN(CO)CA or the TROSY-HN(CO)CA were recorded with 512×12 complex points and 48 scans with a total experiment time of 20 min. The 3D versions of both spectra were measured with $512 \times 24 \times 36$ complex points and 40 scans with a total experiment time of 2 days. The NMR data were processed with NMRPipe, NMRDraw [13] and were analyzed using SPARKY (Goddard, T.D., Kneller, D.G., Sparky 3, University of California, San Francisco). The number of scans necessary for the GB3 sample was eight scans.

2.3. Pulse program description

The schematic pulse sequence of the TROSY-MQ/CRINEPT-HN(CO)CA is outlined in Fig. 1. Briefly, after the first ^{1}H–^{15}N INEPT the magnetization on ^{15}N is transferred via a second INEPT during the delay Δ to ^{13}C′, creating a $4H_zN_zC'_y$ term. During the delay τ magnetization is transferred from ^{13}C′ to ^{13}CA via the one-bond scalar $^1J_{C'-CA}$ coupling and cross-correlated relaxation between dipole–dipole coupling and CSA, creating an $-8H_zN_zC'_xCA_z$ term. The signal amplitude A_I is modulated by the following term [11]:

$$A_I = \sqrt{\sin h(R_C\tau)^2 + \sin(\pi {}^1J_{^{13}C'^{13}CA}\tau)^2}\cdot \exp(-R_I\tau) \quad (1)$$

where

$$R_C = \frac{4}{15}(\gamma_{^{13}C}B_0\Delta\sigma_{^{13}C'})\left(\frac{\hbar\gamma^2_{^{13}C}}{r^3_{^{13}C'^{13}CA}}\right)\tau_C$$

and

$$R_I = \frac{2}{5}\left[\frac{2}{9}(\gamma_{^{13}C}B_0\Delta\sigma_{^{13}C'})^2 + \frac{1}{2}\left(\frac{\hbar\gamma^2_{^{13}C}}{r^3_{^{13}C'^{13}CA}}\right)^2\right]\tau_C + \frac{1}{2T_{1^{13}CA}} + \frac{1}{T_{2^{13}C'}}$$

Here $\gamma_{^{13}C}$ is the gyromagnetic ratio of ^{13}C, $\hbar$ is the Planck's constant divided by 2π, B_0 is the static magnetic field, $\Delta\sigma_{C'}$ is the CSA of ^{13}C′, $r_{C'CA}$ is the distance between the two nuclei, $T_{1\ 13CA}$ is the longitudinal relaxation of CA and $T_{2\ 13C'}$ is the transversal relaxation of ^{13}C′. Multiple quantum coherence is then created by the first 90° pulse on ^{13}CA, creating an $8H_zN_zC'_xCA_y$ or $-8H_zN_zC'_xCA_y$, term, depending on the phase cycle. During t_1 the magnetization evolves with respect to the chemical shift of $^{13}CA_{i-1}$. After the MQ/CRINEPT back-transfer the ^{15}N chemical shift evolves during t_2 in a constant time manner. The TROSY back-transfer from ^{15}N to ^{1}H is compensated for relaxation induced artifacts [14]. For not too large proteins, the artifact suppression can be optimized by empirically adjusting the first two delta delays of this back transfer and ϕ_B as described in Schulte-Herbruggen [14].

3. Results

The TROSY-MQ/CRINEPT-HN(CO)CA was tested on the membrane protein KcsA in DPC. KcsA is a tetrameric potassium channel with a protein/micelle rotational correlation time of ~60 ns, corresponding to the expected molecular weight of ~130 kDa [12]. For comparison of different parameters 2D-^{1}H/^{13}C planes of the TROSY-MQ/CRINEPT-HN(CO)CA and the reference TROSY-HN(CO)CA [18] were measured. We first tested the optimal transfer time τ for the ^{13}CA to ^{13}C′ polarization transfer. For a coupling constant $^1J_{13CA-13C'} = 55$ Hz the theoretical optimum value for the INEPT delay of $1/2J_{13CA-13C'}$ is 9.1 ms + 0.62 ms (the latter value must be added because of the length of the softpulses, see below). A slightly shorter times, which of 9 ms is indeed observed to be experimentally optimal for the TROSY-HNCOCA, while for our modified version the value is slightly further shifted to 8 ms (Fig. 2). This is due to the fact, that during the transfer time τ the TROSY-HNCOCA contains 2 π-pulses on CA which last in total 620 μs, during which the $J_{13CA-13C'}$ coupling is not active. All further comparisons have been measured with these individually optimal τ values.

To test the signal gain of the TROSY-MQ/CRINEPT-HN(CO)CA with respect to the TROSY-HN(CO)CA we measured 3D versions of both spectra on a ^{13}C,^{15}N,^{2}H-labeled KcsA with a τ_c of ~60 ns (Fig. 3). We compared all cross peaks and found a 0.9–3.2 times signal increase, with a mean increase of 1.8 (Fig. 4). In addition, several new resonances appeared.

As mentioned above there are two potential origins of signal gain by the CRINEPT period being: (i) the additional polarization transfer by cross-correlated relaxation (Eq. (1)) and (ii) the use of less pulses reducing signal loss due to B_1 inhomogeneities. When implemented in the TROSY-MQ/CRINEPT-HN(CO)CA experiment, the latter results in 10 less carbon pulses when compared with the reference experiment (Fig. 1). To evaluate between the two the main origin of signal enhancement a comparison between the TROSY-MQ/CRINEPT-HN(CO)CA and the TROSY-HN(CO)CA was measured on the small protein GB3. Since GB3 has a small rotational correlation time τ_c of 4.1 ns it can be assumed that the polarization transfer by cross-correlated relaxation can be neglected and hence the nature of the signal enhancement on GB3 can be attributed to the small number of pulses used. Interestingly, on GB3 an increase in signal enhancement between 1.4 and 2 with an average of 1.7 was obtained, the latter being very similar to the value for KcsA (1.8). Hence, the main signal enhancement of the presented MQ/CRINEPT-HN(CO)CA must be attributed to the use of a significant smaller number of radio-frequency pulses. In addition, the use of less pulses results also in a less severe dependence of the experiment on miss-calibration of pulses. The influence of the latter was tested by a discrete miss-calibration of all ^{13}C pulses by a change of the power by 2700 Hz. While for the TROSY-MQ/CRINEPT-HN(CO)CA this miss calibration resulted in a signal decrease of 20–50% in the reference TROSY-HN(CO)CA experiment at least 70% but for most cross peaks a 100% signal loss is observed.

4. Discussion

MQ versions of triple resonance experiments have been established for the HN(CA)CO experiment without [4] and with [19] the combination of TROSY. Both experiments use a MQ constant time scheme for the $^{13}CA \rightarrow {}^{13}C'$ polarization transfer and ^{13}C′

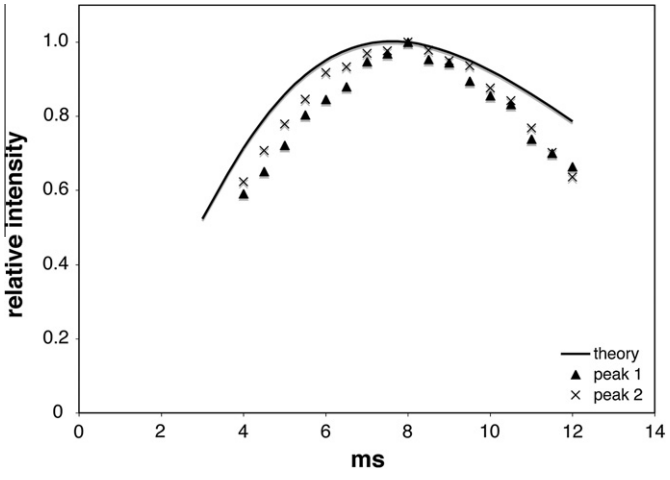

Fig. 2. ^{13}C′–^{13}CA CRINEPT transfer time efficiency. The cross peak intensities for two residues of KcsA at various transfer times are highlighted against a theoretical curve prediction using Eq. (1). For the theoretical calculation the following values were used: $B_0 = 14.1$ T, $^1J_{13C'13CA} = 55$ Hz, $\Delta\sigma_{13C'} = 129.8$ ppm, rotational correlation time $\tau_c = 60$ ns, $T_1 = 1.2$ s, $T_2 = 15$ ms, $r_{13C'13CA} = 0.154$ nm and the angle between the ^{13}CA–^{13}CO bond and the CSA is 38°.

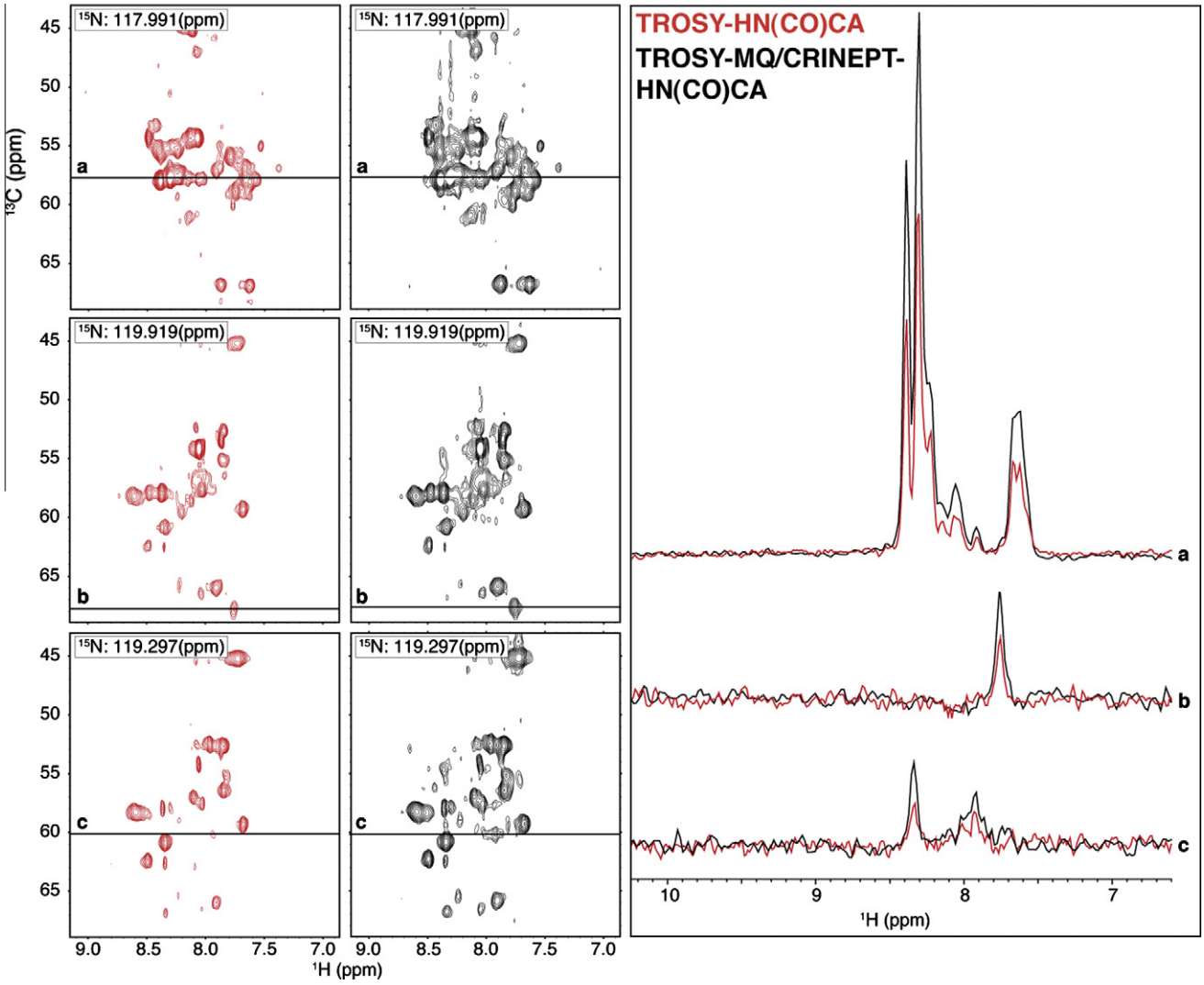

Fig. 3. ^{13}C–^{1}H 2D slices of the 3D TROSY-HN(CO)CA (red) and TROSY-MQ/CRINEPT-HN(CO)CA (black) spectra measured with ^{15}N, ^{13}C, ^{2}H-labeled KcsA. Three cross sections through the 2D planes along the 1H frequency are displayed on the right, again in red for the 3D TROSY-HN(CO)CA and in black for the TROSY-MQ/CRINEPT-HN(CO)CA, respectively. The cross sections are indicated in the planes as solid lines and are labeled with a, b and c both in the planes and the cross sections, respectively. The signal increase of the TROSY-MQ/CRINEPT-HN(CO)CA when compared with the traditional experiment is evident.

frequency labeling. During this constant time period homonuclear $^{1}J_{13CA-13CB}$ couplings are refocused, which is important because otherwise the polarization will also be transferred to the ^{13}CBs. Therefore, the authors of both publications [4,19] argue, that a substantial part of their observed signal gain is due to the refocused $^{1}J_{13CA-13CB}$ coupling. In addition, in the TROSY version [19] the constant time period was modified in a manner, that the $^{1}J_{13CA-13CB}$ coupling was refocused during the $^{13}CA \rightarrow {}^{13}C'$ polarization transfer, while the $^{13}C'$ magnetization was still stored longitudinally. The $^{13}C'$ chemical shift was then recorded during the constant time period, which reduces the time the $^{13}C'$ magnetization spends in the transverse plane. This trick reduces relaxation losses and leads to an additional sensitivity enhancement.

We show here that in an HN(CO)CA experiment a MQ/CRINEPT carbon to carbon transfer is also preferable to a single quantum transfer, even though no $^{1}J_{13CA-13CB}$ coupling has to be refocused. The advantage of a MQ magnetization transfer and evolution time over the corresponding single quantum (SQ) period has three reasons. First, during the MQ polarization transfer period the cross-correlated relaxation between ^{13}C–$^{13}C'$ dipole–dipole coupling and $^{13}C'$ CSA is not suppressed. This leads to polarization build up through the CRIPT mechanism in addition to the polarization transfer by scalar coupling, yielding a CRINEPT transfer. In the SQ case polarization will only be transferred through scalar couplings. In addition, we want to emphasize that the TROSY scheme for ^{1}HN and ^{15}N is not disturbed by the MQ/CRINEPT period because the ^{1}HN and ^{15}N polarizations are stored longitudinally during the entire MQ/CRINEPT interval and neither ^{1}HN nor ^{15}N are decoupled at any time point of the pulse scheme. Second, the MQ period results in a reduced number of carbon pulses, i.e. 10 pulses less then in the SQ case. This appears to be the main origin of the signal enhancement since it reduces signal losses due to pulse imperfections and makes the pulse sequence much less prone to miss-calibrated pulses. Third, during the MQ ^{13}CA chemical shift evolution time the ^{13}CA–$^{13}C'$ dipole–dipole relaxation mechanism is absent. In return, the $^{13}C'$ magnetization is also transversal adding substantially to the relaxation during the evolution time because of the strong CSA of 13C′. This is the reason why we propose a very short ^{13}CA chemical shift evolution time t_1 with the drawback of low resolution along the ^{13}C dimension. The low resolution is only a limited complication for the assignment process, because the HN(CO)CA spectrum is evaluated in combination with the HNCA spectrum. The HNCA has after the HNCO spectrum the highest sensitivity of all 3D triple resonance spectra used for assignment and can

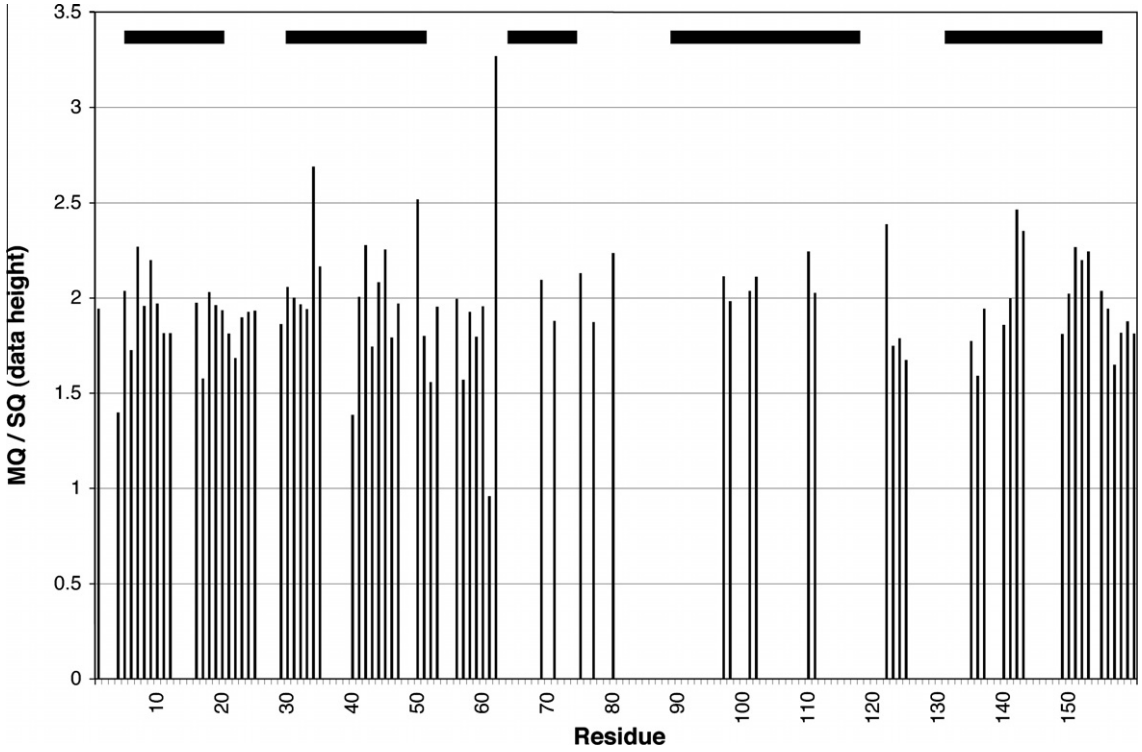

Fig.4. Residue-resolved comparison of the relative signal enhancement of the TROSY-MQ/CRINEPT-HN(CO)CA versus the TROSY-HN(CO)CA measured for KcsA.

therefore be measured with high resolution. The high resolution in the HNCA is also necessary because low resolution may result in severe overlap of $^{13}CA_i$ and $^{13}CA_{i-1}$ resonances. Having the HNCA spectrum, a low resolved HN(CO)CA spectrum adds vital information: For large proteins a substantial part of $^{13}CA_{i-1}$ resonances can be absent in the HNCA spectrum. In this case the low resolved HN(CO)CA spectrum can distinguish between a missing and an overlapped peak or can distinguish between a real and a noise peak. Furthermore, additional $^{13}CA_{i-1}$ resonances may be present in the TROSY-MQ/CRINEPT-HN(CO)CA that are absent in the TROSY-HNCA spectrum as observed for KcsA. For these additional peaks the resolution is indeed low, but for the difficult assignment process of large proteins this imprecise information is to be preferred to no information at all.

The presented TROSY-MQ/CRINEPT-HN(CO)CA was especially designed for large, deuterated proteins. To demonstrate this we used the membrane protein KcsA, which in micelles has an overall molecular weight of ~130 kDa. Using the TROSY-MQ/CRINEPT-HN(CO)CA experiment a signal gain of 0.9–2.7 was observed in comparison to the TROSY-HN(CO)CA. Using this pulse sequence in combination with the TROSY-HNCA and ^{15}N-resolved [^1H, ^1H]-NOESY experiments as well as amino-acid specific labeling, we were able to obtain ~85% of the backbone assignment of KcsA [12]. Similarly, the TROSY-MQ/CRINEPT-HN(CO)CA experiment was vital for the sequential assignment of human VDAC [20] in one of the two NMR studies [20,21] on that membrane protein.

Even though we specifically propose the TROSY-MQ/CRINEPT-HN(CO)CA pulse sequence for large proteins, also small proteins gain from the pulse scheme with respect to the TROSY-HN(CO)CA (as tested here for GB3 as well as ubiquitin, data not shown). It is just of less importance, since for theses proteins already a standard HN(CO)CA yields all expected resonances in the time needed for reasonable resolution. In addition, the TROSY scheme is not necessarily favorable for small proteins.

In conclusion, we present a very simple way to combine the TROSY approach for ^1HN and ^{15}N with a $^{13}C'$–^{13}CA CRINEPT polarization transfer and a MQ ^{13}CA chemical shift evolution period for the HN(CO)CA experiment. This combination improves the sensitivity of the triple resonance experiment significantly, which is especially important for the challenging assignment process of large proteins.

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