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ARTICLE

Improved NMR experiments with ^{13}C-isotropic mixing for assignment of aromatic and aliphatic side chains in labeled proteins

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Abstract Three improved ^{13}C-spinlock experiments for side chain assignments of isotope labelled proteins in liquid state are presented. These are based on wide bandwidth spinlock techniques that have become possible with contemporary cryogenic probes. The first application, the H($C^{ali}C^{aro}$)H-TOCSY, is an HCCH-TOCSY in which all CH_n moieties of a protein are detected in a single experiment, including the aromatic ones. This enables unambiguous assignment of aromatic and aliphatic amino acids in a single, highly sensitive experiment. In the second application, the ^{13}C-detected C^{all}-TOCSY, magnetization transfer comprises all carbons—aliphatic, aromatic as well as the carbonyl carbons—making the complete carbon assignment possible using one spectrum only. Thirdly, the frequently used HC(CCO)NH experiment was redesigned by replacing the long C-carbonyl refocused INEPT transfer step by direct ^{13}C–^{13}C-TOCSY magnetization transfer from side chain carbons to the backbone carbonyls. The resulting HC(CCO)NH experiment minimizes relaxation losses because it is shorter and represents a more sensitive alternative particularly for larger proteins. The performance of the experiments is demonstrated on isotope labeled proteins up to the size of 43 kDa.

Keywords NMR · ^{13}C–^{13}C-TOCSY · Cryogenic probe · Protein side chain assignment · Aromatics · Sample temperature

Introduction

NMR spectroscopy is a well-established technique for the characterization of the structure and dynamics of biomolecules. The basis for NMR studies is the resonance assignment of the target protein (Wüthrich 1986). For resonance assignments of ^{13}C-enriched amino acid side chains, NMR experiments relying on ^{13}C- isotropic mixing show superior sensitivity for medium sized proteins (10–20 kDa) and at medium field strength instruments (500–600 MHz) (Bax and Davis 1985; Bax et al. 1990; Braunschweiler and Ernst 1983; Shaka et al. 1983, 1988), particularly if they are combined with the optimized ^{1}H and ^{13}C sensitivity of cryogenic probes (Kovacs et al. 2005). There are three major experiments for side chain assignments based on isotropic mixing, the H(CC)H-TOCSY, the carbon detected (H)CC-TOCSY and the HC(CCO)NH.

In the H(CC)H-TOCSY experiment (Bax et al. 1990; Logan et al. 1992; Montelione et al. 1992; Wang and Zuiderweg 1995), a ^{1}H coherence is transferred to the attached ^{13}C-spin via INEPT (Morris and Freeman 1979) and spread among ^{13}C-spins within a spin system by ^{13}C–^{13}C-isotropic mixing. Subsequently, the coherence is transferred back to ^{1}H for detection. 2D–4D versions of the experiment are available, providing an ideal basis for side chain assignment (Bax et al. 1990; Hiller et al. 2008; Olejniczak et al. 1992). The experiment is also suitable for

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larger proteins (up to 30–40 kDa) as it can be run with random fractionally deuterated samples. Alternatively, carbon detected approaches like the CC-TOCSY (Bermel et al. 2003) or the ^{1}H-start version (H)CC-TOCSY (Eletsky et al. 2003; Jordan et al. 2006; Serber et al. 2001) can further extend the applicability to larger or perdeuterated proteins. Since the conventional H(CC)H-TOCSY covers only the aliphatic region, additional experiments are needed to link the aromatic resonances to the aliphatic C^{β}. This can be achieved in experiments like the $(H^{\beta})C^{\beta}(C^{\gamma}C^{\delta})H^{\delta}$ and $(H^{\beta})C^{\beta}(C^{\gamma}C^{\delta}C^{\varepsilon})H^{\varepsilon}$ (Yamazaki et al. 1993) for H/C^{δ} and H/C^{ε} individually or, alternatively, for all aromatics in a single spectrum with the AMNESIA approach (Grzesiek and Bax 1995) or the $(H^{\beta})C^{\beta}(C^{\gamma}C^{aro}C^{aro}$-TOCSY)$H^{aro}$ experiment (Löhr et al. 2007). However, these experiments are limited by degeneracy of the C^{β} chemical shift and by their relatively low sensitivity. The problem of degeneracy can be solved by including H^{β} chemical shifts, as is done in the 4D APSY-$H^{\beta}C^{\beta}(C^{\gamma})C^{\delta}H^{\delta}$ experiment (Krähenbühl et al. 2011). Most frequently, however, aromatic assignments are determined using NOESY spectra. These are more sensitive, but ambiguities need to be sorted out in lengthy manual analyses (Lin et al. 2006). Therefore, assignments of aromatic side chains still represent a challenge, in contrast to aliphatic assignments, for which experiments are well established.

A useful complement to H(CC)H-TOCSY is the HC(CCO)NH (Grzesiek et al. 1993; Mobli et al. 2010; Zuiderweg et al. 1996), where, after the initial ^{1}H-^{13}C INEPT step, ^{13}C-coherences on the side chains are transferred through TOCSY mixing to the C^{α}-position and through subsequent INEPT steps via the carbonyl to the amide of the following residue (Logan et al. 1992; Montelione et al. 1992). Here, the side chain assignment is facilitated by the connectivity to the well-dispersed backbone amide nuclei ^{1}H and ^{15}N. Despite sensitivity losses during the long transfer delays, this approach is especially advantageous for large proteins (>30 kDa) as uniform deuteration combined with specific protonation of methyl groups of interest, as well as the TROSY principle, can be exploited (Gardner et al. 1996; Goto et al. 1999; Hilty et al. 2002; Lin and Wagner 1999; Tugarinov and Kay 2003). The combined information from the two experiments H(CC)H-TOCSY and HC(CCO)NH yields side chain assignments of all aliphatic residues and connects these to the backbone.

The TOCSY experiments above are based on isotropic mixing schemes. These consist of pulse trains that suppress the chemical shift differences between nuclei involved in the spinlock. If the Zeeman part of the Hamiltonian is eliminated and the rf (radio frequency)-Hamiltonian of two nuclei have the same value, the Hartmann-Hahn condition is met and magnetization transfer takes place between the two nuclei according to evolution under the strong coupling Hamiltonian (Bax and Davis 1985; Braunschweiler and Ernst 1983). The transfer efficiency of the isotropic mixing schemes is a sum of the transfer efficiencies of each involved spin pair (Cavanagh et al. 2007) which, in turn, depend on the values of the corresponding scalar couplings. The rather large coupling constants of directly bonded ^{13}C-nuclei ($^{1}J_{CC}$) ranging between 35 and 58 Hz, favor the transfer (Wider 1998). In this strong coupling regime, the magnetization transfer is proportional to $sin^{2}(J\pi\tau)$, where J is the coupling constant and τ is the mixing time (Cavanagh et al. 2007; Kupce et al. 1998). The optimal transfer, corresponding to the first maximum of the periodic function, is accomplished at the mixing time of $\tau = 1/2J$ (Braunschweiler and Ernst 1983). In contrast, the time needed for magnetization transfer from one nucleus to the other under free precession, such as refocused INEPT, is $1/J$ (Cavanagh et al. 2007). Another advantage of isotropic mixing is that not only two nuclei are correlated, but all nuclei in a spin system that fulfill the Hartmann Hahn condition. This makes TOCSY experiments highly useful for resonance assignment.

There are, however, limitations to TOCSY experiments regarding the efficiency of the coherence transfer between nuclei of largely different chemical shift. The reduction of the coherence transfer efficiency of a given mixing scheme under off-resonance conditions is expressed in terms of the effective scalar coupling, J_{eff} (Bax et al. 1990; Cavanagh et al. 2007; Kadkhodaie et al. 1991). In proteins, for example, there are two bound carbon pairs with a substantial resonance frequency separation, the C^{β}–C^{γ} pair in aromatic residues and the backbone C^{α}–C' pair. For these pairs of spins, the effective coupling, J_{eff}, may be significantly reduced from the true coupling constant value, $^{1}J_{CC}$ (Bax et al. 1990; Kadkhodaie et al. 1991; Kupce et al. 1998). For the approximation of pure scalar transfer operator to apply, the condition $J_{eff} > 0.5\ J_{CC}$ should be fulfilled (Bax et al. 1990; Peti et al. 2000). Since the coherence transfer through isotropic mixing is a sinusoidal function of the effective coupling constant J_{eff} and the mixing time, τ, a reduced J_{eff} value can be compensated for by a longer mixing time, provided relaxation can be disregarded.

The exact value of the J_{eff} depends on the frequency separation between the two nuclei, the applied spinlock strength, γB_1, and the isotropic mixing scheme. Some of the most efficient mixing options are the numerically optimized DIPSI (Shaka et al. 1988) and FLOPSY (Kadkhodaie et al. 1991) schemes or sequences based on adiabatic pulses (Bennett et al. 2003; Kupce et al. 1998; Peti et al. 2000). The FLOPSY-16 mixing scheme (Cavanagh et al. 2007; Kadkhodaie et al. 1991), in particular, has been optimized with respect to the transfer efficiency under off-

resonance spinlock conditions (see also Suppl. Fig. 1) (Cavanagh et al. 2007). Bandwidths of up to 2.0 times γB_1 can be achieved and the magnetization of spins with chemical shift differences of up to 1.2 times γB_1 can be mixed within the limit $J_{eff} > 0.5\ J_{CC}$ (Cavanagh et al. 2007). Other options for wide off-resonance spinlocks are DIPSI-3 (Shaka et al. 1985, 1988) and isotropic mixing with WURST-type adiabatic pulses (Kupce and Freeman 1995; Kupce et al. 1998). These yield slightly lower values for J_{eff} for spins at large chemical shift separations (Cavanagh et al. 2007).

Up to today hardware based limitations to the spinlock field strengths, giving rise to probe and sample heating, have practically confined the ^{13}C–^{13}C-TOCSY type of experiments to mixing under on-resonance conditions in the aliphatic region and to rather modest mixing times up to 20 ms (Bax et al. 1990). In proteins the coherence transfer to the aromatic ring or to the backbone carbonyl position depends on the value of reduced coupling constant in the critical C^{β}–C^{γ} or C^{α}–C' transfer step. For efficient isotropic mixing in such cases, optimal mixing schemes, highest probe compliant spinlock fields and longer mixing times are mandatory especially at high field NMR instruments, where the separations of the carbon frequencies become larger. To alleviate the hardware related limitations of the otherwise so useful ^{13}C-spinlock based experiments, the contemporary cryogenic probes have been optimized with regard to power handling. The present work focuses on the theoretical and practical aspects of the optimization of wide bandwidth ^{13}C–^{13}C isotropic mixing allowed by such probes. Wide bandwidths are achieved by using short pulses in the range of 14–20 us for the basic 90° mixing pulse, corresponding to spinlock field strengths γB_1 of 17.9–12.5 kHz.

Improvements to three standard experiments, H(CC)H-TOCSY, ^{13}C-detected (H)CC-TOCSY and HC(CCO)NH, are presented. First, we introduce the H($C^{ali}C^{aro}$)H-TOCSY that includes both aliphatic and aromatic spins. With this experiment complete and unambiguous side chain assignments for a protein can be obtained in a single, sensitive experiment. Secondly, we extend this approach to also include carbonyls in the spinlock, leading to ^{13}C-detected C^{all}-TOCSY, where all ^{13}C-correlations of a protein are recorded in one spectrum. Thirdly, we propose a modified HC(CCO)NH experiment, which takes advantage of direct ^{13}C–^{13}C-TOCSY transfer of the side chain ^{13}C-coherences to carbonyl-spins, omitting the relatively long refocused INEPT step from C to carbonyl. This has two advantages. Again, aromatic side chains can be included and, very importantly, the experiment is more sensitive than the conventional HC(CCO)NH experiment, especially for larger proteins that are prone to relaxation losses.

Materials and methods

Calculations

Theoretical ^{13}C–^{13}C-TOCSY coherence transfer efficiencies were calculated with the program ANTIOPE (Deboure-gas and Waugh 1992). In the calculations a Hamiltonian for liquids was used, in which chemical shielding was set to zero for all spins, reflecting an ideal Hartmann-Hahn condition. Reduced scalar coupling constants (J_{eff}) were used to account for off-resonance effects of the FLOPSY-16 mixing scheme (Shaka and Keeler 1987). J_{eff} was calculated from literature $^1J_{CC}$ values (Wider 1998) using scaling factors for a FLOPSY-16 sequence (Suppl. Fig. 1) (Cavanagh et al. 2007). For the spin system of phenylalanine the following J_{eff} values were derived using a carrier frequency of 80 ppm and $\gamma B_1 = 17.9$ kHz for a 700 MHz spectrometer: $^1J(C^{\zeta}C^{\varepsilon}) = {}^1J(C^{\varepsilon}C^{\delta}) = 58$ Hz, $^1J(C^{\delta}C^{\gamma}) = 56$ Hz, $^1J(C^{\gamma}C^{\beta}) = 18$ Hz, $^1J(C^{\beta}C^{\alpha}) = 35$ Hz, $^1J(C^{\alpha}C') = 16$ Hz. For and $\gamma B_1 = 16.7$ kHz for a 600 MHz spectrometer: $^1J(C^{\zeta}C^{\varepsilon}) = {}^1J(C^{\varepsilon}C^{\delta}) = 58$ Hz, $^1J(C^{\delta}C^{\gamma}) = 56$ Hz, $^1J(C^{\gamma}C^{\beta}) = 20$ Hz, $^1J(C^{\beta}C^{\alpha}) = 35$ Hz, $^1J(C^{\alpha}C') = 21$ Hz (Fig. 1, Suppl. Figs. 1, 2). For the spin systems of isoleucine, leucine and valine a FLOPSY-16 sequence with the same spin lock parameters as above for 600 MHz but centred at 95 ppm was assumed, leading to the following J_{eff} values: $^1J(C^{\delta}C^{\gamma}) = {}^1J(C^{\gamma}C^{\beta}) = 37$ Hz, $^1J(C^{\beta}C^{\alpha}) = 35$ Hz, $^1J(C^{\alpha}C') = 22$ Hz.

In these calculations, the case without relaxation is described. The relaxation for individual proteins under study due to overall molecular tumbling during isotropic mixing can be taken into account through multiplication with the factor $exp(-\tau/T_{eff})$ where τ is the mixing time and T_{eff} the effective relaxation time. For the latter applies $1/T_{eff} = w_t/T_2 + (1-w_t)/T_1$, where T_1 and T_2 are the longitudinal and transverse relaxation times and the weighting factor w_t = ca. 0.65 for the FLOPSY-16 mixing scheme (Felli et al. 2009). This simple relation, however, may not properly approximate the relaxation of aromatic rings or methyl groups, as both often undergo internal rotation in addition to the overall molecular tumbling.

Protein samples

The samples were 1.0 mM ^{13}C,^{15}N-labelled ubiquitin (7 kDa) in 50 mM phosphate buffer in a 5 mm NMR tube, 0.4 mM ^{13}C,^{15}N-labelled kRas (21 kDa) sample in a buffer consisting of 20 mM Tris, pH 8.0, 50 mM NaCl and 1 mM $MgCl_2$ in a Shigemi tube and 0.6 mM MBP (maltodextrin binding protein) (43 kDa) sample in 20 mM $NaPO_i$ at pH 6.8 and 50 mM NaCl in a 3 mm NMR tube. The MBP protein was ^2H,^{13}C,^{15}N-labelled with specific protonation

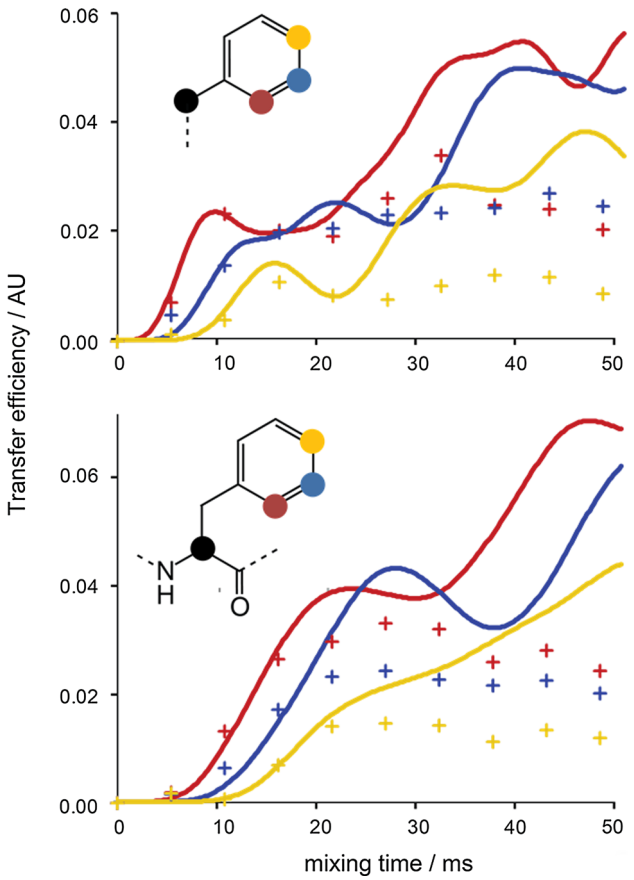

Fig. 1 Theoretical and experimental ^{13}C–^{13}C-TOCSY transfer curves for aromatic spin systems. Transfer efficiencies during ^{13}C-spinlock from aromatic carbons to C^{β} and C^{α} in phenylalanine are shown in the *upper* and *lower* diagrams, respectively. Transfer efficiencies as a fraction of initial magnetization from C^{δ} (*red*), C^{ε} (*blue*) and C^{ζ} (*yellow*), are plotted as a function of mixing time. Theoretical values (*solid lines*) for the spin system of phenylalanine were calculated with the program ANTIOPE (Debouregas and Waugh 1992). Reduced scalar couplings were used that reflect the parameters applied in the measurements, *i.e.* FLOPSY-16 mixing with a spinlock field of 17.9 kHz at 700 MHz. Relaxation was not included in the calculation. Experimental cross peak intensities (+) were measured from 2D [$^{1}H^{aro}$,$^{13}C^{ali}$] planes of a 3D H($C^{ali}C^{aro}$)H-TOCSY spectrum of ubiquitin (Fig. 2). Average values of residues Phe-45 and Phe-4 were used for the analysis and the data were scaled such that the data points of H^{δ} at mixing times 16 ms match the calculated curves. The data of H^{ε} and H^{ζ} of Phe-4 were omitted because of signal overlap (cf. Fig. 2)

of methyl groups of leucine, valine and isoleucine (δ_1-methyl only) (Goto et al. 1999).

NMR spectroscopy

The NMR spectra were recorded on Bruker AVANCE III 600 and 700 MHz spectrometers equipped with TCI and QCI-P CryoProbes™, respectively. All spectra were recorded at 25 °C, the repetition rate was 2 s^{-1}. Below an overview of the conditions is given, details of the individual experiments are found in the respective Figure legends.

In the HCCH-TOCSY experiment, the ^{13}C–^{13}C-spinlock involved aliphatic and aromatic ^{13}C-frequencies (bandwidth ca. 140 ppm). The ^{13}C-offset was set to 80 ppm in order to centre it with respect to the chemical shifts of the C^{γ}–C^{β} pair of spins. The spinlock fields tested were between 12.5 and 16.7 kHz (corresponding to a spinlock pulse of 20–15 μs) at 600 MHz and 12.5–17.9 kHz (corresponding to a spinlock pulse of 20–14 μs) at 700 MHz. Couplings to carbonyl were selectively refocused during the ^{13}C-evolution after spinlock. For the comparisons with the theoretical transfer efficiency the mixing time was incremented stepwise up to 50 ms by increasing the number of basic cycles of the FLOPSY-16 mixing scheme.

In the ^{13}C-detected, ^{1}H-start (H)CC-TOCSY and the modified HC(CCO)NH experiment, the ^{13}C–^{13}C-spinlock covered all ^{13}C-frequencies (bandwidth ca. 180 ppm). In particular, care was taken to keep the effective coupling constant $J_{eff} > 0.5\ J_{CC}$ for the C^{α}-carbonyl pair. For this purpose, the ^{13}C-offset was set to 110 ppm and a spinlock field of 16.7 kHz (corresponding to a spinlock pulse of 15 μs) was used at 600 MHz and a spinlock field of 17.9 kHz (corresponding to a spinlock pulse of 14 μs) was applied at 700 MHz. For the comparison with the theoretical transfer efficiencies, spinlock durations up to 50 ms were used.

For the adiabatic ^{13}C–^{13}C-spinlock a constant adiabaticity (denoted with ca) WURST-2 shape (Kupce and Freeman 1995; Kupce et al. 1998; Tannus and Garwood 1996), with 60 kHz nominal sweep, 70 μs duration and amplitude power index of two, was chosen. The shape was expanded with the p5p9m4 phase cycle (Kupce et al. 1998; Peti et al. 2000), which gave a total duration of 12.6 ms and a total rotation of zero in a 55 kHz wide region. Peak power of 20 kHz was applied, however, the average power of the shape was only 37 %. To determine the optimal mixing time, a series of spectra were recorded at 700 MHz, applying the adiabatic pulse 1–6 times corresponding to spinlock times of 12.6–75.6 ms. To localize the optimal pulse offset, a series of experiments corresponding to offsets 50–120 ppm in steps of 10 ppm, were performed.

Sample temperature

Sample temperature was monitored under ^{13}C–^{13}C-spinlock conditions at 700 MHz magnetic field by applying a cooling gas flow of 600 l/h. The Bruker NMR thermometer™ option was used to display and regulate the sample temperature internally by means of a chemical shift difference of two deuteron signals (Freeman 1970). Three samples in 5 mm tubes with different NaCl concentrations, 0, 200 and 2,000 mM, respectively, were studied. All three

samples also contained 50 mM deuterated sodium acetate in 90 % H_2O/10 % D_2O. In such a sample, the NMR thermometer controls the sample temperature by means of the chemical shift difference between the D_2O and acetate methyl resonances. The separation of the two deuterium signals, 4.7 and 1.8 ppm, respectively, had previously been calibrated against temperature. The internal sample temperature was regulated during the HCCH-TOCSY experiment while applying a 16.7 kHz spinlock field of FLOPSY-16 or an adiabatic mixing with ca (constant adiabaticity)-WURST-2 pulse with peak power of 20 kHz. In both cases, the duration of the spinlock was 50 ms and a 3.5 kHz adiabatic ^{13}C-decoupling was applied during the acquisition time of 183 ms. The target temperature was 25 °C and the repetition rate was 2 s^{-1}.

Results and discussion

H($C^{ali}C^{aro}$)H-TOCSY for complete side chain assignments

The experiment is here called H($C^{ali}C^{aro}$)H-TOCSY as it allows simultaneous observation of through-bond correlations of both aliphatic and aromatic moieties. Thus, complete ^{13}C-assignments of aromatic and aliphatic residues using a single, sensitive experiment become possible.

In a first step, theoretical ^{13}C–^{13}C transfer efficiencies in the phenylalanine spin system were compared with experimental signal intensities as a function of increasing mixing time (Fig. 1). TOCSY-transfer curves as a function of mixing time up to 50 ms were calculated with the program Antiope (Debouregas and Waugh 1992) using effective scalar couplings. If a 16.7 (17.9) kHz spinlock at 600 (700) MHz is assumed, the largest resonance frequency separation in aromatic residues between the C^{γ} and C^{β} frequencies, becomes roughly 16.5 (19.3) kHz. The corresponding effective couplings are still 50 % of the real one-bond coupling constants for the FLOPSY-16 mixing scheme(Cavanagh et al. 2007) (Suppl. Fig. 1). The first maximum of the theoretical magnetization transfer from the aromatic ring $C^{\zeta}/C^{\varepsilon}/C^{\delta}$ to C^{β} and from the aromatic ring $C^{\zeta}/C^{\delta}/C^{\varepsilon}$ to C^{α} is reached at mixing times of 15 and 20 ms, respectively (Fig. 1). The theoretical curves predict further maxima, however, taking relaxation into account, the first maximum is the ideal mixing time.

For the comparison to the calculated transfer efficiencies, two series of H($C^{ali}C^{aro}$)H-TOCSY spectra (Fig. 2) with increasing mixing time were recorded on ^{13}C,^{15}N-labelled ubiquitin using the same 17.9 kHz spinlock field at 700 MHz (Fig. 1) and 16.7 kHz spinlock field at 600 MHz (Suppl. Fig. 2). From these spectra, build-up curves for the cross peak intensities between the aromatic ring carbons $C^{\zeta}/C^{\varepsilon}/C^{\delta}$ and the C^{β} and C^{α} positions, respectively, for residues Phe-4 and Phe-45 in ubiquitin were derived. For Phe-4, the aromatic resonances of H^{ε} and H^{ζ} overlap, therefore these were not used in the analysis (Fig. 2). The calculated and experimental build up curves agree, especially for the $C^{\zeta}/C^{\varepsilon}/C^{\delta}$–$C^{\alpha}$ transfers (Fig. 1, Suppl. Fig. 2). In the experiments with ubiquitin, the first maxima for C^{ζ}–C^{β} and C^{ζ}–C^{α} transfer appear within 20 ms mixing time. The latter is the furthest transfer—it involves six nuclei—and gives rise to the weakest cross signals. The experimental cross peaks between aromatic C^{ε} and C^{δ} to aliphatic C^{β} and C^{α} have higher intensity overall and reach their maximum around 25 ms. Because of the oscillating behaviour of the transfer curves, using mixing times beyond 25 ms does not substantially increase the cross-peak intensity, especially if relaxation is taken into account (Fig. 1). These experiments represent the proof of concept that all correlations between aromatic resonances and C^{β}/H^{β} as well as C^{α}/H^{α} can be obtained in one single ^{13}C–^{13}C-TOCSY spectrum.

In addition, an extensive screen for alternative schemes and optimal parameters was performed using calculations and measurements with ubiquitin as a test sample. The search included alternative mixing schemes (FLOPSY-16, DIPSI-3 and adiabatic mixing) with different spinlock power (17.9–12.5 kHz), and scans of offsets (50–110 ppm) and mixing times (0–50 ms) (Suppl. Figs. 1, 3). In summary, spectra with the highest sensitivity were obtained with the following parameters: FLOPSY-16 at maximum applicable power (here 17.9kHz at 700 MHz and 16.7 kHz at 600 MHz), offset at 80 ppm and 15–20 ms of mixing time depending on the relaxation properties of the protein studied. A second-best alternative was found to be a lower power DIPSI-3 mixing using a spinlock field of 12.5 kHz and duration of 25 ms. Here the signal intensity was roughly two-thirds of the above FLOPSY-16 experiment, the advantage, however, being lower rf-load. Similarly, the virtue of the adiabatic mixing is reduced power. Adiabatic mixing, however, yielded only one-third of the sensitivity of the FLOPSY-16 experiment and showed a more dramatic offset dependence with signal intensity deteriorating rapidly already at ±10 ppm from the optimal ^{13}C-offset of 80 ppm (Suppl. Fig. 3).

To demonstrate the applicability of the H($C^{ali}C^{aro}$)H-TOCSY to systems where relaxation becomes more pronounced, the experiment was recorded on uniformly-^{13}C,^{15}N-labelled kRas (21 kDa). Spectra of good quality were obtained using FLOPSY-16 with a 16.7 kHz spinlock field and 17 ms mixing time at 600 MHz (Fig. 3). For larger proteins with fast transverse relaxation, a high spinlock field should be used in order to keep the mixing time at a minimum. In the case of kRas, strongest cross-peaks between aromatic carbons and C^{β} as well as C^{α} were

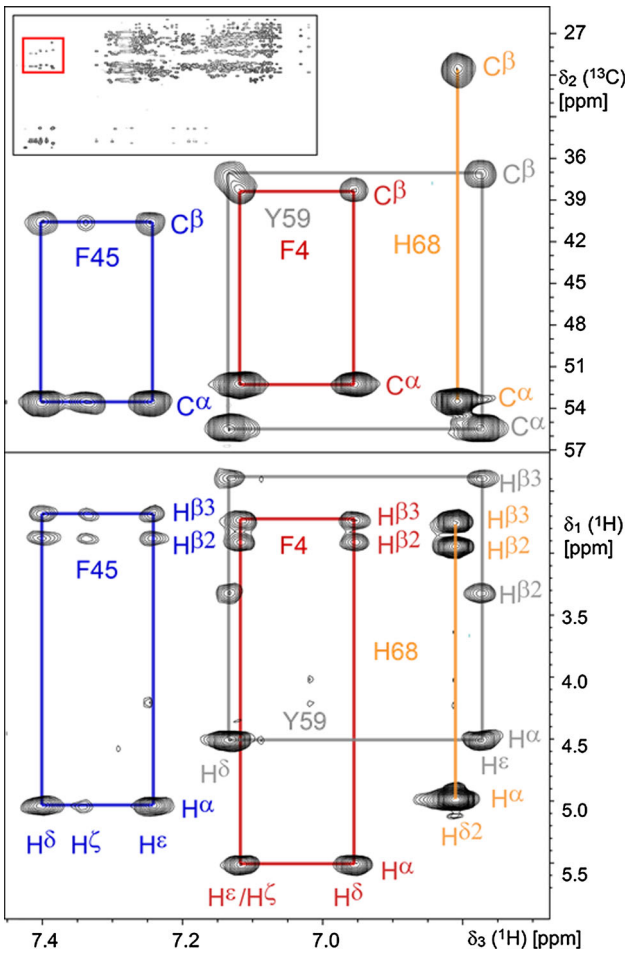

Fig. 2 H($C^{ali}C^{aro}$)H-TOCSY experiment for ubiquitin (7 kDa) recorded at 700 MHz. *Insert:* The 2D [^{1}H, ^{13}C] plane of H($C^{ali}C^{aro}$)H-TOCSY shows aromatic sidechain correlations of ubiquitin. In the two enlarged regions the ring proton correlations to the alpha- and beta-positions of the four aromatic residues in ubiquitin are shown; upper panel [$^1H^{aro}$, $^{13}C^{ali}$], lower panel [$^1H^{aro}$, $^1H^{ali}$]), the color coding: F4 (*red*), F45 (*blue*), Y59 (*grey*) and H68 (*orange*). All correlations, including long range H^{ζ}–H^{α} correlations are clearly visible. The spectra were recorded in 1.5 h using a sample of 1.0 mM ^{13}C,^{15}N-labelled ubiquitin. The static field was 700 MHz, the mixing time was 29.8 ms at 17.9 kHz spinlock field and the ^{13}C-carrier frequency during the spinlock was set to 80 ppm

observed at mixing times between 14 and 20 ms. In the spectra with mixing times over 15 ms, all aromatic resonances could unambiguously be assigned. Importantly, also aliphatic–aliphatic correlations yielded higher sensitivity in the H($C^{ali}C^{aro}$)H-TOCSY compared to a conventional H(CC)H-TOCSY at γB_1 of 10 kHz. This can easily be rationalized based on larger J-scaling factors for FLOPSY-16 at higher power even at large offsets. The H($C^{ali}C^{aro}$)H-TOCSY therefore reduces measurement time because of its higher sensitivity as well as by providing all connectivity's in one TOCSY spectrum. Further, it allows unambiguous assignments due to correlations to C^{α}/H^{α} and C^{β}/H^{β} at the same time, and avoids chemical shift referencing between several spectra.

^{13}C-detected C^{all}-TOCSY for all side chain to backbone carbon correlations

The C^{all}-TOCSY experiment extends the spinlock to the carbonyl frequencies. For this, the spinlock needs to be strong enough to fulfill the condition $^1J(C^{\alpha}C') > 0.5$ (Suppl. Fig. 1). The ^{13}C-detected 2D C^{all}-TOCSY experiment can be performed either as a ^{13}C-start (Bermel et al. 2003) or as ^{1}H-start version (Eletsky et al. 2003; Jordan et al. 2006; Serber et al. 2001). The latter version is usually more sensitive as it profits from an INEPT (Morris and Freeman 1979) transfer of ^{1}H coherences to the bound ^{13}C-nuclei preceding the ^{13}C–^{13}C-isotropic mixing. However, relaxation may undermine the sensitivity advantage of the ^{1}H-start for slow tumbling molecules (Richter et al. 2010) and for the magnetization transferred to the carbonyl carbons at higher magnetic fields as their relaxation is dominated by the chemical shift anisotropy (CSA) mechanism.

The ^{13}C-detected 2D C^{all}-TOCSY spectrum of Fig. 4 illustrates the ^{13}C–^{13}C isotropic mixing between all carbons in ubiquitin. This spectrum was recorded with a spinlock field of 16.7 kHz applied at a ^{13}C-offset of 95 ppm and the duration of 29.8 ms at 600 MHz. A close inspection of the spectrum reveals several notable signals.

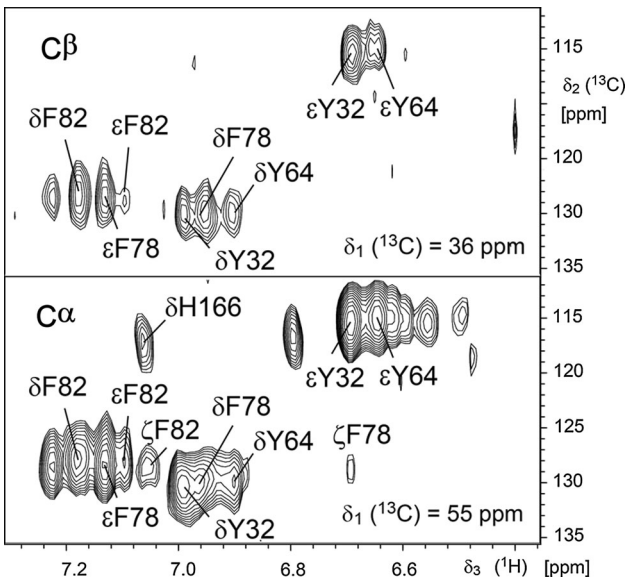

Fig. 3 H($C^{ali}C^{aro}$)H-TOCSY for aromatic assignment of kRas (21 kDa). 2D [$^1H^{aro}$, $^{13}C^{aro}$] planes of a 3D H($C^{ali}C^{aro}$)H-TOCSY recorded on the 21 kDa protein kRas. The connectivity's of aromatic moieties of Y32, Y64, F82 and H116 with beta (C^{β}, δ_1 at 36 ppm) and alpha carbons (C^{α}, δ_1 at 55 ppm) are visible in the *upper* and *lower* panel, respectively. The mixing time was 17.0 ms at 16.7 kHz. The spectrum was recorded in 8 h on a sample of 0.4 mM ^{13}C,^{15}N-labelled kRas on a 600 MHz spectrometer

For instance, the correlation between the carbonyl (173 ppm) and δ_1-methyl group (6.2 ppm) of Ile-23 is visible (red box in Fig. 4). An analysis of the individual couplings involved in the transfer in isoleucine reveals that four carbons are aliphatic and only the transfer involving the C and carbonyl carbon, $^1J(C^{\alpha}C')$, has a reduced effective coupling constant because of the large chemical shift difference of ca. 120 ppm. The fortunately rather large value, 53 Hz, of the true coupling constant is reduced to 34 %, *i.e.* 18 Hz. Selecting a ^{13}C-offset of 110 ppm would yield a reduction to 40 % instead, *i.e.* 21 Hz. Other challenging transfer steps are found in acidic, amidic and aromatic residues. On the one hand there are asparagine, aspartate, glutamine and glutamate residues whose spin systems contain side chain carbonyls. Here, two transfers with a large chemical shift separation of 120–150 ppm and consequently low J_{eff} are involved, namely the backbone $^1J(C^{\alpha}C')$ and the side chain $^1J(C^{\gamma}C^{\beta})$ or $^1J(C^{\delta}C^{\gamma})$. Nevertheless, correlations between side chain and backbone carbonyls are present (blue box in Fig. 4). On the other hand there are the aromatic amino acids. The presence of cross signals between C^{ζ} of phenylalanine or tyrosine and the respective carbonyl demonstrate a transfer over six bonds, including two unfavorable transfers through $^1J(C^{\alpha}C')$ and $^1J(C^{\gamma}C^{\beta})$ with J_{eff} of about 50 % (orange box in Fig. 4). Hence, all ^{13}C–^{13}C-correlations of a protein could be detected, despite transfers involving up to seven carbons with several large chemical shift differences.

The ^{13}C-detected C^{all}-TOCSY experiment becomes particularly useful in connecting the protonated methyl groups to the protein backbone in uniformly ILV-methyl protonated, ^{2}H,^{13}C,^{15}N-labelled proteins.(Jordan et al. 2006) A 2D ^{1}H-start, ^{13}C-detected C^{all}-TOCSY spectrum of the 43 kDa maltodextrin binding protein (MBP) is shown in Fig. 5 (top panel). The protein was uniformly ^{2}H,^{13}C,^{15}N-labelled while the methyl positions of isoleucine-δ_1, leucine and valine were protonated. (Goto et al. 1999) Cross signals between the protonated methyl groups and all other carbons in the amino acid spin systems, even carbonyls, are visible. The spectrum confirms the applicability of the C^{all}-TOCSY for larger systems.

The methyl to carbonyl transfer efficiency for isoleucine-δ_1, leucine and valine as a function of mixing time was also characterized through calculations with the program Antiope (Debouregas and Waugh 1992) assuming a 16.7 kHz spinlock at 600 MHz. Experimental build-up curves under the same conditions were determined for well-separated signals of individual valine, leucine and isoleucine-δ_1 methyl groups. The ^{13}C-start, ^{13}C-detected variant of 2D C^{all}-TOCSY was used for the build-up curves because of higher

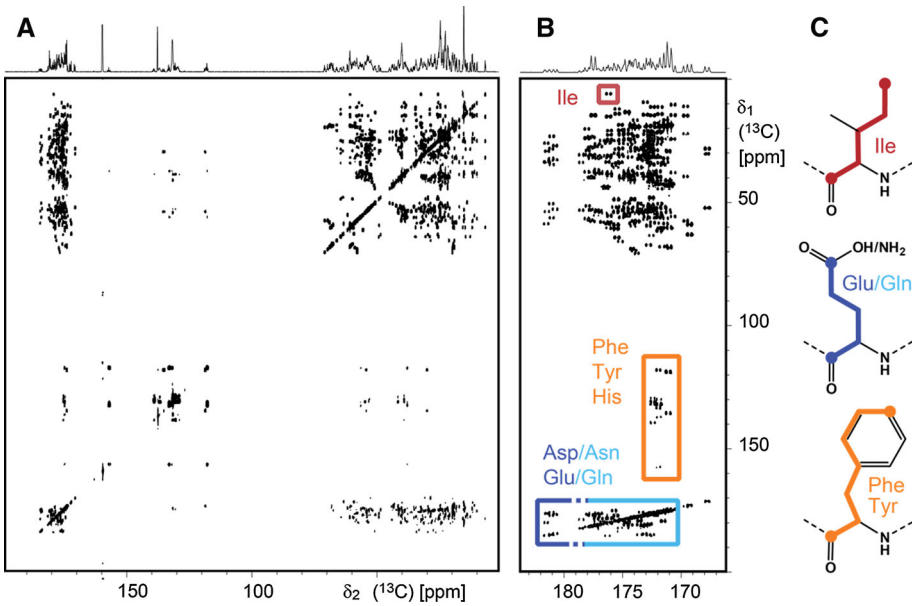

Fig. 4 ^{13}C-detected C^{all}-TOCSY spectrum showing all ^{13}C–^{13}C-correlations in ubiquitin. **a** The complete 2D C^{all}-TOCSY spectrum and (**b**) the enlargement of the carbonyl region are shown. Several exceptional long range through-bond correlations are seen, for instance, the carbonyl–methyl cross-peak of Ile-23 with a chemical shift difference of 171 ppm (25.7 kHz, *red box* in *panel* **b**). The entire spin system of this isoleucine residue is well visible in panel (**a**). A set of correlations between side-chain and backbone carbonyls of aspartate, glutamate and asparagine, glutamine are visible as cross-peaks close to the diagonal (*blue* and *light blue boxes* in *panel* **b**, respectively). The correlations between aromatic side chain carbons and the carbonyl are present (*orange box* in *panel* **b**), including C^{ζ} to carbonyl correlations (signals at 115 ppm for phenylalanine and 160 ppm for tyrosine). In (**c**) the magnetization transfer pathways highlighted in panel (**b**) are depicted in the same *color* on the respective amino acid. The spectrum was recorded at 600 MHz in 6 h on a 1.0 mM sample of ^{13}C,^{15}N-labelled ubiquitin. The mixing time was of 29.8 ms at 16.7 kHz spinlock field and the carrier frequency was set to 95 ppm

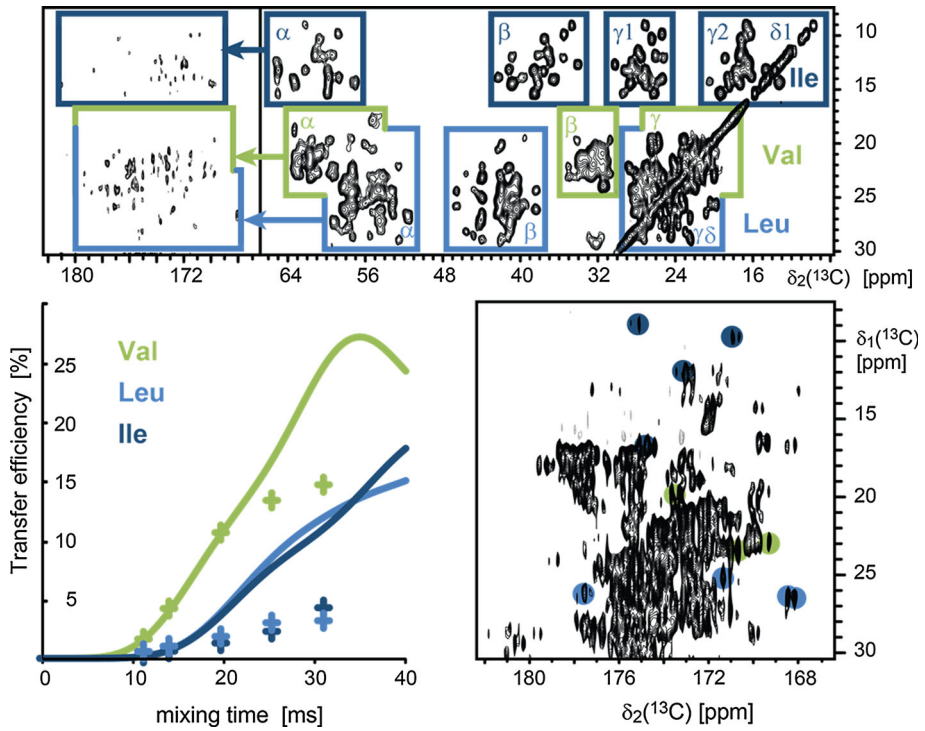

Fig. 5 Theoretical and experimental methyl to carbonyl ^{13}C–^{13}C-spinlock transfer for isoleucine-δ_1, leucine and valine spin systems. Calculated (*solid lines*) and experimental (*crosses*) transfer from methyl groups to carbonyls in isoleucine-δ_1, leucine and valine are shown on the *lower left panel*. A spin lock field of 16.7 kHz centered at 95 ppm was used at a static field of 600 MHz. Here again relaxation was not included in the calculation.The experimental build-up points were obtained from a series of ^{13}C-start,^{13}C-detected 2D C^{all}-TOCSY spectra of a 0.6 mM sample of MBP (43 kDa, u-^{2}H, ^{13}C,^{15}N, ILV-$^{1}H^{methyl}$-labelled) in 9 h per spectrum. The spectrum obtained with 29.8 ms mixing time is shown on the *lower right panel*. The signals integrated for the analysis are indicated with *green*, *light blue* and *dark blue circles* for isoleucine-δ_1, leucine and valine, respectively. Experimental data were uniformly scaled such that the first three data points of valine fit the theoretical *curve*. Differences in theoretical and experimental data can be attributed to relaxation and resonance offset effects. In the *top panel* the ^{1}H-start, ^{13}C-detected H(CC^{all})-TOCSY is shown, that was used for assigning the residue type of the carbonyl signals

intensity of the methyl-carbonyl cross peaks compared to the ^{1}H-start version, although the former spectrum is crowded by the additional isoleucine δ_2-methyl connectivity's. The calculated transfer is compared with the averaged experimental cross peak integrals in Fig. 5. The methyl–carbonyl signals appear at 10 ms of mixing time for valine and at 15 ms for leucine and isoleucine-δ_1. The experimental values increase up to the mixing time of 30 ms. The theoretical curves are in a reasonable agreement with the measured data, indicating a maximum at 35 ms for the transfer from valine methyl groups to the backbone carbon in the 43 kDa MBP. Relaxation was excluded from the calculation because internal rotations of the methyl groups impede the application of a simple relaxation model.

Modified HC(CCO)NH yields higher sensitivity than its classical counterpart

The efficiency of the methyl to carbonyl transfer demonstrated in the C^{all}-TOCSY (Fig. 5) suggests an improvement to the HC(CCO)NH pulse sequence. A shortened HC(CCO)NH experiment is shown in Fig. 6. The modification consists of a direct TOCSY-transfer of magnetization from the side chain carbons to the carbonyl while the refocused C^{α}–C' INEPT step is omitted. Compared to its traditional counterpart, this pulse sequence is roughly 10 ms shorter and contains fewer pulses. Therefore, for larger proteins less relaxation losses and hence improved sensitivity is expected. A comparison of the [$^{13}C^{methyl}$, $^{1}H^{N}$]-planes of the standard and modified HC(CCO)NH experiment is presented in Fig. 7 for the ILV-methyl protonated, ^{2}H,^{13}C,^{15}N-labelled MBP of 43 kDa. The superior sensitivity of the $^{13}C^{all}$-spinlock based experiment over the longer counterpart is evident. In the case of MBP about 60 distinct signals of total 112 were detected in the [$^{1}H^{N}$, $^{13}C^{methyl}$]-plane of the HC(CCO)NH experiment while in the classical counterpart only about 15 signals from the flexible part of the protein were visible. The spectra were recorded at 600 MHz because of a higher sensitivity than at 700 MHz. We ascribe this to an increase of the CSA relaxation of carbonyls at the higher magnetic field.

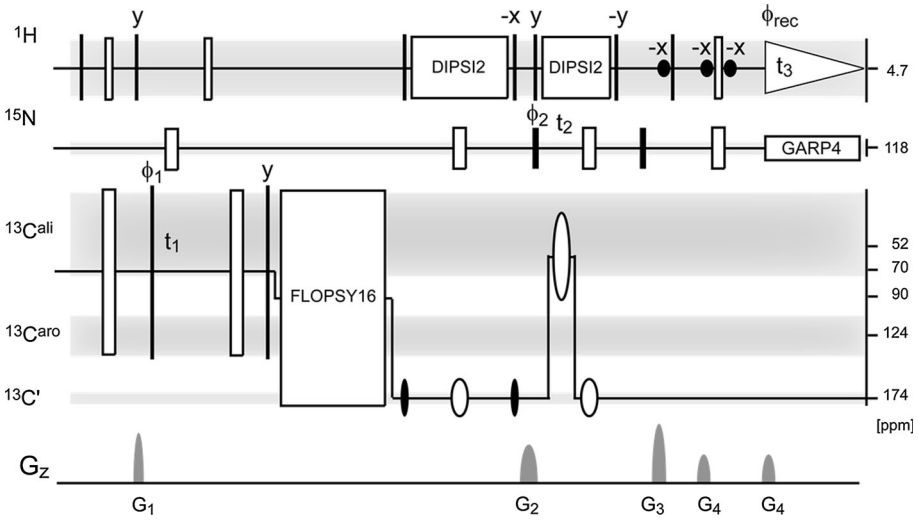

Fig. 6 Pulse sequence for the improved HC(CCO)NH in a representation emphasizing bandwidth and carrier frequencies of rf-pulses. The *grey shaded horizontal rectangles* represent frequency bands of ^{1}H, ^{15}N and different ^{13}C nuclei. The height of the *rectangles* represents the frequency bandwidth of the respective nuclei in Hertz. The ^{13}C bandwidth is subdivided into regions of aliphatic ($^{13}C^{ali}$), aromatic ($^{13}C^{aro}$) and carbonyl ($^{13}C'$) resonances. The bandwidths at 600 MHz are: $^{1}H = 9$ kHz, $^{15}N = 2.4$ kHz, $C^{ali} = 12$ kHz, $C^{aro} = 4$ kHz, $C' = 1$ kHz. Selected ppm values are given on the *right*. *Rectangular* 90° and 180° pulses are indicated by *vertical bars* and *empty rectangles*, respectively, and their phases are indicated above the pulses if they differ from x (= 0°). *Non-rectangular* pulses are shown with *elliptical shape* and *large rectangles* including text represent isotropic mixing sequences or decoupling sequences, their names are indicated (Kadkhodaie et al. 1991; Shaka et al. 1985, 1988). The *vertical length* of the pulses represents their intended excitation bandwidth. The continuous lines on which pulses are centered depict the carrier frequency of each rf-channel, which in the case of ^{13}C is moved several times during the pulse program. Quadrature detection in t_1 (^{1}H) and t_2 (^{13}C) was obtained by alternating ϕ_1 and ϕ_2 according to States-TPPI. Pulsed field gradients along the z-axis (G_z) are represented by *grey shapes*. They consist of smoothed *square shapes* of 1.0 ms duration and are of the relative strengths of 50:40:60:30 for G1:G2:G3:G4, respectively

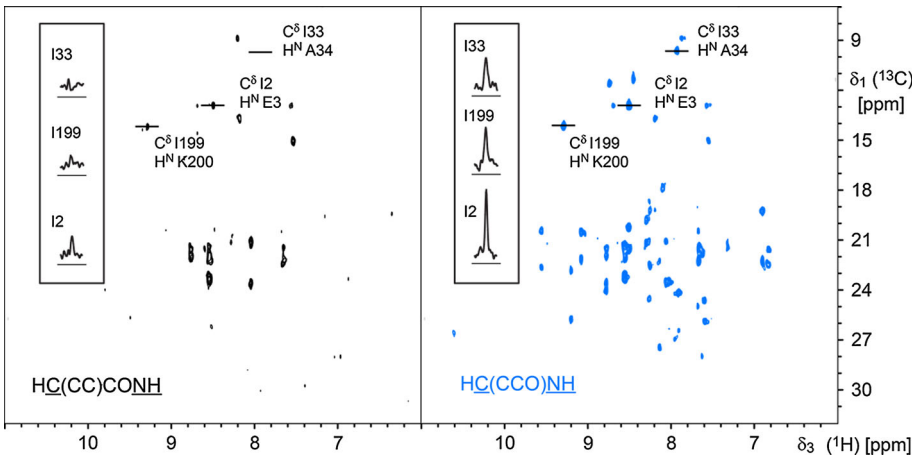

Fig. 7 Improved sensitivity of the modified HC(CCO)NH experiment versus its conventional counterpart for large proteins (MBP, 43 kDa). 2D [$^{1}H^{N}$, $^{13}C^{methyl}$]-planes of the standard (in *black, left panel*) and modified (*blue contours, right panel*) 3D HC(CCO)NH, respectively. The sample was 0.6 mM of ^{2}H,^{13}C,^{15}N, ILV^{methyl} ^{1}H-labelled MBP. In the *blue* spectrum > 60 signals are visible, in the *black* one only about 15, all of which are located in the spectral region of unstructured amino acids. Slices of selected isoleucine signals are shown in inserts on each spectrum. Ile-2 is located in the flexible N-terminus, as reflected in its random coil chemical shift values. Ile-199 is located in a loop and Ile-33 in an α-helical region of MBP. The 2D [$^{1}H^{N}$, $^{13}C^{methyl}$]-planes were recorded in 5 h each, on a 600 MHz spectrometer, using a FLOPSY-16 mixing time of 22.4 and 27.6 ms for the conventional and modified experiment, respectively. The spinlock field strength was 16.7 kHz for both experiments, and the ^{13}C-spinlock frequencies were 39 and 95 ppm for the standard and the modified experiment, respectively. The ^{13}C-carrier frequency was set to 18 ppm during ^{13}C-evolution moved to the aforementioned values for the spinlock and to 173 ppm for the transfer from carbonyl to amide nitrogen

With increasing size of the protein the higher sensitivity of the new, shorter experiment becomes more pronounced. For a 17 kDa protein we observed an average increase in sensitivity of a factor 1.5–2 (data not shown). For the large (43 kDa) deuterated protein MBP improved sensitivity is even more important. For the majority of the correlations

of MBP no signal could be detected in the conventional experiment, whereas distinct signals are seen in the modified HC(CCO)NH spectrum (Fig. 7). In proteins larger than 30 kDa, selectively protonated methyl groups are often the main source of information. The HC(CCO)NH experiment simplifies the methyl assignment and consequently facilitates studies of larger proteins by NMR.

The $^{13}C^{all}$-spinlock of the modified HC(CCO)NH experiment includes also aromatic carbons. For protonated proteins, all types of amino acids are observed in a single spectrum. We recorded aromatic to amide correlations in ubiquitin (Suppl. Fig. 4). In this experiment magnetization is transferred through up to ten different nuclei. This may present an avenue for high throughput assignments relying on a single spectrum for all side chains.

Sample temperature

The spinlock field strength γB_1 is limited by heat dissipation in the NMR probe and the sample. High voltages can lead to electrical discharges and cause damage inside the probe. As for the sample, the oscillating electric field associated with the rf-pulses accelerates mobile ions in the sample solution and lead to heat dissipation (Led and Petersen 1978; Wang and Bax 1993), that might even denature the protein. It has been reported earlier that at a given strength of the electric component of the spinlock field, the absorbed energy increases approximately with the square of the frequency (Wang and Bax 1993). Consequently, the heating effect of a ^{13}C-spinlock is less than that of a ^{1}H-spinlock, and, sample heating can be expected to more pronounce at higher magnetic fields.

To assess sample heating under strong spin lock fields (16.7 kHz for 50 ms) as applied in the current work, the internal sample temperature was monitored and regulated by means of the Bruker NMR thermometer^{TM} in three samples containing 50 mM deuterated sodium acetate and varying concentrations of sodium chloride (0, 200 and 2,000 mM). For the sample with the lowest salt concentration the sample temperature initially rose 0.7 degrees above the target, but reached the stable target value within 3 min after the start of the experiment. A repeat with adiabatic mixing showed practically identical behavior of the temperature, the initial rise being 0.6°. Further, in the samples with 200 and 2,000 mM sodium chloride stabilization at the target temperature was achieved within 3 min in both cases after an initial increase of the sample temperature of 2 and 4°, respectively. For samples containing higher salt concentrations we suggest the use of 3 mm tubes, because the electrical field component that is responsible for heating, is much reduced in the central part of the receiver coil. For samples containing more than roughly 150 mM of total salt, this comes at no loss of sensitivity, since the lower sample noise in the smaller diameter tubes compensates for the lower signal (de Swiet 2005; Voehler et al. 2006).

Concluding remarks

To date the practical limit for the spin lock field strength γB_1 in ^{13}C–^{13}C-TOCSY experiments has been 10–11 kHz with mixing periods of typically 20 ms. This is sufficient to obtain TOCSY spectra of either aliphatic or aromatic resonances (Bax et al. 1990) even at high fields, but not enough to connect aromatic carbons or carbonyls to aliphatic resonances. To overcome this limitation it has earlier been proposed either to increase the bandwidth of ^{13}C–^{13}C-mixing using microcoil probes (Peti et al. 2004) or to design TOCSY sequences with two selective excitation regions (Carlomagno et al. 1996; Coote et al. 2013; Quant et al. 1995; Zuiderweg et al. 1996). The wide bandwidth ^{13}C-spinlock applications presented here are made possible by state-of-the-art cryogenic probe technology. The advantage of the optimized power handling of current cryogenic probes can be exploited in any ^{13}C-spinlock based experiment, such as the ones presented here, or in ^{15}N relaxation dispersion experiments, as recently shown by Ban et al. (2012, 2013). The past limitations of the bandwidth of ^{13}C-spinlock experiments have been ascribed to heating of the probe and heating of the sample itself (Wang and Bax 1993). The sample temperature was controlled internally in our test measurements under the high ^{13}C-spinlock field conditions. After a minor initial rise, a stable temperature in the sample solution was reached typically within 3 min. Our results rely on wideband spinlock fields of 12.5–16.9 kHz at 600–700 MHz magnetic fields. The optimal mixing times for maximal ^{13}C–^{13}C-TOCSY transfer are in the range 15–25 ms, provided a mixing scheme with highly efficient off-resonance transfer is chosen and the carbon offset is centered between the spin pair with the largest chemical shift difference. The rather short optimal mixing times as found here, reflect the focus of the current work on coherence transfer between the bound carbon pairs, disregarding any long-range effects. In the literature, rather soft isotropic ^{13}C–^{13}C-mixing has also been reported relying on long-range coupling constants as low as 1 Hz and mixing times correspondingly beyond 100 ms (Balayssac et al. 2006; Felli et al. 2009; Takeuchi et al. 2010). Such applications of ^{13}C–^{13}C-spinlocks are also expected to benefit from the current probe developments.

Based on these wideband spinlock fields, we improved the commonly used experiments for side chain assignments, namely the H(CC)H-TOCSY, (H)CC-TOCSY and

HC(CCO)NH, to allow the detection of all aliphatic and aromatic ^{13}C-resonances in one single spectrum, even including carbonyls in the latter two cases. This presents several advantages: Firstly, assignment procedures—especially for aromatic side chains—are greatly simplified because ambiguity is reduced and chemical shift mismatches between different spectra are avoided. The resulting data is therefore also ideally suited for automated approaches. Secondly, combining all resonances in a single spectrum leads to reduced measurement times. And thirdly, the shorter HC(CCO)NH experiment offers higher sensitivity than the conventional one, especially for proteins larger than 15 kDa. Thus the modifications generally simplify and speed up side chain assignments and extend the size range of amenable target proteins.

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