Sequential acquisition of multi-dimensional heteronuclear chemical shift correlation spectra with \(^1\)H detection

Peter Bellstedt\(^1\), Yvonne Ihle\(^1\), Christoph Wiedemann\(^1\), Anika Kirschstein\(^1\)\*, Christian Herbst\(^2\), Matthias Görlach\(^1\) & Ramadurai Ramachandran\(^1\)

\(^1\)Leibniz Institute for Age Research - Fritz Lipmann Institute, Department Biomolecular NMR Spectroscopy, Beutenbergstraße 11, 07745 Jena, Germany, \(^2\)Ubon Ratchathani University, Department of Physics, 34190 Ubon Ratchathani, Thailand.

RF pulse schemes for the simultaneous acquisition of heteronuclear multi-dimensional chemical shift correlation spectra, such as \{HA(CA)NH & HA(CACO)NH\}, \{HA(CA)NH & H(N)CAHA\} and \{H(N)CAHA & H(CC)NH\}, that are commonly employed in the study of moderately-sized protein molecules, have been implemented using dual sequential \(^1\)H acquisitions in the direct dimension. Such an approach is not only beneficial in terms of the reduction of experimental time as compared to data collection via two separate experiments but also facilitates the unambiguous sequential linking of the backbone amino acid residues. The potential of sequential \(^1\)H data acquisition procedure in the study of RNA is also demonstrated here.

NMR spectroscopy is a powerful technique for the study of biomolecular structure and dynamics, both in solution as well as in the solid state. In protein NMR a variety of multi-dimensional heteronuclear chemical shift correlation experiments are typically used for resonance assignment and for extraction of \(^1\)H-\(^1\)H distance restraints, e.g. HACANH\(^2\), HNCAHA\(^2\)-\(^5\), HACACONH\(^2\), HNCAHA\(^2\)-\(^5\), HCCNH\(^1\) and \(^1\)N-edited \(^1\)H-\(^1\)H NOESY, respectively. These multi-dimensional spectra are based on different magnetisation transfer pathways and are customarily collected individually. As a result, the time for the acquisition of all required data sets can in many cases become exceedingly long. In this context, a variety of techniques are currently being explored for reducing data acquisition times\(^7\)-\(^10\). One of the approaches that has received considerable attention in the study of proteins, both in solution\(^11\)-\(^14\) and in the solid state\(^15\)-\(^17\), involves the simultaneous collection of different chemical shift correlation spectra. For example, using dual receivers with \(^1\)H and \(^1\)C acquisition in the direct dimension, the simultaneous collection of chemical shift correlation spectra, e.g. \{HACANH & HACACO\} and \{HACACO & HACACONH\}, has been demonstrated employing both parallel\(^12\) and sequential\(^13\) data acquisition procedures. However, as noted already in the literature\(^11\), all other aspects being equal, e.g. magnetisation transfer and relaxation characteristics, the signal intensities seen in the \(^1\)H and \(^1\)C detected data sets would differ because of the difference in the gyromagnetic ratios of the two nuclei. Furthermore, the utility of such experiments is limited as the solution state NMR probes are typically optimised either for \(^1\)H or for \(^1\)C detection only. In addition, many of the chemical shift correlation experiments of interest do not require dual receivers and instead may involve \(^1\)H or \(^1\)C acquisition only. In this context, RF pulse schemes enabling the collection of multi-dimensional data sets with a single receiver is of great interest\(^18\)-\(^21\). Recently we have reported RF pulse schemes involving dual sequential \(^1\)H acquisition with only amide proton detection and making use of dual \(^1\)N-\(^1\)C mixing steps for achieving protein resonance assignment\(^22\). While such sequences can be easily adapted to the study of large \(^2\)H-labeled protein samples, here we present RF pulse schemes that were developed in the context of moderately sized proteins. In such systems the relaxation losses during \(^1\)N-\(^1\)C mixing periods are not expected to be significant even in a fully protonated sample. The pulse sequences presented here make use of the availability of both the HA and HN protons and employ only a single \(^1\)N-\(^1\)C mixing step to achieve sequential resonance assignments in protonated protein samples. The efficacy of the approach is experimentally...
Figure 1 | RF pulse schemes for the simultaneous acquisition of (a) 3D [HA(CA)NH & HA(CACO)NH] (b) 3D [H(N)CAHA & HA(CA)NH] and (c) 3D [H(N)CAHA & H(CC)NH] chemical shift correlation spectra of proteins with dual sequential 1H acquisitions in the direct dimension. Open and filled rectangles represent 180° and 90° pulses, respectively. Phase cycling is as follows: (a) \( \phi_1 = x, -x; \phi_2 = 8(x), 8(-x); \phi_3 = 2(x), 2(-x); \) \( \phi_4 = 4(x), 4(-x); \phi_5 = 2(y), 2(-y); \phi_6 = 4(y), 4(-y); \phi_7 = x, 2(-x), x, -x, 2(x), -x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x). \) (b) \( \phi_1 = x, -x; \phi_2 = 4(x), 4(-x); \phi_3 = 2(y), 2(-y); \phi_4 = 8(y), 8(-y); \phi_5 = 4(y), 4(-y); \phi_6 = x, 2(-x), x, -x, 2(x), -x, 2(-x), x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x). \) (c) \( \phi_1 = x, -x; \phi_2 = 4(x), 4(-x); \phi_3 = 16(y), 16(-y); \phi_4 = 2(y), 2(-y); \phi_5 = 8(y), 8(-y); \phi_6 = 4(y), 4(-y); \) \( \phi_7 = 2(x), 2(-x); \phi_8 = x, 2(-x), x, -x, 2(x), 2(-x), x, -x, 2(x), 2(-x), x, -x, 2(x), -x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x), x, 2(-x). \)

**Results and discussion**

**[HA(CA)NH & HA(CACO)NH].** The triple resonance HACANH experiment is frequently used for sequential resonance assignment of the backbone \( ^1\text{C}_\alpha, ^1\text{N}, ^1\text{H}_N \) and \( ^1\text{H}_Z \) nuclei and involves through-bond magnetisation transfer between the directly coupled nuclei via the pathway \( ^1\text{H}_p \rightarrow ^1\text{C}_\alpha \rightarrow ^1\text{N} \rightarrow ^1\text{H}_N \). *Inter* residue cross peaks arising from transfer of magnetisation from the \( ^1\text{C}_\alpha \) spin of residue (i) to the \( ^1\text{N} \) spin of residue of (i + 1), resulting from \( ^1\text{C}_\alpha \), and are observed in this experiment. *Inter* residue cross peaks can usually be distinguished from *intra* residue cross peaks based on their respective signal intensity. However, to achieve unambiguous resonance assignment, the HACACONH experiment involving magnetisation transfers via the pathway \( ^1\text{H}_p \rightarrow ^1\text{C}_\alpha \rightarrow ^1\text{C}_\alpha \rightarrow ^1\text{N} \rightarrow ^1\text{H}_N \) and leading only to *inter* residue cross peaks is generally carried out in addition. In the HACANH experiment, however, the \( ^1\text{C}_\alpha \rightarrow ^1\text{C}_\alpha \) magnetisation transfer is the critical step as it relies on weak *intra* residue \( ^1\text{C}_\alpha \) couplings (~11 Hz). Although heteronuclear magnetisation transfers are typically carried out via INEPT type transfers, in-phase magnetisation transfers via heteronuclear cross polarization schemes have also been successfully used to enhance sensitivity in triple resonance NMR experiments such as in HACANH. Here, we have implemented RF pulse schemes for the sequential collection of different correlation spectra using \( ^1\text{N} \rightarrow ^1\text{C}_\alpha \) cross polarization schemes. The RF pulse scheme given in Fig. 1a permits the 'one-shot' acquisition of 3D HA(CA)NH and 3D HACACONH data sets. The initial transverse 1H magnetisation generated by the first 90° pulse is allowed to evolve under its chemical shift during the \( t_1 \) (HA)/\( t_1 \) (HA) period and under the one bond heteronuclear \( ^1\text{C}_\alpha \)-1H coupling for a period of 2\( t_3 \) to generate antiphase 1H magnetisation. The anti-phase 1H magnetisation is then converted into antiphase carbon magnetisation by the 90° pulses applied to the two nuclei. The antiphase \( ^1\text{C}_\alpha \) polarisation is allowed to refocus during the interval 2\( t_1 \) to generate \( ^1\text{C}_\alpha \) magnetisation and then subjected to a period of \( ^1\text{C}_\alpha \rightarrow ^1\text{N} \) magnetisation exchange via the application of a band-selective heteronuclear TOCSY mixing sequence. The residual \( ^1\text{C}_\alpha \) transverse magnetisation remaining after the \( ^1\text{C}_\alpha \rightarrow ^1\text{N} \) transfer step is flipped to the z axis and the \( ^1\text{N} \) magnetisation generated after \( ^1\text{C}_\alpha \rightarrow ^1\text{N} \) mixing is allowed to evolve under its chemical shift during the \( t_2 \) (N) period and transferred to the attached proton via an INEPT step and is observed in the \( t_3 \) period under 1H decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum. The WATERGATE sequence is used for water suppression. After the completion the first \( ^1\text{H} \) acquisition, the residual \( ^1\text{C}_\alpha \) magnetisation is brought to the transverse plane and subjected to 13C decoupling to generate the 3D HA(CA)NH spectrum.
Simultaneously acquired (a) 3D HA(CA)NH and (b) 3D HA(CACO)NH spectra of the MCM C-terminal winged helix domain of *Sulfolobus solfataricus* recorded at 600 MHz with 16 transients per *t*₁ increment, 41 *t*₂ increments, 45 *t*₃ increments, spectral widths of 1559 Hz (*H*₁) and 1945 Hz (*¹⁵N*) in the indirect dimensions, respectively, a recycle time of 1.0 s and a proton acquisition time of 60 ms in the direct dimension. The total experimental time was 44 h. The AK2-JCHₐₐmiso and AK2-JCaCₐₐmiso sequences were used for *¹⁵N*-¹³C and *¹³C*-¹⁵N anisotropic cross polarisation, respectively. *¹⁵N*-¹³CO mixing was carried out keeping the *¹³C* RF carrier at 115 ppm, with a peak RF power level of ~11 kHz and for a total duration of 17.92 ms by repeating the basic sequence twice (8.96 ms *×* 2). *¹⁵N*-¹³C mixing was carried out by keeping the *¹³C* RF carrier either at 55 ppm or at 175 ppm for achieving band-selective *¹⁵N*-¹³CA and *¹⁵N*-¹³CO cross polarisations for durations of 25 ms and 50 ms (25 ms *×* 2), respectively. The *¹⁵N*-¹³C mixing sequence with the basic cycle duration of 25 ms was applied employing *¹⁵N/*¹⁵C peak RF power level of ~3.6 kHz, keeping the *¹⁵N* RF carrier at 121 ppm. The *H* RF carrier was kept at 4.3 ppm during *t*₁ and subsequently switched back to the water position (4.7 ppm). Δ₀,₁,₂ = 1.56, 1.56, 2.38 ms were used for INEPT transfers. (c) *H* H-¹5N cross-sections from the HA(CA)NH (blue) and HA(CACO)NH (red) spectra taken at the *¹⁵N* chemical shifts positions indicated and showing the sequential walk along the backbone residues spanning the region E641-K650.

The HA(CACO)NH spectrum is dependent on the amount of residual *¹³C* sub magnetisation present after the *¹³C*-¹⁵N mixing period and hence related to the duration of the mixing period and the performance characteristics of the mixing sequence employed. The residual *¹³C* sub magnetisation has to be kept along the z axis until the first data acquisition is completed and this may affect the signal intensities observed in the HA(CACO)NH spectrum due to relaxation losses. However, in the systems studied here, significant variation in signal intensities were not observed when the residence time of the *¹³C* magnetisation along the z axis was varied over a range of 0–100 ms (Fig. S1). The optimal length of the *¹⁵N*-¹³C het-TOCSY mixing period was found to be ~50 ms (Fig. S2). With this approach we have successfully acquired {3D HA(CA)NH & 3D HA(CACO)NH} spectra of the MCM C-terminal winged helix domain (Fig. 2). Representative cross-sections taken from these 3D data sets are given in the supplementary material (Fig. S3, S4) to indicate spectral quality.

**[HA(CA)NH & H(N)CAHA].** The RF pulse scheme given in Fig. 1b allows to simultaneously collect data from both the HA(CA)NH and H(N)CAHA experiments. Here, unlike the case in the RF pulse schemes given in Fig. 1a, the initial transverse magnetisation generated from both *¹⁵N* and *¹³C* attached protons by the first 90° pulse is allowed to undergo chemical shift evolution during the *t*₁(HN)/*t*₁(TH) period. These evolve under the one bond heteronuclear *¹⁵N*-¹³H and *¹³C*-¹³H scalar couplings during the periods 2Δ₀ and (Δ₀ + Δ₁ − Δ₂), respectively (taking into account the different one bond heteronuclear scalar couplings), to generate the relevant antiphase *H* magnetisation. The antiphase *H* magnetisation are then converted into the corresponding antiphase nitrogen and carbon magnetisation by the 90° pulses applied to the different nuclei. The antiphase *¹⁵N* and *¹³C* polarisation is then allowed to refocus during the interval 2*τ*₁ and 2*τ*₂ to generate (*¹⁵N/¹⁵C*) magnetisation and then subjected to *¹⁵N*-¹⁵C* magnetisation exchange via the application of a band-selective het-TOCSY mixing sequence. Both, the *¹⁵N* and *¹³C* transverse magnetisation present after the *¹⁵N*-¹⁵C transfer step is flipped to the z axis. First, the data from the *¹⁵HN*-¹⁵N/¹⁵N*⁻¹⁵C*-¹⁵CO* pathway [H(N) CAHA] is collected, followed by the acquisition of the signals from the *¹⁵HN*-¹⁵C*-¹⁵CO*-¹⁵N*-¹⁵H* pathway. Sufficient solvent suppression was accomplished by *H* x- and y-purge pulses in combination with gradient pulses just before the *¹³C*-¹³H cross polarisation step and

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Figure 2 | Simultaneously acquired (a) 3D HA(CA)NH and (b) 3D HA(CACO)NH spectra of the MCM C-terminal winged helix domain of *Sulfolobus solfataricus*.
15N-13CA mixing was carried out by keeping the 13C RF carrier at 55 ppm, employing 15N/13C peak RF power level of 3.6 kHz and for a duration of 50 ms by repeating the basic sequence twice (25 ms × 2). The 13C->1H het-TOCSY was carried out with one cycle of the AK2-JCH_{aniiso} sequence having a duration of 7.2 ms, employing H/13CA peak RF power level of -12.5 kHz. The 1H RF carrier was kept at 4.7 ppm. The 1H RF carrier was kept at 3 ppm during t₁ and subsequently switched back to the water position (4.7 ppm). Δt₉=1, 2= 2.58, 1.79, 0.79 ms, 2t₁ = 2Δ₀ and 2t₁ = 3 ms.

With a single 15N-13C mixing step in the RF pulse sequence (Fig. 1b), the simultaneous collection of H(N)CA and HA(CA)NH spectra delivers the chemical shifts of the backbone 13CA and 13C nuclei. Additionally, the (15N,1H) backbone chemical shifts of the adjacent i + 1 residue and the (13CA,1H) chemical shifts of the preceding i−1 residue are also obtained. This facilitates the unambiguous linking of three amino acid residues, i.e. i−1, i and i+1. With this approach we have successfully acquired a combined data set comprising the HA(CA)NH and H(N)CAHA experiment (Fig. 3). Data collected with a cryoprobe are provided in the supplementary material (Fig. S5, S6) to illustrate the performance of the sequence at lower protein concentrations.

(H(N)CAHA & H(CC)NH). In addition to resonance assignment of backbone nuclei a modification of the RF pulse scheme given in Fig. 1b allows to simultaneously acquire the 3D H(CC)NH and H(N)CAHA correlation spectra and to obtain chemical shift information on the protein side chain as well as on the backbone nuclei. In a simple HACAHN experiment the 13Cα magnetisation used for 13Cα->15N mixing arises only from the magnetisation transfer from directly attached 1Hα protons. However, the 13Cα magnetisation in the HCCNH experiment is also generated starting from the side chain protons via the 1HSC->15N->13Cα magnetisation transfer pathway. This is achieved by introducing a 13C->1H longitudinal TOCSY mixing period just before the heteronuclear cross polarisation step (Fig. 1c), with the remainder of the pulse sequence essentially the same as in Fig. 1b. Obviously, one can design the RF pulse scheme to obtain either 1H or 13C side chain chemical shift information. The spectral widths in the indirect dimension of the two data sets can also be independently adjusted by appropriate scaling of the t₂ (CA)/t₉ (N) increments and spectral folding in one data set does not lead to resonance overlaps in the other, as the two data sets are effectively independent. As in the case of HACAHN experiment, interresidue side chain cross peaks arising from transfer of magnetisation from 13Cα spin of residue (i) to the 15N spin of residue of (i + 1) are observed in the HCCNH spectrum. The HCCNH and HNAHA spectra (Fig. 4) were acquired in one shot via the pulse scheme given in Fig. 1c.

The results presented here clearly demonstrate that it is possible to achieve simultaneous acquisition of multidimensional data sets in solution using the sequential data acquisition procedure, akin to recently reported solid state NMR studies of proteins. The unambiguous sequential linking of backbone nuclei (i−1, i, i + 1) is achieved in one shot. The basic strategy with sequential data acquisition procedure is that two different experiments leading to correlation spectra arising from different magnetisation transfer pathways are simultaneously started and at a defined intermediate stage the relevant magnetisation belonging to one of the pathways is kept along the z axis. Depending on the type of data to be sequentially collected, it can be either 15N or the 13C nuclei that are to be kept as longitudinal polarisation. After this, magnetisation transfers followed by the first data acquisition are carried out to complete the experiment via the first pathway. Subsequently, appropriate mag-
Simultaneously acquired 3D correlation spectra via the (a) 3D H(N)CAHA and (b) 3D H(CC)NH experiments. These spectra of the MCM C-terminal winged helix domain of *Sulfolobus solfataricus* recorded at 600 MHz with 16 transients per $t_1$ increment, 36 $t_1$ increments, 50 $t_2$ increments, spectral widths in the indirect dimensions of 3598 Hz ($^1$H), 5278 Hz ($^{13}$C), 2639 Hz ($^{15}$N), a recycle time of 1.0 s and a proton acquisition time of 60 ms in the direct dimension. Total experimental time was 42 h. The AK2-JCH$_{\text{min}}$ sequence was used for both $^{15}$N-$^{13}$CA and $^{13}$C-$^{1}$H anisotropic cross polarisation transfers. The $^{15}$N-$^{13}$CA mixing was carried out by keeping the $^{13}$C RF carrier at 55 ppm, employing $^{15}$N/$^{13}$C peak RF power level of $\sim$3.6 kHz and for a duration of 50 ms by repeating the basic sequence twice (25 ms * 2). The $^{13}$C-$^{1}$H het-TOCSY was carried out with one cycle of the AK2-JCH$_{\text{min}}$ sequence having a duration of 7.2 ms, employing $^{1}$H/$^{13}$CA peak RF power level of $\sim$12.5 kHz. Longitudinal $^{13}$C-$^{13}$C mixing in the aliphatic region was carried out employing the AK2-JCC sequence, with a peak $^{13}$C RF power level of 10 kHz and for a duration of 9.6 ms by repeating two times the basic cycle of duration 4.8 ms (4.8 ms * 2). The RF carrier was kept at 35 ppm during $^{13}$C-$^{13}$C mixing and at 55 ppm for $^{13}$CA-$^{15}$N band-selective mixing. The $^{1}$H RF carrier was kept at 3 ppm during $t_1$ and subsequently switched back to the water position at 4.7 ppm. $\Delta_0, 1, 2 = 2.58, 1.79, 0.79$ ms, $2\tau_1 = 2\Delta_0$ and $2\tau_2 = 3$ ms were used for INEPT transfers. (c) $^{1}$H$_a$-$^{1}$H$_a$ spectral cross-sections from the H(N)CAHA spectrum taken at the $^{15}$N chemical shifts positions indicated and showing the sequential walk along the backbone residues spanning the region E641-K650. (d) $^{1}$H$_a$-$^{1}$H$_a$ spectral cross-sections from the H(CC)NH spectrum taken at the $^{15}$N chemical shifts positions indicated and showing the connectivities between the adjacent backbone residues.
et al. The States procedure\textsuperscript{a} was applied for phase-sensitive detection in the indirect dimensions. Standard phase cycling procedures were employed to select signals arising from desired magnetisation transfer pathways.

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**Author contributions**

P.B., Y.I. and R.R. jointly conceived the study and wrote the manuscript. R.R. implemented the idea and collected data. P.B. evaluated data and prepared the figures. Y.I. prepared the RNA sample and analysed data, C.W. prepared the protein sample and analysed data, A.K. and C.H. designed NMR mixing sequences, M.G. corrected the manuscript and supervised the study. All authors reviewed the manuscript.

**Additional information**

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