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Enhancing the resolution of multi-dimensional heteronuclear NMR spectra of intrinsically disordered proteins by homonuclear broadband decoupling†

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Limited spectral resolution in the proton dimension of NMR spectra is a severe problem in intrinsically disordered proteins. Here we show that homonuclear broadband proton decoupling of the direct and indirect dimensions of multi-dimensional NMR spectra significantly enhances their resolution.

One-dimensional NMR spectra are rarely sufficient for the structural analysis of small molecules, since they can provide only limited information about through-bond and through-space connectivities and their resolution is not adequate for natural products and even less for biomacromolecules like proteins. In contrast two- and multidimensional heteronuclear-correlated spectra not only contain important information about connectivity but also show increased signal dispersion. The large majority of two- and multi-dimensional NMR spectra contain one or more proton dimensions, which typically show the lowest resolution. This results from a combination of their relatively small spectral range (~10 ppm) together with the extensive proton–proton scalar couplings. Limited spectral resolution is a particularly severe problem in intrinsically disordered proteins (IDPs),[1] whose chemical shifts are often close to random coil values. The low resolution encountered in NMR spectra of intrinsically disordered proteins can be partially overcome by using carbon-detected experiments[2] or by employing higher-dimensionality (>3D) NMR spectra, typically in combination with fast acquisition methods like non-uniform sampling,[3] GFT,[4] projection reconstruction[5] or APSY.[6] However, these experiments are often time-consuming and/or require more elaborate data processing schemes. Broadband homonuclear decoupling has been suggested to be a solution to the resolution problem of proton NMR spectra for small molecules.[7] Broadband homonuclear decoupling can be achieved using several approaches: constant-time experiments (applicable for defined, typically heteronuclear coupling constants),[8,9] time reversal experiments,[10] the diagonal signals of z-COSY spectra,[11] projections of J-resolved 2D spectra,[12] BIRD-based decoupling[13,14] and frequency-selective decoupling during a weak gradient field (also called the Zangger–Sterk or ZS method).[7,15,16] All of these methods enable decoupling of the indirect dimension of multidimensional spectra. If 1D spectra or decoupling in the acquisition dimension are desired the actual decoupled spectra have to be constructed using different post-processing algorithms. This additional "decoupling dimension" significantly reduces the achievable signal to noise ratio per unit time. Very recently it has been shown that BIRD-based decoupling of protons bound to carbon at natural abundance as well as slice-selective decoupling during a weak gradient field can also be obtained during acquisition in a single scan.[17,18] All other methods for decoupling are not amenable for decoupling during acquisition as they do not physically decouple the protons but only extract the decoupling information from the special appearance of the respective spectra or after combining spectra obtained with different pulse phases. Decoupling during acquisition allows the spectra to be processed like regular NMR data and it significantly increases the sensitivity per unit time. So far pure shift spectra have only been reported for one- and two-dimensional experiments on small molecules. The use of conventional homonuclear decoupled experiments on biomacromolecules was typically prevented by low sensitivity. The enormous sensitivity gain per time achieved by homonuclear decoupling during acquisition finally lifts this shortcoming. For protons in uniformly ^{13}C-labeled molecules, the frequency-selective decoupling during a weak gradient field is the only option for achieving broadband homonuclear proton decoupling during the acquisition. For this method a selective pulse is applied during a weak pulsed field gradient, which results in the excitation of different signals in different slices of the NMR sample.[16,19,20] Homonuclear broadband decoupling is then achieved by a combination of hard and selective 180° pulses, which are again applied during the same weak gradient. In each slice, the signal that was originally excited by the selective pulse is then decoupled from all other protons (for more detailed explanations see ref. 7, 16, 18 and 21). Here we show that by using this type of broadband decoupling both in the direct and indirect dimensions of

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† Electronic supplementary information (ESI) available: Experimental details, pulse sequence schematic of homonuclear decoupled H(CCO)NH experiment, NMR spectra and chemical shifts table. See DOI: 10.1039/c3cc48135b

multidimensional NMR spectra, the resolution in the proton-dimensions of intrinsically disordered proteins (IDPs) can be significantly enhanced. The number of known IDPs is rapidly growing and it is currently believed that 45–50% of all eukaryotic proteins contain long ID regions.[22,23] They are involved in various physiological functions, like signalling, cell-cycle control or molecular recognition, but they are also implicated in various disorders like Alzheimer's and Parkinson's diseases, cancer or type II diabetes. Due to the extensive signal overlapping, IDPs benefit the most from homonuclear decoupling and on the other hand their relatively long transverse relaxation times do not produce artifacts because of the interrupted acquisition. Combining instant broadband proton decoupling in the ω_2-dimension with constant-time carbon decoupling in ω_1 results in 2D ^{1}H, ^{13}C HSQC spectra with unprecedented resolution. These high definition (HD) HSQC spectra are perfectly suited to monitor weak interactions between intrinsically disordered proteins by chemical shift mapping. The pulse sequence for the HD ^{1}H, ^{13}C HSQC experiment is shown in Fig. 1. In order to assign the signals which could be resolved only by broadband homonuclear proton decoupling, a set of three-dimensional HCCONH spectra,[24] homonuclear decoupled in the indirect proton or carbon dimension, were used. Using this approach we investigated the interaction of the two intrinsically disordered proteins α-synuclein and SERF1a.[25,26] The pathogenic amyloidogenesis of the Parkinson's-associated protein α-synuclein is accelerated using SERF1a through transient interaction with the negatively charged C-terminal region of α-synuclein.[25]

Assignment of side chain resonances of well-structured proteins can be readily achieved by triple resonance experiments. The HCCCONH experiment is widely used to correlate sidechain ^{13}C and ^{1}H resonance frequencies with the amide ^{15}N and ^{1}H resonances. Two 3D experiments, namely H(CCCO)NH and (H)C(CCO)NH are frequently used for resonance assignment.[24]

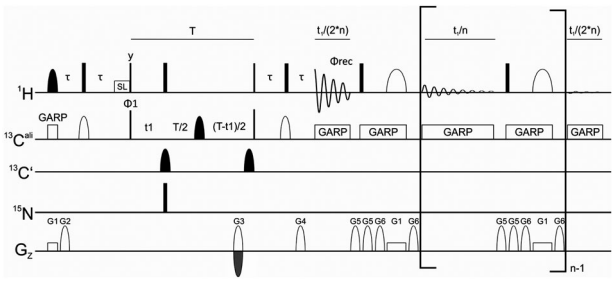

Fig. 1 Pulse scheme of the HD ^{1}H, ^{13}C HSQC experiment. The pulse sequence is based on the sequence for a constant time (CT) ^{1}H, ^{13}C HSQC, which has been described earlier,[27] and has been modified to accomplish homonuclear decoupling in the acquisition dimension as follows: the first 90° pulse has been replaced by a slice selective 90° pulse. Gradients 2, 3 and 4 are used to explicitly select magnetization which has been slice-selectively excited and subsequently transferred to ^{13}C. Acquisition is interrupted every 10–20 ms for decoupling, which is achieved by a combination of a hard and a slice selective 180° pulse as described elsewhere.[18] ^{13}C decoupling during slice-selective excitation and decoupling is achieved using a GARP decoupling scheme. Narrow and wide rectangles represent 90° and 180° pulses. Filled and open half-ellipsoids denote 90° and 180° pulses respectively. SL represents a spin-lock pulse to suppress residual HDO resonance.

However, the resolution in the indirect dimension of these experiments is limited to >20 Hz for ^{1}H and >60 Hz for ^{13}C due to homonuclear couplings. Since resonance frequencies of many methyl groups only differ by a few Hz, a reliable, unambiguous assignment cannot be obtained using the conventional 3D pulse-sequences. In order to obtain high resolution methyl assignments we recorded broadband homonuclear decoupled versions of the experiments. Homonuclear decoupling in the H(CCCO)NH experiment was achieved by placing a ZS-decoupling scheme in the middle of the ^{1}H evolution period (ESI,† Fig. S1). To allow for ^{13}C homonuclear decoupling in the (H)C(CCO)NH a constant time evolution period was used instead of the commonly implemented real-time evolution. For those amino acids which have more than one methyl group (Val, Leu, Ile) the HD ^{1}H, ^{13}C HSQC was used to identify the correct ^{1}H, ^{13}C resonance pairs. In the very few cases where the HD ^{1}H, ^{13}C HSQC spectra do not resolve either of the two methyl groups, a 3D HC(CCON)H acquisition decoupled in both the indirect carbon and proton dimensions can be utilized. With this approach >95% of the methyl groups of α synuclein could be assigned unambiguously. Since the linewidths of the decoupled dimension are less than 4 Hz, assignments can be easily transferred to a HD ^{1}H, ^{13}C HSQC spectrum (ESI,† Fig. S2). Thus, the use of high definition spectra for resonance assignment renders the exhaustive acquisition and analysis of 4D and higher dimensional spectra unnecessary. In cases where the resolution in such decoupled 3D spectra is not sufficient, broadband homonuclear decoupling can of course also be combined with fast NMR methods like non-uniform sampling (NUS) or reduced dimensionality experiments. To ensure optimal resolution in all indirect dimensions we used a NUS scheme with 5–20% of the regular sampling. Spectra were processed using MDDNMR.[28,29]

Despite the high resolution in the indirect dimension of a CT ^{1}H, ^{13}C HSQC most of the resonances of α synuclein are overlapping and hardly any peak can be assigned unambiguously (Fig. 2). The HD ^{1}H, ^{13}C HSQC, which is decoupled in both frequency dimensions, displays much less signal overlapping. Slice selective decoupling is associated with a loss of sensitivity determined by $\Delta\omega/(\gamma G^* s)$, with the excitation bandwidth $\Delta\omega$, the gyromagnetic ratio γ, the gradient strength G and the length of the active sample volume s. Note that this equation is only valid for $\Delta\omega \leq \gamma^* G^* s$. The loss of sensitivity is thus proportional to the spectral window which is covered in the decoupled dimension and is partially compensated for by the collapse of multiplets into singlets due to decoupling. To restrict the sensitivity loss we recorded two spectra covering the Hα and the H-methyl regions of the HSQC spectrum (Fig. 2 and Fig. S3, ESI†). To achieve this, the carrier frequency was set to 1 or 4 ppm and selective pulses with 80 Hz bandwidths in combination with a gradient of 0.2 or 0.4 Gauss per cm were used for excitation and decoupling of H-methyl or Hα respectively. The actual loss in the sensitivity of the HD HSQC is therefore on the order of 80%. Fig. 2 shows the Val methyl region of α synuclein, which is rather crowded in the CT ^{1}H, ^{13}C HSQC with only very few peaks being resolved. In contrast the HD ^{1}H, ^{13}C HSQC eliminates most of the overlapping: out of the 19 Val present in α synuclein more than 70% show at least one of the methyl groups being resolved. In addition, overlapping was greatly reduced for Ala-methyl groups

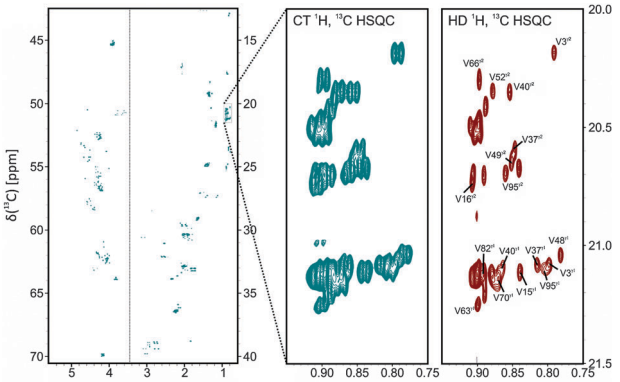

Fig. 2 (left) CT ^{1}H, ^{13}C HSQC of α synuclein. Despite the high resolution in the ^{13}C dimension, resonances are not well resolved due to the poor signal dispersion inherent to spectra of intrinsically disordered proteins. (right) Close-up view of the valine methyl region. Whereas the conventional CT ^{1}H, ^{13}C HSQC (left) shows significant resonance overlapping most of the peaks are well separated in the HD ^{1}H, ^{13}C HSQC experiment and can be unambiguously assigned.

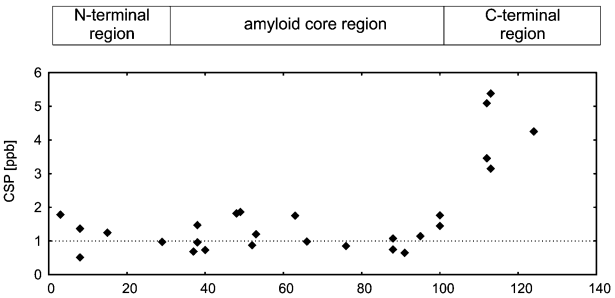

Fig. 3 Plot of α synuclein methyl CSPs (in parts per billion, ppb) upon titration with SERF1a. The C-terminal region experiences the largest perturbations. CSPs are represented as $((\delta\Delta(^1\mathrm{H}))^2 - (\delta\Delta(^{13}\mathrm{C})/4)^2)^{1/2}$.

as well so that 9 out of 19 Ala methyl groups are baseline separated in the high definition HSQC. This high level of peak separation, which can very well compete with that of a ^{15}N-HSQC, enables the HD ^{1}H, ^{13}C HSQC to serve as the basis for NMR titration experiments. In contrast to the ^{1}H, ^{15}N HSQC which is very well suited to monitor interactions involving the protein backbone, ^{1}H, ^{13}C HSQCs can detect in particular hydrophobic interactions which often involve methyl groups. Upon addition of SERF1a, methyl-groups of residues located at the C-terminus of α synuclein show the most prominent chemical shift perturbations (CSP, Fig. 3 and Fig. S4, ESI†).

This is in line with the binding site, which has been identified previously by ^{1}H, ^{15}N-HSQC titrations.[25] Additional smaller changes above the threshold occur at the N-terminus and in the amyloid core region of α synuclein. Those changes may be attributed to release of long range interactions of the amyloid core region with aliphatic sidechains of the C-terminus of α synuclein upon binding to SERF1a and have not been observed previously by ^{1}H, ^{15}N HSQCs since they involve mainly hydrophobic sidechain interactions. An exposure of the amyloid core region would enhance aggregation into amyloid fibrils and explain how binding of SERF1a to the C-terminus of α synuclein enhances its aggregation into amyloid fibrils.

In conclusion we have shown that broadband homonuclear proton decoupling by frequency-selective pulses during a weak gradient field can be applied both in the direct and indirect dimensions of two- and multidimensional NMR spectra. It is perfectly suited for intrinsically disordered proteins which suffer from low resolution. Using 2D ^{1}H, ^{13}C-HSQC as well as 3D HCCONH spectra, homonuclear broadband proton decoupling in the direct and/or indirect dimensions greatly simplifies assignment of side chain resonances. Furthermore, methyl chemical shift perturbations traced by HD ^{1}H–^{13}C HSQC can provide valuable information which might not be picked up by conventional NMR titration based on ^{1}H, ^{15}N HSQCs.

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