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A new strategy for sequential assignment of intrinsically unstructured proteins based on ^{15}N single isotope labelling

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ABSTRACT

We describe a new efficient strategy for the sequential assignment of amide resonances of a conventional ^{15}N–^{1}H HSQC spectrum of intrinsically unfolded proteins, based on composite NOESY–TOCSY and TOCSY–NOESY mixing times. These composite mixing times lead to a Hα-proton mediated unidirectional transfer of amide to amide proton. We have implemented the composite mixing times in an HSQC–NOESY–HSQC manner to obtain directional connectivity between amides of neighbouring residues. We experimentally determine the optimal mixing times for both transfer schemes, and demonstrate its use in the assignment for both a fragment of the neuronal tau protein and for α-synuclein.

1. Introduction

NMR is a most powerful experimental approach to study intrinsically unfolded proteins (IUPs) [1] yielding time-averaged parameters that can be interpreted in terms of conformational sampling of the individual amino acids [2–8], mapping the interactions with (bio)molecular partners [9–12] and even analyzing post-translational modification patterns in terms of identification and quantification of the PTM pattern and its structural consequences [13–18]. An absolute prerequisite for all these studies is the assignment of the ^{1}H–^{15}N HSQC spectrum. The reduced amide proton chemical shift range and reduced deviation of the carbon chemical shifts from their random coil values make this assignment more difficult than for a folded protein. Exploiting the NOE effect between adjacent amide protons, but encoding them by their associated ^{15}N amide frequency, thereby has early on been proposed as strategy to assign unfolded proteins [19]. More recently, the HNCaNNH experiment [20,21] was developed, that bases the transfer of magnetization between adjacent amide functions not on the NOE effect of its protons, but on the scalar coupling between nitrogen and carbon atoms. This experiment equally allows to connect a given amide signal to the nitrogen frequency of its two neighbouring amino acids. The good ^{15}N spread for the amide nitrogen and the limited magnetization losses due to the reduced line width of the resonances in an IUP make this experiment particularly useful, and it therefore is invariable part of the assignment strategy of IUPs. Other developments including techniques of higher dimensionality [22] and non-uniform sampling of the carbon dimensions [23], have allowed a certain level of automatisation of the assignment process [24,25]. As a result, the assignment, both of larger and more repetitive IUPs, such as the 441 amino acids long microtubule associated protein Tau [25] has been accomplished, even though these approaches are not yet routine procedures in most laboratories.

One drawback of the HNCaNNH experiment is that it lacks directionality. Because of the non-vanishing coupling constant between the ^{15}N of residue i and the Cα of residue $i-1$, one indeed observes 3 resonances in the third dimension. Another limitation is that it only gives information on the nitrogen frequency of the neighbouring amide functions. If we could equally access the proton frequency, one could imagine to walk through the spectrum in a straightforward manner.

The ROESY–TOCSY mixing scheme was implemented to cross the glycosidic linkage in complex glycan molecules [26,27], and was later implemented as a NOESY–TOCSY mixing to assist in the assignment of small folded proteins [28]. A proton NOESY mixing time combined with a carbon TOCSY transfer was equally proposed to transfer magnetization from one to the next amino acid [19]. We propose here the use of a proton-only composite mixing time to connect in a directional manner sequential amide nitrogen or hydrogen frequencies in an IUP. The experiment exploits a mixing period composed of two periods, whereby magnetization transfer is based alternatively on NOE dipolar relaxation (as in a regular NOESY spectrum) and on scalar coupling (as in a regular TOCSY experiment). We explore the optimal mixing times to be used for efficient transfer from amide to amide function, and apply the sequence to two IUPs, a functional TauF4 fragment that we previously have defined [29] and α-Synuclein [9,30], another IUP

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that has been extensively studied. We implement the sequence as 4 separate 3D sequences, with particular attention to water suppression and the minimizing of antidiagonal artefacts. Finally, we show that a nearly complete assignment of these IUPs can be obtained on the sole basis of the hereby presented pulse sequences.

2. Results and discussion

The magnetization transfer during the composite mixing times can be easily seen on a hypothetic tripeptide (Fig. 1). Amide proton magnetization of $(i+1)$th residue will be transferred to the H^{α} proton of the upstream residue (i) during the NOE period. The resulting $H^{\alpha}(i)$ magnetization will then be transferred to its own amide proton $H^{N}(i)$ via the TOCSY transfer, resulting in a net transfer from the $H^{N}(i+1)$ proton to the $H^{N}(i)$. In the 2D spectrum with this composite mixing time, cross peaks will hence show between a given amide proton in the direct dimension and its downstream neighbour. If we invert the order of NOESY and TOCSY mixing times, the transfer would be from $H^{N}(i-1) \rightarrow H^{\alpha}(i-1) \rightarrow H^{N}(i)$, resulting in a cross peak between a given amide proton in the direct detection proton dimension and its upstream neighbour.

Fig. 2 shows the pulse sequence designed based on the 4D HSQC–NOESY–HSQC pulse sequence [31], where we have implemented the composite mixing times. We have performed these two 4D sequences as four 3D experiments (3D-NNH_N-NOESY–TOCSY, 3D-H_NNH_N-NOESY–TOCSY, 3D-NNH_N-TOCSY–NOESY, 3D-H_NNH_N-TOCSY–NOESY) on a sample of α-Synuclein, to obtain unidirectional correlations between proton and nitrogen frequencies of backbone amides of neighbouring residues. Whereas in the original NOESY–TOCSY experiment [28], a 4 step phase cycle was used, we extended it to 8 in order to increase signal to noise.

Because amide protons in IUPs are in fast exchange with water due to their easy accessibility, the first block of the pulse sequence is implemented as a water flip-back HSQC [32,33]. When encoding nitrogen in the first dimension ($t1$) for the 3D-NNH_N-NOESY–TOCSY or 3D-NNH_N-TOCSY–NOESY schemes, and denoting the amide proton or nitrogen magnetization terms by I and S, respectively, and the water magnetization by H, the magnetization after this first HSQC block (time point a in Fig. 2) is $-Iy\cos(\omega_N\, t1) + Hz$ or $-Iy\sin(\omega_N\, t1) + Hz$, according to the phase selection by the nitrogen pulse φ1. The 90° hard pulse along the $-x$-axis preceding the composite mixing period transforms these terms into $Iz\cos(\omega_N\, t1) + Hy$ or $Iz\sin(\omega_N\, t1) + Hy$. The water term is then brought back to the z axis in an active manner by a flip back pulse with the opposite phase of the 90° hard pulse (time point b in Fig. 2), before the gradient pulse G5 defocuses all residual magnetization terms in the xy plane.

Before considering the evolution of these terms in the composite mixing time, we describe how the same scheme can be used for encoding the amide proton frequency in $t2$ (3D-H_NNH_N-NOESY–TOCSY and 3D-H_NNH_N-TOCSY–NOESY experiments). The drawback of encoding protons in an indirect dimension is the requirement of many complex points to obtain a satisfying resolution. Because in the present case, we focus on IUPs, the reduced amide proton window can be sampled with a small number of points, especially as the first HSQC block efficiently acts as a band pass filter for only those protons that are directly bound to a ^{15}N nucleus. Therefore, at time point a (Fig. 2), we switch the proton frequency to the middle of the amide proton region ensuring that the proton evolution during the $t2$ period will be centered at this offset. One nevertheless should take into account the NH2 side chain protons, that will be folded in this dimension when too small a window is chosen. We therefore typically used a 2.4 ppm window centered at 7.7 ppm. The proton evolution in $t2$ is followed by a proton pulse with phase φ2 that is still centered at the same offset in the middle of the amide proton region. After the $t2$ encoding, the resulting magnetization can be written as $-Iy\cos(\omega_H\, t2) + Ix\sin(\omega_H\, t2) + Hz$. Phase selection in the $t2$ dimension is performed by the phase φ2 of the 90° pulse preceding the mixing time, that will select the cosine or sine term, resulting in $Iz\cos(\omega_H\, t2) + Ix\sin(\omega_H\, t2) + Hy$ or $-Iy\cos(\omega_H\, t2) + Iz\sin(\omega_H\, t2) + Hx$. After this pulse, at time point b, we switch back the proton carrier to the water frequency, and apply the water flip back pulse with the opposite phase as the φ2 pulse. The G5 gradient will destroy all in-plane magnetization, leaving as only terms at the beginning of the mixing time $Iz\cos(\omega_H\, t2) + Hz$ or $Iz\sin(\omega_H\, t2) + Hz$. This ensures a pure Z component before the composite mixing period, and avoids any amplitude differences in the cosine and sine terms that might cause anti-diagonals peaks [34]. A gradient G4 at the end of the composite mixing period assures that only magnetization terms along the Z axis enter the second half of the pulse sequence that we implement as a sensitivity enhanced HSQC sequence [35].

The composite mixing time is aimed to transfer with optimal efficacy the magnetization from one amide proton to its next neighbour (upstream or downstream, depending on the order of NOESY and TOCSY blocks) via the H^{α} proton. We therefore first sought to optimize the durations of both mixing times, by considering the transfer from $H^{\alpha}(i-1)$ to H^{N} in a simple NOESY experiment or from $H^{\alpha}(i)$ to $H^{N}(i)$ in a TOCSY experiment. In order to optimize these delays experimentally for our systems, we ran the 2D H–H planes of a TOCSY–HSQC and NOESY–HSQC pulse sequence with varying mixing times on the TauF4 sample, and considered the projection of the H^{α} protons. Fig. 3a shows the sum of the columns (F_1 dimension) of the proton–proton plane of the NOESY–HSQC corresponding to amide proton chemical shifts between 8.7 and 7.7 ppm, extracted from 2D experiments at different mixing times. The maximum intensity in the Hα region is obtained for a mixing time of 200 ms (Fig. 3b). Because pH and temperature might alter this values for different samples, we ran the same experiments on a α-synuclein sample at 10 °C and pH 7.4 (see below). We again found the same flat maximum with optimal NOE mixing time between 200 and 300 ms. Using only a NOE based transfer period to directly connect consecutive amide protons

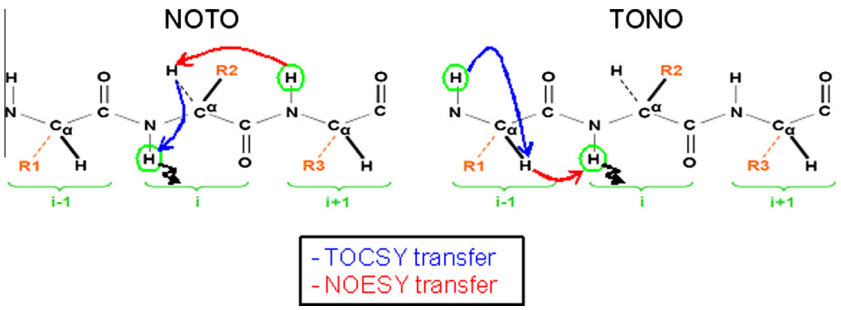

Fig. 1. Magnetization transfer pathways during NOESY–TOCSY and TOCSY–NOESY composite mixing times: The NOESY–TOCSY mixing time leads to correlations between ith and $(i+1)$th residues while the TOCSY–NOESY scheme gives opposite directionality, and the the correlations between ith and $(i-1)$th can be obtained.