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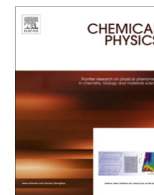

# Pseudo 5D HN(C)N experiment to facilitate the assignment of backbone resonances in proteins exhibiting high backbone shift degeneracy

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ABSTRACT

Assignment of protein backbone resonances is most routinely carried out using triple resonance three-dimensional NMR experiments involving amide $^{1}H/^{15}N$ resonances. However for intrinsically unstructured proteins, alpha-helical proteins or proteins containing several disordered fragments, the assignment becomes problematic because of high-degree of backbone shift degeneracy. In this backdrop, a novel reduced-dimensionality (RD) experiment –(5,3)D-hNCO-CANH- is presented to facilitate/validate the sequential backbone resonance assignment in such proteins. The proposed 3D NMR experiment makes use of the modulated amide $^{15}N$ chemical shifts (resulting from the joint sampling along both its indirect dimensions) to resolve the ambiguity involved in connecting the neighboring amide resonances (i.e. $H_iN_i$ and $H_{i-1}N_{i-1}$) for overlapping amide-NH peaks. The experiment -in combination with routine triple resonance 3D-NMR experiments involving backbone amide ($^{1}H/^{15}N$) and carbon ($^{13}C^{\alpha}/^{13}C'$) chemical shifts- will serve as a powerful complementary tool to achieve the nearly complete assignment of protein backbone resonances in a time efficient manner.

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## 1. Introduction

Over the decades, NMR has emerged as a powerful technique for studying the structure and dynamics of proteins and their complexes in solution. Further, it is the technique of choice for studying conformational properties of intrinsically unstructured proteins (IDPs), and their interactions with their physiological binding partners in solution [1–3]. For various such studies on proteins by NMR, the very first and key requirement is the sequence specific assignment of backbone ($^{1}H$, $^{15}N$, $^{13}C'$ and $^{13}C^{a}$) resonances [4,5]. The well-established and most routinely used assignment strategies involve the use of $^{15}N$, $^{1}H^{N}$ resolved triple resonance experiments sequentially linking $^{13}C^{\alpha\beta}$, $^{13}C'$ or $^{15}N$ shifts [6–22] and many proteins have been assigned this way (evident from the Biological Magnetic Resonance Bank: http://www.bmrb.wisc.edu). However for proteins exhibiting high degree of backbone amide and carbon shift degeneracy (e.g. α-helical proteins or proteins containing disordered fragments including IDPs), getting this information in an unambiguous and time-efficient manner has always remained problematic and challenging. Therefore new or alternative NMR methods and strategies -for rapid and efficient assignment of backbone resonances in such proteins- are required.

Several efforts have been made in the past to resolve this problem [8,13,14,16,20,22–25,12,26–33] involving either (a) the use of $H^{a}$-detected NMR experiments [27,34] or (b) $^{13}C$ detected NMR experiments [24,29,23], or (c) higher dimensionality (4D or 5D) NMR experiments [29,31–33]. Of them, $H^{\alpha}$-detected experiments require protein samples in deuterated solvents, while $^{13}C$-detected experiments suffer from reduced sensitivity due to the lower gyromagnetic ratio of $^{13}C$ with respect to $^{1}H$ [16]. Further if the assignment process has already been started acquiring the conventional $^{15}N$, $^{1}H^{N}$ resolved triple resonance 3D NMR experiments, one has to acquire the completely different set of NMR experiments to resolve the ambiguities. Therefore, the approach not only increases the demand for NMR instrument time but may elaborate the analysis as well. Of-course, the $^{15}N$, $^{1}H^{N}$ resolved higher dimensionality ($\geqslant$3D) NMR experiments (e.g. $^{15}N/^{13}C$ edited 4D HNCOCANH)

*Abbreviations:* NMR, Nuclear Magnetic Resonance; HSQC, Heteronuclear Single Quantum Correlation; CARA, Computer Aided Resonance Assignment; RD, reduced dimensionality; BMRB, Biological Magnetic Resonance Bank; ESM, Electronic Supplementary Material.

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can easily resolve the problems arising because of overlapped resonances, however, a 4D/5D spectrum with reasonable high-resolution typically requires weeks-to-months' time for data collection with conventional acquisition routines which severely limits the utility of NMR for studying unstable proteins. In such situations, the higher dimensionality information can be obtained using "unconventional" acquisition schemes involving the indirect dimensions, such as non-uniform sampling (NUS) [35,36] or reduced dimensionality approach [37,38] (both these methods can also be combined to further reduce the overall experiment time). Of them, NUS allows rapid acquisition of multi-dimensional NMR spectra by reducing the sampling of data points in indirect evolution time space [35,36] and the approach has recently been employed for studying several challenging and complex protein systems [24,26,29,31–33,35,36,39–42]. However, the only limitation associated with NUS approach is that it requires good signal-to-noise ratio (SNR) for optimal processing of higher-dimensionality (4D, 5D) non-uniform sampling NMR experiments [43,44] and therefore cannot be applied to protein systems exhibiting low SNR (either because of their higher MW or reduced solubility in aqueous solution). In such situations, the reduced dimensionality (RD) NMR [37,38] offers the most viable and feasible solution to reduce the acquisition time by an order of magnitude. It provides higher dimensional information in a reduced dimensionality spectrum [45] through joint sampling of two or more chemical shifts in a single indirect dimension, thus provides considerable reductions in the measurement time. In addition to affording savings in measurement time, the sums and differences of chemical shifts - resulting from joint sampling- span a wider spectral range than the individual shifts themselves. However, the important consequence of the linear combination of chemical shifts is that it provides better dispersion and randomness to resolve the ambiguities arising because of degenerate chemical shifts while establishing the sequential connectivities in backbone assignment process [46,47]. The idea has been used earlier by Atreya et al. [8] to solve the backbone assignment problem for proteins with very high shift degeneracy –based on 5D spectral information encoded in $G^2$FT NMR experiments. These experiments, although, make use of backbone $^{15}$N and $^{13}$C′ shifts, that remain well dispersed for most of the folded and unfolded proteins, also include other nuclei with poorer dispersion, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$, which may adversely affect the assignment process. As an alternative, a novel reduced dimensionality 3D NMR experiment –(5,3)D h<u>NCO</u>-<u>CAN</u>H- has been presented here which makes full use backbone $^{15}$N and carbon ($^{13}$C′ and $^{13}C^{a}$) chemical shifts. The resulted spectrum offers relatively higher chemical shift dispersion and randomness simultaneously (a) to break the backbone shift degeneracy and (b) to resolve the problems arising because of overlapping amide NH resonances. The performance of the experiment and the application of the method have been demonstrated here using (i) a 15.4 kDa size folded protein named tgADF (*Toxoplasma gondii* ADF, a 118 amino acid protein with an additional N-terminal twenty-one residue purification tag) and (ii) a 12 kDa size unfolded protein named CFP-10 (i.e. culture filtrate protein of *Mycobacterium tuberculosis H37Rv*, a 100 amino acid protein with an additional C-terminal thirteen residue purification tag).

## 2. Materials and methods

The proposed reduced dimensionality experiment -(5,3)D-h<u>NCO</u>-<u>CAN</u>H was developed and tested successfully first using a standard $^{15}$N/$^{13}$C labeled protein sample of chicken SH3 domain (final concentration ~1.5 mM, dissolved in 50 mM Sodium phosphate buffer containing 90% $H_2O$/10% $D_2O$, pH 6.5 in high quality NMR tube sealed under inert atmosphere purchased from Cambridge Isotope Laboratories, Inc., USA: http://www.isotope.com/cil/). The application of the experiment has been demonstrated here on (i) a 15.4 kDa size folded protein referred here as tagged TgADF (i.e. *T. gondii* ADF, a 118 amino acid protein with an additional N-terminal twenty-one residue purification tag from vector pET16b) expressed and purified as described here [48] without cleaving the additional twenty-one residue purification tag and (ii) a 113 amino acid long unfolded protein referred here as tagged CFP-10 (i.e. culture filtrate protein of *Mycobacterium tuberculosis H37Rv* with an additional C-terminal thirteen residue purification tag from vector pET28b) expressed and purified as described here [49] without cleaving the additional thirteen residue purification tag. The purpose of keeping terminal tags in both the cases was to induce the spectral complexity. The final $^{13}$C/$^{15}$N labeled samples of both the proteins (1.0 mM in concentration) were prepared in 50 mM Sodium phosphate buffer (pH 6.0) containing 150 mM NaCl and 90% $H_2O$/10% $D_2O$. All the experiments have been performed on a Bruker Avance III 800 MHz NMR spectrometer equipped with a Cryoprobe (acquisition parameters and measurement times for the different experiments are provided in Table S1 of the Supporting Information). Along each indirect dimension, frequency selection has been achieved using standard States-TPPI method [50] where quadrature detection has been performed for $^{15}$N signal. Since all the spectra are acquired in a RD manner [37,38], the raw data does not require any pre-processing and are processed using routine method used for processing conventional 3D FT-NMR spectra. All the NMR data was processed using Topspin 2.1 (Bruker software: http://www.bruker.com/) and analyzed using CARA [51].

## 3. Results and discussion

### 3.1. Pulse sequence and magnetization transfer in (5,3)D-h(<u>NCO</u>)-(<u>CAN</u>)H experiment

The pulse sequence for (5,3)D-h(<u>NCO</u>)-(<u>CAN</u>)H experiment has been shown in Electronic Supplementary Material (ESM, Fig. S1). It has been derived from the previously described HN(C)N [14] pulse sequence by tweaking both the indirect evolutions involving $^{15}$N nuclei (i.e. $t_1$ and $t_2$) according to reduced dimensionality (RD) NMR approach [37,38]. Fig. 1 traces the magnetization transfer pathway along with the respective frequency labeling schemes in this new pulse sequence. As explicitly depicted in Fig. 1, the $t_1$ evolution involves joint sampling of backbone $^{15}N_i$ and $^{13}C'_{i-1}$ chemical shifts, whereas $t_2$ evolution involves joint sampling of backbone $^{15}N_{i/i-1}$ and $^{13}C^{\alpha}_{i-1}$ chemical shifts. For each indirect dimension, $^{15}$N chemical shifts are detected in quadrature whereas backbone carbon ($^{13}$C′ or $^{13}C^{\alpha}$) chemical shifts modulate the transfer amplitude. Depending upon the carbon ($^{13}$C′ or $^{13}C^{\alpha}$) chemical shift involved in $^{15}$N frequency modulation, the $F_1$ and $F_2$ dimensions

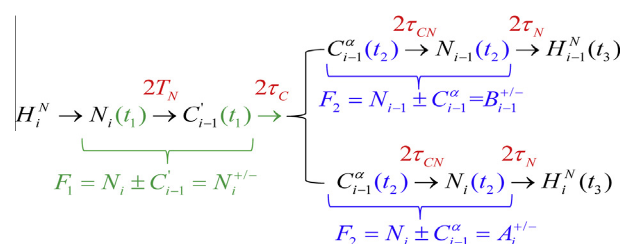


**Fig. 1.** Schematic illustrations of the magnetization transfer pathway along with the frequency labeling of the appropriate nuclei employed in reduced dimensionality tailored HN(C)N experiment –(5,3)D-h(<u>NCO</u>)-(<u>CAN</u>)H. The delays -$2T_N$, $2\tau_C$, $2\tau_{CN}$, and $2\tau_N$ - during which the transfers indicated by the arrows take place in the pulse sequence– were set to 28, 9, 25 and 27 ms, respectively.

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