Incorporating $^1$H chemical shift determination into $^{13}$C-direct detected spectroscopy of intrinsically disordered proteins in solution

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Abstract

Exclusively heteronuclear $^{13}$C-detected NMR spectroscopy of proteins in solution has seen resurgence in the past several years. For disordered or unfolded proteins, which tend to have poor $^1$H-amide chemical shift dispersion, these experiments offer enhanced resolution and the possibility of complete heteronuclear resonance assignment at the cost of leaving the $^1$H resonances unassigned. Here we report two novel $^{13}$C-detected NMR experiments which incorporate a $^1$H chemical shift evolution period followed by $^{13}$C-TOCSY mixing for aliphatic $^1$H resonance assignment without reliance on $^1$H detection.

1. Introduction

Recent advances in cryogenic probe technology, most notably the incorporation of cryogenically cooled carbon coils, have resulted in the re-emergence of $^{13}$C-direct detection spectroscopy as a tool for studying proteins in solution [1]. An extensive suite of so called “protonless” 2D and 3D NMR experiments now exist for complete heteronuclear ($^{13}$C and $^{15}$N only) chemical shift assignment of proteins, based primarily on experiments detected through the backbone carbonyl carbon ($^{13}$C$_{\text{O}}$) [2]. Sequential backbone connectivity is most readily determined in protonless spectroscopy by experiments which correlate the inter-residue $^{13}$C$_{\text{O}}^{-}/$ intra-residue ($^1$H$_{\text{O}}$) $^{13}$C$_{\text{C}}$, in analogy to familiar residue-hopping in $^1$H-detected triple resonance NMR experiments [3]. One key feature of these protonless spectra, preserved in the novel experiments presented here, is the incorporation of “virtual decoupling” to eliminate the effects of $^{13}$C$_{\text{O}}^{-}/$ $^{13}$C$_{\text{C}}$ scalar coupling in the direct-detect dimension [1]. This feature provides a signal enhancement great enough to make these experiments practical.

In addition to the protonless $^{13}$C-direct detection experiments, sporadic attempts have been made to combine indirectly recorded proton chemical shift dimensions with direct $^{13}$C observation. An “out-and-stay” version of the HACACO, correlating the $^1$H$_{\text{F}}$, $^{13}$C$_{\text{C}}$, and $^{13}$C$_{\text{O}}$, works reasonably well, but suffers from splitting by the $^{13}$C$_{\text{C}}$-$^{13}$C$_{\text{O}}$ scalar coupling in the direct-detect dimension [4]. A second multiple-quantum variant of the HACACO, reported along with a protocol for post-processing of the spectrum to “decouple” the direct-detect dimension, circumvents this problem and restores full resolution [5]. Most recently, 2D $^{13}$C-start $^{13}$C-detected TOCSY spectra were extended with a third $^1$H dimension, yielding side chain $^1$H resonance information in a $^{13}$C-aliphatic detection format [6].

$^{13}$C detected spectra of paramagnetic, very large, or intrinsically disordered proteins (IDPs) offer serious advantages in resolution and, potentially, sensitivity over more conventional $^1$H-amide detected spectra because the unfavorable relaxation properties of the $^1$H nucleus can be partially or entirely avoided. NMR spectroscopy is a uniquely powerful tool for studying the dynamic structural ensembles of IDPs, which have emerged as a critical class of functionally diverse biomolecules [7,8]. Even so, study of IDPs by NMR has been limited by the extremely poor $^1$H-amide chemical shift dispersion typically observed. Direct detection through the $^{13}$C, which tends to retain greater resonance dispersion in IDPs, led to the observation that protonless NMR is a valuable tool for the study of these molecules [9]. For IDPs, unlike the paramagnetic proteins for which modern protonless experiments were originally developed, there is no reason to avoid (1) the gain in sensitivity arising from utilizing the proton equilibrium polarization at the beginning of an experiment or (2) the recording of $^1$H chemical shifts in an indirect evolution period for further use. Underscoring the timely nature of this point, the use of proton polarization to enhance sensitivity of protonless spectroscopy has just been reported [10]. As yet, no effort to incorporate $^1$H chemical shift evolution into modern $^{13}$C detected experiments, yielding carbon-detected “triple resonance” spectroscopy, has been reported. Proton assignment in amide detected spectroscopy is often achieved through a combination of the H(CC)CONH [11,12] and the $^{15}$N-TOCSY-HSQC.
Here we report two novel pulse sequences developed from the CCCO and CCCON [13] to accomplish complete aliphatic \(^1\)H resonance assignment. The utility of these experiments, which we name the H(CC)CO–IPAP and H(CC)CON–IPAP, is demonstrated with the 83 residue intrinsically disordered C-terminal acidic region of FCP1 [14].

2. Results and discussion

A variety of protonless experiments have been developed for amino acid type determination and the generation of complete heteronuclear assignments [1]. Our experiments, based on the pulse sequences shown in Fig. 1, complement these by incorporating a \(^1\)H chemical shift evolution in place of one indirect \(^{13}\)C evolution period in two of the most commonly employed \(^{13}\)C-carbonyl detected experiments. The H(CC)CO–IPAP (Fig. 1A) yields \(H^{t_0}N^{t_1}C^{t_2}C^{t_3}_i\) and \(H^aN^{t_1}C^{t_2}C^{t_3}_i\) correlations that, coupled with acquisition of the purely heteronuclear CCCO–IPAP, generate complete resonance assignment of the aliphatic side chains. More directly analogous to the heteronuclear CCCO–IPAP, generate complete resonance assignment. The utility of these experiments, which we name the H(CC)CO–IPAP and H(CC)CON–IPAP, is demonstrated with the 83 residue intrinsically disordered C-terminal acidic region of FCP1 [14].

The desire to study paramagnetic metalloenzymes motivated development of the current generation of \(^{13}\)C-direct detect experiments [15]. As such, these experiments have been kept entirely protonless in order to prevent efficient relaxation of the desired signal by proximity to the paramagnetic centers. These experiments have also seen rapid adoption for investigations of intrinsically disordered or chemically unfolded proteins [9,16]. Their utility is due to overcoming the lack of dispersion in the \(^1\)H–amide resonances, which has long been one of the fundamental spectral limitations impeding NMR investigation of IDPs. The ability to assign and work with purely heteronuclear experiments comes at the cost of losing all proton derived information. The experiments reported here restore \(^1\)H resonance evolution periods to the \(^{13}\)C detected spectra, thus retaining the advantages of \(^{13}\)C detection without loss of \(^1\)H information. 2D \(^1\)H–\(^{13}\)C planes from the H(CC)CO–IPAP and H(CC)CON–IPAP spectra collected on a 1 mM sample of the intrinsically disordered C-terminal acidic region of FCP1 are shown in Fig. 2. The extreme degeneracy of several important \(^1\)H resonances can be seen in the regions circled for the Leu \(^1\)H (solid), Ala \(^1\)H (dashed), and Pro \(^1\)H (dotted). However, the observed chemical shift dispersion of the \(^{13}\)C dimension is sufficient to allow satisfactory resolution of nearly all spin systems in the full 3D versions of the spectra.

Use of the new \(^1\)H incorporated, \(^{13}\)C detected experiments for amino acid type verification and complete \(^1\)H resonance assignment is illustrated in Fig. 3 for Leu 953, Pro 902, and Ala 901 of FCP1. Representative strips are shown from the H(CC)CO–IPAP and H(CC)CON–IPAP; as well as the \(^1\)H-amide detected H(CC)CONH and \(^1\)H-TOCSY-HSQC of the same sample. Leu 953 (Fig. 3A) was selected for illustration because it is one of the very few residues well enough resolved in the \(^1\)H,\(^{13}\)N-HSQC to allow display of reasonably clear strips from the comparison \(^1\)H detected spectra. All of the expected correlations, based on \(^1\)H detected strips, are found in the \(^{13}\)C detected spectra recorded with the pulse programs presented here. Also, the sharpened \(^{13}\)C line width, relative to the \(^1\)H-
amide line width, is clearly seen. An additional advantage of the 
$^{13}$C detected experiments is that proline correlations are present 
in the H(CC)CON–IPAP, unlike the H(CC)CONH in which they are 
absent. For example, the $^1$H resonances of Pro 902 are clearly 
assignable (Fig. 3B) from the H(CC)CON–IPAP. Most notably, pro-
line residues do not disrupt the walk along the backbone, which 
is important for proline rich IDPs, as illustrated for Ala 901 that 
correlates with the backbone $^{15}$N of Pro 902 (Fig. 3B), making proper 
placement of these residues into the primary sequence of FCP1 
trivial. These factors, coupled with the increased residual chemical 
shift dispersion previously mentioned, have allowed nearly complete 
resonance assignment of FCP1, which was not possible based on $^1$H-amide detected spectroscopy alone [17].

3. Conclusions

Recently reported protonless $^{13}$C-direct detected experiments, 
acquired on spectrometers equipped with cryogenic probes opti-
mized for $^{13}$C detection, have emerged as valuable tools for study-
ing paramagnetic or intrinsically disordered proteins. Here, we 
have extended the existing suite of protonless $^{13}$C-direct detected 
experiments through introduction of indirect $^1$H evolution periods 
to produce “triple resonance” $^{13}$C-direct detected spectra. The 
completeness of aliphatic $^1$H spin system detection by these spec-
tra is comparable to that which is observed for the handful of 
residues well resolved in $^1$H-amide detected spectra of the intrin-
sically disordered protein FCP1, as demonstrated for Leu 953. Addi-
tion of the H(CC)CO–IPAP, H(CC)CON–IPAP experiments to the 
standard set of protonless experiments allows nearly complete 
assignment of the $^1$H, $^{13}$C, and $^{15}$N resonances of intrinsically disor-
dered proteins without the need for analysis of the poorly dis-
pered $^1$H-amide detected experiments.

4. Experimental

The $^{15}$N/$^{13}$C human FCP1 sample was prepared and purified as 
previously reported [17]. The sample was 1 mM FCP1 in 20 mM so-
dium phosphate, pH 7.0, 100 mM NaCl, 0.02% (w/v) NaN$_3$, 10% (v/v) 
D$_2$O. All experiments were recorded on an 11.7 T Bruker AVANCE-3 
spectrometer operating at 500.13 MHz $^1$H frequency equipped 
with a TCI cryoprobe, allowing high sensitivity acquisition of 
$^{13}$C-direct detected spectra. All spectra were recorded at 298 K. 
Reported spectra were collected with eight scans and, after re-
combination of the in-phase and anti-phase sub-spectra yielded 
data matrices of 128$^2$/C$^2$ = 64$^2$/C$^2$ = 1024 data points for the H(CC)CON– 
IPAP and 64$^2$/C$^2$ = 128$^2$/C$^2$ = 1024 data points for the H(CC)CO–IPAP spec-
trum. Total acquisition time was approximately two days per 3D 
experiment.

The H(CC)CO–IPAP and H(CC)CON–IPAP spectra were collected 
using the pulse sequences shown in Fig. 1. In each pulse sequence, 
$^1$H magnetization evolves in a semi-constant time period prior to 
the first 90° carbon pulse. This period simultaneously incorporates 
the $T_1$ evolution period and INEPT transfer of polarization from the 
$^1$H nuclei to produce antiphase magnetization with respect to the 
directly attached $^{13}$C nuclei [12,18]. Following the first 
90° carbon pulse, the $^1$H–$^{13}$C couplings are allowed to refocus dur-
ing a short refocusing delay $D_2$. From this point forward, the pulse 
sequences bear significant resemblance to their protonless forms 
and the reader is referred to the original reports for further discus-
sion [2,13].

Narrow and wide rectangular pulses correspond to 90° and 180° 
hard pulses. Pulse widths were 10.27 and 31 l for hard $^1$H and $^{15}$N 
90° pulses, respectively. Narrow and wide black filled shapes cor-
respond to 90° (Q3; duration 384 µs) and 180° (Q5; duration 307 µs) band selective $^{13}$C pulses [19]. The grey filled shapes on 
$^{13}$C$^{13}$C$^{13}$ correspond to a 180° (Q3; duration 1 ms) band 
selective inversion pulse and an adiabatic inversion pulse (smoothed Chirp, 500 µs, sweep width 60 kHz, 20% smoothing

![Fig. 2. 2D $^1$H–$^{13}$C planes from the (A) H(CC)CO–IPAP (B) H(CC)CON–IPAP. Spectra were acquired on the intrinsically disordered C-terminus of FCP1 with an 11.7 T spectrometer equipped with a TCI cryoprobe for $^{13}$C detection. As an illustration of the resolution gained through $^{13}$C detection, the Leu $^1$H, Ala $^1$H, and Pro $^1$H resonances are enclosed in solid, dashed, and dotted lines, respectively. Three resonances featured in Fig. 3 are indicated as * = L953 $^1$H, † = A901 $^1$H, and ‡ = P902 $^1$H.](image-url)
respectively. Pulsed field gradients (PFG) are also indicated by shapes. The 1H and 15N carriers were placed at 4.7 and 124 ppm, respectively. The 13C carrier was changed at the positions indicated by vertical arrows to 13C\(_a\) = 39 ppm, 13C\(_b\) = 54 ppm, and 13C\(_0\) = 172 ppm. Sweep widths were set to 1H = 16 ppm, 13C\(_a\) = 40 ppm, 13C\(_b\) = 24 ppm, and 15N = 40 ppm. The FLOPSY-16 13C spin lock was applied with a 10 kHz field strength for 22 ms. Composite pulse decoupling during acquisition and as indicated during the pulse programs is achieved with the WALTZ-16 (1H) and GARP-4 (15N) sequences applied at 3.1 and 1.25 kHz field strength, respectively. Experiments were acquired with recycle delays of 1.0 s and acquisition times of 170 ms. Further delay values and phase cycle information are provided in the legend to Fig. 1.

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References


