Journal of Magnetic Resonance 200 (2009) 354–358

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Communication

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

Bernie O'Hare, Alan J. Benesi, Scott A. Showalter *

Department of Chemistry, The Pennsylvania State University, 104 Chemistry Building, University Park, PA 16802, USA

ARTICLE INFO

Article history:
Received 1 June 2009
Revised 3 July 2009
Available online 17 July 2009

Keywords:
^{13}C-direct detection
^{1}H chemical shift
Intrinsically disordered proteins
IPAP

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′) [2]. Sequential backbone connectivity is most readily determined in *protonless* spectroscopy by experiments which correlate the inter-residue $^{13}C_i^{\alpha}$ and intra-residue (with respect to the directly observed $^{13}C_i'$) $^{13}C_{i+1}^{\alpha}$, 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 $^{13}C^{\alpha}$–$^{13}C'$ 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 $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, and $^{13}C'$, works reasonably well, but suffers from splitting by the $^{13}C^{\alpha}$–$^{13}C'$ 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.

* Corresponding author. Fax: +1 814 865 3314.
E-mail addresses: bvo100@psu.edu (B. O'Hare), alan@chem.psu.edu (A.J. Benesi), sas76@psu.edu (S.A. Showalter).

1090-7807/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jmr.2009.07.014

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_i^{ali}–C_i^{α}–C_i' and H_i^{α}–C_i^{α}–C_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 amide detected H(CC)CONH [11,12], the H(CC)CON–IPAP (Fig. 1B) yields H_i^{ali}–N_{i+1}–C_i' and H_i^{α}–N_{i+1}–C_i' correlations. Therefore, this spectrum contributes to sequential assignment by crossing the amide bond spanning residues i and $i+1$.

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 $^1H^{\delta}$ (solid), Ala $^1H^{\beta}$ (dashed), and Pro $^1H^{\delta}$ (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 ^{15}N-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,^{15}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-

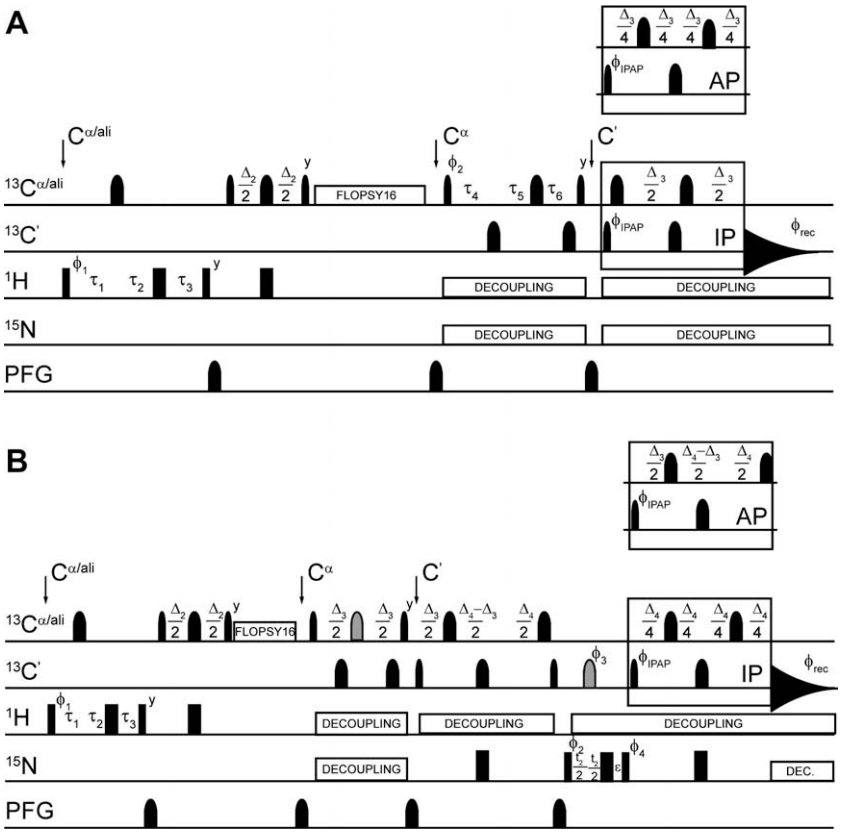

Fig. 1. Pulse sequences for (A) H(CC)CO–IPAP and (B) H(CC)CON–IPAP. The delays are $\Delta_1 = 3.4$ ms, $\Delta_2 = 2.2$ ms, $\Delta_3 = 9.0$ ms, $\Delta_4 = 25$ ms, and $\varepsilon = t_2(0)$. ^{1}H(t_1) chemical shift evolution is performed using a semi-constant time period with delays $\tau_1 = (\Delta_1 + t_1)/2$, $\tau_2 = (1 - \Delta_1/t_{1\max})t_1/2$, $\tau_3 = (1 - t_1/t_{1\max})\Delta_1/2$; as is $^{13}C^{\alpha}(t_2)$ chemical shift evolution in the H(CC)CO–IPAP with delays $\tau_4 = (\Delta_3 + t_2)/2$, $\tau_5 = (1 - \Delta_3/t_{2\max})t_2/2$, $\tau_6 = (1 - t_2/t_{2\max})\Delta_3/2$. The phase cycle is (A) $\varphi_1 = x, -x$; $\varphi_2 = x, x, -x, -x$; $\varphi_3 = 4(x), 4(-x)$, $\varphi_{IPAP}(IP) = x$, $\varphi_{IPAP}(AP) = -y$, $\varphi_{rec} = x, -x, -x, x$; (B) $\varphi_1 = x, -x$; $\varphi_2 = x, x, -x, -x$; $\varphi_3 = 4(x), 4(-x)$, $\varphi_4 = 8(x), 8(-x)$, $\varphi_{IPAP}(IP) = x$, $\varphi_{IPAP}(AP) = -y$, $\varphi_{rec} = x, -x, -x, x, x, -x, -x, x, -x, x, x, -x, -x, x, x, -x$. Quadrature detection was obtained in both experiments by States-TPPI incrementing of φ_1 and φ_2.

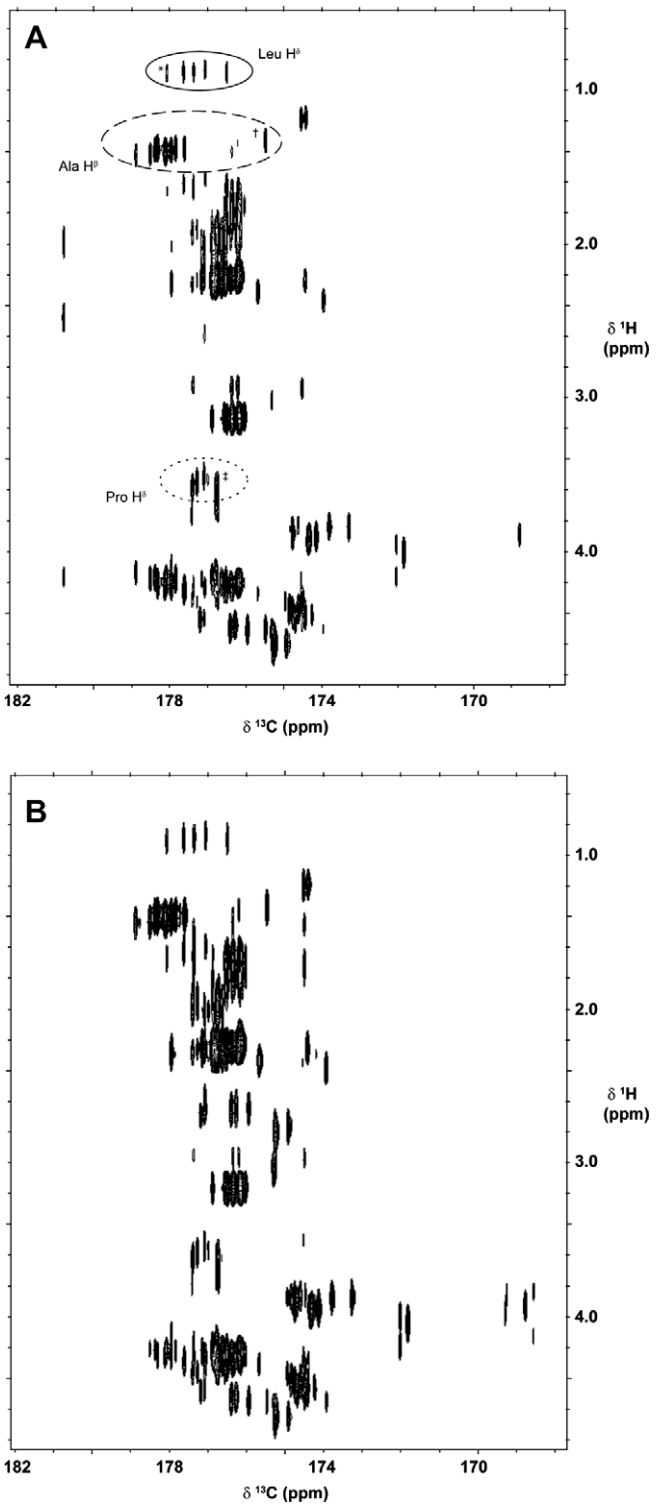

Fig. 2. 2D ^{1}H–$^{13}C'$ planes from the (A) H(CC)CO–IPAP (B) H(CC)CON–IPAP. Spectra were acquired on the intrinsically disordered C-terminus of FCP1 with an 11.7 T spectrometer equipped with a TCI cryoprobe for ^{13}C detection. As an illustration of the resolution gained through $^{13}C'$ detection, the Leu $^{1}H^{\delta}$, Ala $^{1}H^{\beta}$, and Pro $^{1}H^{\delta}$ resonances are enclosed in solid, dashed, and dotted lines, respectively. Three resonances featured in Fig. 3 are indicated as * = L953 $^{1}H^{\delta}$, † = A901 $^{1}H^{\beta}$, and ‡ = P902 $^{1}H^{\delta}$.

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, proline 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 optimized for ^{13}C detection, have emerged as valuable tools for studying 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 spectra is comparable to that which is observed for the handful of residues well resolved in ^{1}H-amide detected spectra of the intrinsically disordered protein FCP1, as demonstrated for Leu 953. Addition 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 disordered proteins without the need for analysis of the poorly dispersed ^{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 sodium phosphate, pH 7.0, 100 mM NaCl, 0.02% (w/v) NaN_3, 10% (v/v) D_2O. 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 recombination of the in-phase and anti-phase sub-spectra yielded data matrices of $128 \times 64 \times 1024$ data points for the H(CC)CON–IPAP and $64 \times 128 \times 1024$ data points for the H(CC)CO–IPAP spectrum. 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 $^{13}C^{ali/\beta}$ and $^{13}C^{\alpha}$ spins [12,18]. Following the first 90° carbon pulse, the ^{1}H–^{13}C couplings are allowed to refocus during a short refocusing delay Δ_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 discussion [2,13].

Narrow and wide rectangular pulses correspond to 90° and 180° hard pulses. Pulse widths were 10.27 and 31 μs for hard ^{1}H and ^{15}N 90° pulses, respectively. Narrow and wide black filled shapes correspond to 90° (Q3; duration 384 μs) and 180° (Q5; duration 307 μs) band selective ^{13}C pulses [19]. The grey filled shapes on $^{13}C^{\alpha/ali}$ and $^{13}C'$ 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

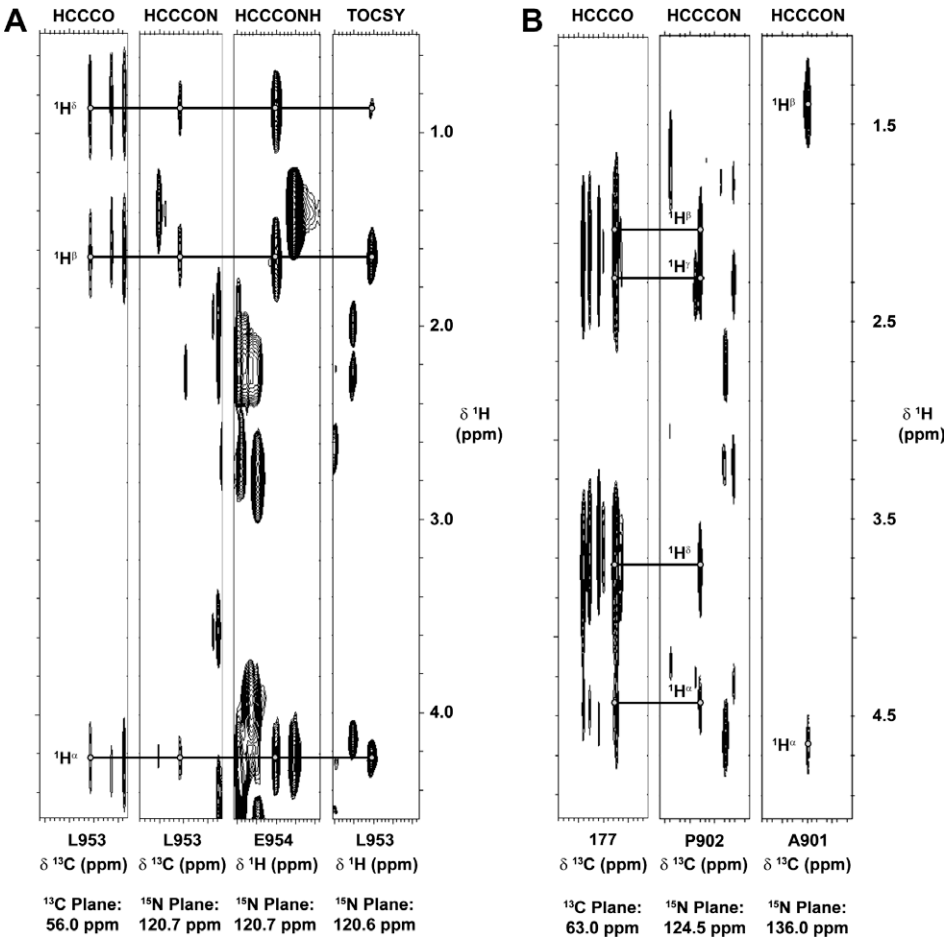

Fig. 3. Representative strips for (A) Leu 953 from the ^{13}C–^{1}H or ^{1}H–^{1}H planes of (from left to right) the H(CC)CO–IPAP, H(CC)CON–IPAP, H(CC)CONH, and ^{15}N-TOCSY-HSQC and (B) Pro 902 from the ^{13}C–^{1}H planes of the H(CC)CO–IPAP and H(CC)CON–IPAP. Resonances corresponding to Leu 953 are highlighted in (A), showing that the completeness of assignment attainable by the ^{13}C-direct detected experiments is comparable to that of the ^{1}H detected methods. A unique advantage to these spectra is the ease of determining ^{1}H resonances for Pro residues and for the residues which precede Pro, as illustrated in (B) for Pro 902 and Ala 901.

[20]), respectively. Pulsed field gradients (PFG) are also indicated by shapes. The ^{1}H and ^{15}N carriers were placed at 4.7 and 124 ppm, respectively. The ^{13}C carrier was changed at the positions indicated by vertical arrows to $^{13}C^{\alpha/ali(\beta)}$ = 39 ppm, $^{13}C^{\alpha}$ = 54 ppm, and $^{13}C'$ = 172 ppm. Sweep widths were set to ^{1}H = 16 ppm, $^{13}C^{\alpha}$ = 40 ppm, $^{13}C'$ = 24 ppm, and ^{15}N = 40 ppm. The FLOPSY-16 ^{13}C 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 (^{1}H) and GARP-4 (^{15}N) 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.

Acknowledgment

This work was supported by start-up funds from the Pennsylvania State University to SAS.

References

[1] W. Bermel, I. Bertini, I.C. Felli, M. Piccioli, R. Pierattelli, ^{13}C-detected protonless NMR spectroscopy of proteins in solution, Prog. Nucl. Magn. Reson. Spectrosc. 48 (2006) 25–45.
[2] W. Bermel, I. Bertini, L. Duma, I.C. Felli, L. Emsley, R. Pierattelli, P.R. Vasos, Complete assignment of heteronuclear protein resonances by protonless NMR spectroscopy, Angew. Chem. Int. Ed. 44 (2005) 3089–3092.
[3] W. Bermel, I. Bertini, I.C. Felli, R. Pierattelli, P.R. Vasos, A selective experiment for the sequential protein backbone assignment from 3D heteronuclear spectra, J. Magn. Reson. 172 (2005) 324–328.
[4] Z. Serber, C. Richter, D. Moskau, J.M. Bohlen, T. Gerfin, D. Marek, M. Haberli, L. Baselgia, F. Laukien, A.S. Stern, J.C. Hoch, V. Dotsch, New carbon-detected protein NMR experiments using CryoProbes, J. Am. Chem. Soc. 122 (2000) 3554–3555.
[5] K. Pervushin, A. Eletsky, A new strategy for backbone resonance assignment in large proteins using a MQ-HACACO experiment, J. Biomol. NMR 25 (2003) 147–152.
[6] K.F. Hu, B. Vogeli, K. Pervushin, Side-chain H and C resonance assignment in protonated/partially deuterated proteins using an improved 3D(13)C-detected HCC-TOCSY, J. Magn. Reson. 174 (2005) 200–208.
[7] V.N. Uversky, C.J. Oldfield, A.K. Dunker, Showing your ID: intrinsic disorder as an ID for recognition, regulation and cell signaling, J. Mol. Recognit. 18 (2005) 343–384.
[8] H.J. Dyson, P.E. Wright, Intrinsically unstructured proteins and their functions, Nat. Rev. Mol. Cell Biol. 6 (2005) 197–208.
[9] V. Csizmok, I.C. Felli, P. Tompa, L. Banci, I. Bertini, Structural and dynamic characterization of intrinsically disordered human securin by NMR spectroscopy, J. Am. Chem. Soc. 130 (2008) 16873–16879.
[10] W. Bermel, I. Bertini, V. Csizmok, I.C. Felli, R. Pierattelli, P. Tompa, H-start for exclusively heteronuclear NMR spectroscopy: the case of intrinsically disordered proteins, J. Magn. Reson. 198 (2009) 275–281.
[11] S. Grzesiek, J. Anglister, A. Bax, Correlation of backbone amide and aliphatic side-chain resonances in C-13/N-15-enriched proteins by isotropic mixing of C-13 magnetization, J. Magn. Reson. B 101 (1993) 114–119.
[12] T.M. Logan, E.T. Olejniczak, R.X. Xu, S.W. Fesik, A general-method for assigning nmr-spectra of denatured proteins using 3d Hc(Co)Nh-Tocsy triple resonance experiments, J. Biomol. NMR 3 (1993) 225–231.
[13] W. Bermel, I. Bertini, I.C. Felli, R. Kummerle, R. Pierattelli, Novel C-13 direct detection experiments, including extension to the third dimension, to perform the complete assignment of proteins, J. Magn. Reson. 178 (2006) 56–64.

[14] J. Archambault, G.H. Pan, G.K. Dahmus, M. Cartier, N. Marshall, S. Zhang, M.E. Dahmus, J. Greenblatt, FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase IIO, J. Biol. Chem. 273 (1998) 27593–27601.
[15] W. Bermel, I. Bertini, I.C. Felli, R. Kummerle, R. Pierattelli, C-13 direct detection experiments on the paramagnetic oxidized monomeric copper, zinc superoxide dismutase, J. Am. Chem. Soc. 125 (2003) 16423–16429.
[16] W. Bermel, I. Bertini, I.C. Felli, Y.M. Lee, C. Luchinat, R. Pierattelli, Protonless NMR experiments for sequence-specific assignment of backbone nuclei in unfolded proteins, J. Am. Chem. Soc. 128 (2006) 3918–3919.
[17] S.A. Showalter, NMR assignment of the intrinsically disordered C-terminal region of *Homo sapiens* FCP1 in the unbound state, Biomol. NMR Assign. (2009), in press.
[18] S. Grzesiek, A. Bax, Amino-acid type determination in the sequential assignment procedure of uniformly C-13/N-15-enriched proteins, J. Biomol. NMR 3 (1993) 185–204.
[19] L. Emsley, G. Bodenhausen, Optimization of shaped selective pulses for NMR using a quaternion description of their overall propagators, J. Magn. Reson. 97 (1992) 135–148.
[20] J.M. Bohlen, G. Bodenhausen, Experimental aspects of CHIRP NMR-spectroscopy, J. Magn. Reson. A 102 (1993) 293–301.