Communication

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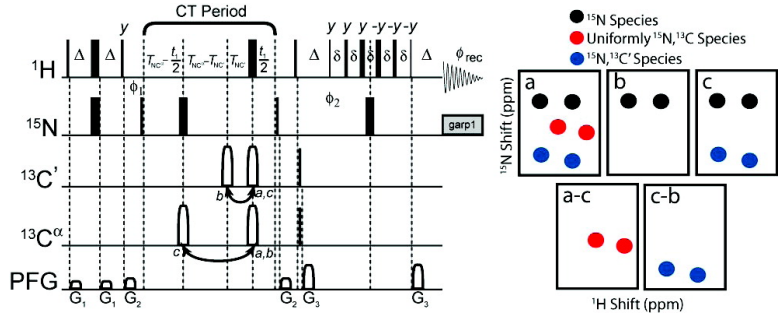

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Simultaneous Detection and Deconvolution of Congested NMR Spectra Containing Three Isotopically Labeled Species

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Analyses of protein fingerprint NMR spectra (e.g., 2D $^{1}H-^{15}N$ correlated spectra) yield atomic portraits of the conformational and dynamic changes that accompany binding events, such as ligand−protein, protein−protein, protein−DNA, and protein−RNA interactions.[1−6] The introduction of NMR techniques such as TROSY (transverse relaxation optimized spectroscopy)[7] and CRINEPT (cross-relaxation enhanced polarization transfer)[8] has extended these approaches to biomolecules as large as 1 MDa.[9] A common strategy in such experiments is to label only one binding partner with ^{15}N so that its signal is detected while that from the unlabeled partner remains "NMR silent". Then, a complex is prepared with another partner labeled so that its signals can be observed. However, it would be ideal to detect all species using one experiment and one sample preparation, so as to avoid the cost of preparing a second sample and to eliminate inconsistencies between sample preparations. Recently, Bermel et al. proposed such an approach for a two-component complex: one partner is ^{15}N-labeled and the other is double-labeled with ^{13}C and ^{15}N, and a modified HNCO experiment is used to simultaneously detect and obtain subspectra of the two species.[10] With a ternary complex (e.g., enzyme−substrate−inhibitor), however, this approach would leave one species NMR silent.

In this communication, we present an approach for the simultaneous detection and deconvolution of $^{1}H/^{15}N$ correlations in a ternary mixture of isotopically labeled proteins. This approach will support the molecular analysis of the myriad biological systems that involve multiple species, such as disruptions of protein−protein complexes by peptides; competitive, cooperative, and allosteric binding; and protein assemblies.

To separate the spectrum from each species in a ternary mixture, we used species-selective isotopic labeling in concert with a new pulse sequence based on spin−echo filtering,[11,12] dual carbon label selective (DCLS) $^{1}H/^{15}N$ HSQC (Figure 1A). The labeling scheme for the ternary mixture is as follows: (species A) uniformly ^{15}N labeled, (species B) uniformly ^{13}C and ^{15}N labeled, and (species C) ^{15}N,$^{13}C'$ labeled. The DCLS-$^{1}H/^{15}N$-HSQC enables the filtering of each species from detection by exploiting differences in ^{1}J scalar coupling of ^{15}N nuclei during a constant time (CT) period (Figure 1A). A series of three interleaved experiments are acquired in parallel. First, a reference data set is collected with simultaneous 180° selective pulses on $^{13}C'$ and $^{13}C^{\alpha}$ concurrent with a 180° square pulse on $^{1}H^{N}$ during the ^{15}N CT evolution (all pulses at positions "***a***"). This scheme results in suppression of frequency modulation arising from $^{1}J_{N-C'}$, $^{1}J_{N-C\alpha}$, and $^{2}J_{N-C\alpha}$ scalar couplings, and ^{15}N signals are thus decoupled from any bonded ^{13}C nucleus. In this case, all $^{1}H/^{15}N$ correlations are observed as in a conventional decoupled $^{1}H/^{15}N$ HSQC spectrum. Next, the first suppression data set is acquired by using a pulse sequence in which

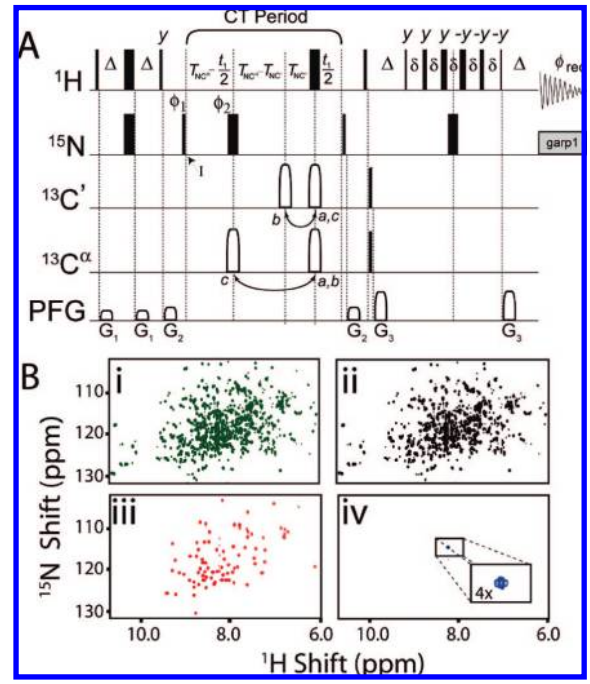

Figure 1. (A) The DCLS-HSQC pulse sequence. Optimal suppression of both $^{13}C^{\alpha}$ and $^{13}C'$ attached NH groups use $T_{NC\alpha}$ and $T_{NC'}$ delays of 24.5 and 16.4 ms, respectively (CT period of 49 ms). Further pulse sequence details in Supporting Information. (B) Representative spectra obtained at 37 °C on a Varian VNMRS 800 MHz spectrometer equipped with a cryogenic probe: i. full ternary mixture consisting of 1 mm [U−^{2}H, U−^{15}N]MBP, 0.8 mM [U−^{13}C, U−^{15}N]-ubiquitin, and 0.8 mM [$^{13}C'$]-Ala4, [^{15}N]-Ser5 Kemptide; ii. MBP subspectrum; iii. ubiquitin subspectrum; iv. Kemptide subspectrum.

the 180° selective pulse on $^{13}C'$ is moved from position **a** to position ***b***. At the beginning of the CT delay (point I), $^{1}H-^{15}N$ magnetization is present as $2H_zN_{x/y}$ (depending on the phase of the preceding ^{15}N 90° pulse). During the CT delay, frequency modulation of ^{15}N magnetization due to active $^{1}J_{NC'}$ coupling occurs, and any ^{15}N resonance attached to $^{13}C'$ evolves into antiphase magnetization ($4H_zN_{x/y}C'_z$). The residual $2H_zN_{x/y}$ term is modulated by $\cos(2\pi^{1}J_{NC'}T_{NC'})$ and can be completely suppressed by setting $T_{NC'}$ to $1/4^{1}J_{NC'}$, that is, 16.4 ms.[11] The antiphase terms are either dephased by gradient G_2 (for the $4H_zN_xC'_z$ term) or converted to unobservable multiple quantum coherence during the reverse INEPT transfer (for the $4H_zN_yC'_z$ term). This suppression scheme provides selective observation of signals from species A, whose NH groups are not attached to a $^{13}C'$. Finally, a second suppression experiment is acquired by moving the 180° selective pulse on $^{13}C^{\alpha}$ from position ***a*** to position ***c***. Under active $^{1}J_{NC\alpha}$ and $^{2}J_{NC\alpha}$ coupling, $2H_zN_{x/y}$ magnetization arising from ^{15}N,$^{13}C^{\alpha}$-labeled species evolves into various antiphase magnetization terms

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($4H_zN_{x/y}C^{\alpha(i)}{}_z$, $4H_zN_{x/y}C^{\alpha(i-1)}{}_z$, $8H_zN_{x/y}C^{\alpha(i)}{}_zC^{\alpha(i-1)}{}_z$) that are either dephased by the G_2 gradient or converted into unobservable multiple quantum coherences during the reverse INEPT transfer. Any observable signal arising from the residual $2H_zN_{x/y}$ term is modulated by the function $\cos(2\pi^1J_{NC\alpha}T_{NC\alpha})\cdot\cos(2\pi^2J_{NC\alpha}T_{NC\alpha})$. Since $^1J_{NC\alpha}$ is larger than $^2J_{NC\alpha}$, the $2H_zN_{x/y}$ term can be suppressed by setting $T_{NC\alpha}$ to $1/4^1J_{NC\alpha}$. However, whereas $^1J_{NC'}$ varies little with conformation, $^1J_{NC\alpha}$ and $^2J_{NC\alpha}$ show small dependence on protein backbone conformation.[13,14] Wirmer and Schwalbe[14] reported values of 10.8 ± 0.8 Hz for $^1J_{NC\alpha}$ and 7.7 ± 1.3 Hz for $^2J_{NC\alpha}$ for ubiquitin, with a positive correlation between the two coupling constants (i.e., larger values of $^1J_{NC\alpha}$ correspond to larger values of $^2J_{NC\alpha}$). On the basis of this, the suppression of signals for a $^{15}N,^{13}C^{\alpha}$-labeled species is best achieved by setting $T_{NC\alpha}$ to 24.5 ms. This completely suppresses signals from small values of $^1J_{NC\alpha}$, while signals with large values of $^1J_{NC\alpha}$ are inverted with residual intensities less than 4% (see Supporting Information). The only observable correlations will be those without $^{13}C^{\alpha}$ labeling (species A and C).

As a proof of concept, we applied this labeling scheme and pulse sequence to a ternary mixture of noninteracting proteins: maltose binding protein (MBP, 370 residues), ubiquitin (76 residues), and Kemptide (7 residues). Recombinant proteins were expressed in *Escherichia coli* BL21(DE3) to obtain MBP uniformly labeled with ^2H and ^{15}N and ubiquitin uniformly labeled with ^{13}C and ^{15}N. Kemptide was prepared by solid-phase peptide synthesis using standard Fmoc chemistry. Labeling was introduced by incorporating $^{13}C'$-labeled Fmoc-alanine (at residue 4) and ^{15}N-labeled Fmoc-serine(*t*Bu) (at residue 5). The NMR sample contained 1 mM MBP, 5 mM Kemptide, and 0.8 mM ubiquitin.

Resulting spectra acquired with the DCLS-HSQC pulse sequence are in Figure 1B. All $^1H-^{15}N$ correlations were observed in the ^{13}C-decoupled DCLS-HSQC reference spectrum (Figure 1Bi). As expected, the first suppression experiment resulted in the filtering of both $^{13}C'$-labeled species from detection, and only correlations attributed to MBP were observed (Figure 1Bii). The second suppression experiment selectively filtered ubiquitin from detection. Separate subspectra for ubiquitin and Kemptide were obtained by linear combinations of the data sets: subtraction of the reference spectrum from the second suppression spectrum ($^{15}N-^{13}C^{\alpha}$ filtered detection) provided a subspectrum for ubiquitin (Figure 1Biii), and subtraction of the second suppression spectrum ($^{15}N-^{13}C^{\alpha}$ filtered) from the first suppression spectrum provided a subspectrum for Kemptide (Figure 1Biv). In this manner, subspectra were obtained from a single sample for each individual component of the ternary mixture, and all of the resonances could be resolved for chemical shift analysis.

Introduction of selectively labeled $^{13}C'$ can be accomplished in recombinant proteins by using ^{15}N- and $^{13}C'$-labeled amino acids or, in a more cost efficient manner, by using 1-^{13}C pyruvate and ^{13}C-labeled $NaHCO_3$ as the sole carbon sources.[15] Alternatively, selective $^{13}C^{\alpha}$ labeling can be achieved by using 2-^{13}C glucose as the sole carbon source.[16] In either case, a boost in sensitivity can be obtained using perdeuteration.[17,18]

Our method offers advantages over the recently proposed HNCO-based approach.[10] First, it enables the detection and deconvolution of spectra from three different species simultaneously. Second, the pulse sequence introduced here does not rely on complete refocusing of $^{13}C'-^{15}N$ coupled nuclei for detection; thus the CT period in which ^{15}N magnetization is transverse is shorter, alleviating losses due to T_2 relaxation (49 vs 66 ms). If the HNCO-based approach were adapted to observe $^{15}N-^{13}C^{\alpha}$ species, the CT period would be ~100 ms. In addition, the $^{13}C'$-filtered subspectrum for the ^{15}N-labeled species (A) has a S/N ratio that is $\sqrt{2}$ higher than the corresponding HNCO-based spectrum. Due to spectral editing, however, the subspectra for the two ^{13}C-labeled species (B and C) have S/N ratios comparable to the HNCO-based approach for $^{15}N-^{13}C'$-labeled species.[10] Therefore, the largest protein in the complex under investigation should only be ^{15}N labeled. Finally, additional labeling patterns should allow the possibility to resolve separate spectra from other species. A variety of selective labeling technologies, including asymmetric methyl labeling (Ile vs Val plus Leu),[19] segmental labeling,[20] or cell-free labeling,[21] could be combined with the DCLS-HSQC scheme to support NMR investigations of assemblies containing five or more species.

In summary, the highly sensitive pulse sequence and labeling scheme presented here supports the simultaneous detection and deconvolution of spectra from individual members of a ternary mixture of proteins. Many important biological pathways involve dynamic interactions among members of multicomponent protein assemblies, and this approach offers a powerful way to monitor such processes.

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Supporting Information Available: Plot of the relative peak intensities as a function of $^1J_{NC\alpha}$ and $^2J_{NC\alpha}$ for the optimization of the constant time period, and details of the pulse sequence. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Betz, M.; Saxena, K.; Schwalbe, H. *Curr. Opin. Chem. Biol.* **2006**, *10*, 219–225.
(2) Kay, L. E. *J. Magn. Reson.* **2005**, *173*, 193–207.
(3) Clarkson, J.; Campbell, I. D. *Biochem. Soc. Trans.* **2003**, *31*, 1006–1009.
(4) Sitkoff, D.; Case, D. A. *J. Am. Chem. Soc.* **1997**, *119*, 12262–12273.
(5) Revington, M.; Holder, T. M.; Zuiderweg, E. R. *J. Biol. Chem.* **2004**, *279*, 33958–33967.
(6) Matsuo, H.; Walters, K. J.; Teruya, K.; Tanaka, T.; Gassner, G. T.; Lippard, S. J.; Kyogoku, Y.; Wagner, G. *J. Am. Chem. Soc.* **1999**, *121*, 9903–9904.
(7) Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366–12371.
(8) Riek, R.; Wider, G.; Pervushin, K.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4918–4923.
(9) Sprangers, R.; Kay, L. E. *Nature* **2007**, *445*, 618–622.
(10) Golovanov, A. P.; Blankley, R. T.; Avis, J. M.; Bermel, W. *J. Am. Chem. Soc.* **2007**, *129*, 6528–6535.
(11) Vuister, G. W.; Wang, A. C.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 5334–5335.
(12) Tonelli, M.; Masterson, L. R.; Hallenga, K.; Veglia, G.; Markely, J. L. *J. Biomol. NMR* **2007**, *39*, 177–185.
(13) Delaglio, F.; Torchia, D. A.; Bax, A. *J. Biomol. NMR* **1991**, *1*, 439–446.
(14) Wirmer, J.; Schwalbe, H. *J. Biomol. NMR* **2002**, *23*, 47–55.
(15) Hansen, D. F.; Vallurupalli, P.; Lundstrom, P.; Neudecker, P.; Kay, L. E. *J. Am. Chem. Soc.* **2008**, *130*, 2667–2675.
(16) Lundstrom, P.; Teilum, K.; Carstensen, T.; Bezsonova, I.; Wiesner, S.; Hansen, D. F.; Religa, T. L.; Akke, M.; Kay, L. E. *J. Biomol. NMR* **2007**, *38*, 199–212.
(17) Fiaux, J.; Bertelsen, E. B.; Horwich, A. L.; Wuthrich, K. D.; Fierke, C. A. *J. Biomol. NMR* **1995**, *5*, 339–344.
(18) Pitto Venters, R. A.; Huang, C. C.; Farmer, B. T., II.; Trolard, R.; Spicer, D.; Fierke, C. A. *J. Biomol. NMR* **1995**, *5*, 339–44.
(19) Traaseth, N. J.; Verardi, R.; Veglia, G. *J. Am. Chem. Soc.* **2008**, *130*, 2400–2401.
(20) Xu, R.; Ayers, B.; Cowburn, D.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 388–393.
(21) Yabuki, T.; Kigawa, T.; Dohmae, N.; TakioK.; Terada, T.; Ito, Y.; Laue, E. D.; Cooper, J. A.; Kainosho, M.; Yokoyama, S. *J. Biomol. NMR* **1998**, *11*, 295–306.

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