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Speeding Up Three-Dimensional Protein NMR Experiments to a Few Minutes

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Multidimensional (nD) NMR provides the required high spectral resolution to resolve individual nuclear sites in a macromolecule, a prerequisite for the study of molecular structure, dynamics, and kinetics. An important drawback of nD NMR is that it requires long measurement times associated with the collection of hundreds or thousands of scans (repetitions of the basic pulse scheme). Acquisition times for three-dimensional (3D) NMR spectra typically range from hours to days. As a large number of different spectra need to be recorded during the NMR investigation of a macromolecule, such as a protein, this imposes a high time stability on the protein samples. It is also a major limitation for real-time NMR studies of kinetic molecular processes such as hydrogen−deuterium exchange, conformational changes occurring during biochemical reactions, or protein folding. Therefore, there is an increasing interest in new acquisition schemes that speed up multidimensional NMR data collection. A first class of methods allows reducing the number of scans. Examples are reduced dimensionality or projection NMR, Hadamard-type or spatially encoded frequency labeling, and nonlinear time domain sampling, combined with appropriate data processing algorithms.[1] An alternative way of reducing acquisition times is to shorten the delay between consecutive scans to achieve higher repetition rates. Longitudinal ^{1}H relaxation optimization techniques ensure high sensitivity for short scan times.[2] Interestingly, the two types of approaches can be easily combined to new hybrid techniques to further reduce experimental times.[3]

Recently, we have introduced SOFAST-HMQC,[4] a pulse scheme that allows recording of two-dimensional (2D) ^{1}H−^{15}N correlation spectra of proteins within a few seconds. Here we extend this approach of increasing the signal-to-noise (S/N) ratio for high repetition rates to 3D H−N−C correlation experiments. We demonstrate that the sensitivity provided by these experiments is sufficient to record 3D H−N−CO and 3D H−N−CA spectra of ^{13}C/^{15}N-labeled proteins within a few minutes of data collection. The new Band-selective Excitation Short-Transient (BEST) HNCO/CA pulse sequences are shown in Figure 1. The coherence transfer pathway is identical to standard sensitivity-enhanced HNCO/CA. Selective ^{1}H manipulation ensures minimal perturbation of the proton spins resonating outside the excited spectral window. Dipolar interactions between the detected amide and the unperturbed aliphatic protons then ensure efficient spin-lattice (T_1) relaxation between consecutive scans, yielding an increased sensitivity for high repetition rates.[2−4] The shaped ^{1}H pulses labeled (1) to (3) in Figure 1 are centered on the amide proton spectral region (∼6 to 10 ppm). The 180° square pulses (4), required for ^{1}H decoupling, are applied as broadband ^{1}H inversion (BIP) pulses to reduce signal loss due to B_1-field inhomogeneities.[5a] In this way, more than 60% of the aliphatic ^{1}H spin polarization is recovered at the end of the sequence in the fast-pulsing regime (see Figure S2). The sensitivity of BEST-HNCO/CA is plotted in Figure 2 as a function of the recycle delay and compared to results obtained for a standard pulse scheme using two different ^{13}C/^{15}N-labeled protein samples: ubiquitin and SiR-FP18.[6] Signal gains of up to a factor of 2.8 are observed for both

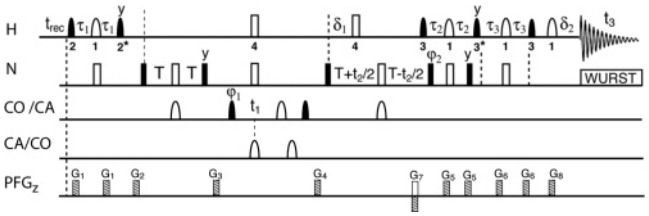

Figure 1. BEST-HNCO/CA pulse sequences to record HNCO or HNCA correlation spectra of proteins. Filled and open pulse symbols indicate 90° and 180° rf pulses. All selective ^{1}H pulses are centered at ∼8.0 ppm, covering a bandwidth of 4.0 ppm, with the following shapes: (1) REBURP,[5b] (2) PC9,[5c] and (3) EBURP-2.[5b] A star indicates a flip back pulse obtained by time and phase inversion. The particular pulse shapes have been chosen to minimize signal loss because of transverse spin relaxation and B_1-field inhomogeneities. Open squares on ^{1}H indicate BIP-720−50−20 pulses.[5a] CO pulses have the shape of the center lobe of a sin *x*/*x* function, whereas CA pulses are applied with a rectangular shape and zero excitation at the CO frequency. The relative durations of G_7 and G_8 are given by the gyromagnetic ratios $G_7/G_8 = \gamma_H/\gamma_N$. Quadrature detection in t_1 and t_2 is obtained by time-proportional phase incrementation φ_1 = x,y and φ_2 = x,−x, respectively. In addition, the sign of G_7 is inverted for experiments with $\varphi_2 = -$x. Further experimental details and delay settings at 600 MHz are given in the Supporting Information.

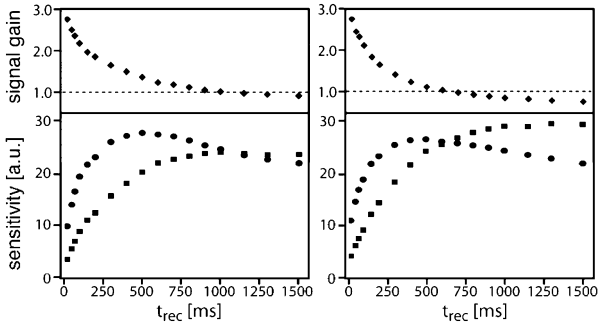

Figure 2. Signal-to-noise ratio per unit time (sensitivity) measured using different recycle delays, t_{rec}, for BEST-HNCO/CA (circles), and standard wfb-se-HNCO/CA (squares), as implemented in the Varian BioPack. The intensity ratios (BEST over standard) are plotted on top. 2D ^{1}H−^{15}N data sets were recorded at 600 MHz for (left curves) ubiquitin (8.6 kDa, pH 6.2, 25 °C) and (right curves) SiR-FP18 (18 kDa, pH 7.5, 30 °C). Examples of spectra are shown in Figure S3 of the Supporting Information. Each point corresponds to the sum of all amide cross-peak intensities.

proteins when using the BEST sequences with high repetition rates. As deuteration is now widely used when studying larger proteins, we have also evaluated the performance of BEST-HNCO/CA for a deuterated (∼75%) sample of SiR-FP18. Despite the small number of remaining protons, a significant sensitivity gain is observed (see Figure S4) that encourages the use of BEST-HNCO/CA when studying partially deuterated protein samples.

To demonstrate that the sensitivity provided by the BEST sequences is sufficient to record 3D HNCO and HNCA spectra in a few minutes of acquisition time on standard high-field NMR spectrometers, we have performed experiments on small- to medium-sized ^{13}C/^{15}N-labeled proteins at millimolar concentration.

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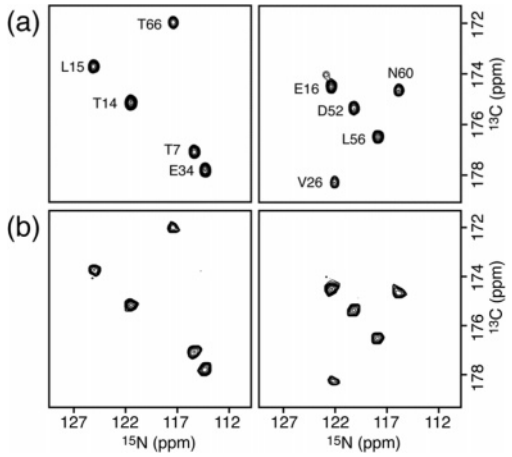

Figure 3. N−CO planes extracted from (a) a 3D BEST-HNCO spectrum recorded in 10 min and (b) a BEST-HNCO spectrum reconstructed using a minimal value algorithm[7] from a set of six 2D projections recorded in an overall time of only 2 min. For spectrum (a), 30 (^{13}C) × 27 (^{15}N) complex points were acquired with t_{rec} = 50 ms. 2D projections were obtained by setting $\Delta t_1 = \cos(\alpha)/SW_{tilt}$ and $\Delta t_2 = \sin(\alpha)/SW_{tilt}$, with α the projection angle and SW_{tilt} the spectral width in the projected dimension. All data were recorded at 600 MHz, 25 °C on the 1.9 mM ubiquitin sample.

Figure 3a shows N−CO planes extracted from a 3D H−N−CO correlation spectrum recorded for ^{13}C/^{15}N ubiquitin on a 600 MHz spectrometer equipped with a room-temperature probe. The data set was acquired with 1 scan per (t_1,t_2) increment in an experimental time of only 10 min. Note that the BEST sequences yield good water suppression within a single scan. To obtain spectra of comparable S/N ratio using a standard sequence, either a ∼5-times longer t_{rec} or a ∼7-times longer acquisition time using the same t_{rec} would have been required (see Figure 2a). All expected cross-peaks were observed in this BEST-HNCO spectrum, with an average S/N ratio of 50:1. In addition, we have recorded a set of six 2D projections for angles α = 0°, ±30°, ±60°, and 90° in an acquisition time of 20 s per projection. A first possibility of extracting the correlated frequency triplets from these projections is to reconstruct the 3D spectral space using retro-projection techniques.[7] Figure 3b shows the same N−CO planes as plotted in (a) extracted from the reconstructed 3D H−N−CO spectrum, obtained in a total acquisition time of only 120 s. An alternative strategy consists of extracting the frequency information directly from a set of projection spectra using symmetry criteria, as implemented in several assignment programs.[8] For small globular proteins, two or three projection spectra are generally sufficient to retrieve the complete 3D spectral information.[9] This approach further reduces the required acquisition time to less than one minute. To further investigate the applicability of BEST-HNCO/CA to larger proteins in the 100−200 residues range, we recorded a 3D HNCA spectrum of SiR-FP18 (167 residues). The experiment was performed in an experimental time of 15 min. All expected intra-, and most interresidue correlation peaks were observed in this spectrum. Figure 4 shows a series of H^N-CA strips illustrating the potential of sequential resonance assignment using fast 3D BEST-HNCA. Additional BEST-HNCA data of ubiquitin (see Figure S6) recorded on an 800 MHz spectrometer, equipped with a cryogenic probe, show the compatibility of BEST-HNCO/CA and high repetition rates with the use of cryogenic probes.

In summary, we have shown that 3D H−N−CO and H−N−CA protein correlation spectra can be recorded within a few minutes, sometimes even less, of data collection. We have also shown that the BEST experiments are compatible with projection NMR techniques to further reduce acquisition times. It is equally possible

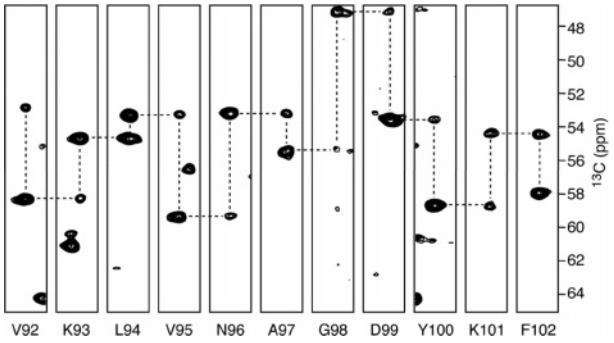

Figure 4. H^N−CA strips (V92 to F102) extracted from a 3D BEST-HNCA spectrum of 1.8 mM ^{13}C/^{15}N-labeled SiR-FP18 (30 °C). 30 (^{13}C) × 27 (^{15}N) complex points were acquired with t_{rec} = 150 ms in only 15 min.

to combine BEST-HNCO/CA with other fast acquisition techniques, such as Hadamard-type or spatially-encoded ^{15}N and/ or ^{13}C frequency labeling. The BEST sequences can be easily extended to other correlation experiments involving only amide protons, for example, sequential, intraresidue, and bidirectional H−N−CA, H−N−CO, and H−N−CB correlation experiments required for backbone resonance assignment, as well as scalar and residual dipolar coupling, and auto- and cross-correlated relaxation rate constant measurements. It can be expected that the BEST experiments will prove very useful for NMR investigations of the structure and dynamics of unstable protein samples. One can also envisage application to real-time studies of kinetic processes by adding a third frequency dimension and, thus, further increasing spectral resolution with respect to ^{1}H−^{15}N correlation spectra. For example, a well-chosen pair of H−N−CO projections from the known resonance frequencies may allow identification of most nuclear sites in a larger protein and still provide a time resolution of about 30 s to follow some kinetic event by real-time 3D NMR.

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Supporting Information Available: Further experimental details on the BEST-HNCO/CA sequences and additional NMR data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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