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Fast Two-Dimensional NMR Spectroscopy of High Molecular Weight Protein Assemblies

Carlos Amero,[†] Paul Schanda,[†,‡] M. Asunción Durá,[†] Isabel Ayala,[†] Dominique Marion,[†] Bruno Franzetti,[†] Bernhard Brutscher,[†] and Jérôme Boisbouvier*[,†]

Institut de Biologie Structurale Jean-Pierre Ebel CNRS-CEA-UJF, 41 rue Jules Horowitz, 38027 Grenoble, France, and Laboratorium für Physikalische Chemie, ETH Zürich, 8093 Zürich, Switzerland

Received December 18, 2008; E-mail: jerome.boisbouvier@ibs.fr

Recent progress in nuclear magnetic resonance (NMR) spectroscopy and advancements in isotope labeling techniques have enabled the study of high molecular weight protein assemblies in terms of local structure and dynamics.[1] Selective protonation of methyl groups in fully perdeuterated proteins, combined with transverse relaxation optimized methyl spectroscopy (methyl-TROSY[2]) and the use of high-field NMR magnets equipped with cryogenically cooled probes, has greatly enhanced sensitivity and spectral resolution and allowed the detection of NMR signals of individual nuclear sites in very large molecules. Protein assemblies often act as molecular machineries involved in various cellular processes, such as protein folding and degradation, signal transduction, etc. Because of its atomic resolution capabilities, it is appealing to use multidimensional NMR spectroscopy to monitor in real-time and site-specifically the structural and dynamic changes a molecular machine undergoes while performing its biological action. So far, fast real-time 2D NMR techniques have been successfully applied to study the folding pathways and hydrogen exchange kinetics[3] in small proteins on the seconds to minutes time scale. Here we show that similar real-time NMR studies are also feasible for large molecular assemblies. An optimized NMR experiment that combines the advantages of methyl-TROSY[2] and SOFAST-HMQC[4] allows the recording of high quality $^{1}H-^{13}C$ methyl spectra of protein assemblies of several hundred kDa in a few seconds acquisition time. This is demonstrated for the TET2 protein, an aminopeptidase involved in protein degradation in the Archaea bacterium *Pyrococcus horikoshii*, forming a homo-dodecamer of 468 kDa.[5]

The SOFAST-methyl-TROSY experiment is shown in the insert of Figure 1a. Selective excitation of methyl protons preserves the favorable relaxation properties of fast rotating CH_3 groups in large, highly deuterated proteins, while the variable flip angle capability of the first ^{1}H pulse allows adjustment of the excitation angle to the so-called Ernst angle for optimal sensitivity.[6] Combined these two effects significantly enhance the proton steady-state polarization for high repetition rates. Figure 1a shows sensitivity curves measured for a sample of U-[^{15}N,^{12}C,^{2}H], Ile-δ_1-[$^{13}CH_3$] labeled TET2 in D_2O. These curves provide a measure of the relative signal-to-noise ratio (*S*/*N*) obtained for different experiments in the same acquisition time. Despite the high level of deuteration (>95% at all ^{1}H sites except Ile-δ_1), a small but substantial longitudinal relaxation enhancement effect is still observed for this protein. This effect is manifest in a shift of the maximum intensity of the 90° SOFAST-methyl-TROSY curve with respect to the standard methyl-TROSY toward shorter recycle delays. This enhancement is expected to become even more pronounced for molecules with slower tumbling rates (increased molecular size), as demonstrated by numerical simulations of the methyl ^{1}H longitudinal relaxation in the presence of a 3% residual protonation level (Figure 1b). Additional simulations (Figure S2) indicate that the enhancement effect also increases for higher levels of residual protonation. No enhancement effect is expected for proteins with isotropic correlation times, τ_c, of less than ~10 ns. This has been experimentally confirmed for the small (8.6 kDa) protein ubiquitin. The use of small excitation-pulse flip angles further shifts the maximum of the sensitivity curves toward short recycle delays with a concomitant

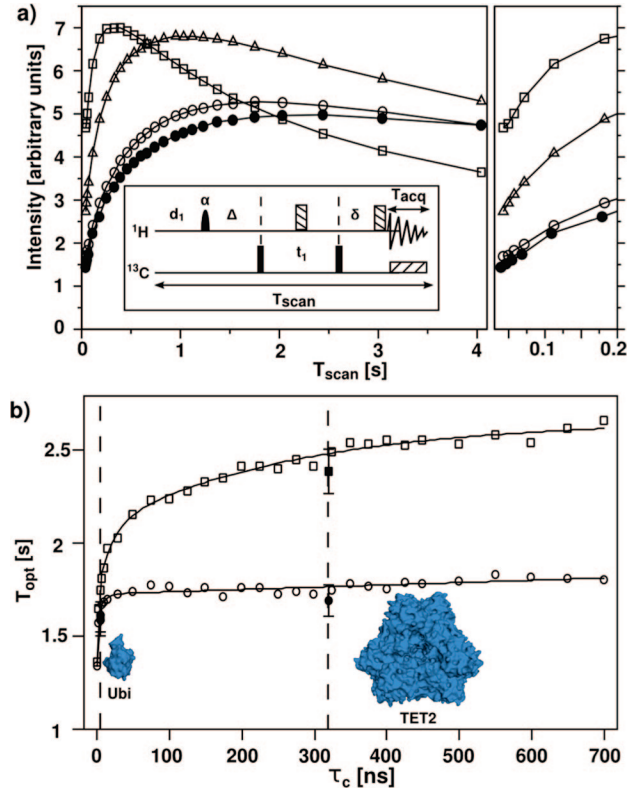

Figure 1. (a) Sensitivity (*S*/*N* per unit experimental time) as a function of the scan time (T_{scan}) measured for a sample of U-[^{15}N,^{12}C,^{2}H], Ile-δ_1-[$^{13}CH_3$] TET2 using standard (●), and SOFAST methyl-TROSY experiments with $\alpha = 90°$ (○), $\alpha = 60°$ (△), and $\alpha = 30°$ (□). The SOFAST-methyl-TROSY pulse sequence is shown as an insert (experimental details are provided in the Supporting Information). (b) Simulation of the optimal scan time, T_{opt}, as a function of the molecular tumbling correlation time, τ_c. T_{opt} is the value of T_{scan} that leads to optimal sensitivity per unit acquisition time (Figure S1). Numerical simulations were performed for standard (□) and SOFAST (○) methyl-TROSY assuming a 3% residual protonation level and $\alpha = 90°$. The experimentally determined T_{opt} for TET2 (315 ns) and ubiquitin (4 ns) using SOFAST (●) and standard methyl-TROSY (■) are shown.

[†] IBS Grenoble.
[‡] ETH Zürich.

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sensitivity increase of ~40%. Similar results are obtained for a second sample of U-[^{15}N,^{12}C,^{2}H] Ala-β-[^{13}CH$_3$]-labeled TET2.

The SOFAST-methyl-TROSY experiment yields a significant sensitivity gain, especially in the context of very fast data acquisition, such as is required for real-time NMR studies, where short recycle delays of a few milliseconds are used. In this fast-pulsing regime the *S/N* is increased by a factor of ~3 compared to a conventional methyl-TROSY pulse scheme. Figure 2 shows

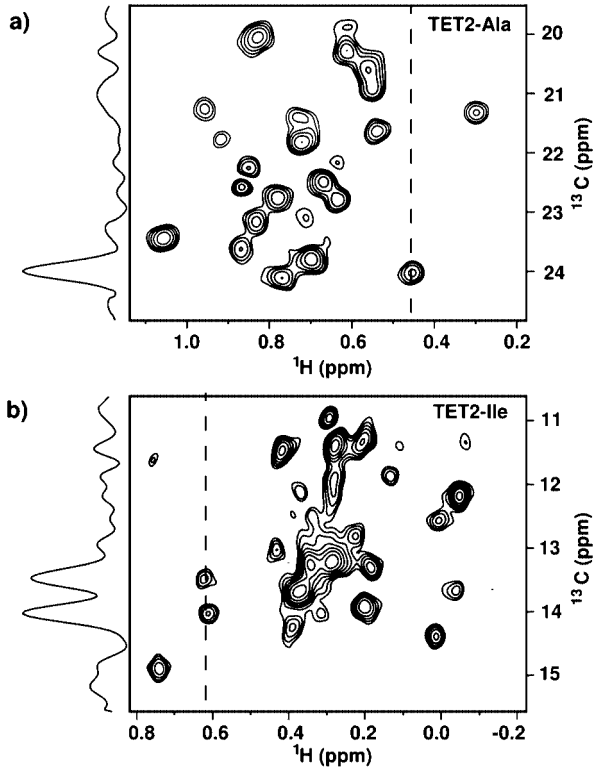

Figure 2. SOFAST-methyl-TROSY spectra acquired on ~80 μM samples of the 468 kDa TET2 protease in D_2O (~1 mM of protomer). (a) Ala methyl spectra[7] recorded on the U-[^{15}N,^{12}C,^{2}H], Ala-β-[^{13}CH$_3$]-TET2 sample. (b) Ile-δ_1 methyl spectra[8] recorded on the U-[^{15}N,^{12}C,^{2}H], Ile-δ_1-[^{13}CH$_3$]-TET2 sample. Both spectra have been acquired in 3.4 s at 37 °C on a 800 MHz spectrometer, with a 1 ms d_1 delay, 30 ms acquisition time (T_{acq}), and 16 complex points in the ^{13}C dimension (t_1). The variable flip angle was set to 25° (a) and 18° (b). Additional 1D traces are shown on the left.

SOFAST-methyl-TROSY spectra of the TET2 protease recorded in a total acquisition time of ~3 s on a 800 MHz spectrometer equipped with a cryogenic triple-resonance probe. 80 μM samples of the 468 kDa oligomeric TET2 protease were used with selective methyl group isotope labeling of either 30 Ala[7] or 34 Ile (δ_1 position) residues.[8] High quality methyl NMR spectra are obtained for both samples, indicating that this method provides useful spectroscopic probes, both close to the polypeptide backbone (Ala methyls) and at the extremity of long and generally more flexible side chains (Ile δ_1 methyls).

The average *S/N* for individual peaks in these spectra is ~10:1, as can be appreciated from the 1D traces shown in Figure 2. Under these conditions, the SOFAST-methyl-TROSY experiments are still in the sampling limited regime where the acquisition time is dictated by the number of data points that need to be sampled along the t_1 time evolution dimension. Therefore alternative sampling and processing techniques[9] may be used in to reduce the number of repetitions (scans) and, thus, to achieve even shorter experimental times for recording 2D methyl correlation spectra. Examples of methyl spectra of the TET2 protein recorded using limited time-domain sampling in both the ^{1}H and ^{13}C dimensions and 2D maximum entropy[10] spectral reconstruction (instead of Fourier transformation) are shown in Figure S4 of the Supporting Information. Such studies allow the time resolution of real-time NMR to be reduced to less than a second.[3] These data illustrate that SOFAST-methyl-TROSY enables monitoring fast molecular kinetics of reactions catalyzed by macromolecular machineries simultaneously at multiple (methyl) sites within the molecule. Such real-time NMR studies may provide a comprehensive picture of the molecular mechanisms involved in enzymatic function.

In summary we have shown that selective excitation of CH_3 groups accelerates the recovery of methyl proton polarization in high molecular weight protein assemblies in the presence of small residual protonation levels. Combined with Ernst angle excitation, this effect allows the recycle delay between successive scans to be reduced to a few milliseconds. SOFAST-methyl-TROSY NMR combines the advantages of longitudinal and transverse spin-relaxation optimization to enable acquisition of high quality methyl ^{1}H$-^{13}$C correlation spectra of protein assemblies of several hundreds of kDa in a few seconds acquisition time. This time requirement can be further reduced to <1 s using sparse time-domain sampling schemes combined with advanced nonlinear data processing methods. SOFAST-methyl-TROSY-based real-time NMR offers new opportunities for the study of structural and dynamic changes occurring in molecular nanomachines while they perform their biological function *in vitro*. This technique may also prove useful for real-time investigation of macromolecular folding and self-assembly. Therefore we believe that the NMR method presented here provides an important new analytical tool for the investigation and characterization of complex biomolecular assemblies and their implication in various biochemical processes in the cell.

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Supporting Information Available: Description of Methyl SOFAST-TROSY pulse sequence, protocols for preparation of protein samples, simulations of methyl proton polarization recovery for different protonation levels, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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