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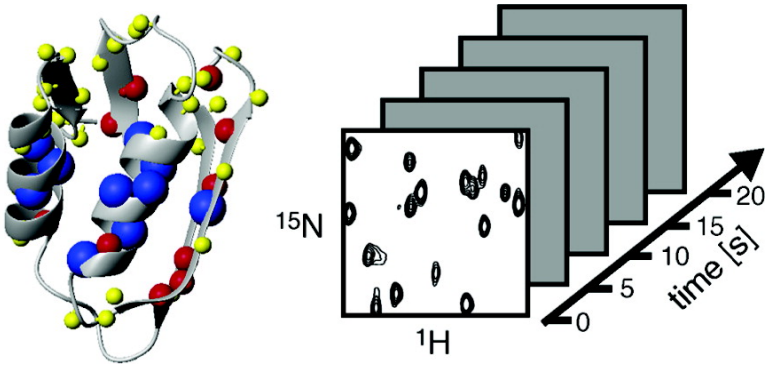

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Very Fast Two-Dimensional NMR Spectroscopy for Real-Time Investigation of Dynamic Events in Proteins on the Time Scale of Seconds

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One-dimensional NMR has proven in the past to be a powerful method for real-time experimental investigations of slow dynamic processes in chemistry and biochemistry such as reaction kinetics, conformational interconversion, and protein folding.[1] However, because of its intrinsically low spectral resolution, 1D NMR yields only very limited atomic resolution when applied to macromolecules. On the other hand, recording a series of 2D spectra, which provides the high spectral resolution, is time-demanding because each spectrum requires at least several minutes of data acquisition. Line shape analysis of cross-peaks in the indirect dimension provides information on the dynamic events occurring during 2D data acquisition,[1c] but the quality of the spectra is reduced by line broadening and line distortions, thus limiting the reliability of such an analysis. Therefore, there is a strong interest in developing fast 2D NMR techniques, where the dynamic information is encoded in the peak intensities and positions, to follow dynamic processes in proteins on a per-residue basis with a time resolution of a few seconds. Recently, Frydman and co-workers[2] introduced "single-scan" NMR spectroscopy, which in an ingenious way allows acquiring any multidimensional data set within a single scan. Unfortunately, the applicability of single-scan NMR to protein samples is currently limited by its inherently low sensitivity and demanding spectrometer hardware requirements. Here we present an attractive alternative for the recording of 2D $^{1}H-^{15}N$ (or $^{1}H-^{13}C$) correlation spectra of proteins within only a few seconds of data acquisition. The new band-Selective Optimized Flip-Angle Short-Transient heteronuclear multiple quantum coherence (SOFAST-HMQC) experiment relies on standard data sampling in the indirect dimension and is easily implemented on any commercial NMR spectrometer. The experiment yields significantly increased sensitivity for short acquisition times when compared to other existing techniques.[2−4] This has allowed us to obtain well-resolved $^{1}H-^{15}N$ correlation spectra with a high signal-to-noise (S/N) ratio for several proteins in the 1 to 2 mM concentration range. As a first application of interest we have applied the new experiment to the study of fast amide hydrogen−deuterium (H/D) exchange in the small protein fragment MerAa (67 residues) of the mercuric reductase MerA from *Ralstonia metallidurans*.[5]

The new SOFAST-HMQC experiment is shown in Figure 1. The pulse sequence has been optimized for very short interscan delays (t_{rec}), as required for fast data acquisition. HMQC-type coherence transfer has the advantage of using less radio frequency (rf) pulses compared to HSQC-based sequences, which reduces signal loss due to B_1-field inhomogeneities and pulse imperfections, and limits the rf power deposited in the NMR probe. In addition, HMQC offers the possibility of Ernst-angle excitation.[6] The S/N per unit experimental time for arbitrary flip-angle excitation is given by $M_0\{[1 - \exp(-T_{rec}/T_1)] \sin \beta\}/\{[1 - \exp(-T_{rec}/T_1) \cos \beta]\sqrt{-(nT_{scan})}\}$, where M_0 is the thermal equilibrium magnetization, $\beta = \alpha - 180°$ is the effective flip angle, T_1 is the proton spin−lattice relaxation time, T_{rec} is the effective recycle delay ($t_{rec} + t_2^{max} + \Delta + t_1/2$), and T_{scan} is the time required for a single transient. As has been demonstrated previously,[3] the use of optimized flip angles in HMQC experiments allows an increase in the repetition rate of the experiment without losing much S/N per unit time. The sensitivity of the experiment is further increased by enhanced ^{1}H spin−lattice relaxation. It has been shown[7] that the selective manipulation of a subset of proton spins in a protein, while leaving all other protons unperturbed, yields significantly shortened relaxation times T_1. This effect is exploited in the sequence of Figure 1 by the use of band-selective ^{1}H pulses. Here we use a polychromatic PC9 pulse shape for adjustable flip-angle band-selective excitation.[8a] The PC9 pulse yields quite uniform excitation over the desired bandwidth for flip angles in the range $0° < \alpha < 130°$. For larger flip angles, the excitation profile becomes more distorted. The phase dispersion is linear and can be easily corrected to pure phase by properly adjusting the delay δ to account for spin evolution during the PC9 pulse.

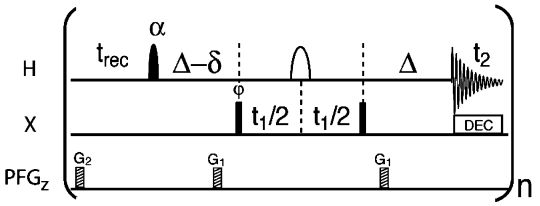

Figure 1. SOFAST-HMQC experiment to record $^{1}H-X$ (X = ^{15}N or ^{13}C) correlation spectra of proteins. Filled and open pulse symbols indicate 90° and 180° rf pulses, except for the first ^{1}H excitation pulse applied with flip angle α. The variable flip-angle pulse has a polychromatic PC9 shape,[8a] and band-selective ^{1}H refocusing is realized using an r-SNOB profile.[8b] The transfer delay Δ is set to $1/(2J_{HX})$, the delay δ accounts for spin evolution during the PC9 pulse, and t_{rec} is the recycle delay between scans. Adiabatic WURST-2 decoupling[8c] is applied on X during detection. Quadrature detection in t_1 is obtained by phase incrementation of φ according to TPPI-STATES.

Figure 2 shows a SOFAST-HMQC $^{1}H-^{15}N$ correlation spectrum recorded at 800 MHz ^{1}H frequency on a 2 mM sample of $^{13}C,^{15}N$-labeled ubiquitin in an overall experimental time of 5 s. The high S/N obtained in this spectrum can be appreciated from the 1D trace extracted along the ^{15}N dimension as indicated in the figure. To compare the performance of SOFAST-HMQC with other existing $^{1}H-^{15}N$ correlation experiments, we have recorded 1D spectra for a series of recycle delays. These spectra provide a measure of the relative average S/N ratio obtained by the different experiments for a given overall experimental time. As shown in Figure S1 of the Supporting Information, for the fast repetition rates we are interested in ($T_{scan} \approx 100$ ms), SOFAST-HMQC yields a sensitivity increase of about 300% with respect to standard techniques (sensitivity-enhanced HSQC, fast-HMQC,[3] or FHSQC[4]). If one takes into account recent experimental results reported by Pelupessy[9] indicating a decrease in S/N by about a factor of 7 for single-scan HSQC with respect to standard HSQC, one may also conclude that SOFAST-HMQC presents a significant sensitivity advantage with

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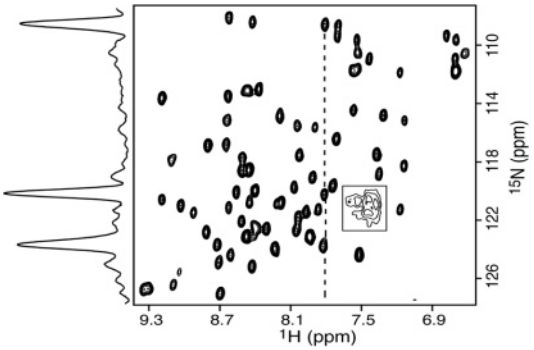

Figure 2. ^{1}H−^{15}N correlation spectrum (central part) of ubiquitin (2 mM, pH 4.7) recorded at 800 MHz in only 5 s using the sequence of Figure 1. The acquisition parameters were set to: $\alpha = 120°$, $\Delta = 5.4$ ms, $\delta = 1.2$ ms, $t_2^{max} = 40$ ms, and $t_{rec} = 1$ ms. Forty complex points ($n = 80 + 4$ dummy scans) were recorded for $t_1^{max} = 22$ ms. The band-selective ^{1}H pulses were centered at 8.0 ppm covering a bandwidth of 4.0 ppm. SOFAST-HMQC yields good water suppression in a single transient. The peak pattern surrounded by a box arises from Arg side chain resonances.

respect to current single-scan methods. SOFAST-HMQC spectra obtained for other proteins varying in size and concentration are shown in Figure S2 of the Supporting Information. This is, to the best of our knowledge, the first time that high-quality 2D correlation spectra of proteins have been recorded in a few seconds of experimental time, which opens the way for 2D-based real-time NMR studies of protein kinetics on a seconds time scale.

The measurement of site-specific amide H/D exchange rates provides insight into both structural and dynamic properties of proteins. Applications of H/D exchange measurements comprise the identification of H-bond networks in proteins, the characterization of the stability of structural elements in native states, folding intermediates, or partially denatured states of proteins, and the identification of ligand-binding sites.[10] Recently, H/D exchange has also been proposed as an additional dimension for mass spectrometry-assisted NMR resonance assignment.[11] H/D exchange rates in proteins cover a wide range of time scales, from milliseconds to hours or days. Very fast H/D exchange with characteristic time constants τ_{ex} up to a few seconds can be monitored by NMR methods based on selective perturbation of the water resonance. Slow H/D exchange is commonly measured by dissolving the protein in D_2O and following the peak intensity in the NMR spectrum over time. Such studies typically rely on ^{1}H−^{15}N correlation experiments, and the time resolution is therefore determined by the acquisition time of a single 2D data set, typically a few minutes. The new SOFAST-HMQC experiment, which allows 2D data acquisition in only a few seconds, thus offers a way to close the missing time gap.

Figure 3 shows the results of H/D exchange measurements performed on MerAa. For this 67-residue protein fragment, H/D exchange at pH 7.5 is completed for all amides only a few minutes after dilution of the protein in D_2O. For the NMR experiments, the protein was concentrated in 100 μL of H_2O solution, and H/D exchange was initiated by adding 400 μL of D_2O, yielding a final protein concentration of 2.1 mM. The mixing was achieved outside the NMR spectrometer, resulting in a dead time of about 40 s between mixing and the actual start of the NMR experiments. Figure 3 illustrates the performance of the SOFAST-HMQC experiment to measure fast exchange rates on a per-residue basis down to a characteristic time constant of about $\tau_{ex} = 15$ s. Even faster exchange may be quantified by reducing the dead time using a rapid mixing device.[1a] The measured H/D exchange data indicate

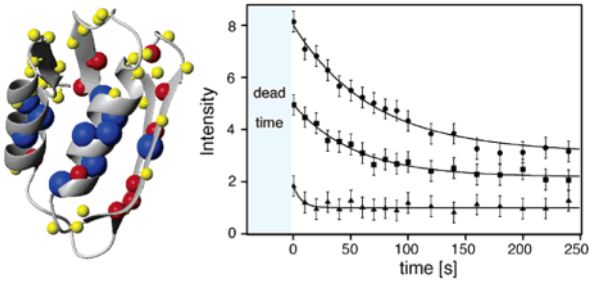

Figure 3. H/D exchange kinetics measured in the small protein MerAa (pH 7.5). On the right, the cross-peak intensities measured in a series of SOFAST-HMQC spectra are plotted as a function of exchange time for V25 (■), L53 (●), and A43 (▲). A fit to the function $I(t) = a_0 \exp(-t/\tau_{ex}) + a_1$ yields exchange time constants $\tau_{ex} = 69$ s for V25 and $\tau_{ex} = 53$ s for L53. No reliable fit is possible for A43, but the upper limit can be estimated to $\tau_{ex} < 15$ s. On the left, the measured exchange rates, divided in three classes (i) $\tau_{ex} < 15$ s (yellow small spheres), (ii) 15 s $< \tau_{ex} < 40$ s (red medium-size spheres), and (iii) $\tau_{ex} > 40$ s (blue large spheres), are represented on the ribbon structure of MerAa.[5]

differential solvent protection for amides along the protein backbone. Interestingly, solvent protection in α-helices is found to be higher than that in β-sheets. The intensities of cross-peaks belonging to amides in loop regions have decreased to their plateau value (20% of the initial peak intensity) during the dead time.

In summary, we have shown that 2D ^{1}H−^{15}N correlation spectra of ^{15}N-labeled proteins can be recorded in only a few seconds of data acquisition using a very simple new pulse sequence (SOFAST-HMQC) that provides the required high sensitivity. This experiment allows real-time site-resolved NMR studies of kinetic processes in proteins on a time scale of seconds. Here, we have demonstrated its applicability to the measurement of fast H/D exchange rates. Other applications include the real-time study of protein folding kinetics and the observation of short-lived folding intermediates. The experiment may also become valuable for high-throughput characterization of protein samples and screening of ligand binding to target proteins by NMR.

Supporting Information Available: ^{1}H−^{15}N SOFAST-HMQC spectra of several proteins, acquisition and processing details, and a sensitivity comparison of different pulse schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

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