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Published on Web 12/24/2005

Real-Time Monitoring of Chemical Transformations by Ultrafast 2D NMR Spectroscopy

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Abstract: An approach enabling the acquisition of 2D nuclear magnetic resonance (NMR) spectra within a single scan has been recently proposed. A promising application opened up by this "ultrafast" data acquisition format concerns the monitoring of chemical transformations as they happen, in real time. The present paper illustrates some of this potential with two examples: (i) following an H/D exchange process that occurs upon dissolving a protonated protein in D_2O, and (ii) real-time in situ tracking of a transient Meisenheimer complex that forms upon rapidly mixing two organic reactants inside the NMR observation tube. The first of these measurements involved acquiring a train of 2D $^1H-^{15}N$ HSQC NMR spectra separated by ca. 4 s; following an initial dead time, this allowed us to monitor the kinetics of hydrogen exchange in ubiquitin at a site-resolved level. The second approach enabled us to observe, within ca. 2 s after the triggering of the reaction, a competition between thermodynamic and kinetic controls via changes in a series of 2D TOCSY patterns. The real-time dynamic experiments hereby introduced thus add to an increasing family of fast characterization techniques based on 2D NMR; their potential and limitations are briefly discussed.

1. Introduction

Nuclear magnetic resonance (NMR) offers a number of avenues toward monitoring dynamic chemical and biochemical processes.[1−6] Insight into kinetics occurring on a $10^{-7}-10^{-3}$ s time scale can be accessed by a variety of relaxation time measurements; environmental changes in the $10^{-2}-10^{1}$ s range can be revealed by line shape variations in appropriate one- and two-dimensional (1D and 2D) NMR experiments; longer time scales can be probed simply by monitoring changes in the appearance of the NMR spectra as a function of time. The latter approach has become a standard route to examine the kinetics of chemical, biochemical, and in vivo processes, owing in part to sensitivity advances that have enabled the rapid acquisition of pulsed NMR spectra. And yet, over the years, the limitations of conventional 1D NMR spectroscopy to tackle complex systems with multiple overlapping lines have also become clear. A solution to this overlap problem is afforded by higher-dimensional NMR spectroscopy, which in either homo- or heteronuclear acquisition modes provides sufficient spectral dispersion to tackle otherwise overtly complex systems. A penalty, however, is associated with the acquisition of these higher-dimensional spectral sets: as multiple dimensions are explored via the collection of numerous time-incremented scans, exponentially longer acquisition times become an inherent feature of the measurement. This can deprive kinetic studies from many of the benefits of real-time NMR acquisitions. Only in rare instances have kinetic measurements been able to benefit from the insight that 2D NMR can provide — in such instances, the impact which kinetic 2D NMR could have in the realm of biomolecular transformations was unambiguously evidenced.[4,7,8]

In view of the unique opportunities which real-time 2D NMR could open up, it is not surprising that this has remained a topic of active methodological investigations. This progress has been stimulated by ongoing improvements in NMR's sensitivity, as well as by developments during recent years on the topic of accelerated multidimensional acquisitions.[9−11] Bougault et al., for instance, have recently demonstrated that a 2D Hadamard-encoded version of HSQC NMR spectroscopy running on a cold probehead could be employed to monitor H/D amide exchange rates in ubiquitin at rates of about 1 min^{-1}.[12] Schanda and Brutscher also showed that an ingenious modification of a conventional 2D HMQC scheme, involving a combination of frequency-selective and partial excitation pulses, enables one to eliminate long recycle delays while preserving suitable sensitivities at 18.8 T.[13] Kinetic studies of H/D exchange

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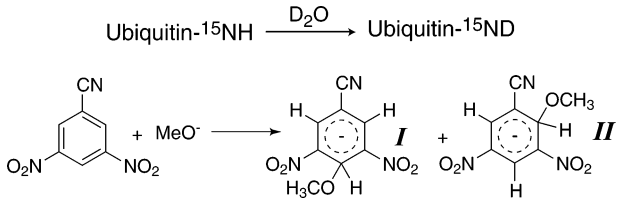
Scheme 1



in proteins at ~5 s repetition times were demonstrated in this manner. As part of this ongoing progress, we discuss here some of our efforts in this area. These have been based on ultrafast 2D NMR, a protocol that we have recently put forward to enable the acquisition of multidimensional spectra within a single scan.[14,15] This approach replaces the indirect-domain parametric time encoding with a spatial one, which unlike the traditional encoding mode can be imparted and completed in its entirety within a single shot. We believe that following rapid transient processes could constitute one of the main areas of application of such an "ultrafast" 2D NMR approach.

2. Materials and Methods

Two types of ultrafast 2D NMR sequences were assayed in this study: a heteronuclear ^{1}H−^{15}N HSQC version and a homonuclear ^{1}H−^{1}H TOCSY one. Both sequences were implemented on a Varian Inova 500 MHz console equipped with a conventional triple-axis HCN probe head. The HSQC experiment was applied to monitor a hydrogen−deuterium exchange process occurring when a protonated ubiquitin powder was suddenly dissolved in D_2O,[17,18] whereas TOCSY was used to follow the in situ formation of a transient Meisenheimer complex. Scheme 1 and Figure 1 schematize the reactions that were analyzed and the pulse sequences used in this study. The spatial encodings underlying the latter were implemented by pairs of RF pulses exciting/storing spins over the full length L of the sample, sweeping the sample over intervals $t_1^{max}/2$ while under the action of $\pm G_e$ external gradients. The offsets of such pulses were thus chirped over a $\pm\gamma G_e L/2$ span, and their amplitudes calibrated as effective $\pi/2$ nutations by being set to $\gamma B_1 \approx 0.25(|2\gamma G_e L/t_1^{max}|)^{1/2}$. Further experimental details on setting up and processing these experiments are given in refs 14−16, as well as in the paragraphs below.

3. Results

H/D Exchange Studies on Ubiquitin. In an initial test on the capabilities of ultrafast 2D NMR to follow dynamic processes, attention was focused on the H/D exchange process characterizing amide groups in human ubiquitin. This presents a well-characterized NMR target,[12,13,19] readily available in an ^{15}N-enriched form. Figure 2 compares conventional and ultrafast HSQC acquisitions collected at 500 MHz on a 3.25 mM solution of a lyophilized His-tagged powder (Asla Biotech), dissolved in a 90%/10% H_2O/D_2O phosphate buffer (pH = 6.5). Under these conditions of concentration and field strength, the sequence employed was unable to deliver quality ultrafast 2D kinetic data on the amide protons' disappearance within a single scan. Two scans with ±180° cycling of the initial ^{15}N-chirped excitation and of the receiver phase were needed for suppressing the otherwise intense water peak that was left over after the Watergate, and two such pairs were co-added for improving the overall signal-to-noise ratio. This latter co-addition was not

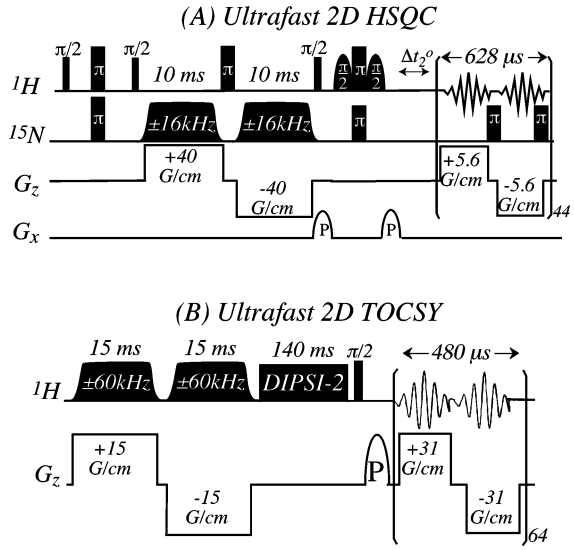


Figure 1. Scheme and details of the two ultrafast 2D NMR pulse sequences used to carry out real-time characterizations of chemical dynamics. Sequence A was utilized for monitoring H/D exchange in ^{15}N-labeled ubiquitin, while sequence B served to monitor the formation of a transient organic complex. Both pulse sequences are akin to those described in ref 16. Sequence A incorporated a ±180° phase-cycling of the initial ^{15}N-chirped pulse and of the receiver phase as well as a ^{1}H Watergate pulse[20] (aided by the non-encoding x-gradient) for the sake of a better solvent suppression, and a delay $\Delta t_2°$ that was set to approximately 0 and $\Delta t_2/2$ in alternate scans for the sake of data interleaving.

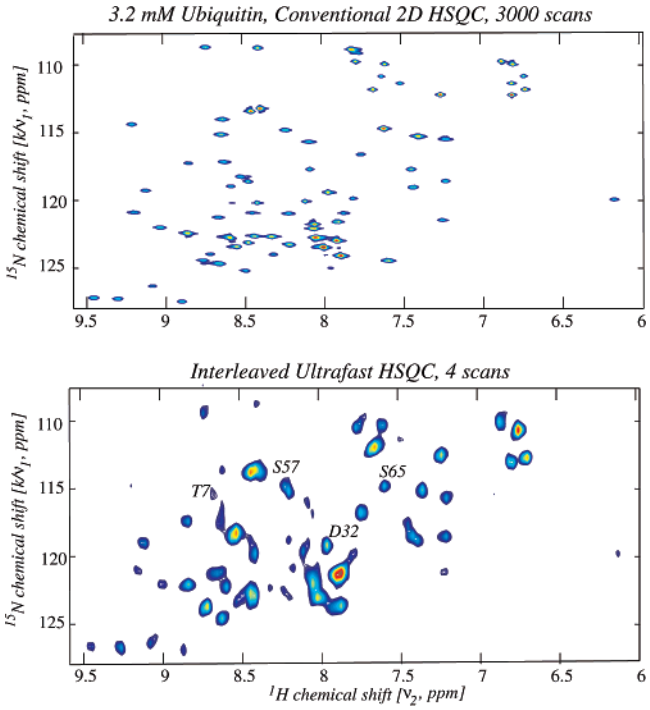


Figure 2. Comparison between conventional and ultrafast 2D HSQC ^{15}N−^{1}H NMR spectra recorded at 11.7 T on a 3.25 mM ubiquitin solution. The conventional spectrum involved 250 t_1 increments collected with an eight-scan phase-cycling (plus four dummy scans); the ultrafast set resulted from four scans and involved the data interleaving of two phase-cycled acquisitions collected under the conditions depicted in Figure 1A. A similar procedure was adopted for the kinetic 2D acquisitions. Labeled are peaks arising from fast-exchanging amide sites, whose kinetics are detailed in Figure 4.

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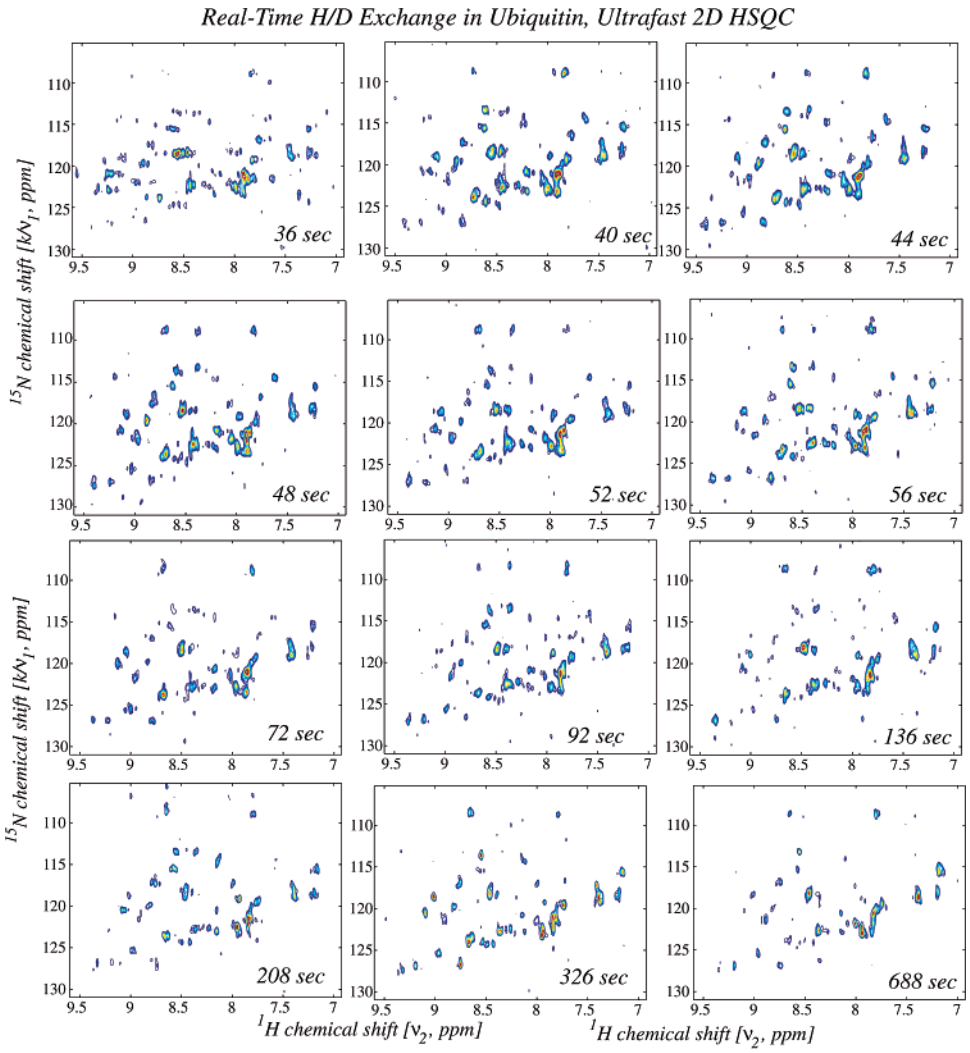

Figure 3. Representative series of real-time 2D HSQC NMR spectra recorded on a ~3.2 mM ubiquitin solution, following the dissolution of an initially fully protonated lyophilized powder onto a D_2O-based 50 mM phosphate buffer. The times indicated in each frame correspond to the approximate delay elapsed since the powder was initially dissolved (something which happened outside the magnet) and the average time of the data acquisition. In the actual experiment a full series of spectra separated by ~4 s was recorded over a 20 min interval; only a subset of these spectra is shown.

carried out in the usual signal-averaging fashion but rather in an interleaved form, that is, after waiting half a direct-domain dwell time ($\Delta t_2° \approx \Delta t_2/2$) prior to beginning the actual data digitization.[15] This allows one to effectively double the gradient oscillation period T_a without compromising on the achievable direct-domain spectral width SW_2; this in turn enables a halving of the acquisition gradient G_a without reducing the effective indirect-domain bandwidth $SW_1 \propto G_aT_a$. Thus, as spectral noise grows proportionally to $G_a^{1/2}$, interleaving two such t_2-shifted data sets and processing the resulting matrix in the usual ultrafast fashion leads to a doubling of the overall sensitivity rather than to the traditional $2^{1/2}$ enhancement. Such data interleaving was adopted throughout the present investigation. As can be appreciated from the resonances presented in the four-scan 2D correlation spectrum shown in Figure 2, this procedure enabled the resolution of multiple residues from the protein, which could then be identified on the basis of literature data.[21]

Utilizing this approach, we attempted to follow the rate of H/D exchange at a site-resolved level by monitoring how the signals of an originally protonated ubiquitin sample disappeared as the protein resided in D_2O. Ideally this could have been followed by inserting a lyophilized powdered sample inside the NMR tube and then recording a series of 2D NMR scans as D_2O was added within the NMR magnet. Although this appears to be a straightforward procedure, the kinetics thus measured were not a genuine reflection of the H/D amide exchange but rather of the powder dissolution process. Hence, the adopted procedure involved dissolving thoroughly within an Eppendorf vial a lyophilized sample of the protonated protein in D_2O, pipetting the resulting solution into a Shigemi NMR tube, and only then inserting the tube into the NMR magnet. At this stage the spectrometer was already collecting 2D HSQC NMR spectra while running in an unlocked mode, at a rate of one phase-cycled (and interleaved) spectrum every 4 s. This dissolution/insertion/acquisition approach suffered from an initial dead time, during which the fastest exchanging sites lost their $^1H-^{15}N$ NMR resonances. Repeated optimization of this procedure allowed us to reduce this blind time to ca. 30−40 s.

Figure 3 illustrates a series of 2D spectra collected in this fashion. On comparing the plot recorded in protic water with the initial data sets that appear in this series, it was clear that some of the peaks have already disappeared. The most notable

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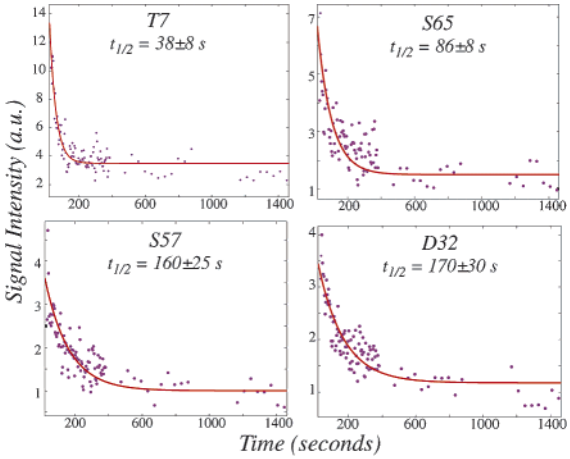

Figure 4. H/D exchange kinetics resolved for the sites indicated in Figure 2, resulting from monitoring their integrated peak intensities (in magnitude mode) as a function of time. Curves indicate the best fit of the data to a single decaying exponential, leading to the half-life times indicated in each inset. Also indicated are the confidence intervals arising from the fits, whose r^2 values ranged between 0.6 and 0.8. These peaks were chosen for display owing to their relatively fast kinetics.

examples of these were the amine peaks (~6.5−7 ppm ^{1}H region), whose exchange lifetimes under the conditions used were <1 s. Conversely, other peaks arising from amide sites and possessing exchange lifetimes of hours barely changed their appearance over the course of the experimental series. The most interesting instances arose from those sites whose residence times were within minutes, for example amide residues S57, S65, T7, and D32 (labeled resonances in Figure 2). The disappearance of such sites would be hard to track by conventional 2D methods but could be followed by the four-scan HSQC approach; an example of the kinetic behavior observed for them is presented in Figure 4. The H/D exchange rates observed in this fashion for sites S57, S65, and D32 (as well as for several others) were in good quantitative agreement with those previously monitored by Bougault et al. via Hadamard HSQC.[12] Ultrafast NMR also allowed us to follow the kinetics of certain fast-exchanging sites such as T7, whose rates were slightly higher than could be accommodated by the Hadamard spectroscopy.

Ultrafast 2D NMR of a Transient Species. A major limitation of the procedure just depicted resides in its initial dead time. During this period, interesting dynamics might occur but their details will be lost to the 2D acquisition — not due to an inherent spectroscopic limitation but rather due to an artifact of the mixing approach employed. In an effort to solve this problem, a number of rapid-mixing devices were assembled and assayed. One of these incorporated a hollowed plastic device mounted on top of a 5 mm NMR tube, connecting a plastic pipe to a thin capillary and capable of flowing chemicals onto the bottom of the NMR tube either manually or via a console-driven stepping-motor assembly. A suitable spin pre-polarization of the two chemicals about to react could thus be achieved by holding the individual reactants in either the NMR tube or the insert capillary for a suitable period prior to their actual injection/mixing and data recording. The kinetic dead time of such an assembly was then mainly given by the settling delay of the turbulent fluid, which on the basis of tests carried out with colorants was estimated at 1−2 s. This delay is sufficiently short

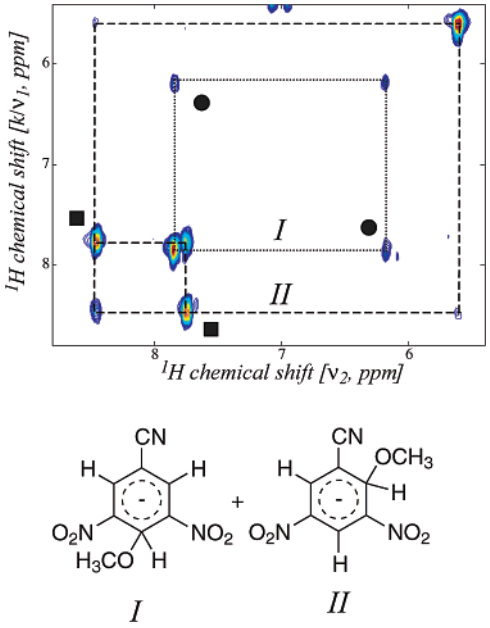

Figure 5. Ultrafast 2D TOCSY ^{1}H NMR spectrum resulting upon rapidly mixing in situ 200 μL of a 125 mM 3,5-dinitrobenzonitrile solution in DMSO-d_6 with 200 μL of a 125 mM methoxide ion solution in 87.5% DMSO-d_6/12.5% MeOD (v/v). The first of these solutions was loaded into a 5 mm NMR tube; the second was also pre-polarized by holding it within a capillary prior to its sudden injection. Data were collected at 16 °C ca. 4.6 s following the mixing using the sequence in Figure 1B, clearly revealing the coexistence of Meisenheimer complexes **I** and **II**.

to enable the detection of certain transient intermediates and of faster kinetics in general by single-scan 2D NMR.

To test this hypothesis, a simple chemical reaction was chosen: the formation of the Meisenheimer complexes resulting upon the reaction of a methoxide ion onto 1-cyano-3,5-dinitrobenzene.[22,23] As illustrated in Scheme 1, such addition can happen on either position 4 (**I**) or 2 (**II**) of the phenyl ring, yielding in turn 2D TOCSY NMR patterns which can be readily differentiated (Figure 5). The 4-methoxy derivative dominates the kinetics at short reaction times yet is thermodynamically unstable, interconverting into the 2-methoxy form within a few seconds. The short lifetime of **I** hence offers a good test of the methodology's capabilities. Moreover, as the concentrations that can be achieved for these organic reactants are at least an order of magnitude higher than in the protein scenario, this reaction could be followed on a single-scan basis. Ultrafast 2D TOCSY acquisitions were thus implemented, monitoring spectra every 2.3 s and as the reaction took place inside the NMR tube. Figure 6 illustrates the temporal progression of one such 2D acquisition series, while Figure 7 summarizes the kinetic results that were obtained by the integration of different peak volumes identified for derivatives **I** and **II**. Overall these results are within the kinetic range originally reported by Fyfe et al.,[23] lending support to the potential of this 2D NMR approach.

4. Discussion and Perspective

Opening new routes to follow the real-time kinetics of biomolecular processes is one of the main promises inherent in

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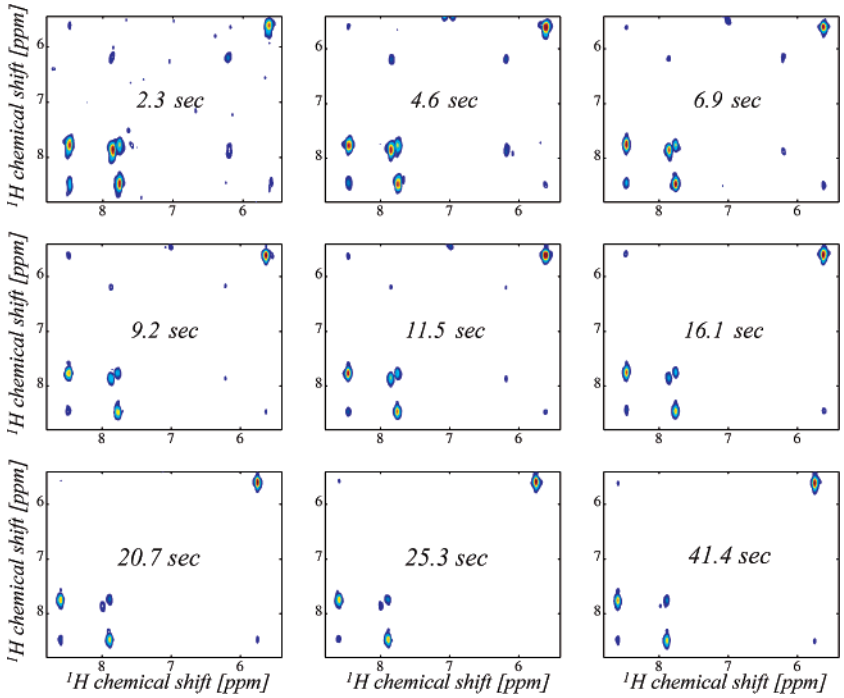

Figure 6. Representative spectra extracted from a series of 2D ^{1}H TOCSY NMR spectra recorded on a system akin to that in Figure 5, as a function of the time elapsed since the triggering of the reaction. In contrast to the approach taken to monitor H/D exchange in ubiquitin, the mixing of the reactants actually happened inside the NMR magnet. Notice the eventual disappearance of the TOCSY pattern arising from **I**.

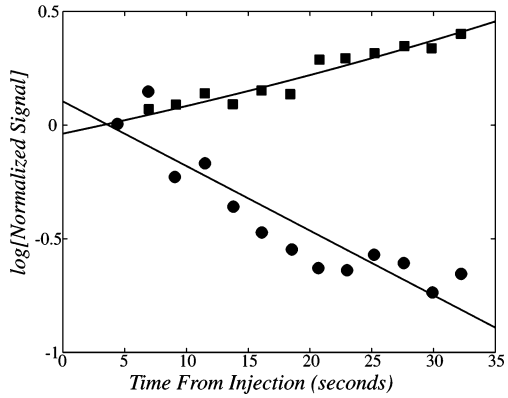

Figure 7. Results stemming from the integration of cross-peaks indicated in Figure 5, when monitored as a function of reaction time. Curves indicate the best fit of the magnitude-mode data to a decaying exponential according to $I_o \exp(-t/\tau) + C$, and are plotted in a normalized log scale. At this temperature, complex **I** is found to decay with a half-life time of 8 s (2.5 s standard deviation).

the growing array of fast 2D magnetic resonance methods that have emerged over the past few years. Much of this impetus is undoubtedly provided by developments in the related area of 2D NMR imaging, where the advent of ultrafast acquisition methodologies has led to unexpected insights about cognitive and other transient functionalities.[24,25] When considering the 2D spectroscopy scenario, it is clear that unique insight into important biophysical and metabolic processes could result from monitoring at high repetition rates the environments of multiple sites within folding proteins or nucleic acids, or the fate of multiple metabolites undergoing irreversible changes. Given the results presented in the preceding section, it appears that the ultrafast 2D NMR methodology could assist in fulfilling this potential. Yet it is also evident that a number of obstacles remain to be overcome in order to facilitate such a role. One entails the sensitivity of the spectra illustrated in Figures 3 and 6, which not being optimal lead to a relatively large scattering in the resulting kinetic curves. Fortunately, there appears to be sufficient room for improvement in these acquisitions to remain optimistic on such matters; progress could come from changing the ultrafast protocol to include sensitivity enhancement procedures, from operating at higher B_o magnetic fields, or from relying on cryogenic probeheads. Additional sensitivity gains could come at no expense in either time or spectral resolution by combining the approach hereby described with other emerging acquisition techniques, for instance, by coupling ultrafast NMR to rapid relaxation-enhanced acquisition techniques.[13] A second main obstacle to overcome arises from the potentially long dead times associated with the triggering of the dynamic processes themselves, something which, as noted earlier, could conceal many of the most important changes being sought. To alleviate this constraint, a number of approaches could be taken, including the rapid mixing of the reactants within the NMR tube. This approach gave satisfactory results when assayed on organic solutions, yet when it was attempted on aqueous protein systems inconsistent results were obtained at the shortest times. Repetitive tests revealed foaming as one of the factors responsible for such artifacts: by virtue of its spoiling of the high-resolution shimming conditions necessary to collect quality solvent-suppressed data, this phenomenon precluded an efficient quantification of the more minor protein signals. We are currently attempting to eliminate this problem by further refining the mode of rapid mixing and by exploring various approaches to laminarly flow the resulting solution into the detection region. We trust that, in unison with the additional spectroscopic

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improvements detailed above, we shall be able to further advance the use of ultrafast 2D NMR as a new approach to track biophysical dynamics.

Acknowledgment. We are grateful to Mr. Boaz Shapira for assistance during the initiation of these experiments. This work was supported by the U.S. National Institutes of Health (GM-72565), the Israel Academy of Sciences (ISF 1206/05), and the German-Israel Fund for Research (GIF 56/2003).

JA0564158