Protein folding and unfolding studied at atomic resolution by fast two-dimensional NMR spectroscopy

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Atom-resolved real-time studies of kinetic processes in proteins have been hampered in the past by the lack of experimental techniques that yield sufficient temporal and atomic resolution. Here we present band-selective optimized flip-angle short transient (SOFAST) real-time 2D NMR spectroscopy, a method that allows simultaneous observation of reaction kinetics for a large number of nuclear sites along the polypeptide chain of a protein with an unprecedented time resolution of a few seconds. SOFAST real-time 2D NMR spectroscopy combines fast NMR data acquisition techniques with rapid sample mixing inside the NMR magnet to initiate the kinetic event. We demonstrate the use of SOFAST real-time 2D NMR to monitor the conformational transition of α-lactalbumin from a molten globular to the native state for a large number of amide sites along the polypeptide chain. The kinetic behavior observed for the disappearance of the molten globule and the appearance of the native state is monoexponential and uniform along the polypeptide chain. This observation confirms previous findings that a single transition state ensemble controls folding of α-lactalbumin from the molten globule to the native state. In a second application, the spontaneous unfolding of native ubiquitin under nondenaturing conditions is characterized by amide hydrogen exchange rate constants measured at high pF by using SOFAST real-time 2D NMR. Our data reveal that ubiquitin unfolds in a gradual manner with distinct unfolding regimes.

Results and Discussion

SOFAST Real-Time 2D NMR Spectroscopy. To overcome the inherent time limitation of 2D NMR spectroscopy, two conceptually different approaches can be envisaged. First, the number of scans required to sample the multidimensional time space may be reduced by using spectral aliasing, nonlinear data sampling techniques, spatial frequency encoding, or Hadamard-type frequency-space spectroscopy (7, 8). Second, accelerating the recovery of spin polarization between scans by means of optimized pulse sequences (9–11) or the addition of relaxation agents to the sample (12, 13) allows higher repetition rates of the pulse sequence. Here, we propose a fluid turbulence-adapted (FTA) version of 1H-15N 2D SOFAST-heteronuclear multiple quantum coherence (HMQC) (10, 14) to follow a kinetic process for individual amide sites in a protein [see supporting information (SI) Fig. 4].

Author contributions: V.F. and B.B. designed research; P.S. performed research; V.F. contributed new reagents/analytic tools; P.S. analyzed data; and P.S. and B.B. wrote the paper.

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Abbreviations: FTA, fluid turbulence-adapted; HMQC, heteronuclear multiple quantum coherence; MG, molten globule; SOFAST, band-selective optimized flip-angle short transient.

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Conformational Transition Kinetics of α-Lactalbumin from an MG to the Native State Studied in Real Time. The protein α-lactalbumin serves as a model system to investigate the structural transition from a partially folded to the native state by using SOFAST real-time 2D NMR. This protein has been used extensively as a model system for protein-folding studies. The structure of native α-lactalbumin (14 kDa) comprises two domains, one containing four α-helices and a short 3_{10} helix, and the other containing a three-stranded β-sheet and another 3_{10} helix. Here, we focused on the Ca-free (apo) form of α-lactalbumin. Under destabilizing conditions such as low pH, addition of cosolvents, high temperature, or combinations thereof, apo-α-lactalbumin exists in an MG state. The MG state is characterized by the absence of long-lived tertiary structure, but it still contains a high degree of secondary structure (20, 21), with a radius of gyration only ~10% larger than the native state (22, 23). The MG state of α-lactalbumin has recently attracted considerable interest when it was realized that it may act as an important component causing apoptosis of tumor cells (24).

Pioneering work on the use of time-resolved NMR methods for monitoring protein refolding was performed on α-lactalbumin (5, 23, 25–27). One-dimensional 1H NMR spectra recorded during the refolding reaction of apo-α-lactalbumin provided evidence that the MG state, accumulated during the early stages of the folding reaction, shows similar spectral characteristics (poorly dispersed and broad resonances) and therefore similar dynamic properties of the conformational ensemble to those observed for the MG state stabilized at acidic pH (25). Additional diffusion-edited and NOE transfer NMR measurements allowed monitoring the compactness of the protein and the establishing of native tertiary contacts, respectively (23, 27). The folding kinetics of apo-α-lactalbumin have also been studied previously at a residue level by line shape analysis of individual cross-peaks detected in a single 2D NMR spectrum recorded during the refolding event (26). This technique, however, is limited to relatively slow kinetics (several minutes) and is prone to large experimental uncertainties. In the study of Balbach et al. (26), folding rates for a total of 25 backbone amides could be quantified with an estimated experimental error of ~25%. Because of the small number and the high uncertainties of the measured rate constants, these data did not allow definite
folding are annotated. (10.9-s duration). Peaks corresponding to the MG state that disappear during injection (23, 26, 27), the intensity decay of five cross-peaks characteristic for the MG state could be quantified. Under the experimental conditions chosen (15°C, pH 8, Ca²⁺-free), the NMR intensity decay and buildup curves can be fitted to monoexponential functions. Fitting the data to more complex, e.g., biexponential or stretched exponential functions, does not yield statistically significant improvements. The folding time constants measured for individual amide sites of the native protein and the MG state are identical (τ₁ = 109 ± 5 s) within the experimental uncertainty. The obtained folding kinetics are consistent with results from fluorescence measurements (SI Fig. 7), yielding a characteristic time constant of 110 ± 10 s for the establishment of native tertiary structure. The good agreement with the fluorescence measurements demonstrates the accuracy obtained by SOFAST real-time 2D NMR experiments.

The MG state of apo-α-lactalbumin explores a large conformational space, possibly including the presence of native and nonnative secondary structures (20). Therefore, the transition from this MG state to the native state requires a large decrease in conformational entropy, known as entropy bottleneck effect (28, 29), which is the main reason for the significantly slower folding kinetics observed for the apo form compared with the holo form of α-lactalbumin (30). The high precision of rate constants (error of ≈5–10%) obtained in our work for a large number of individual amide sites allows us to draw some conclusions on the energy landscape and folding pathways. The finding of equal kinetic rates for the buildup of the native state and the disappearance of the MG state indicates a transition between only two states, the MG ensemble representing a large number of conformational substates interconverting on the micro- to millisecond time scale, and the native state. In other words, the establishment of the native tertiary structure is not accompanied by a change in the structural and dynamic properties of the MG ensemble as folding proceeds. These observations are in agreement with the assumption of a smooth energy landscape where the folding rate is controlled by a single transition state ensemble originating from the conformational entropy bottleneck effect. The existence of a unique transition state in agreement with conclusions drawn from other biophysical folding experiments (30).

Ubiquitin Unfolding Kinetics Under Equilibrium Conditions Revealed by EX1 Amide Hydrogen Exchange Measurements. To illustrate further the potential of SOFAST real-time 2D NMR to detect differential kinetic behavior along the polypeptide chain, we have investigated the unfolding kinetics of human ubiquitin under non-denaturing equilibrium conditions by amide hydrogen/deuterium (H/D) exchange methods. H/D exchange is a powerful tool for the study of protein structure, folding, unfolding, and binding (31, 32). The exchange between amide and solvent hydrogens requires that the amide group is in an exchange-competent, solvent-accessible conformation, which can be achieved by local, subglobal, or global protein unfolding fluctuations (“opening reactions”) that transiently break the exchange-protecting structures, e.g., hydrogen bonds. The measurement of amide H/D exchange rates thus provides residue-specific information on the otherwise invisible manifold of partially or globally unfolded excited conformational states populated at a very low level. Despite their low population under native conditions, such high-energy states may be crucial for

![Fig. 2. Folding of α-lactalbumin studied by SOFAST real-time 2D NMR.](Image)

exclusion of the presence of differential folding kinetics along the polypeptide chain. To illustrate the improvements provided by SOFAST real-time 2D NMR for the measurement of residue-specific folding rates, we monitored the same refolding reaction as Balbach et al. from the MG state, formed at pH 2, to the native state. Refolding was initiated by a sudden pH jump (from 2 to 8), and the reaction was monitored by a series of FTA-SOFAST-HMQC spectra recorded at a 0.1 s⁻¹ rate. Spectra acquired before injection, immediately after, and 2 min after injection are shown in Fig. 2a. The center spectrum in Fig. 2a provides a snapshot of the transient MG state under native conditions. The broad and weak signals in this spectrum are indicative of large-scale dynamics occurring on a micro- to millisecond time scale. These data are in agreement with this transient MG state being a compact, highly dynamic ensemble of conformational states. All NMR signals observed during the folding process can be assigned either to the MG or the native state. No additional peaks indicative of a significantly populated folding intermediate were detected, in agreement with previous findings (23, 26, 27). The refolding kinetics could be quantified for 92 of 121 backbone amide sites in the protein from intensity measurements of well resolved cross-peaks in the 1H-15N correlation spectra (Fig. 2b). In addition, and in contrast to the studies by Balbach et al. (23, 26, 27), the intensity decay of five cross-peaks characteristic for the MG state could be quantified. Under the experimental conditions chosen (15°C, pH 8, Ca²⁺-free), the NMR intensity decay and buildup curves can be fitted to monoexponential functions. Fitting the data to more complex, e.g., biexponential or stretched exponential functions, does not yield statistically significant improvements. The folding time constants measured for individual amide sites of the native protein and the MG state are identical (τ₁ = 109 ± 5 s) within the experimental uncertainty. The obtained folding kinetics are consistent with results from fluorescence measurements (SI Fig. 7), yielding a characteristic time constant of 110 ± 10 s for the establishment of native tertiary structure. The good agreement with the fluorescence measurements demonstrates the accuracy obtained by SOFAST real-time 2D NMR experiments.

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protein function (33, 34), or they may represent intermediate states on the protein folding pathway (35). Especially interesting are H/D exchange measurements in the so-called EX1 regime, where the measured exchange rate constants directly reflect the kinetics of the unfolding reaction at individual amide sites (32). EX1 conditions are generally reached at high pH. However, for most proteins, H/D exchange at high pH is too fast to measure by conventional 2D real-time NMR. Therefore, previous EX1 studies had to focus on a small number of well protected amide sites. It has been shown that the EX1 exchange rates measured for a few slowly exchanging amide sites in ubiquitin correspond to the rate of global unfolding observed with other biophysical methods (36). The SOFAST real-time 2D NMR method, presented here, allows extension of the H/D exchange measurements under EX1 conditions to a significantly larger number of amide sites, yielding a more comprehensive picture of the potentially heterogeneous unfolding processes along the polypeptide chain. Representative exchange curves measured for the 76-residue protein ubiquitin at pH 11.95 and 25°C are shown in Fig. 3. For all amides, even in the most protected parts of the protein, exchange time constants $\tau_{ex} < 200$ s were observed. The high quality of the data allowed accurate quantification of exchange kinetics down to $\tau_{ex} \approx 5$ s, with a detection limit of $\tau_{ex} \approx 1$ s. This time resolution proved to be sufficient to measure exchange rates for amides in secondary structural elements, whereas H/D exchange in loop regions was generally completed during the dead time of the experiment. In the top drawing of Fig. 3, the measured exchange rates are color-coded on the ribbon structure of ubiquitin. The slowest H/D exchange is observed for residues in strands I and II of the $\beta$-sheet (residues 3–5 and 15) and the central part of the $\alpha$-helix (residue 27), whereas amide hydrogens located in other parts of the molecule, comprising strands III–V, exchange much faster. Interestingly, a gradual increase in the kinetics of opening fluctuations across the $\beta$-sheet is observed, indicating noncooperative unfolding events in this $\beta$-sheet, reminiscent of a hydrophobic zipper folding mechanism (37).

Over the past 20 years, ubiquitin has been used as a model system for the study of protein stability and folding with a variety of biophysical techniques, denaturation methods, protein engineering studies, and computational approaches (38). Therefore, it is interesting to compare the results presented here with previous findings on the thermodynamic stability, folding, and unfolding kinetics of ubiquitin under different experimental conditions. H/D exchange rates measured under EX2 conditions (39, 40) provide a measure of the thermodynamic equilibrium constant $K = k_{open}/k_{close}$, rather than a kinetic rate constant as obtained under EX1 conditions. Interestingly, the residue-specific equilibrium constants also show differential stability among the secondary structural elements of ubiquitin, with notably the first two $\beta$-strands and the $\alpha$-helix being the most stable parts. The kinetic information obtained from H/D exchange measurements under EX1 conditions complements these former results, indicating that the differential thermodynamic stability observed for ubiquitin is mainly determined by the unfolding kinetics (opening rates). Differential noncooperative unfolding has also been observed by NMR studies of ubiquitin under denaturing conditions. The populations of nonnative conformational states can be enhanced by changing the temperature, pressure, or pH, or by adding alcohol cosolvents. A cold-denaturation study by Wand et al. (41, 42) shows for reverse micelle-encapsulated ubiquitin that the mixed $\beta$-sheet is progressively destabilized from the C-terminal side in the temperature range from $-20°$ to $-30°C$. In an NMR study by Cordier and Grzesiek (43), the change in hydrogen bond strength with increasing temperature was measured. The N terminus of $\beta$-strand V was found to be the least thermally stable, whereas strands I and II remain stable even at elevated temperatures. Spectral changes observed upon high-pressure denaturation at ambient temperature indicate the presence of a partially unfolded intermediate in which the C-terminal part (residues 70–76) as well as residues 33–42 and residue 8 are denatured (44). The higher stability of the secondary structural elements in the N-terminal part of ubiquitin ($\beta_1$-$\beta_4$ strand and $\alpha$-helix) is also evidenced by the observation of a partially structured state (A state) at low pH in a 60%/40% methanol/water mixture (45). In the A state, the N-terminal part of ubiquitin retains a native-like secondary structure, whereas the C-terminal part undergoes a conformational transition forming the native state to an ensemble of conformational states with high helical propensity (46). Finally, a recent time-resolved infrared (IR) study (47) revealed a complex thermal unfolding behavior of ubiquitin spanning a wide range of time scales, indicating a gradual, rather than a cooperative, unfolding process. All experimental data from both
achieve high time resolution while retaining good spectral quality. The C-terminal part, comprising \( \beta \)-strands III–V, is significantly less stable, with unfolding rates that are up to 2 orders of magnitude higher than those observed for \( \beta \)-strands I and II, and the \( \alpha \)-helix in the N-terminal part. The observed conformational dynamics under native conditions and the presence of partially unfolded conformational states may be of importance for the biological function of ubiquitin because it has been shown that incorporation of a disulfide bridge between residues 4 and 66, stabilizing the connection between strands I and V of the \( \beta \)-sheet, leads to a 70–80% decrease of activity in signaling proteolysis (48).

SOFAST real-time 2D NMR closes the gap (seconds to minutes) on the kinetic time scale (see Fig. 1), which makes possible H/D exchange measurements for a large number of amide sites at any desired pH by 2D NMR methods. The combined interpretation of H/D exchange rates measured under EX1 as well as the more common EX2 conditions presents an attractive tool to access residue-specific kinetic folding and unfolding rates of proteins under native (physiological) conditions. In concert with other NMR data, providing the same atomic resolution, e.g., spin relaxation measurements or NMR spectra recorded under denaturing conditions, H/D exchange measurements will help us to gain a deeper insight into the nature of partially unfolded conformational states present under native conditions and the rates of interconversion between those excited states and the native state.

In summary, we have demonstrated that SOFAST real-time 2D NMR allows the accurate measurement of kinetic rate constants up to \( \sim 1 \text{s}^{-1} \) simultaneously for a large number of amide sites in a protein. The time frame ranging from a few seconds to roughly a minute used to be inaccessible to conventional 2D NMR methods. We have illustrated the potential of SOFAST real-time 2D NMR to provide accurate residue-specific kinetic information on protein folding and unfolding events either by direct real-time studies of folding/unfolding or indirectly by real-time measurements of H/D exchange kinetics. The kinetic rates measured for the refolding of \( \alpha \)-lactalbumin are uniform throughout the protein, indicating a smooth energy landscape for the transition from the MG to the native state. On the contrary, the kinetic data collected for the protein ubiquitin indicate a highly noncooperative unfolding behavior under native conditions, in agreement with previous results from NMR and IR spectroscopy. The methodology presented here can be easily applied to protein systems with molecular masses ranging between \( \sim 5 \) and \( \sim 20 \text{kDa} \) where deuteration is not a prerequisite for obtaining well resolved \( ^{1} \text{H} - ^{15} \text{N} \) correlation spectra. Here, we have focused on the application of SOFAST real-time 2D NMR to the study of protein folding and unfolding, but the method also offers opportunities for residue-specific kinetic measurements of other unidirectional events occurring on a time scale of seconds, such as binding, enzymatic reactions, and chemical exchange. Combined with recent advances toward NMR studies of proteins inside intact living cells (49, 50), SOFAST real-time 2D NMR may also become a powerful experimental tool for in situ time- and site-resolved observation of kinetic events, such as protein folding and post-translational modifications in a cellular environment.

Materials and Methods

SOFAST Real-Time 2D NMR. All NMR experiments were performed on an INOVA spectrometer (Varian, Palo Alto, CA) operating at 800-MHz \( ^{1} \text{H} \) frequency and equipped with a cryogenic probe. To keep the dead time as short as possible, the acquisition of the first spectrum was started after the dead time of the mixing required to stabilize the fluid turbulence, which was \( \sim 2 \text{s} \) (see SI Fig. 6). To achieve high time resolution while retaining good spectral quality without the need of advanced spectral processing, we used alternate phase cycling in subsequent spectra as follows. Each experiment was performed with only one scan per increment in \( t_{1} \), ensuring highest repetition rates of experiments, and the sign of the \( ^{15} \text{N} \) excitation pulse phase and receiver phase in FTA-SOFAST-HMQC was alternated between subsequent experiments. Adding two succeeding spectra (1 and 2, 2 and 3, 3 and 4, . . . ) along the kinetic time dimension removes spectral artifacts such as axial peaks or \( t_{1} \) noise at the water frequency and improves the base line, allowing a more accurate and precise measurement of spectral parameters, such as peak positions and intensities. This procedure does not change the kinetic information contained in the data and the time resolution as illustrated in SI Fig. 8. The only price to pay is an effective dead time that is longer by half the duration of one basic 2D experiment. All data were extracted as peak intensities and fit to a three-parameter exponential function. Uncertainties in the obtained fit parameters were estimated from Monte Carlo simulations by using 100 data sets generated by varying the intensity value of each data point within three times the spectral noise.

Real-Time Folding of Bovine \( \alpha \)-Lactalbumin. Nitrogen-15 labeled bovine \( \alpha \)-lactalbumin (M90V) was expressed and purified as described previously (51). The MG state was prepared by dissolving 8.8 mg of protein in 350 \( \mu \text{L} \) of \( \text{H}_{2} \text{O} / \text{D}_{2} \text{O} \) (10:1) and adjusting the pH to 2.0 with 1 M HCl solution. Fifty microliters of a refolding buffer solution [\( \text{H}_{2} \text{O} / \text{D}_{2} \text{O} \) (10:1)] containing 800 mM Tris and 80 mM EDTA (pH 9.1) was loaded in the injection device, and refolding was initiated inside the spectrometer by rapid injection into the protein solution. The folding reaction was followed by a series of FTA-SOFAST-HMQC experiments of 10.9-s duration with a scan time of 130 ms and 40 complex data points in \( t_{1} \) (80 scans per 2D spectrum). The final pH measured after the folding reaction was 8.0, and the final protein concentration was 1.55 mM. NMR assignments were taken from ref. 27. The reproducibility of the real-time folding data has been evaluated by repeating the experiment twice. The difference in the rate constants obtained from the two measurement series is smaller than the experimental error estimated from Monte Carlo simulations of a single data set.

Amide Hydrogen Exchange Measurements in Ubiquitin. For the SOFAST real-time 2D NMR amide hydrogen exchange measurements, 2.5 mg of human ubiquitin was dissolved in 50 \( \mu \text{L} \) of \( \text{H}_{2} \text{O} \) buffer containing 50 mM glycine and loaded into the injection device. H/D exchange was initiated by injection of the protein solution into 350 \( \mu \text{L} \) of D\(_{2}\)O buffer, resulting in a final protein concentration of 0.7 mM. The resulting pH, corrected for the electrode isotope effect, was 11.95. The decay of peak intensities caused by exchange was followed by recording a series of FTA-SOFAST-HMQC spectra of 6.7-s duration with a scan time of 112 ms, and recording 30 complex data points (60 scans). NMR assignments at pH 11.95 were obtained from published data at pH 6.6 (52), and a series of \( ^{1} \text{H} - ^{15} \text{N} \) SOFAST-HMQC spectra was recorded in the pH range 7–12.

SOFAST NMR makes use of longitudinal amide \( ^{1} \text{H} \) relaxation enhancement to increase the experimental sensitivity for high repetition rates of the experiment (10, 14). Shorter spin-lattice relaxation times are achieved by using amide-proton selective radiofrequency pulses that leave the polarization of other \( ^{1} \text{H} \) spins, e.g., aliphatic and water \( ^{1} \text{H} \), unperturbed. The energy put into the system is then efficiently dissipated within the dipolar-coupled \( ^{1} \text{H} \) spin network by spin diffusion (NOE) effects. In the course of H/D exchange monitored by real-time 2D NMR, amide hydrogens are progressively exchanged for deuterons. This exchange alters the \( ^{1} \text{H} \) spin coupling network in the protein from one 2D experiment to the other, which influences the relaxation properties of the amide \( ^{1} \text{H} \) spins and raises the question about the accuracy of the measured exchange rates by using SOFAST real-time 2D NMR. We have addressed this issue by both
experiment and computer simulations. The results, shown in SI Fig. 9, indicate that the systematic error introduced by the changing $^1$H spin network remains small compared with the statistical uncertainty and can be safely neglected.

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Supporting Information

Files in this Data Supplement:
- SI Figure 4
- SI Figure 5
- SI Figure 6
- SI Figure 7
- SI Figure 8
- SI Figure 9

Fig. 4. Fluid turbulence-adapted band-selective optimized flip-angle short transient heteronuclear multiple quantum coherence (FTA-SOFAST-HMQC) experiment used for SOFAST real-time 2D NMR measurements. The small angle (\(<90^\circ\)) \(^1\)H pulse (filled symbol) is applied with a PC9 shape (1), whereas for the \(^1\)H 180° pulses (open symbol) an iSNOB-5 shape (2) is used. The angle is typically set between 40° and 60° depending on the scan time chosen. The transfer delays \(D\) are set to \(1/(2J_{\text{HN}})\), and the small delay \(d\) takes into account spin evolution during the PC9 pulse and is adjusted to half of the PC9 pulse length. For \(^{15}\)N chemical shift editing in \(t_1\), the basic sequence is repeated \(n\) times using short interscan delays (between 1 and 50 ms) resulting in scan times of \(~100\) ms. Heteronuclear decoupling is performed by using WURST-40 (3). The pulsed-field gradients (PFG\(_Z\)) are applied for 100 ms with a strength of typically 30 G/cm (1 G = 0.1 mT). In the original SOFAST-HMQC pulse sequence (4, 5), the delay between the gradients encloses the indirect evolution time \(t_1\), with typical maximum values of 20-30 ms. Therefore, turbulence of the solution, which stems from rapid injection, leads to incomplete refocusing and thus to loss of magnetization, which means that the signal in a series of experiments builds up only slowly after injection (see SI Fig. 6), a fact that clearly compromises spectral quality and interpretation of kinetic data. In contrast, in the FTA-SOFAST-HMQC sequence shown here, the pulsed-field gradients are moved to the end of the pulse sequence, and an additional \(^1\)H refocusing pulse is added. While retaining excellent water suppression and high sensitivity, this modification reduces the time delay between gradients to 1-2 ms. The pulse sequence code in Varian language is available from the authors on request.

Fig. 5. Practical implementation of rapid mixing inside the NMR spectrometer. (a) Sketch of the fast injection device used for SOFAST real-time 2D NMR applications. (b) Photographs of the NMR sample tube taken after fast injection of 50 ml of blue dye into 350 ml of water with a rapid injection device. Homogeneous mixing is completed within \(\sim100\) ms.
**SI Figure 6**

**Fig. 6.** Buildup of signal intensity after rapid sample injection measured by a gradient-echo NMR pulse sequence with different dephasing times. Fifty microliters of a 2 M glycine solution (in D$_2$O) was injected in 350 ml of D$_2$O, and the signal intensity of the glycine Ha peak was followed as a function of time by using a 1D gradient spin-echo pulse sequence (90° pulse-PFG$_z$-t/2-180° pulse-t/2-PFG$_z$-acquisition). This sequence is sensitive to translational diffusion effects and thus to the fluid turbulences created by the fast injection. It mimics the situation found in more complex NMR experiments such as SOFAST-HMQC. Results are shown for dephasing times of $t = 20$ ms (filled squares), corresponding to the situation in standard SOFAST-HMQC, and $t = 1$ ms (open circles), corresponding to the situation in FTA-SOFAST-HMQC. Stable conditions are reached in less than ~2 s with the short dephasing time, whereas ~15 s elapse until equilibrium is reached with the longer delay, emphasizing the interest of FTA-SOFAST-HMQC when using a rapid injection device.

**SI Figure 7**

**Fig. 7.** Comparison of folding kinetics of $\alpha$-lactalbumin measured by fluorescence (black squares) and SOFAST real-time 2D NMR spectroscopy (red circles). The protocol used for the fluorescence measurements (protein concentration, pH jump, solvent) is identical to that used for the time-resolved NMR experiments, except for the initiation of the folding reaction. For the fluorescence measurements, manual mixing yielded a dead time of ~1 min. The excitation wavelength is 295 nm (1-nm bandwidth) and the fluorescence change is monitored at 340 nm (3-nm bandwidth). Nonoptimal sample volume and the resulting small detection window, as well as autoquenching effects caused by the high sample concentration, explain the relatively low signal-to-noise ratio obtained by the fluorescence experiment. A high protein concentration was chosen to mimic the NMR conditions, resulting in a small volume for the available amount of 2 mg of $^{15}$N-labeled protein. The same recombinant $^{15}$N-labeled $\alpha$-lactalbumin has been used for NMR and fluorescence experiments because the recombinant protein (expressed in *Escherichia coli*) is less stable than the natural protein, and this has an effect on the refolding kinetics (6). The experimental data were fitted to monoexponential decay shown as red and black lines for NMR and fluorescence data, respectively.

**SI Figure 8**

**Fig. 8.** Additional spectral processing used in SOFAST real-time 2D NMR. (a) Coaddition of subsequent 2D spectra using alternate phase cycling. (*Left* and *Center*) First two FTA-SOFAST-HMQC spectra recorded at time 0 and $D_t$ after injection of ubiquitin into a H$_2$O solution. The phases of the $^{15}$N excitation pulse and the receiver have been alternated for subsequent experiments. As a result of this phase cycling, artifacts have opposite sign in the two spectra, whereas the signals of interest are of the same sign. Addition of the two spectra thus removes the artifacts (*Right*). If the series of recorded spectra is replaced by the sum of successive spectra, e.g. (1 + 2), (2 + 3), (3 + 4), ..., this results in cleaner spectra without affecting the time resolution of the kinetic data. (b) Effect of this coaddition approach on the extracted rate constants for monoexponential intensity changes. The black solid line represents the exponential change in peak intensity that is sampled at a rate determined by the acquisition time of one spectrum, as indicated by black circles. Coadding two successive spectra corresponds to replacing two data points by their linear average and assigning this
summed value to the central time point in between the experimentally measured data points. These data points (which for reasons of clarity were scaled in the figure) are indicated as green circles, and the linear approximation connecting the experimental data points is indicated by black straight lines. The monoexponential rate determined by these coadded data points corresponds exactly to the actual rate, which is explained by the fact that these data points are shifted by a constant offset independent of time.

SI Figure 9

**Fig. 9.** Evaluation of the accuracy of measured H/D exchange time constants $t_{\text{ex}}$ using SOFAST real-time 2D NMR. (a) $t_{\text{ex}}$ values measured by using the new FTA-SOFAST-HMQC sequence of SI Fig. 4 are compared with results obtained by using standard HSQC-based 2D NMR methods. H/D exchange was monitored for 24 h for ubiquitin at 25°C at two different pH values: 7.3 (black circles) and 9.8 (red circles). HSQC (experiment time, 3 min 15 s; recycle delay, 0.9 s) and FTA-SOFAST-HMQC (experiment time, 25 s; recycle delay, 50 ms) spectra were recorded in an interleaved manner resulting in a 0.14 min$^{-1}$ sampling rate of the exchange process. H/D exchange was initiated as explained in "Materials and Methods." Under these experimental conditions, the data recorded at pH 9.8 (red circles) represent the 15 most slowly exchanging amides, whereas the data measured at pH 7.3 allowed quantification of the exchange rates for less protected amide sites. The good correlation of these data (correlation coefficient of $R = 0.997$) illustrates the high accuracy obtained by the SOFAST approach despite the changing $^1$H spin-coupling network arising from the partial deuteration of amide sites during the exchange process. (b) The experimental results shown in (a) may be rationalized by computer simulations. The buildup of amide $^1$H polarization has been calculated for ubiquitin by numerical integration of the Solomon equations describing the spin dynamics in the dipolar-coupled $^1$H network (7) assuming a magnetic field strength of 18.8 T and different rotational correlation times $t_c$ of the protein. Two extreme cases have been considered: (i) all backbone amide sites are protonated (solid curves), and (ii) only the observed amide site is protonated while all others are deuterated (dashed curves). The result of these simulations is that polarization recovery is slightly faster for the second case. This can be understood by the fact that fewer amide protons are excited in the molecule, and therefore less energy has to be evacuated from the spin system to restore the equilibrium polarization. Nevertheless, for the fast-pulsing conditions used here (indicated by an arrow) and the tumbling correlation time of ubiquitin at 25°C ($t_c = 4$ ns), the difference in the restored polarization is negligible (<1%). For larger proteins, the difference increases (see curves obtained for $t_c = 15$ ns) because of the more efficient spin diffusion. However, if higher repetition rates (shorter recovery times) are used for such large proteins, the difference becomes again negligible.

Fig. 4. FTA-SOFAST-HMQC experiment used for SOFAST real-time 2D NMR measurements. The small angle ($\alpha < 90^\circ$) $^1$H pulse (filled symbol) is applied with a PC9 shape (1), while for the $^1$H 180° pulses (open symbol) an iSNOB-5 shape (2) is used. The $\alpha$ angle is typically set between 40° and 60° depending on the scan time chosen. The transfer delays $\Delta$ are set to 1/(2J_{HN}), and the small delay $\delta$ takes into account spin evolution during the PC9 pulse, and is adjusted to half of the PC9 pulse length. For $^{15}$N chemical shift editing in $t_1$, the basic sequence is repeated n-times using short inter-scan delays (between 1 and 50 ms) resulting in scan times of ~100ms. Heteronuclear decoupling is performed using WURST-40 (3). The pulsed field gradients (PFG$_z$) are applied for 100 µs with a strength of typically 30 G/cm. In the original SOFAST-HMQC pulse sequence (4, 5), the delay between the gradients encloses the indirect evolution time $t_1$, with typical maximum values of 20-30ms. Therefore, turbulence of the solution, that stems from rapid injection leads to incomplete refocusing and thus to loss of magnetization. This means that the signal in a series of experiments builds up only slowly after injection (see SI Fig 6), a fact that clearly compromises spectral quality and interpretation of kinetic data. In contrast, in the FTA-SOFAST-HMQC sequence shown here, the pulsed field gradients are moved to the end of the pulse sequence and an additional $^1$H refocusing pulse is added. While retaining excellent water suppression and high sensitivity this modification reduces the time delay between gradients to 1-2 ms. The pulse sequence code in Varian language is available from the authors upon request.
Fig. 5. (a) Sketch of the fast injection device used for SOFAST real-time 2D NMR applications. (b) Photographs of the NMR sample tube taken after fast injection of 50 µL blue dye into 350 µL of water using a rapid injection device. Homogeneous mixing is completed within approximately 100 milliseconds.
Fig. 6. Buildup of signal intensity after rapid sample injection measured by a gradient-echo NMR pulse sequence using different dephasing times. 50 µL of a 2M glycine solution (in D₂O) were injected in 350 µL D₂O and the signal intensity of glycine's Hα-peak was followed as a function of time using a 1D gradient spin-echo pulse sequence (90° pulse – PFGz – τ/2 - 180° pulse – τ/2 – PFGz – acquisition). This sequence is sensitive to translational diffusion effects, and thus to the fluid turbulences created by the fast injection. It mimics the situation found in more complex NMR experiments such as SOFAST-HMQC. Results are shown for dephasing times of τ = 20 ms (filled squares), corresponding to the situation in standard SOFAST-HMQC, and τ = 1 ms (open circles), corresponding to the situation in fluid-turbulence-adapted FTA-SOFAST-HMQC. Stable conditions are reached in less than ~2 s using the short dephasing time, while ~15 s elapse until equilibrium is reached when using the longer delay, emphasizing the interest of FTA-SOFAST-HMQC when using a rapid injection device.
Fig. 8. Comparison of folding kinetics of α-lactalbumine measured by fluorescence (black squares) and SOFAST real-time 2D NMR spectroscopy (red circles). The protocol used for the fluorescence measurements (protein concentration, pH-jump, solvent...) is identical to that used for the time-resolved NMR experiments, except for the initiation of the folding reaction. For the fluorescence measurements manual mixing yielded a dead time of about 1 minute. The excitation wavelength is 295 nm (bandwidth: 1 nm) and the fluorescence change is monitored at 340 nm (bandwidth: 3 nm). Non-optimal sample volume, and the resulting small detection window, as well as auto-quenching effects because of the high sample concentration explain the relatively low signal-to-noise ratio obtained by the fluorescence experiment. A high protein concentration was chosen to mimic the NMR conditions, resulting in a small volume for the available amount of 2 mg $^{15}$N-labeled protein. The same recombinant $^{15}$N-labeled α-lactalbumin has been used for NMR and fluorescence experiments because the recombinant protein (expressed in E. Coli) is less stable than the natural protein, and this has an effect on the refolding kinetics (6). The experimental data were fitted to mono-exponential decay shown as red and black lines for NMR and fluorescence data, respectively.
Fig. 7. Additional spectral processing used in SOFAST real-time 2D NMR. (a) Co-addition of subsequent 2D spectra using alternate phase cycling. Left and central panel: first two FTA-SOFAST-HMQC spectra recorded at time 0 and $\Delta t$ after injection of ubiquitin into a $\text{H}_2\text{O}$ solution. The phases of the $^{15}\text{N}$ excitation pulse and the receiver have been alternated for subsequent experiments. As a result of this phase cycling, artifacts have opposite sign in the two spectra, whereas the signals of interest are of the same sign. Addition of the 2 spectra thus removes the artifacts (right panel). If the series of recorded spectra is replaced by the sum of successive spectra, e.g. $(1+2), (2+3), (3+4), \ldots$, this results in cleaner spectra without affecting the time resolution of the kinetic data. (b) Effect of this co-addition-approach on the extracted rate constants for mono-exponential intensity changes. The black solid line represents the exponential change in peak intensity that is sampled at a rate determined by the acquisition time of one spectrum, as indicated by black circles. Co-adding two successive spectra corresponds to replacing two data points by their linear average and assigning this summed value to the central time point in between the experimentally measured data points. These data points (which for
**Fig. 9.** Evaluation of the accuracy of measured H/D exchange time constants $\tau_{ex}$ using SOFAST real-time 2D NMR. (a) $\tau_{ex}$ values measured using the new FTA-SOFAST-HMQC sequence of Fig. 4 are compared to results obtained using standard HSQC-based 2D NMR methods. H/D exchange was monitored during 24 hours for ubiquitin at 25°C at two different pH values: 7.3 (black circles), and 9.8 (red circles). HSQC (expt. time: 3 min 15 s, recycle delay: 0.9 s) and FTA-SOFAST-HMQC (expt. time: 25 s, recycle delay: 50 ms) spectra were recorded in an interleaved manner resulting in a 0.14 min$^{-1}$ sampling rate of the exchange process. H/D exchange was initiated as explained in the Methods section. Under these experimental conditions, the data recorded at pH 9.8 (red circles) represent the 15 most slowly exchanging amides, whereas the data measured at pH 7.3 allowed quantifying the exchange rates for less protected amide sites. The good correlation of these data (correlation coefficient of $R=0.997$) illustrate the high accuracy obtained by the SOFAST approach despite the changing $^1$H spin coupling network arising from the partial deuteration of amide sites during the exchange process. (b) The experimental results shown in (a) may be rationalized by computer simulations. The buildup of amide $^1$H polarization has been calculated for ubiquitin by numerical integration of the Solomon equations describing the spin dynamics in the dipolar-coupled $^1$H network (7) assuming a magnetic field strength of 18.8 T, and different rotational correlation times $\tau_c$ of the