Monitoring the Kinetics of Ion-Dependent Protein Folding by Time-Resolved NMR Spectroscopy at Atomic Resolution

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Abstract: The kinetics of protein refolding have been monitored by time-resolved NMR spectroscopy. It is shown that refolding of metal binding proteins can be induced by photolysis of photo labile ion chelators, the subsequent release of Ca^{2+} ions can induce protein folding, and the changes in resonance positions can be monitored by time-resolved NMR spectroscopy. The feasibility of the approach is demonstrated by characterizing the refolding of α-lactalbumin, or protein containing a Ca^{2+} binding site, unfolded in 4 M urea at pH 7 in the absence of Ca^{2+}. The refolding kinetics of the methyl groups of residues Leu15 and Val21 in the core of the protein have been determined to be mono-exponential with rates of 0.28 s^{-1} and 0.23 s^{-1}, respectively at 300 K.

Introduction

A variety of different spectroscopic techniques has been developed to study the kinetics of protein folding. Recently, also direct NMR spectroscopic detection of the kinetics of protein refolding has been reported. Experimental approaches have been developed to induce folding by rapidly injecting high concentrations of unfolded protein into folding buffer[1] or by mixing unfolded protein and folding buffer in specially designed NMR probes.[2]

An alternative approach is to initiate protein folding[3] by exploiting the differences in protein stability in the presence and absence of stabilizing cofactors[4] such as Ca^{2+} and Mg^{2+}.[5,6] Ion-dependent protein folding steps can be initiated by rapid release of Ca^{2+} ions caged in photolabile calcium cage compounds[7] like the EDTA-analogue DM-nitrophen which has a $K_D = 5 \times 10^{-9}$ M for Ca^{2+} binding.[8,9] This approach involves the combination of the application of short laser pulses[10] with the subsequent observation of an NMR signal. The following criteria have to be met to follow protein refolding by time-resolved NMR spectroscopy:

- The release time of the caged compound is a property of the chelator and should be as short as possible.
- The irradiation time $\tau_{irradiate}$ is a property of the sample geometry, the concentration of the caged compound in solution and the power output of the laser at the wavelength of the absorption maximum of the photo labile compound (in W). $\tau_{irradiate}$ has to be shorter or of the order of the folding time.
- Conditions have to be found in which the protein conformation can be triggered by the rapid release of the caged ion. This includes that the dissociation constant of the chelator/ion complex is orders of magnitude lower than the dissociation constant of the protein/ion complex.
- The released byproducts of the uncaged chelator must not interfere with the folding to be studied.

Here we present the first experimental data in which the kinetics of protein refolding have been characterized by coupling rapid ion release to high-resolution NMR spectroscopy. We have studied the refolding of α-lactalbumin as a model system. Calcium free α-lactalbumin is unfolded in 4 M urea, while the Ca^{2+} bound form is nativelike under these conditions as evidenced by NMR and circular dichroism (CD) spectra.

The developed general approach should allow application of time-resolved NMR spectroscopy to the characterization of folding steps or reactions that can be triggered using photochemical reactive caged compounds.

Material and Methods

A number of different photolabile ion chelators[11] have been developed. We have chosen DM-nitrophen for our studies because of its favorable photochemical properties (compare Figure 1).[12] The

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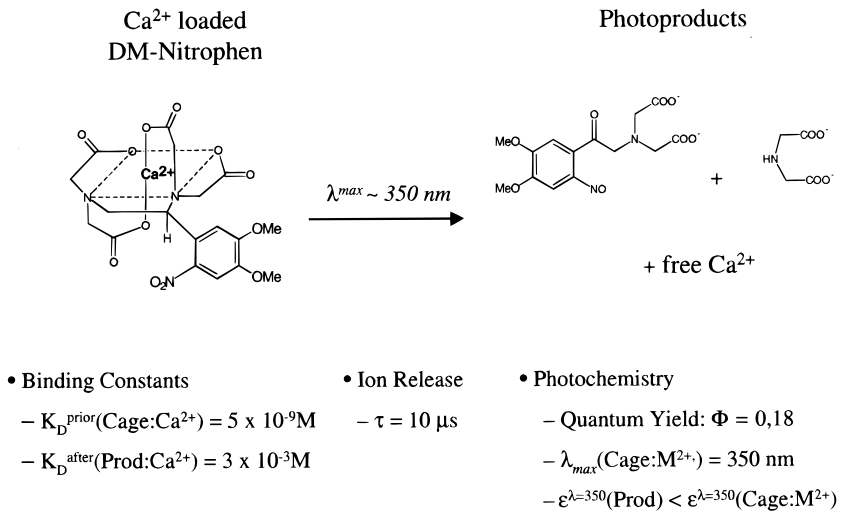

Figure 1. Photochemical properties and dissociation constants of DM-nitrophen and its photo products after laser irradiation.

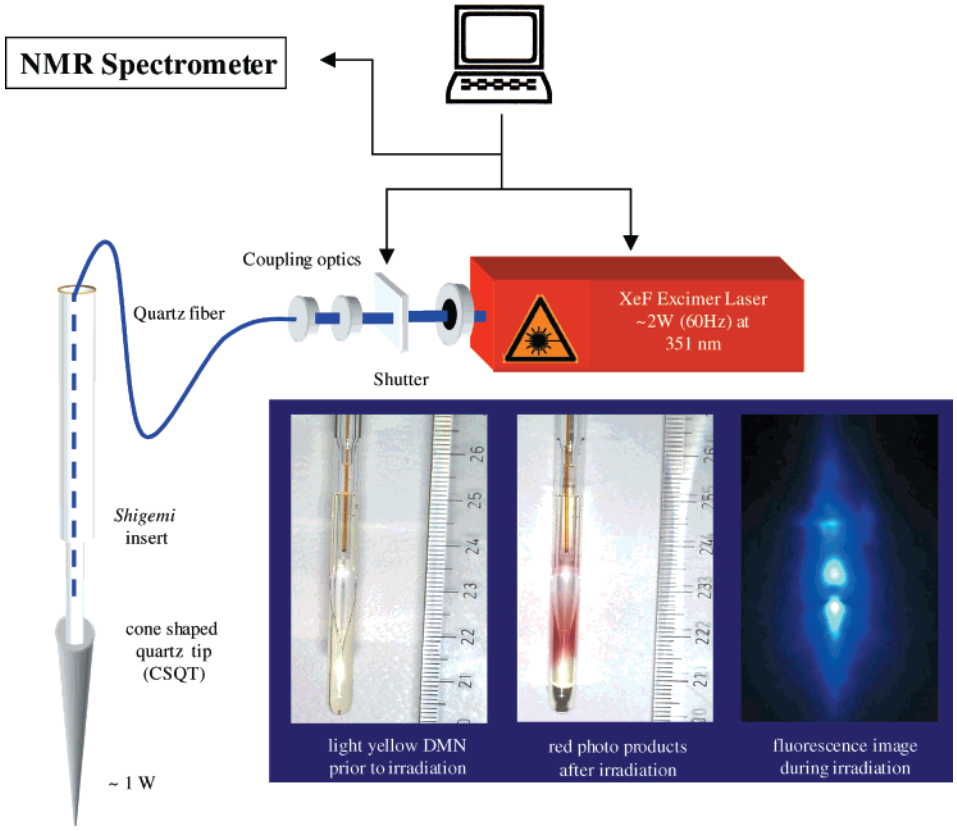

Figure 2. Setup for the laser−NMR coupling. As a light source a Lambda Physik EMG 101 MSC excimer laser was used with a XeF gas mixture. The emitted wavelength was 351 nm. The laser pulse energy 35 mJ per 20 ns pulse at 60 Hz. The laser output was coupled into the sample in the NMR tube via a quartz fiber optic. The coupling attenuation was less than 45%. Laser pulses or an electronic shutter respectively were controlled by a TTL signal from the spectrometer console.

dissociation constant of DM-nitrophen is sufficiently small to bind Ca^{2+} in the presence of α-lactalbumin (K_D^{prior}(Cage:Ca^{2+}) = 5×10^{-9} M) and the K_D of the photo products after cleavage is 6 orders of magnitude higher (K_D^{after}(Prod:Ca^{2+}) = 3×10^{-3} M). The ion release time is short compared to the NMR acquisition period of approximately 100 ms. The quantum yield for photocleavage is smaller than that for 4-(2-nitrophenyl)-3,13-bis-[(ethoxycarbonyl)methyl]-6,9-dioxa-3,12-diaza-tetradecanedioic acid (nitrophenyl-EGTA) (Φ = 0.20). A prominent absorption maximum of DM-nitrophen is near 351 nm, while the absorption maximum of the photo products is near 290 nm and the absorption coefficient of the photo products at 351 nm is smaller than for DM-nitrophen.[13] A modified synthesis of DM-nitrophen is described in detail in the Supporting Information.

Laser Setup. In the laser experiment shown schematically in Figure 2, a Lambda Physik EMG 101 MSC excimer laser filled with a Xe/F_2 gas mixture was used to tune the emitted wavelength to 351 nm. The 60 Hz pulsed laser beam was guided through a self-made electronically controlled shutter and was focused on a multimode silica/silica fiber (1500 µm core, 1800 µm cladding, CERAM OPTIC). For coupling of the pulsed laser beam into the NMR tube we used a cone-shaped quartz tip (CSQT) that was mounted to a fiber guiding quartz tube of 4.8 mm diameter, which itself was attached inside a standard 5 mm H_2O/D_2O

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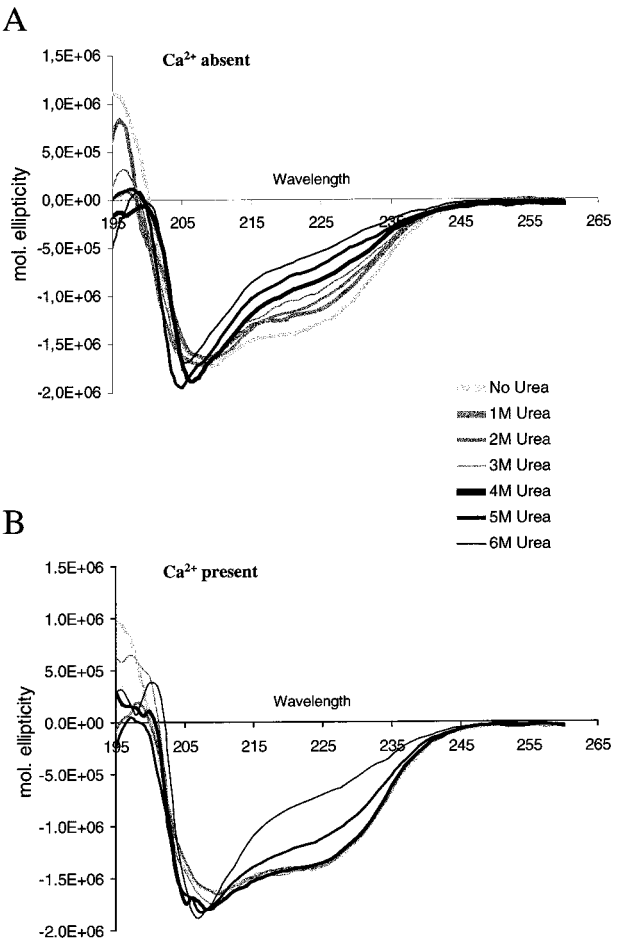

Figure 3. CD titration spectra of bovine α-lactalbumin and increasing concentrations of urea at pH 7.0 in the presence and absence of calcium ions. The protein remains structured in the presence of equimolar amounts of Ca^{2+} even in 4 M urea. In the absence of Ca^{2+}, α-lactalbumin is unfolded at a concentration of 4 M urea at pH 7. Due to the urea absorbance below about 210 nm, spectra in the presence of urea can only be interpreted for wavelengths above 210 nm.

Shigemi NMR tube. The coupling efficiency between the CSQT and the fiber was optimized using the fluorescence image of the scattered light. Losses due to scattering were minimized by using methanol as a mediator between the diffraction indices of the two quartz types (fiber and tip). The CSQT was completely covered by the solution and was fixed to the tube in such a way that the end of the tip was located 2 mm below the center of the rf coil. Gradient shimming was applied to the setup after a gradient map for each specific sample was recorded. In irradiation experiments performed outside the NMR spectrometer, no diffusion of the red photo products was observed over several seconds (compare Figure 2, after irradiation). Diffusion of released calcium occurring during the experiment should therefore be negligible.

CD Study of α-Lactalbumin. Urea titration experiments followed by CD of bovine milk α-lactalbumin (Fluka) were performed on a JASCO J710 CD spectrometer. To perform equilibrium urea titration experiments on Ca^{2+} free and Ca^{2+} loaded α-lactalbumin, the protein was separated by ultrafiltration using a YM1000 Amicon membrane and washed three times with 50 mL of a desalting solution of 50 mM EDTA in 100 mM Tris/HCl pH 7.0. The protein was filtered five times in 50 mL of 100 mM Tris/HCl at pH 7.0 to equilibrate in EDTA free buffer. A 100 μM solution of this Ca^{2+} free protein was used for urea titration CD experiments in 0−6 M urea and 100 mM tris/HCl at pH 7.0. To study the Ca^{2+}-loaded form of α-lactalbumin, solutions of 100 μM Ca^{2+} free α-lactalbumin in 200 μM $CaCl_2$ and 100 mM Tris/HCl at pH 7.0 with urea concentrations varying from 0 to 6 M were prepared. The results are shown in Figure 3. All of the scanning near UV CD experiments were recorded using a 0.01 cm quartz cuvette.

Stopped flow CD and fluorescence data were acquired on an Applied Photophysics π* 180 stopped flow spectrometer at 300 K and with final concentrations (after mixing) of 100 μM α-lactalbumin in 4 M urea, 100 mM Tris/HCl at pH 7.0 and 200 μM $CaCl_2$ which was obtained by mixing of 100 μM α-Lactalbumin in 4 M urea and 100 mM Tris/HCl at pH 7.0 against 200 μM $CaCl_2$ in 4 M urea and 100 mM Tris/HCl at pH 7.0. Urea background spectra were subtracted from the kinetic traces. The general buffer was 100 mM Tris/HCl at pH 7 to ensure the same conditions as in the NMR experiments.

Results and Discussion

CD Titration Experiments. α-Lactalbumin in its Ca^{2+} free form is unstructured in 4 M urea at pH 7.0, whereas the presence of calcium stabilizes the native state of the protein. As can be seen in Figure 3a the molar ellipticity increases with the increase of the urea concentration around 222 nm in the absence of calcium, indicating a decrease in α-helicity. With increasing urea concentration in the calcium free form, α-lactalbumin becomes less structured. This is clearly not the case in the presence of calcium (Figure 3b). Up to a concentration of about 4 M urea, the CD spectra do not differ significantly from the Ca^{2+} free form of the protein in the absence of urea. The largest structural change, according to CD data, will be expected upon addition of equimolar amounts of calcium to an α-lactalbumin solution in 4 M urea at pH 7.

Laser-Coupled NMR Spectroscopy. In a standard 5 mm Shigemi H_2O tube 0.88 mg of α-lactalbumin (0.5 mM) was dissolved in 250 μl of 4 M urea solution in 90% H_2O/10% D_2O, 2 mM DM-nitrophen, and 1.5 mM $CaCl_2$ in 100 mM Tris/HCl at pH 7.0. The laser/NMR coupling was performed as described above. The experiments were performed on a Bruker DRX 600 MHz spectrometer equipped with a *z* gradient TXI probe. The signal-to-noise ratio of this probe measured by using standard ethylbenzene (thin wall tube) is greater than 1250:1. The laser/lightguide coupling was optimized as well as the lightguide/CSQT joint with respect to the emitted light energy. The following experiments were carried out at 300 K.

The NMR spectrum of the methyl group region of the protein prior to calcium release is shown in the lower part of Figure 4. The spectrum is characteristic of an unfolded protein: the methyl groups (and the amide region, not shown) resonate at the random coil shifts, and the observed peaks are sharp. The upper part of Figure 4 shows the single scan NMR spectrum after release of calcium ions in the spectrometer with individual resonance positions assigned. The folding transitions between the two forms are reversible. Addition of EDTA after photo-destruction of the DM-nitrophen unfolds the protein as evidenced by NMR spectra after each kinetic experiment. α-Lactalbumin could be recovered in its native form by extensive dialysis and proved to be unmodified according to ESI-MS spectral analysis. The effects of the photo products of DM-nitrophen on the folding kinetics of α-lactalbumin were not specifically tested. However, since 1D NMR spectra of the protein, folded by calcium released from photocleavage are indistinguishable from those of α-lactalbumin folded by calcium addition prior to the experiment, we do not expect changes in refolding kinetics in the presence or absence of submillimolar amounts of photo products. Temperature changes during laser irradiation were not measurable using the standard Bruker temperature control unit in the probe, and the lock signal dropped only marginally, if at all, during irradiation.

For the kinetic NMR experiment, the following sequence has been recorded. A single scan jump and return echo FID[14] was

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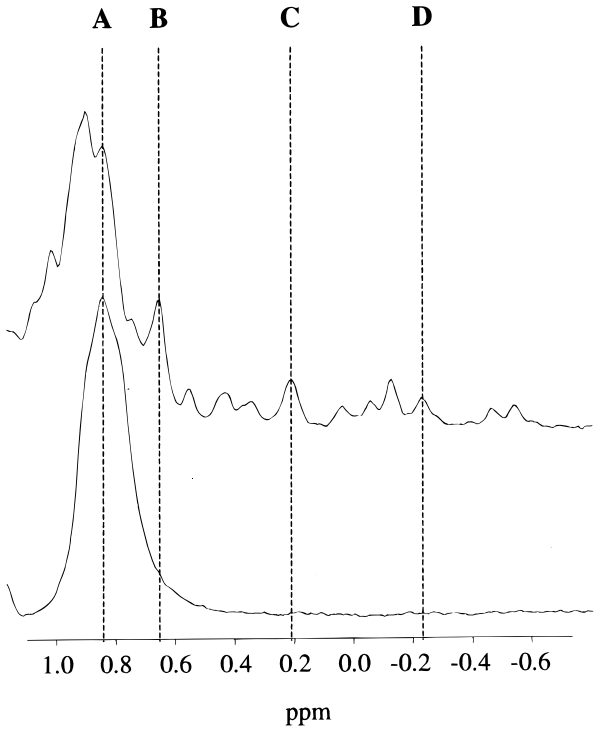

Figure 4. (Bottom) Single scan 1D NMR spectrum of the methyl group region of the denatured form of α-lactalbumin in 4 M urea with access of "caged calcium" prior to irradiation (initial state). The spectrum of the protein is identical to the 1D spectrum of α-lactalbumin in calcium-free buffer in 4 M urea. (Top) Single scan NMR spectrum of the methyl group region of refolded α-lactalbumin after irradiation and Ca^{2+} release. It can be seen that distinct peaks are arising in the Me-group region. The spectrum is identical to the protein spectrum in 4 M urea in the presence of calcium. A, B, C, and D indicate the peaks for which individual refolding kinetics are discussed in text and Figure 7.

acquired (further referred to as *dark_1*) as a reference experiment. After a relaxation delay of 1.5 s, the sample was irradiated with a wavelength of 351 nm by 30 laser pulses with a rate of 60 Hz and a pulse duration of 20 ns each, the high voltage at the laser electrodes was set to 24.6 kV. The total irradiation time $\tau_{\text{irradiate}}$ was therefore 500 ms. This relatively long irradiation period prooved to be unproblematic since protein folding can still be detected for several seconds thereafter. Also no major phase shifts between the rising single residue methyl peaks and the decaying intensity of the methyl bulk peak could be detected, as they would be expected if the rate constants of refolding increase and thereby induce a frequency change during acquisition.[15] Therefore we conclude that the reaction is too slow to result in a major signal change during the acquisition time and significant changes are measurable in difference experiments as described here. Nevertheless, it is desirable to work with shorter irradiation times for improved dead times; however, it proved inapplicable here due to insufficient laser energy and therefore unsatisfactory signal-to-noise ratio in experiments with shorter irradiation time. Energetic measurements before and after the NMR experiment revealed that the laser was running in a stable mode and that the pulse energy was steady during the kinetic NMR experiment. Pulse energies of 35 mJ where coupled into the fiber at the same conditions and with the same gas mixture as that used during the NMR experiment. The light energy per laser irradiation in the sample during the experiment was approximately 1 W, taking into account all of the losses due to scattering at the coupling site and attenuation in the fiber (about 50%).

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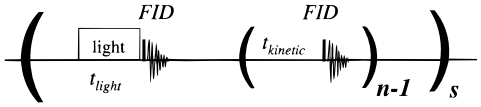

Figure 5. The pulse sequence of the time-resolved NMR experiment, including the irradiation period. Herein, t_{light} is the duration of a light pulse, t_{kinetic} is the time between two acquisitions plus the acquisition time (for best time resolution, t_{kinetic} should be as small as possible), and *n* represents the number of scans after single laser application. This inner loop (*n*) therefore contains non-equilibrium kinetic information. Each of these *n* experiments is repeated *s* times. The outer loop holds thermodynamic (equilibrium) information. Therefore, each individual signal of the inner loop represents the same state of folding after initiation by the light pulse. In this case, for example the second signal after the light always represents the state of folding after the acquisition time of the first FID plus t_{kinetic}.

Directly after the irradiation, a series of seven 1D experiments (*dark_2* to *dark_8*) were recorded. Two thousand complex data points were recorded per FID, the recovery time after each acquisition was set to 1.5 s. This experiment was repeated 20 times (*dark_9* would be identical to *dark_1* of the next series) (compare Figure 5). The resulting FIDs were transformed using the MSI program *Felix98*. The resulting 3D file was split up into the individual FIDs which are described above.

This gives rise to several sets (*s*) of series ($n = 8$) of 8 FIDs. The trace of any given spectrum (*n*) over the 20 sets yields thermodynamic information since it resembles a titration curve. This is the case since only a fraction of the Ca^{2+} is released by one laser irradiation period apparently and each of the FIDs is a sum of the fraction of folded protein and the fraction of unfolded protein. The trace over the whole series of spectra (*n*) within each set (*s*), however, provides kinetic information since it monitors the events directly following a single train of laser pulses. The signal-to-noise-ratio in such a single trace is too poor to yield reasonable kinetic data. Therefore, the kinetic traces where averaged as described below.

The individual experiments were stored as two-dimensional data arrays given in eq 1:

$$dark\_1\_mat: = \begin{pmatrix} dark\_1_1^{trans} \\ dark\_1_2^{trans} \\ \vdots \\ dark\_1^{trans} \end{pmatrix}, dark\_2\_mat: = \begin{pmatrix} dark\_2_1^{trans} \\ dark\_2_2^{trans} \\ \vdots \\ dark\_2^{trans} \end{pmatrix}, \ldots dark\_8\_mat: = \begin{pmatrix} dark\_8_1^{trans} \\ dark\_8_2^{trans} \\ \vdots \\ dark\_8^{trans} \end{pmatrix} \quad (1)$$

The subscript refers to the number of the irradiation cycle (*s*), the superscript *trans* indicates that subsequent data manipulation was performed on the Fourier transformed data set, and *dark*_1 resembles the first FID ($n = 1$) of an irradiation cycle (*s*).

Inspection of the spectra reveal the time point for which the deliberately added excess of DM-nitrophen has been consumed by the observation of the starting point of decreasing methyl group peak intensity of the unstructured form. In the experimental series shown in Figure 6 this occurs after about two laser irradiations. In the same way it is possible to determine the time point for which all of the protein is folded or for which all Ca^{2+} is released (methyl group peak intensity reaching

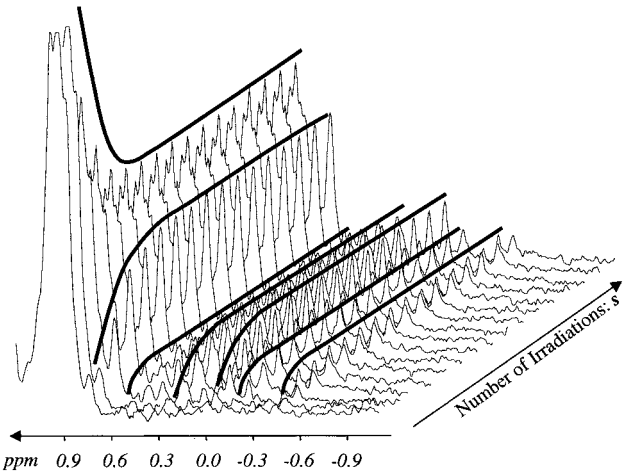

Figure 6. Equilibrium information: stack plot of the methyl group region. The 3D representation was obtained by constantly plotting the eighth spectrum after the laser pulse train as a function of the number of irradiation cycles (*s*) (only spectra of every 2nd laser irradiation are plotted here). Since only a certain amount of Ca^{2+} is released after a single laser application, the experiment represents a calcium titration experiment.

steady-state asymptotically). The part between these characteristic points where the slope of methyl group peak intensity vs number of irradiation cycles ≠ 0, we call the *relevant area*. In the three independent experiments with different samples conducted here, the relevant area extended from set $s = 2-10$, $s = 3-14$, and $s = 2-13$, respectively.

Series of spectra were subtracted, and the result was summed to improve the signal-to-noise ratio. Only spectra of irradiation cycles within the relevant area were used, thereby avoiding the unnecessary summation of noise. An explanation of this is given by eq 2:

$$diffspectrum1: = \sum_{s=2}^{10}(dark\_2_s^{trans} - dark\_1_s^{trans})$$

$$diffspectrum2: = \sum_{s=2}^{10}(dark\_3_s^{trans} - dark\_1_s^{trans}), \text{ etc.} \quad (2)$$

Herein, *s* resembles the number of the irradiation, and the relevant area is between the secone and tenth laser irradiation. A 2D matrix of the resulting difference spectra (*dark*_2 − *dark*_1 up to *dark*_8 − *dark*_1) was stored for subsequent kinetic data analysis.

Comparison of Folding Kinetics Revealed by Stopped Flow CD, Fluorescence, and NMR Spectroscopy. We were able to follow the kinetics of several distinct methyl groups and lowfield NH groups. Best fits to kinetic functions of these data reveal different time constants for individual methyl groups which indicates discriminative folding patterns in different regions of the molecule. Kinetic data is shown in Figure 7. The overall decay of the methyl-bulk peak follows a single-exponential kinetic (A → B) with $k = 0.33\ s^{-1}$ and a half time of $\tau_{1/2} = 2.10$ s (Figure 7a). The decreasing integral of the methyl-bulk peak clearly indicates the formation of secondary structure in the protein. The chemical shifts of the methyl groups start to disperse upon structural formation. Most of the other traces gain intensity over time, since they correspond to the formation of distinct structural features in the molecule. Their kinetics differ from each other as well as from the kinetics of the overall methyl peak decay. All of the clearly resolved peaks reveal mono-exponential refolding rates.

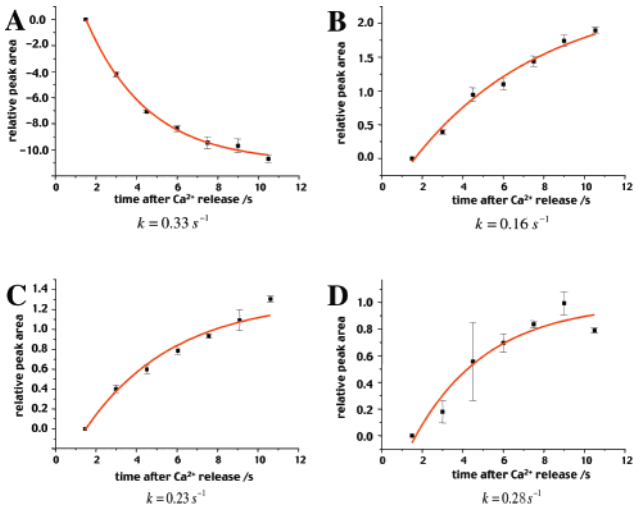

Figure 7. Kinetic traces of individual peaks in the methyl group region. Selected assigned peaks are annotated:[17] (A) decay of the methyl group bulk peak, indication of overall folding; (B) rise of the residual peak at 0.66 ppm in the methyl group area; (C) folding kinetics of the single Val21 residue in α-lactalbumin at 0.21 ppm; (D) kinetic trace for the Leu15 moiety in α-lactalbumin at −0.23 ppm.

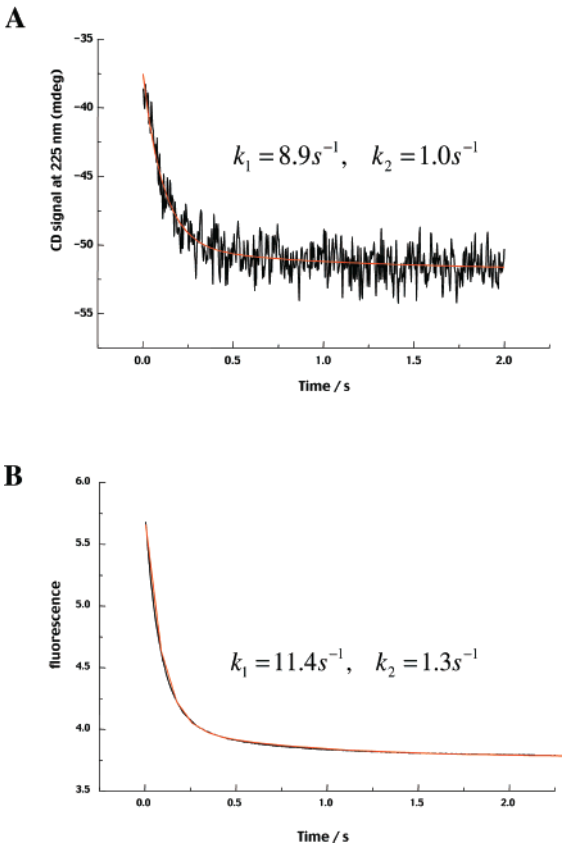

Figure 8. Stopped flow data for the refolding of α-lactalbumin from 4 M urea upon the addition of an equimolar Ca^{2+} solution. Experimental data and fitted data are shown. (A) CD kinetic trace recorded at 225 nm, indicating the formation of α helical structure in the protein. The kinetics were fitted to a mono-exponential decay. (B) Fluorescence kinetic trace of the tryptophan residues: excitation at 295 nm, cutoff before detection: 320 nm. The kinetics were fitted to a biexponential decay.

The decay of the unfolded protein, as evidenced by the decay of the bulk of the methyl groups, is faster than the build up of the native methyl signal that evidences native tertiary contacts within the protein. The rates of formation of native tertiary

contacts vary from $k = 0.16\ s^{-1}$ for the peak at 0.66 ppm (Figure 7b) through $k = 0.23\ s^{-1}$ for the Val21 residue at 0.21 ppm (Figure 7c), to $k = 0.28\ s^{-1}$ for the Leu15 moiety at −0.23 ppm (Figure 7d). The difference between the rate constants of the slower single residues and the rate constant of the fast decay of the methyl bulk peak is statistically significant.

The kinetic data obtained from high resolution NMR spectroscopy differs significantly from the data obtained by stopped flow CD and fluorescence data at the same temperature. For stopped-flow CD, a double exponential rate for the formation of α helical structure (CD signal at 225 nm) $k_1 = 8.9\ s^{-1}$ and $k_2 = 1.0\ s^{-1}$ is observed. The overall change of the fluorescence signal (excitation at 295 nm, cutoff 320 nm) shows biphasic behavior with $k_1 = 11.4\ s^{-1}$ and $k_2 = 1.3\ s^{-1}$. Kinetic traces are shown in Figure 8. These two methods probe for the formation of − or for the change in secondary structure. The results obtained here with our new NMR experiment are sensitive to changes in side-group interactions presumably by the formation of tertiary contacts. The decreasing peak integral of the methyl peak at 0.84 ppm arises from the bulk of the methyl groups in the unfolded state of α-lactalbumin. The signals of upfield shifted methyl groups in the folded state of α-lactalbumin arise from native contacts of these methyl groups presumably with aromatic amino acids in the interior of the proteins. The slower rates for these processes monitored by NMR, compared to the results obtained by time-resolved CD and fluorescence, therefore indicate a faster rate for secondary structure formation than for tertiary structure formation in the case of α-lactalbumin. The fact that different residues show different refolding rate constants and that those rate constants can be fitted to a two-step kinetic process favors the idea of a nucleation model[16] in the case of the calcium-induced refolding of α-lactalbumin from 4 M urea.

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Conclusions

Triggering of the refolding of α-lactalbumin by rapid photo release of Ca^{2+} from DM-nitrophen and the subsequent NMR spectroscopic detection of the reaction allowed the measurement of refolding rates as fast as ∼0.3 Hz with atomic resolution. The data presented stem from three independent experiments carried out with 0.5 mM sample in 250 μL of solution on a 600 MHz spectrometer. The data therefore shows that the kinetics of biopolymer folding and reactions, for example, enzyme-catalyzed reaction, can be triggered and subsequently monitored with high sensitivity by time-resolved NMR spectroscopy. A plethora of structural and mechanistic questions in structural biology should be addressable using this approach of rapid release of photochemically caged functionality.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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