Millisecond Time Resolved Photo-CIDNP NMR Reveals a Non-Native Folding Intermediate on the Ion-Induced Refolding Pathway of Bovine α-Lactalbumin**

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Proteins adopt their native conformation within milliseconds to seconds after the synthesis of the polypeptide chain. This timescale suggests that protein folding follows predetermined folding pathways.[1] Folding intermediates have been detected for a number of proteins along these pathways.[2] Theoretical studies have proposed that folding intermediates may resemble folding traps.[3] The question, therefore, as to whether folding proceeds through well-defined folding intermediates or whether a large number of independent pathways leads to the native structure is of current interest.[4] There are few methods that provide structural insight into folding intermediates with high temporal and site resolution. Herein we report on the kinetic investigation of the Ca^{2+}-induced refolding of α-lactalbumin (α-LA) and NMR characterization at atomic resolution of a folding intermediate populated after 200 ms.

For the calcium binding protein α-LA ($K_D = 2 \times 10^7$ M at 37°C)[5], a partially folded and highly dynamic state of α-LA, the so-called "molten globule", can be stabilized under acidic conditions (pH 2);[6] this molten globule has been proposed to resemble a folding intermediate. Indeed, previous kinetic experiments by Dobson and co-workers revealed a close similarity between the molten globule and a folding intermediate populated during the refolding caused by the dilution from 6 M to 0.54 M guanidinium hydrochloride (GdnCl).[7]

Recently, the application of time-resolved NMR methods to follow protein refolding has gained renewed interest.[8, 9] Comparison of the NMR spectral fingerprint of the aromatic region obtained from NMR refolding experiments of α-LA using time-resolved photo-CIDNP[10] (using tenfold dilution from 6 M GdnCl in the absence of Ca^{2+} ions) suggested a close similarity between the intermediate populated after a few seconds and the static photo-CIDNP spectrum of the acid-stabilized molten globule state of α-LA.[11]

A different approach to the study of refolding of α-LA has been investigated in our research group: Ca^{2+} ions have little effect on the structure of the native state of α-LA,[12] but they stabilize the protein against denaturants such as GdnCl and urea, high temperature, or pressure.[5] The refolding of α-LA can be induced at a constant urea concentration by the addition of Ca^{2+} ions. Figure 1b shows a time–concentration profile of unfolded (U), intermediate (I), and folded (F) states of α-LA derived from kinetic analysis of stopped-flow fluorescence measurements (Figure 1a) of the Ca^{2+}-induced refolding in 2 M urea (α-LA:Ca^{2+} 1:2 final concentrations). When fitted to a three-state model, an intermediate is found to be populated to 70% at $\tau = 350$ ms, as evidenced both by stopped-flow fluorescence (Figure 1a) and CD data. This result is in agreement with the observation of the rise and decay of a non-native NMR signal in the time-resolved experiments discussed below.

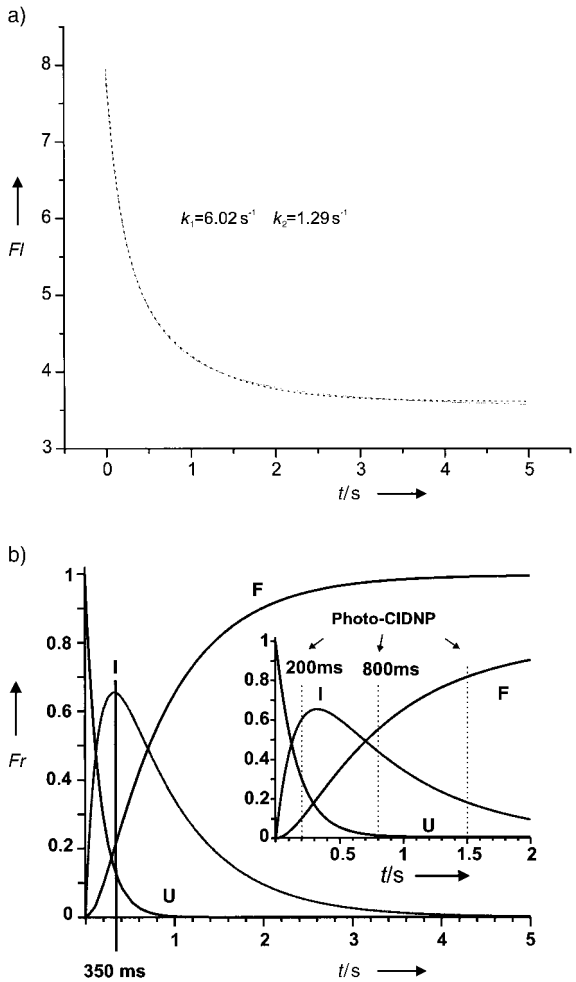

Figure 1. a) Stopped-flow fluorescence data ($\alpha^{exc} = 289$ nm) for the refolding of α-LA in 2 M urea upon the addition of two equivalents of Ca^{2+} ions (Fl = fluorescence, gray line = experimental data, for the exact conditions see the Experimental Section). Data are averages from ten measurements. The urea background was subtracted from the kinetic trace. The fluorescence remains unchanged in blank dilution experiments in the absence of Ca^{2+} ions. The fitting of the data to double exponential decay (assuming three-state kinetics) yields $k_1 = 6.02 \pm 0.06$ s^{-1}, $A_1 = 2.06 \pm 0.02$, $k_2 = 1.29 \pm 0.02$ s^{-1}, $A_2 = 2.15 \pm 0.02$, $R^2 = 0.99993$, and $\chi^2 = 0.00047$ (fit shown as black dots). b) Time–concentration profile of the fraction (Fr) of unfolded (U), folded (F), and intermediate (I) as calculated from k_1 and k_2 by using the software package Maple. The time points at which the kinetic photo-CIDNP NMR spectra have been recorded are indicated in the inset.

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In the NMR experiments reported here, refolding is induced by the release of Ca^{2+} ions from the photolabile chelator DM-Nitrophen (DMN) within 100 ms $< \tau <$ 200 ms at constant denaturant concentration.[8, 13] Our initial studies focused on the characterization of the kinetics for the build-up of the signals corresponding to the methyl groups. The acquisition of the signals for the methyl groups in the native tertiary conformation is a factor of ten slower than the formation of secondary structure of the protein.[8]

Herein refolding of α-LA is studied using time-resolved NMR spectroscopy in conjunction with photo-CIDNP excitation of aromatic residues as pioneered by Hore and coworkers.[11] The experiments described here combine the advantages of photochemical initiation of folding[8] and photo-CIDNP NMR signal detection:[10, 11] namely, the dead-time of the experiment is only governed by the signal-to-noise ratio of the NMR experiment, mixing inhomogeneities are avoided, and the resolution and intensities of aromatic resonances (such as Trp, Tyr, and His) that are in van der Waals contact with CIDNP dyes, such as flavin-mononucleotide (FMN), are enhanced.

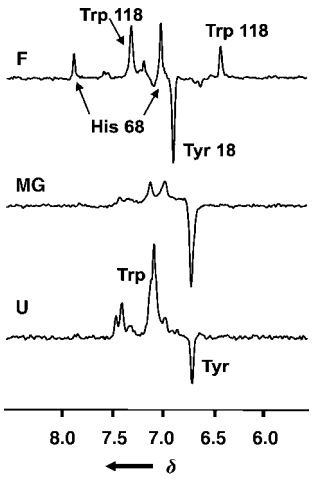

Figure 2. Static photo-CIDNP NMR spectra of the unfolded (U), molten globule (MG), and folded state (F) of bovine α-LA. Spectra are single-scan spectra using 0.5 mM α-LA and 100 ms laser irradiation (sample conditions are given in the Experimental Section). All NMR spectra were analyzed using the software package Felix (MSI).

Figure 2 shows the static photo-CIDNP spectra of the three states of α-LA. Absorptive signals are observed for Trp and His residues; Tyr signals are emissive. For a given amino acid, the signal intensity in the folded state of α-LA can be correlated with the solvent accessibility (SA).[14] In agreement with previous measurements by Hore and coworkers,[11] only Trp 118 (SA = 0.134), Tyr 18 (SA = 0.302), and His 68 (SA = 0.818) lead to a photo-CIDNP effect and contribute to the CIDNP spectrum of folded α-LA. Whereas the spectra of the folded and the unfolded state exhibit sharp lines, broader line widths are characteristic for the molten globule state of α-LA. A distinctive fingerprint for each of the three states can, therefore, be observed using photo-CIDNP spectroscopy.

By applying the combination of ion release and photo-CIDNP, faster ion-induced refolding kinetics can be characterized by NMR spectroscopy with a very small amount (< 0.3 mg, $M_r = 14200$) of protein, and the changes in the organization of the aromatic core during folding can be monitored with site specificity. The correlation of the photo-CIDNP efficiency may also provide a link to the changes in solvent accessibility during folding.[15]

The experimental setup and the pulse sequence used for these experiments are shown in Figure 3 a and b, respectively.

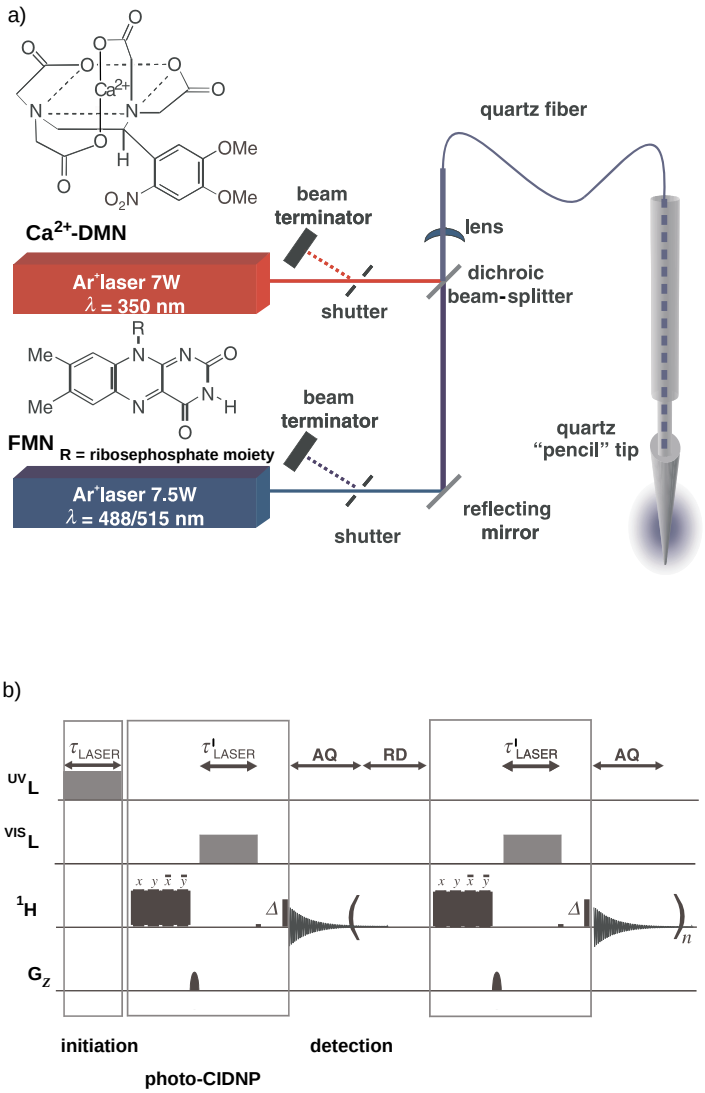

Figure 3. a) Set-up used for laser-NMR coupling. Two argon ion lasers emitting at 350 (7 W) and 488/515 nm (7.5 W) were coupled in the NMR tube through a quartz fiber optic. The coupling efficiency was 70 % for both lasers, as measured by an optical power meter before and after the actual NMR experiment. b) Experimental scheme used for the time-resolved photo-CIDNP experiments. ^{UV}L represents the laser operating at 350 nm for ion release, ^{VIS}L represents the laser operating at 488/515 nm for photo-CIDNP. Equilibrium ^1H magnetization is destroyed prior to irradiation with visible light by using trim pulses and a z-gradient (G_z); excitation of the signal and water suppression was done using a jump-return echo pulse sequence. $\tau_{LASER} = 200$ ms, $\tau'_{LASER} = 100$ ms, $\Delta = 5$ ms, AQ = 56 ms, RD = 440 ms or 1140 ms, $\tau_{GRADIENT} = 1$ ms (50 G cm^{-1}). AQ = aquisition time, RD = relaxation delay.

Ca^{2+} ions were released from DMN inside the NMR spectrometer by irradiation at 350 nm. The accessibility of aromatic side chains was subsequently probed by means of a photochemical reaction (photo-CIDNP). In order to improve the time resolution of the experiment, which is governed by the protein concentration and the metal:α-LA binding constant, a concentration of 0.1 mM (in 160 µL of solution) of α-LA was used. An irradiation time of 200 ms was applied to release 0.2 mM Ca^{2+} ions (of a total of 0.5 mM caged Ca^{2+} ions) to fold the protein. The FMN concentration used in the time-resolved experiments had to be kept low, since higher concentrations were found to suppress ion release.

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The results of the time-resolved photo-CIDNP experiments on the ion-induced refolding of α-LA are shown in Figure 4. Native signals of Trp 118 (absorptive signals at $\delta = 6.4$ and 7.42) and Tyr 18 (emissive signal by $\delta = 6.95$) are observed

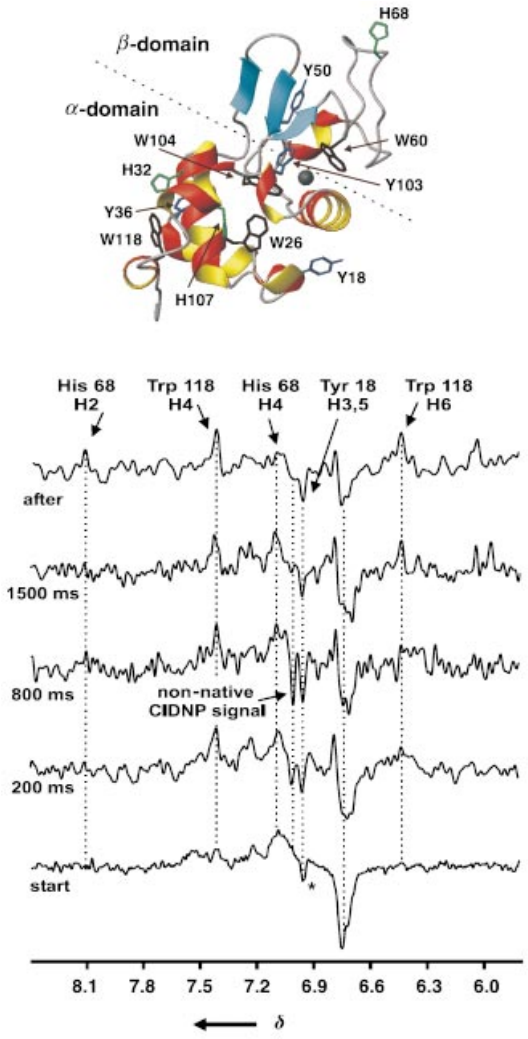

Figure 4. Time resolved photo-CIDNP. The spectra shown are averaged over eight samples. The first and last trace are photo-CIDNP spectra before and after kinetic experiments, respectively; * denotes a native signal present at the start of the experiment. The ribbon representation of the X-ray structure (1HFZ in pdb)[17] of α-LA with tyrosine (blue), tryptophan (black), and histidine (green) residues is indicated. The figure was prepared using the program MOLMOL.[18]

200 ms after initiation of the folding in the first kinetic experiment. Those signals do not significantly change in intensity at later times. A sharp emissive signal at $\delta = 7.05$ arising from one of the three additional Tyr residues in α-LA is present after 200 ms and disappears between 800 and 1500 ms. We assign this signal to the folding intermediate that is also observed in the kinetic analysis of the stopped-flow fluorescence and CD experiments. The chemical shift of the signal does not correspond to any Tyr signal in either the native (F), the unfolded (U), or the molten globule state of α-LA (see Figure 2 and additional NMR data of the native state[16]). It can, therefore, be assigned to an intermediate with a non-native environment.

The assignment of this emissive signal at $\delta = 7.05$ to one of the three remaining Tyr residues (Tyr 36, 50, and 103) cannot be made with certainty. However, the following conclusion can be drawn: It is unlikely that the signal arises from Tyr 36 or Tyr 103 because both Tyr residues are part of hydrophobic clusters formed between Tyr 36 and Trp 118 and between Tyr 103, Trp 60, and Trp 104. Tyr 36 and Tyr 103 are both part of the α-domain in α-LA. In contrast, Tyr 50 is in the β-sheet of the β-domain of α-LA. The assignment of the non-native signal to Tyr 50 would imply that the hydrophobic core in the α-domain of α-LA is locked in its nativelike environment,[7] while parts of the β-domain, maybe those involved in β-sheet formation, would undergo slower structural reorganization.

It is important to note that the emissive Tyr signal seen in the intermediate does not arise from a Tyr residue in a native environment. This result implies that at least part of the polypeptide chain samples non-native conformations during protein refolding. In addition, the emissive signal observed for the folding intermediate populated under the specific experimental conditions has no characteristics of a molten globule. Additional experiments will need to be conducted to further establish the dependence of these observations on the exact folding conditions and to gain further insight into structural aspects of protein folding intermediates at atomic resolution.

Experimental Section

Photo-CIDNP NMR spectroscopy: Two argon lasers (models 2085 and 2017, Spectra Physics) emitting at 350 nm (7 W) and 488/515 nm (7.5 W) were coupled into a 750 MHz home-built NMR spectrometer. The laser beams were guided through an electronic shutter (Uniblitz, Vincent Associates) and combined using a dichroic beam splitter (CVI Laser-optics). The combined laser beam was focused on a multimode silica/silica fiber (1500 μm core, Ceram Optecs). Laser coupling into the NMR spectrometer was performed as described.[8] The overall sample volume used in this setup was 160 μl. Sample conditions were as follows: Kinetic photo-CIDNP: 0.1 mM bovine α-LA, 0.5 mM $CaCl_2$, 0.5 mM DMN, 0.05 mM FMN, 2 M urea, and 50 mM cacodylate buffer in D_2O, pH* 6.7 (pH*: measured in D_2O, uncorrected). The synthesis of DMN and the Ca^{2+} depletion of bovine α-lactalbumin was performed as described.[8] All chemicals were purchased from Fluka.

Static photo-CIDNP: All samples: 0.5 mM α-LA, 0.2 mM FMN, 35 °C in D_2O, U: pH* 2, 8 M urea; MG: pH* 2; F: 50 mM cacodylate, 10 mM $CaCl_2$, pH 6.7.

Stopped-flow fluorescence: Refolding kinetics were acquired using an Applied Photophysics π*-180 stopped-flow spectrometer at 35 °C ($\alpha^{exc} =$ 289 nm, $\alpha^{em} > 320$ nm). Refolding was initiated by mixing 100 μM α-LA against 200 μM $CaCl_2$, both in 2 M urea and 50 mM Tris/HCl at pH 7.0; dead time of the experiment was 1 ms (Tris = tris(hydroxymethyl)aminomethane).

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Immobilization of Olefin Metathesis Catalysts on Monolithic Sol – Gel: Practical, Efficient, and Easily Recyclable Catalysts for Organic and Combinatorial Synthesis**

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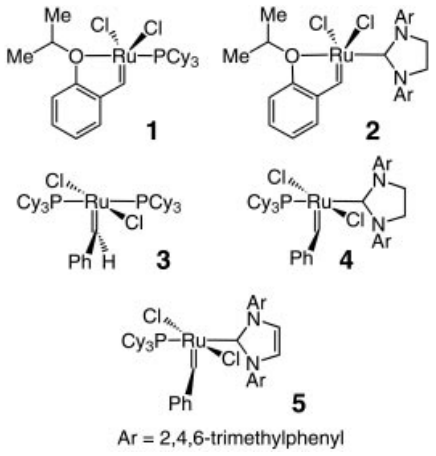
Environmental issues and recent developments in chemistry and biology have introduced a number of compelling requirements that must be met in the development of practical catalysts. Recyclability is one important attribute. Retrievable catalysts that are recovered inexpensively and without significant waste generation efficiently deliver products of higher purity and lower toxicity. The emerging significance of combinatorial chemistry demands that a catalyst promote reactions efficiently and selectively while being easily adaptable to 96-well and higher density formats; repeated weighing of catalysts or substrates for a library synthesis and subsequent purification of each mixture is costly and time-consuming.

Herein we disclose the synthesis and activity of Ru complexes supported by monolithic (smallest dimension = 1 mm) samples of porous sol – gel glass that effectively promote various olefin metathesis reactions.[1] These catalysts can be easily employed in a library synthesis format without multiple weighings, in air and with undistilled commercial reagent-grade solvents. Catalyst recovery is simply carried out with a pair of tweezers; it does not require filtration and generates minimal solvent waste. The catalyst retains its activity after multiple cycles (>15), affording products that are of high (often analytical) purity without recourse to any purification steps.

Recent reports from our laboratories relate to the chemistry of recyclable monomeric metathesis catalysts (**1** and **2**).[2] A key feature of these systems is the isopropyl styrenyl ether;



this bidentate ligand favors efficient metal recovery for entropic reasons, allowing the promotion of olefin metathesis by a release/return mechanism.[2] These Ru complexes can also offer reactivity and chemo- and stereoselectivity profiles[3] which differ from the alternative catalysts **3**,[4] **4**,[5] and **5**.[6]

Although **1** and **2** are robust and recyclable, catalyst retrieval generates substantial amounts of silica gel and solvent waste. Based on the release/return mechanism, Ru – carbenes **1**,[7] **3**,[8] and **5**[9] were subsequently attached to insoluble cross-linked and monoporous polystyrene polymers as well as to a soluble polyethylene glycol (PEG) resin. However, these supported catalysts typically suffer from one or more of the following shortcomings: 1) There are no reports of catalyst utility in the synthesis of trisubstituted olefins; efficient processes involve only ring-closing metathesis (RCM) reactions with terminal olefin substrates or those that benefit from entropic factors.[7–9a] 2) Diminished

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