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Detection of a Transient Intermediate in a Rapid Protein Folding Process by Solid-State Nuclear Magnetic Resonance

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Experimental and computational studies of protein folding indicate that small, single-domain proteins are capable of folding on the microsecond or even submicrosecond time scale. Since kinetics experiments rely on spectroscopic parameters that may not be sensitive to all details of the molecular structure[1] and computationally folded proteins do not have the exact experimentally determined structures of the fully folded state,[2] uncertainty exists about the state of a rapidly folding protein after only microseconds of evolution from an unfolded state. Here we describe experiments in which rapid freezing (on the 10−20 μs time scale) from an unfolded state at 90 °C to a frozen state creates a trapped intermediate state of the 35-residue villin headpiece subdomain[3] (HP35) for structural analysis by solid-state NMR. Two-dimensional (2D) NMR spectra of freeze-quenched HP35 show two components but are not simply superpositions of unfolded and fully folded spectra, indicating the existence of a transient intermediate with native secondary structure but incomplete tertiary structure.

Figure 1 shows 2D NMR spectra of HP35 with ^{13}C labels at specific sites, either frozen slowly from 24 °C (Figure 1a) or frozen rapidly from 90 °C, which is above the 74 °C unfolding midpoint temperature in the glycerol/water solvent (Figure 1b). Figure 1a shows a single set of cross-peaks with CO, Cα, and Cβ chemical shifts that are consistent with the native α-helical secondary structure at V50 and L69 (helical segments: H1 = residues 44−50; H2 = residues 55−58; H3 = residues 63−72). Importantly, the Cα/Cγ and Cβ/Cγ cross-peaks for V50 are doublets, reflecting a 3.5 ppm separation of the two V50 methyl signals. This splitting, which disappears in the earliest stages of chemical denaturation of HP35,[4] depends on an atypical V50 side-chain conformation[5] and is therefore an empirical signature of the fully folded structure. The cross-peaks in Figure 1b are broadened and can be decomposed into helical (unprimed) and nonhelical (primed) chemical shift components. Thus, rapid freeze-quenching from 90 °C traps a nonequilibrium structural state of HP35. Moreover, this state is not a mixture of unfolded and fully folded HP35 molecules, as one might expect in a simple two-state folding process, because even the V50 signals with helical Cα and Cβ chemical shifts do not exhibit a splitting of Cγ signals. The line widths for the helical components are also greater in Figure 1b, indicating greater conformational disorder. The 2D spectrum in Figure 1b therefore indicates a mixture of strongly unfolded molecules and molecules with native secondary structure but incomplete tertiary structure. The latter ensemble of molecules is the intermediate structural state.

Details of our rapid freeze-quenching method are given in the Supporting Information. Briefly, 150 μL of 6−8 mM HP35 solution in glycerol/water was contained in 1.0 mm i.d. stainless steel tubing within a heated copper tube at 90 °C. The solution was expelled at 25 μL/s through a 20 μm aperture by 2000 psi pressure from an HPLC pump, producing a jet of hot solution with a velocity of 8 $\times$ 10^3 cm/s that traveled 1.0 cm to the surface of a stirred isopentane bath maintained at −145 °C (Figure S1 in the Supporting Information). Upon striking the isopentane surface, the jet broke into particles with ∼10 μm average diameter, as visualized by optical microscopy, producing a calculated average freezing time of ∼15 μs (Figure S2). After the resulting slurry of frozen particles was allowed to settle, the excess isopentane was removed by pipetting and blotting with cold filter paper. The slurry was packed in a cold 6 mm magic-angle spinning (MAS) rotor and transferred to a precooled MAS NMR probe for NMR measurements at −120 °C. Direct measurements with a K-type thermocouple indicated jet temperatures of 86, 80, and 74 °C at 0.0, 1.0, and 2.0 cm flight distances, implying that cooling occurred primarily in the isopentane, not during the flight through air. 1D NMR spectra (Figures S3 and S4) verified that increasing the flight distance from 1.0 to 2.0 cm did not affect the freeze-quenched intermediate state, that no folding occurred during the NMR measurements, and that rapid (or slow) freeze-quenching itself did not cause unfolding.

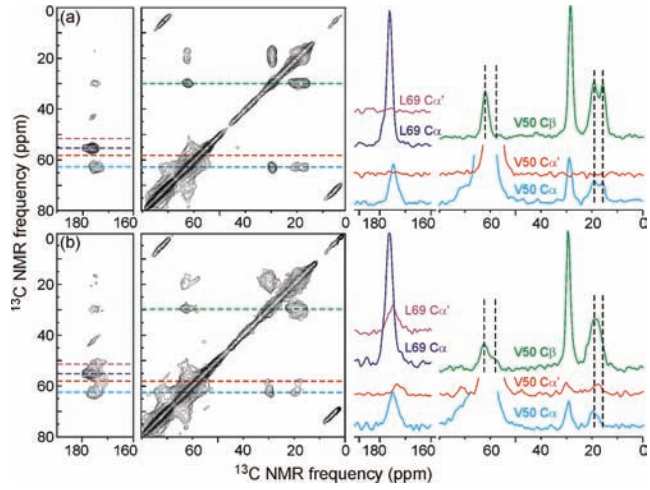

Figure 1. 2D solid-state ^{13}C−^{13}C NMR spectra of HP35 in 3:2 (w/w) glycerol/water in (a) the fully folded state obtained by freezing from 24 °C in ∼5 s by immersion in liquid nitrogen and (b) the freeze-quenched state obtained by spraying a thin, high-velocity jet of solution at 90 °C into cold isopentane. All of the V50 carbon sites and the CO and Cα sites of L69 were ^{13}C-labeled. 1D slices are shown at the right, with unprimed slices being the helical, folded-state shifts and primed slices being the nonhelical, strongly unfolded shifts. Spectra were obtained at a sample temperature of −120 °C and a ^{13}C NMR frequency of 100.8 MHz with 6.70 kHz MAS.

Figure 2 shows 2D ^{13}C−^{13}C NMR spectra of a second HP35 sample prepared with uniform ^{13}C labeling of V50, G52, T54, A57, F58, and L63. The V50 signals were the same as those in Figure 1, indicating reproducibility. Other resolved signals showed two components when the HP35 solution was rapidly frozen from 90 °C (Figure 2b), with one component (unprimed) having nearly the same CO, Cα, and Cβ chemical shifts as in the slowly frozen

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spectrum (Figure 2a) but generally larger linewidths. The second component (primed) appeared at non-native chemical shifts. The ratios of partially folded to strongly unfolded populations, determined by fitting the cross-peaks in Figures 1b and 2b with pairs of 2D Gaussian functions, are approximately 60:40 for V50, 70:30 for A57 and L69, and 50:50 for T54 (Table S1). Site-specific variations in these ratios indicate a nonuniform population of the native secondary structure along the polypeptide chain.

The nonequilibrium state of HP35 prepared from the thermally unfolded state by rapid freeze-quenching differs significantly from the chemically denatured state examined in earlier solid-state NMR studies. In particular, the chemically denatured state in a 7 M solution of guanidine hydrochloride in frozen glycerol/water contains negligible helical secondary structure, according to both ^{13}C chemical shifts and quantitative backbone torsion angle data.[4]

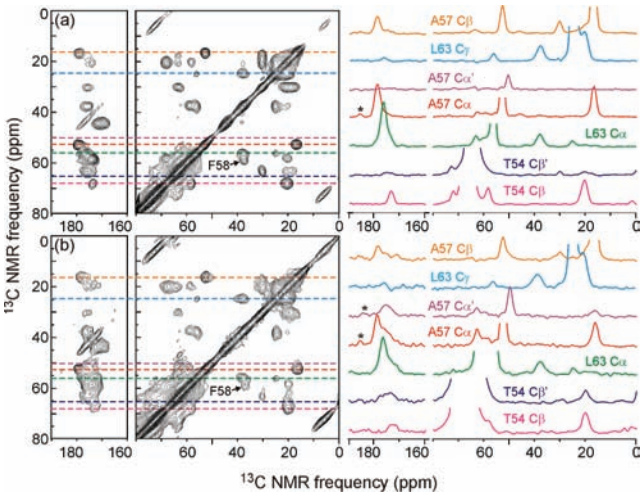

Figure 2. Same as Figure 1, but with uniform ^{13}C labeling of V50, G52, T54, A57, F58, and L63. Asterisks denote MAS sideband signals.

Figure 3 summarizes the qualitative picture that emerges from our data. Under our experimental conditions, on the 10−20 μs time scale, thermally unfolded HP35 converts primarily (50−70%) to an intermediate state with native secondary structure. The tertiary structure, with an ordered hydrophobic core consisting of the side chains of L42, F47, V50, F51, F58, L61, L69, and L75, is incomplete in the intermediate ensemble. Ordered tertiary structure develops more slowly, requiring rearrangements and optimization of side-chain packing and concomitant adjustments of backbone conformation. Broad ^{13}C NMR lines (indicating disorder) for residues in the loop between H1 and H2 and attenuation of ^{13}C spin-polarization transfers from F58 to L69 (Figure S5) provide additional evidence for incomplete tertiary structure in the partially folded intermediate state.

Kubelka et al.[1b] have reported a folding rate constant (k_f) of 2×10^5 s^{-1} for HP35 in water that is nearly independent of temperature from 300 to 355 K. Data of Cellmer et al.[6] indicate $k_f \approx [3/(0.8 + \eta)] \times 10^5$ s^{-1}, where η is the solvent viscosity (cP). Taking into account the temperature-dependent viscosity of glycerol/water (see http:/www.dow.com/glycerine/) and our calculated cooling rates (Figure S2), we estimate from these results that the folded fraction should be ∼54% in our experiments, in rough agreement with the *partially* folded fractions determined above.

Lattice-model and all-atom simulations of protein folding by Kussell et al.[7] indicate that full structural ordering can occur on a longer time scale than structural nucleation, especially for residues outside the nucleus. A longer time scale for side-chain ordering may contribute to discrepancies between the folding kinetics determined for HP35 by optical[1b,6] and liquid-state NMR[8] methods. Simulations of HP35 folding in which helix formation precedes tertiary structure formation[9] may be consistent with our data, although these simulations do not clearly show a slower phase of side-chain ordering after the three helices form.

The experiments described herein are the first example of solid-state NMR spectroscopy of a short-lived transient state in protein folding. In future work, additional solid-state NMR techniques can be used to obtain quantitative structural constraints on transient states, and the time course of folding can be elucidated by adding a variable delay at an intermediate temperature.

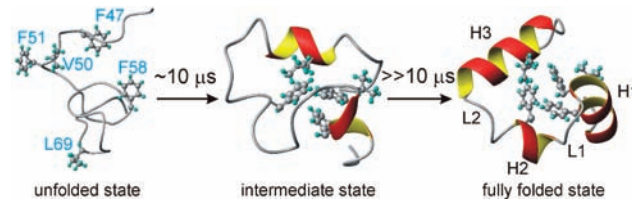

Figure 3. Schematic llustration of the HP35 folding process. Starting with a thermally unfolded ensemble that lacks helical secondary structure, a rapid temperature drop causes rapid conversion to an intermediate ensemble with nearly native secondary structure but disordered tertiary structure. The fully folded state, with helical segments joined by ordered loops and with an ordered hydrophobic core, forms on a longer time scale.

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Supporting Information Available: Methods description; figures showing the freeze-quenching apparatus, calculation of the freezing time, the rf pulse sequence for double-quantum-filtered 1D ^{13}C NMR, 1D spectra from control experiments, and additional 2D and 1D spin diffusion data; and a table of fits to cross-peaks in the 2D spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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