Published in final edited form as:
J Am Chem Soc. 2010 January 13; 132(1): 24–25. doi:10.1021/ja908471n.

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 scales. Since kinetic experiments rely on spectroscopic parameters that may not be sensitive to all details of molecular structure1 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 from an unfolded state at 90° C to a frozen state, on the 10-20 μs time scale, creates a trapped intermediate state of the 35-residue villin headpiece subdomain[3] (HP35) for structural analysis by solid state NMR. 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.

Fig. 1 shows 2D NMR spectra of HP35 with ^{13}C labels at specific sites, either frozen slowly from 24° C (Fig. 1a) or frozen rapidly from 90° C (Fig. 1b), above the 74° C unfolding midpoint temperature in the glycerol/water solvent. Fig. 1a shows a single set of crosspeaks 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, Cα/Cγ and Cβ/Cγ crosspeaks 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 sidechain conformation5 and is therefore an empirical signature of the fully folded structure. Crosspeaks in Fig. 1b are broadened and can be decomposed into helical (unprimed) and non-helical (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. Linewidths for the helical components are also greater in Fig. 1b, indicating greater conformational disorder. The 2D spectrum in Fig. 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-quench method are given in 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

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

μm aperture by 2000 psi pressure from an HPLC pump, producing a jet of hot solution with 8 × 10^3 cm/s velocity that traveled 1.0 cm to the surface of a stirred isopentane bath maintained at −145° C (Supporting Fig. S1). Upon striking the isopentane surface, the jet broke into particles with ~10 μm average diameters, as visualized by optical microscopy, producing a calculated average freezing time ~15 μs (Supporting Fig. S2). After allowing the resulting slurry of frozen particles to settle, 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 indicate jet temperatures of 86, 80, and 74° C at 0.0, 1.0, and 2.0 cm flight distances, implying that cooling occurs primarily in the isopentane, not during flight through air. 1D NMR spectra (Supporting Figs. S3 and S4) verify that increasing the flight distance from 1.0 cm to 2.0 cm does not affect the freeze-quenched intermediate state, that no folding occurs during the NMR measurements, and that rapid (or slow) freeze-quenching itself does not cause unfolding.

Fig. 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. V50 signals are the same as in Fig. 1, indicating reproducibility. Other resolved signals show two components when the HP35 solution is rapidly frozen from 90° C (Fig. 2b), with one component (unprimed) having nearly the same CO, Cα, and Cβ chemical shifts as in the slowly frozen spectrum (Fig. 2a), but generally larger linewidths. The second component (primed) appears at non-native chemical shifts. Ratios of partially folded to strongly unfolded populations, determined by fitting crosspeaks in Figs. 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 (Supporting Table S1). Site-specific variations in these ratios indicate nonuniform population of native secondary structure along the polypeptide chain.

The non-equilibrium state of HP35 prepared by rapid freeze-quenching from the thermally unfolded state differs significantly from the chemically denatured state examined in earlier solid state NMR studies. In particular, the chemically denatured state at 7 M 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]

Fig. 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-like secondary structure. Tertiary structure, with an ordered hydrophobic core comprised of sidechains 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 sidechain 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 provide additional evidence for incomplete tertiary structure in the partially folded intermediate state (Supporting Fig. S5).

Kubelka *et al.*[1b] report a folding rate $k_f = 2 \times 10^5\ s^{-1}$ for HP35 in water, nearly independent of temperature from 300 K 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 solvent viscosity (cP). Taking into account the temperature-dependent viscosity of glycerol/water (see http:/www.dow.com/glycerine/) and our calculated cooling rates (Supporting Fig. 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. indicate that full structural ordering can occur on a longer time scale than structural nucleation, especially for residues outside the nucleus.[7] A longer time scale for sidechain ordering may account for

discrepancies between 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 sidechain ordering after the three helices form.

Experiments described above 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. We thank W.A. Eaton and D.A. Torchia for helpful discussions.

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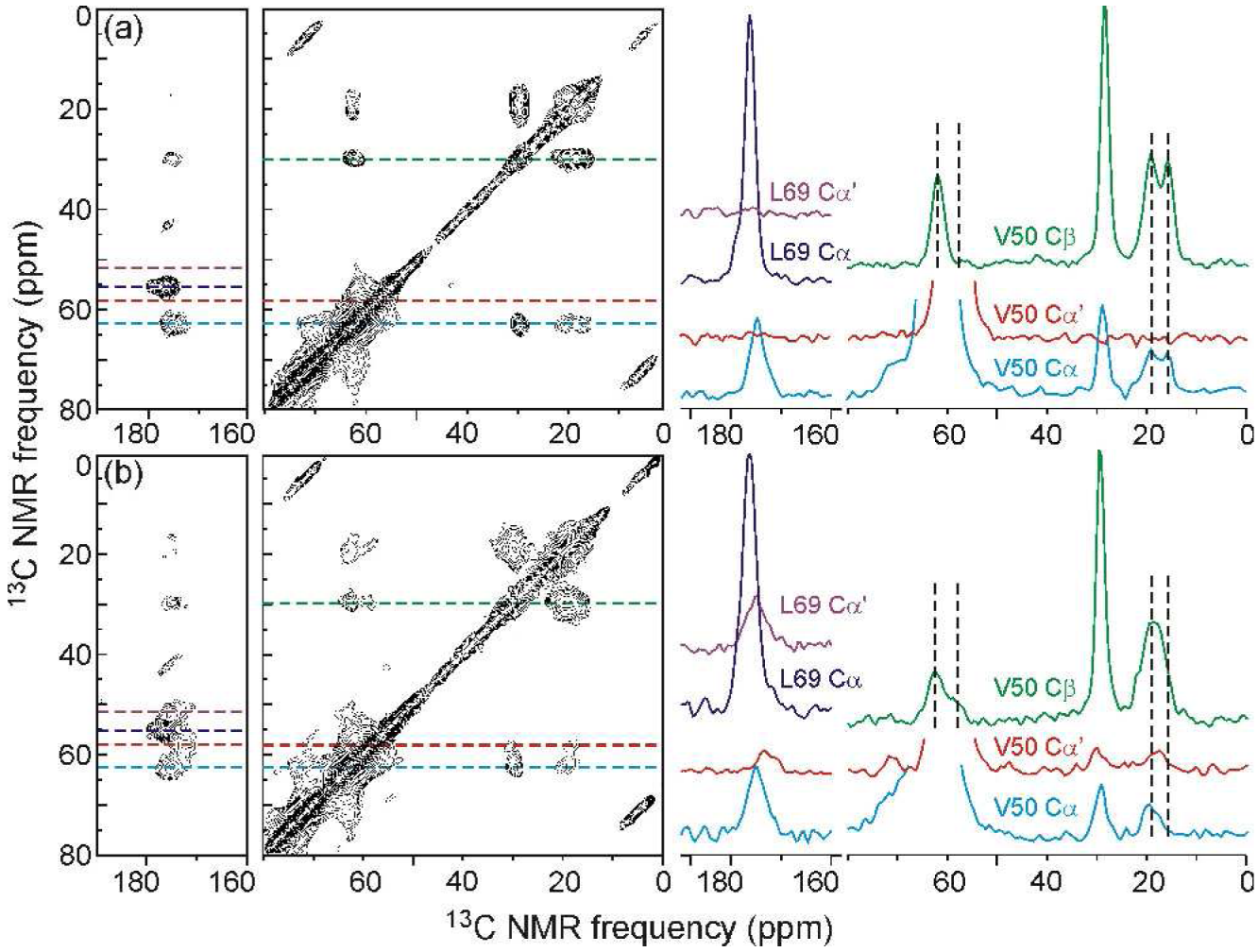

Figure 1.

2D solid state ^{13}C-^{13}C NMR spectra of HP35 in glycerol/water (3:2 w/w) 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 V50 carbon sites and the CO and Cα sites of L69 are ^{13}C-labeled. 1D slices are shown at the right, with unprimed slices being the helical, folded-state shifts and primed slices being non-helical, strongly unfolded shifts. Spectra were obtained at −120° C sample temperatures, 100.8 MHz ^{13}C NMR frequency, and 6.70 kHz MAS.

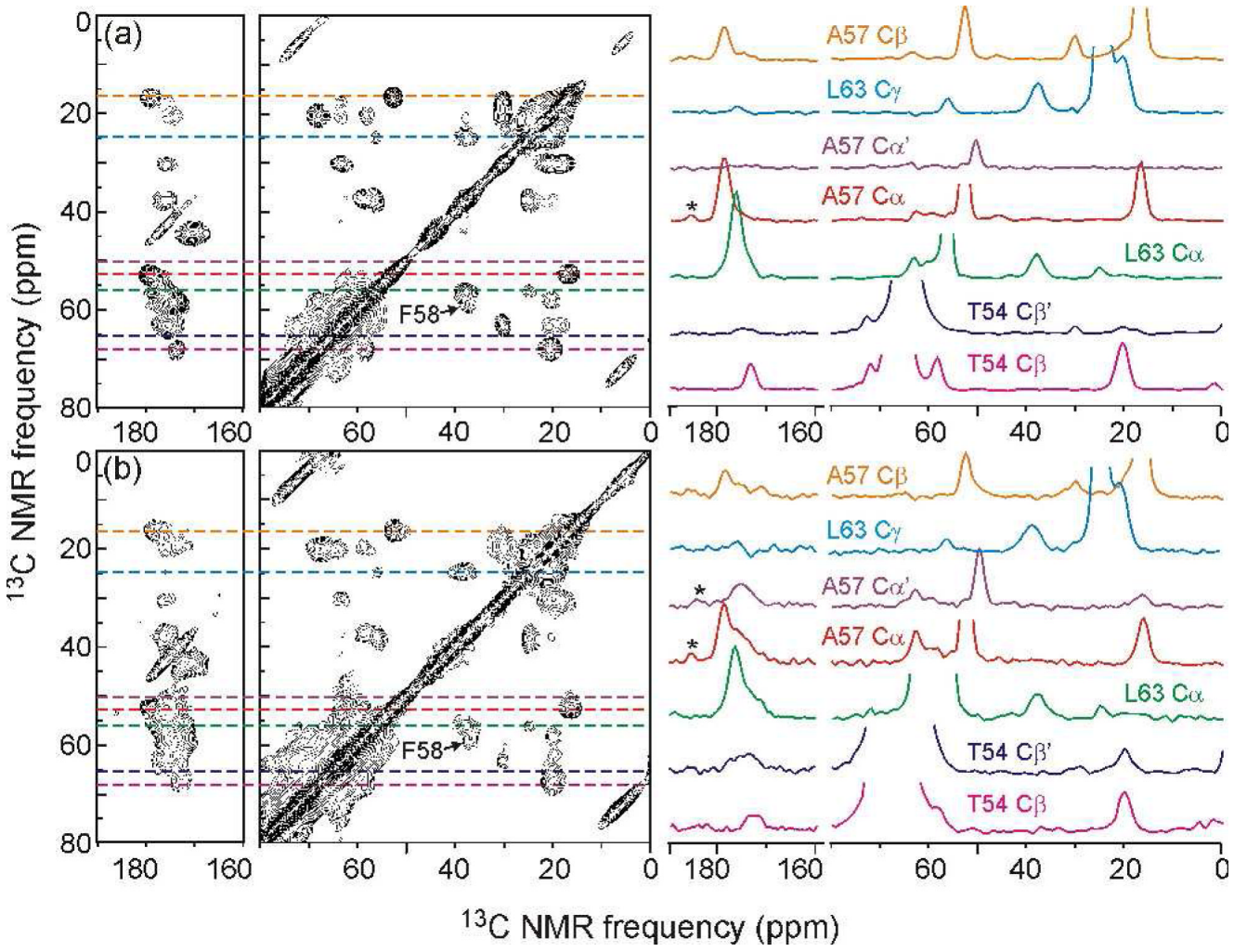

Figure 2.
Same as Fig. 1, but with uniform ^{13}C labeling of V50, G52, T54, A57, F58, and L63. Asterisks denote MAS sideband signals.

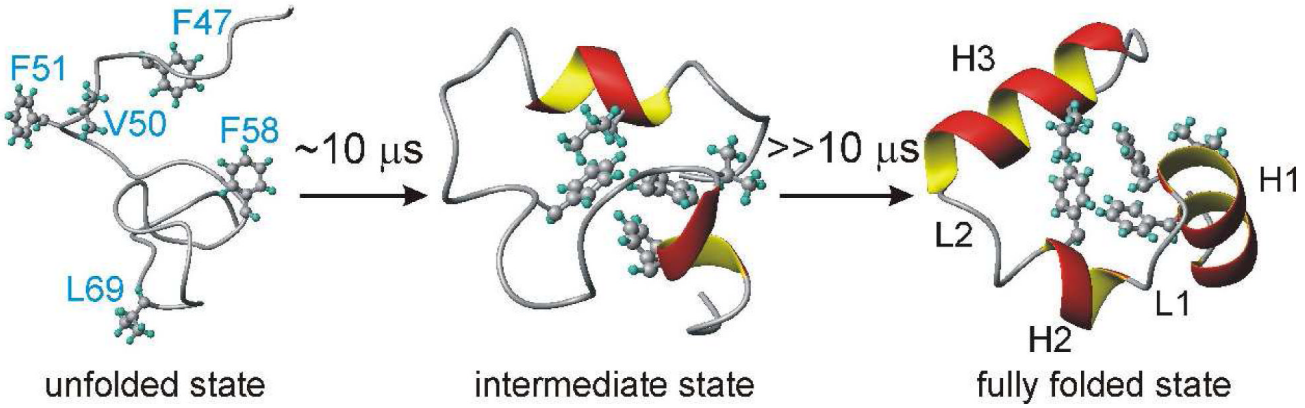

Figure 3.

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.