Two-State Folding Observed in Individual Protein Molecules

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The folding thermodynamics and kinetics of small proteins are often described in terms of a two-state model[1,2] in which only two populations of molecules exist (folded and unfolded) separated by a single free energy barrier. The simplicity of the corresponding time-dependent behavior should be reflected in the stochastic dynamics of individual proteins. Here we report single-molecule folding/unfolding trajectories of a protein that meets all criteria of the two-state model.[3,4] Förster resonance energy transfer (FRET) measurements were obtained from individual protein molecules trapped in surface-tethered lipid vesicles. The trajectories demonstrate the predicted bistable behavior with very fast steplike transitions between folded and unfolded conformations. The results provide the opportunity to examine the correspondence between single-molecule and ensemble measurements quantitatively. They stand in contrast to the unexpected complexity observed in previous reports of folding trajectories of individual proteins[5,6] and offer the first model-free demonstration of two-state protein-folding dynamics.

The cold-shock protein from *Thermotoga maritima* (Csp*Tm*) was labeled with a green-fluorescing and a red-fluorescing dye, serving as FRET donor and acceptor, respectively.[4] FRET upon excitation by a laser beam allows folded and unfolded molecules to be distinguished on the basis of the strong distance dependence of energy transfer between the chromophores. Previous experiments on single molecules freely diffusing in solution indicated that Csp*Tm* exhibits only two populations of molecules in its FRET efficiency distributions, corresponding to the folded and unfolded states.[4,7] To keep the labeled molecules in the laser beam long enough to observe multiple folding and unfolding events, they were individually encapsulated in unilamellar lipid vesicles, which were surface-tethered using biotin−avidin chemistry [5,8] (Figure 1). This method of immobilization was recently developed to allow long observation times for individual proteins while minimizing their surface interactions.[8] Encapsulation was carried out in aqueous buffer containing 2 M of the denaturant guanidinium chloride. Under these solution conditions, folded and unfolded states are equally populated.[4] A sample-scanning confocal microscope was used to locate individual molecules of Csp*Tm* and record fluorescence intensity traces. Donor and acceptor photons were collected separately, allowing the calculation of FRET efficiencies as a function of time. Single-molecule fluorescence depolarization experiments did not give any indication for interactions of trapped proteins with vesicular walls.

Two such measurements are shown in Figure 2, A and B. The data in these trajectories were binned in 20 ms intervals. Steady levels of FRET efficiency are followed by rapid jumps (seen in insets as anti-correlated changes of donor and acceptor intensities), until photobleaching of one of the dyes occurs. On the basis of previous FRET experiments with Csp*Tm*,[4] we identified high transfer efficiencies with the folded state and low transfer efficiencies with the unfolded state of the protein. Consequently, abrupt changes in the FRET efficiency (with an average amplitude of 0.46
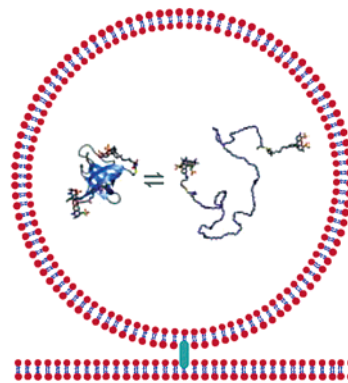

Figure 1. Schematic of Csp*Tm* encapsulated within a surface-tethered vesicle (relative dimensions not to scale).

± 0.16) represent folding or unfolding events. The actual transitions were too rapid to be time-resolved and occurred within the time span of one or two time bins, even when the data were analyzed with a much smaller integration time of 100 μs. One example for such an analysis is shown in Figure 2C. Due to the high FRET efficiency of the folded protein, the donor essentially stops emitting after the transition (green arrow). It can then be estimated that the transition occurs within 100−200 μs of the last registered donor photon. A statistical analysis was performed in which the likelihood to find a specific experimental realization of donor and acceptor photon sequences was evaluated, taking into account the photon emission probabilities before and after the transition. This analysis confirmed the visual estimate.

To quantitatively compare our results to ensemble experiments, we built a histogram of all time intervals preceding folding and unfolding transitions in the trajectories of 43 individual protein molecules (54 transitions overall, Figure 3). An exponential fit to this histogram gave a rate constant of 0.62 ± 0.26 s^{-1} (95% confidence bounds). This rate is in agreement with the folding rate constant of 0.39 ± 0.02 s^{-1} measured in a bulk experiment under identical solution conditions.[4]

It is a characteristic of two-state systems that, upon perturbation of an ensemble of molecules, the distribution between the two states relaxes with an exponential time course, the rate of which is determined by the height of the barrier. The single-molecule equivalent of this behavior is stochastic but fast jumping between the two states.[9] In the latter case, the observable determined by the barrier height is the frequency of transitions. The steplike folding and unfolding behavior seen in our experiment is exactly what would be predicted from this notion and is in quantitative agreement with the ensemble result. The second important parameter, apart from the transition frequency, is the actual time the molecule spends crossing the barrier. Our results provide an upper bound of ∼200 μs for this transit time, which is in agreement with the simple assumptions made in previous analyses of protein folding.[10] In a Kramers-type formulation of escape over a barrier,[11] the barrier-
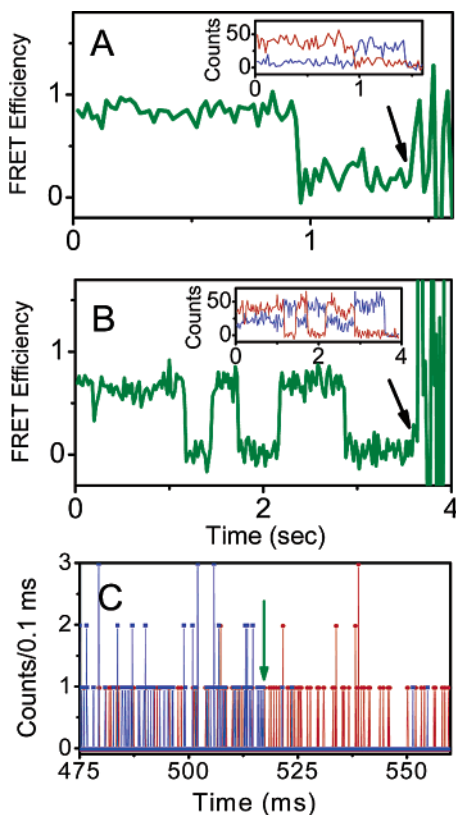
Figure 2. (A,B) FRET efficiency trajectories of individual Csp*Tm* molecules trapped in vesicles at 2 M GuHCl showing (A) a single or (B) multiple abrupt folding/unfolding transitions with a binning time of 20 ms. Arrows indicate the time of photobleaching of one of the dyes. (Inset) Fluorescence intensity trajectories from which FRET efficiencies were calculated. (Blue) Donor signal. (Red) Acceptor signal. (C) A single-molecule trajectory analyzed with 100-μs bins. Green arrow indicates time of folding transition.
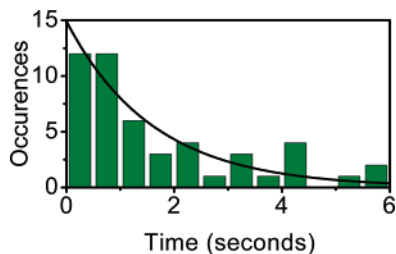

Figure 3. Histogram of time intervals preceding transitions in Csp*Tm* single-molecule trajectories, fit to a single-exponential decay (black). The rate constant obtained from the fit, 0.62 ± 0.26 s^{-1} (95% confidence bounds), agrees well with the ensemble-averaged folding rate constant.

crossing time is a function not only of the effective diffusion coefficient of the folding molecule as it traverses the barrier but also of the shape of the barrier. The direct measurement of transition times in single-molecule experiments of the type described here will therefore provide essential constraints on these fundamental parameters of the free energy barrier that are difficult to obtain from ensemble-averaged experiments.[12-14]

In previous work on single-molecule protein folding, correlation analysis [15] and histograms[4,7,15,16] constructed from measurements on freely diffusing or immobilized proteins were used to demonstrate the presence of the two populations of molecules expected from ensemble experiments. However, none of these approaches allowed the direct observation of folding transitions in individual molecules. Additionally, it was shown that single-molecule histograms can hide more complex behavior.[5] The prototypical two-state behavior observed here in Csp*Tm* trajectories is in contrast with recent reports of folding trajectories of individual proteins. Rhoades et al., using single-molecule FRET spectroscopy, showed that some of the folding/unfolding transitions seen in a large protein, adenylate kinase, were very slow and took up to a few seconds to complete.[5] Fernandez and Li investigated covalent heptamers of ubiquitin molecules using force-clamp microscopy and found slow, continuous folding trajectories.[6] However, in monomeric two-state proteins such as Csp*Tm*, the involvement of non-native interactions is minimal, and the resulting robustness of their folding behavior may contribute to eliminating the additional complexity observed in previous experiments.

Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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