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Protein folding studied by single-molecule FRET

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A complete understanding of a protein-folding mechanism requires description of the distribution of microscopic pathways that connect the folded and unfolded states. This distribution can, in principle, be described by computer simulations and theoretical models of protein folding, but is hidden in conventional experiments on large ensembles of molecules because only average properties are measured. A long-term goal of single-molecule fluorescence studies is to time-resolve the structural events as individual molecules make transitions between folded and unfolded states. Although such studies are still in their infancy, the work till now shows great promise and has already produced novel and important information on current issues in protein folding that has been impossible or difficult to obtain from ensemble measurements.

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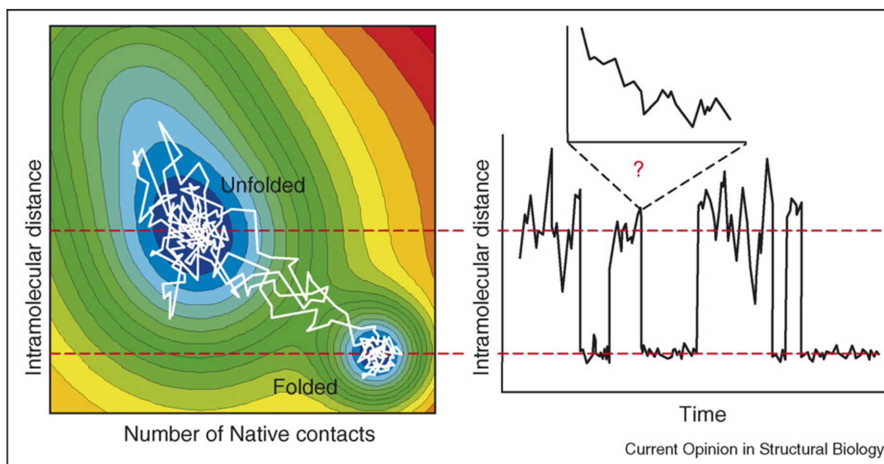
Introduction

A major technological development in recent years has been the capability of investigating protein folding and unfolding at the single-molecule level. Two principal methods are currently being used: force-probe techniques and fluorescence. Experiments using atomic force microscopy and laser tweezers have provided important and previously inaccessible information on the mechanical stability and folding of proteins, and the reader is referred to a number of recent reviews on this topic [1–5]. Here we focus on the investigation of single-molecule protein folding using the fluorescence method that has produced the most important and interesting results so far, Förster resonance energy transfer (FRET) [6–8], first demonstrated in single molecules by Ha *et al.* [9] and applied to protein folding in pioneering studies by Hochstrasser and coworkers [10,11] and Weiss and coworkers [12]. Recent applications to protein folding using other single-molecule fluorescence techniques and the closely related method of fluorescence correlation spectroscopy can be found elsewhere [13,14].

The exciting prospect of watching individual molecules fold has been the major motivating factor for single-molecule FRET studies. Additional motivation comes from computer simulations and analytical models of folding, which are playing an increasingly important role in investigations of protein-folding mechanisms. If accurate, everything that one could possibly know about a protein-folding mechanism is contained in a complete set of folding trajectories from atomistic molecular dynamics calculations. Such calculations have in fact just recently become possible for microsecond-folding proteins using distributed computing [15]. Moreover, most of what one would want to know about mechanisms is contained in the trajectories of coarse-grained representations of proteins [16]. Although ensemble experiments have provided a wealth of experimental data to test the accuracy of theoretical calculations, the experimental results alone yield relatively little information on actual folding mechanisms.

This point can be made clear by considering the case of a protein exhibiting two-state behavior (Figure 1). No matter what the probe, the same relaxation rates will be observed in kinetic experiments. The measured property at any time, moreover, will be a simple linear combination of the averaged property of the folded and unfolded states, and the time course will be exponential. The reason for this simple behavior is that there is a separation of time scales; that is, interconversion among all of the conformers in each of the two states is rapid compared to the rate of interconversion between the two states. Experimental information concerning the mechanism by which the protein proceeds between the folded and unfolded states comes primarily from the systematic analysis of folding kinetics, in particular ϕ values [17]. This approach uses measurements of the relative effect of mutations on rates and equilibria and their interpretation with extra-thermodynamic relations to infer the average structure surrounding a mutated residue in the ensemble of structures that make up the transition state. There is, with few exceptions [18], no structural information at any other point along the reaction coordinate. Such ensemble experiments therefore provide relatively little, though important information concerning mechanism, which for protein folding means describing the sequence of structural events in the microscopic pathways that

Figure 1



Two-state folding dynamics of an individual protein molecule illustrated as a diffusive process on a two-dimensional free energy surface (left) with a corresponding equilibrium folding/unfolding trajectory (white). An intramolecular distance (corresponding to the distance between a donor and an acceptor fluorophore in a single-molecule FRET experiment) is plotted as a function of time (right), showing rapid jumps between folded and unfolded states. An ultimate goal of single-molecule experiments is to time-resolve these transitions (expanded scale, top right).

connect the folded and unfolded states and the distribution of these pathways, as can be obtained from both simulations [15,19] and the solution to the kinetic equations of analytical models [20] (ER Henry, WAE, unpublished results).

In contrast to ensemble studies, the investigation of individual molecules promises direct access to information on microscopic pathways. The ultimate goal of single-molecule FRET studies is the time-resolved observation of individual protein-folding events, which will allow the acquisition of trajectories of FRET efficiency — and therefore distance — versus time as the molecule transits between the unfolded and folded states. Such data should provide new insights and be much more demanding tests of both simulation and analytical models, thereby speeding progress toward a quantitative and more complete understanding of protein-folding mechanisms.

Although single-molecule FRET studies are still in their infancy, step-by-step progress toward the goal of watching single molecules fold has already produced novel and important information which has been either impossible or difficult to obtain from ensemble measurements. Such information includes the ability to count thermodynamic states from the distribution of FRET efficiencies, the separate measurement of properties of subpopulations, such as distance distributions and dynamics of the unfolded state in the presence of an excess of the folded state, and the determination of equilibrium constants and rate coefficients from FRET trajectories by measuring the mean residence times in folded and unfolded states at equilibrium. Examples of each of these will be presented, with brief discussions of their relevance to current issues in protein folding.

Accuracy of distances from single-molecule FRET

Unlike most optical methods, FRET provides quantitative information on intermolecular distances (Box 1). An immediate question that arises in using the large chromophores that are necessary for single-molecule FRET studies is:how accurate is this distance information? This question was first addressed for polypeptides by Schuler *et al.* [22], who used polyproline as a spacer between donor and acceptor chromophores, as did Stryer and Haugland in their classic study which established FRET as a ‘spectroscopic ruler’ in biophysical research [23]. Schuler *et al.* measured the FRET efficiency of molecules that produce a burst of photons upon freely diffusing into and out of the illuminated volume of a confocal microscope (Figure 2). They found that the average FRET efficiency at short distances was slightly lower than predicted by Förster theory, but found that it was much higher than predicted for distances longer than R_0, assuming that polyproline is a rigid rod in the all-*trans* conformation. Two possible explanations for the small discrepancy at short distances are the lack of complete orientational averaging during the shortened donor lifetime to result in an orientational factor $\kappa^2 < 2/3$, and the breakdown of the point dipole approximation of Förster theory, which requires that the interchromophore distance be long compared to the size of the chromophores. To explain the much larger discrepancy for longer polyproline spacers, they carried out molecular dynamics simulations using an atomistic representation of polyproline in implicit solvent (i.e. Langevin simulations), which

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