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Protein folding at single-molecule resolution

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Abstract

The protein folding reaction carries great significance for cellular function and hence continues to be the research focus of a large interdisciplinary protein science community. Single-molecule methods are providing new and powerful tools for dissecting the mechanisms of this complex process by virtue of their ability to provide views of protein structure and dynamics without associated ensemble averaging. This review briefly introduces common FRET and force methods, and then explores several areas of protein folding where single-molecule experiments have yielded insights. These include exciting new information about folding landscapes, dynamics, intermediates, unfolded ensembles, intrinsically disordered proteins, assisted folding and biomechanical unfolding. Emerging and future work is expected to include advances in single-molecule techniques aimed at such investigations, and increasing work on more complex systems from both the physics and biology standpoints, including folding and dynamics of systems of interacting proteins and of proteins in cells and organisms.

1. Introduction

The proper folding of proteins is critically important in cellular activities, and involves substantial interesting physics and complexity. Hence, this physical chemical process has generated tremendous interest in the multidisciplinary field of protein science. The protein folding field has advanced greatly over the past 50 years due to knowledge acquired through a multitude of experimental, theoretical and computational approaches [1–8]. Experimental strategies have included ensemble methodologies such as NMR, fluorescence and CD spectroscopy, small-angle x-ray scattering, x-ray crystallography and protein engineering, among many others. In comparison, the application of single-molecule techniques in protein folding studies is relatively new, with many early single-molecule protein folding experiments being proof of principle studies or direct validations of previous ensemble observations. As we discuss here, important new insights are now increasingly being obtained from single-molecule folding studies, highlighting the potential impact and unique contributions that single-molecule applications can provide to this area of research that has engaged several generations of protein scientists (Fig. 1).

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1.1 Protein folding problems

The field of protein folding can be thought of in terms of three related but separate problems [3]. The first is the conformational thermodynamics issue, *i.e.*, for a given amino acid sequence and protein environment, what determines a protein's native structure? The second deals with the kinetics, mechanisms and energy landscapes of folding. And last is the task of predicting protein native structures. Along these lines, several more specific questions (Fig. 1) are addressed in the field, including:

- **a.** What are the thermodynamic states involved as a protein folds? Are there intermediates in the folding pathway? Are the intermediates on- or off-pathway? What are the structural properties of each thermodynamic state? What does the transition state look like?
- **b.** What are the sizes of the denatured state ensemble? Does it resemble a random coil, or is there significant residual structure, resulting in more collapsed conformations?
- **c.** What are the dynamics or fluctuations involved in each of the thermodynamic states? How much conformational fluctuations are present in the native state ensemble?
- **d.** What is the shape of the protein free energy landscape associated with folding? How rugged or smooth are the contours in the funnel-shaped landscape? What energy barriers are involved in the folding transitions? Can fast protein folders achieve global downhill folding?
- **e.** What is the nature of the interactions involved in protein folding? What are the enthalpic and entropic contributions to folding?
- **f.** How does interaction with ligands, molecular chaperones, protein partners or small molecule solutes such as salts and osmolytes change the energy landscape? How do parameters and conditions relevant to the cell affect protein folding?

The answers to many of these questions are critically important to function in the cell, and are encoded in a generalized energy landscape for protein binding and folding. Importantly, they will shed deep insight into fundamental biology, nanotechnology and protein design, and provide new ideas for strategies in combating protein misfolding diseases.

1.2 Early events in the history of single-molecule protein folding studies

Single-molecule techniques have the advantage of allowing the detailed examination of heterogeneous populations without ensemble averaging. Single-molecule measurements also eliminate the necessity of synchronization, which is required in some ensemble experiments. Research into single-molecule protein folding is a natural by-product of the recent technological advancements in single-molecule instrumentation, particularly in single-molecule fluorescence (especially FRET) and force techniques (*i.e.*, atomic force microscopy (AFM), and optical and magnetic tweezers). Applications of these single-molecule techniques range from the study of protein and nucleic acid folding and dynamics to structural investigations of protein complexes and other biomolecular machines (including the nucleosome, ribosome and motor proteins), and investigations in live cells [9–19].

The first reported single-molecule 'unfolding' measurements were performed using optical tweezers and AFM on the muscle protein titin, whose physiological function naturally involves unfolding/stretching [20–22]. Single-molecule fluorescence experiments on denaturant-induced protein unfolding soon followed [23,24]. Early work on protein folding that employed single-molecule FRET (smFRET) were performed using simple systems [23,24]. For example, an early study on chymotrypsin inhibitor 2 (CI2) demonstrated the

direct observation of folded and unfolded populations for a two-state system, and also showed that data acquired at the single-molecule level are consistent with bulk or ensemble data [23]. Early single-molecule unfolding experiments on a membrane protein were performed using AFM by Oesterhelt *et al.* [25], and presented the interesting observation that the transmembrane α-helices of individual bacteriorhodopsin molecules unfold and dissociate from the membrane in a pairwise and sequential manner. More recently, there has been a surge in single-molecule protein folding studies. This review is not meant to be comprehensive; rather it discusses several examples of information about the protein folding problem that are available from single-molecule studies, providing brief descriptions of common single-molecule and supporting techniques to orient the reader. The reader is also directed to several other reviews and original papers in the field for more information, several of which are cited throughout this review.

2. Single-molecule techniques

Here, we briefly introduce a couple of techniques that have been used in several single-molecule protein folding studies, and also discuss the use of computation and supporting ensemble methods. For additional technical details of instrumentation and theoretical aspects of single-molecule fluorescence and force techniques, the reader is referred to the following reviews: [9–19,26–32].

2.1 Single-molecule FRET

Förster/fluorescence resonance energy transfer (FRET) is the non-radiative transfer of singlet excitation energy from donor to acceptor chromophores. This transfer has a strong distance dependence in the 30–70 Å range, making it well-suited for probing conformational properties of proteins and other biomolecules. There are two methods for applying FRET at single-molecule resolution (smFRET): experiments can be carried out on “immobilized” or “freely diffusing” molecules [26]. In the latter technique, numerous single molecules freely diffusing in solution are observed one at a time, from which histograms and population distributions are generated. In comparison, experiments on immobilized proteins follow signals from single molecules for longer times (e.g., seconds), from which equilibrium and kinetic information can be derived. The free diffusion method has the advantage over immobilized methods in minimizing the potential for surface interaction artifacts. On the other hand, the immobilized methods have the ability to monitor longer time trajectories of conformational fluctuations. To circumvent surface interaction problems with the immobilized methods, Rhoades *et al.* [33,34] have used encapsulation of protein molecules in lipid vesicles. And recently, Chung *et al.* [35] have tackled significant issues in the immobilized methods for protein folding studies through careful analysis of fluorescent dye properties. smFRET methods provide information about several key molecular characteristics of particular interest to understanding protein folding, including conformational fluctuations, structural distributions and stochastic transitions of the protein within and between unfolded, intermediate and native ensembles. Furthermore, a range of timescales may be probed ranging from millisecond (or slower) to nanosecond (or faster) times (using correlation-type analyses as described later), covering regions of importance for local to global fluctuations and structural transitions. In addition, smFRET measurements can be carried out both *in vitro* and in cells, adding to its wide applicability.

2.2 Force methods (AFM and tweezers)

A number of single-molecule protein folding studies have been performed using atomic force microscopy (AFM), with a few key ones using optical tweezers (Fig. 2) [27,36]. With AFM, proteins are held between a flexible cantilever and a surface, are pulled on, and the response of the molecule in terms of extension can be studied. Similarly, with optical

tweezers, the molecule is held between two beads and the extension can be studied while applying forces to the molecule. These methods have the advantage of having more direct control on the reaction coordinate (which can be defined as the end-to-end distance), and the potential disadvantage in the necessity for the attachment of bulky "handle" surfaces or molecules. Changing pulling geometries can result in a high degree of anisotropy in the unfolding energy landscape [37], allowing a detailed exploration of folding energy landscapes [38]. The pulling force exhibits directionality, which can be controlled to mimic that which can naturally occur *in vivo*, for example, during the unfolding of muscle proteins or the action of the ATPase motors [39]. The use of force-clamp AFM instrumentation has opened the way for protein folding studies because it has enabled protein folding to be studied in reversible conditions, which is necessary for equilibrium thermodynamics investigations [22,40]. More recently, the use of lock-in single-molecule force spectroscopy has increased the force sensitivity to sub-pN regime [41,42]. There are fewer studies on protein folding using optical tweezers, but it has the advantage over AFM in that low forces and loading rates can often be used, resulting in increased spatial resolution.

2.3 In silico single-molecule protein folding

Progress in single-molecule protein folding research was accelerated by insights from computation and molecular dynamics (MD) simulations [6,43–48]. For example, steered molecular dynamics (SMD) simulations have validated and predicted many of the results from force manipulation of biomolecules [49]. By default, these simulations already start off using single-molecule proteins. In most single-molecule studies, the assignments or characterization of the structural or conformational changes down to atomic resolution could only be done *in silico*. In some instances, these simulations have been predictive, such as in the importance of the linkers in spectrin where mutations in these regions were found to cause disease [49]. In another recent example, collaboration between single-molecule experimental and computational groups has resulted in new insight into the folding of the dimeric Rop protein [50].

2.4 Essential experimental controls

Because single-molecule techniques are still relatively new, evolving and non-standard in most laboratories, it is necessary to use controls to check the reliability of the results acquired using single-molecule methods. In most cases, standard ensemble measurements should be used as controls for comparison and can give hints as to whether something is amiss [23,27]. In other cases, protein engineering methods can be used to advantage, such as in studying the different modules of titin and the corresponding AFM sawtooth curves [51] and the use of point mutations to validate models and methods [23]. For example, the presence of an intermediate can be further validated by making a point mutation to alter the population of the intermediate [52,53]. It is also sometimes necessary to perform ensemble measurements to separate out the contribution of artifacts related to dye properties, such as that performed in Citrine unfolding to check for the presence of an intermediate [54].

3. Equilibrium and kinetic information from single-molecule protein folding experiments

3.1 Thermodynamic and kinetic parameters

Protein energy landscapes can be fully described by the structural states involved, by the rates of folding and unfolding, and the transition activation barriers. Population distributions can be examined without significant model-dependent analysis and the free energy of unfolding ($\Delta G°_{N\text{-}D}$) can be obtained directly from smFRET population histograms of the native and the denatured ensembles as a function of increasing denaturant, as first

demonstrated by Deniz *et al.* for CI2 [23]. This aspect is most useful in the study of more complex proteins, as is the case when multiple coexisting states complicate simple ensemble analyses [55–57]. Also in an early study, Schuler *et al.* [58] presented limits to the activation energy barrier to folding from the initial estimates of polypeptide reconfiguration time according to Kramers theory, providing experimental information about the elusive pre-exponential factor [28]. A clever aspect of this study was the use of polyproline as a well-defined distance standard for more facile analysis of the data; however, even these molecules were later reported by these and other authors to be somewhat conformationally heterogeneous [59,60], which underlines the difficulty of obtaining “ideal” distance standards.

Unfolded states show a large degree of conformational fluctuations occurring in a wide range of timescales. For example, the natively unfolded proteins Sup35-NM prion and α-synuclein were demonstrated to exhibit high degrees of conformational fluctuations in the ns-μs timescales, as measured by FCS and FCS-FRET techniques [55,61]. The width of the transfer efficiency histogram peaks can reveal the underlying chain dynamics involved [28], taking into account contributions from shot noise. In the work by Nettels *et al*. [62], a reconfiguration time of ~50 ns was extracted from photon statistics for unfolded Csp, which places the free energy barrier at ~10 k_BT. Furthermore, using two parameters (shape of the end-to-end distance distributions and the effective end-to-end diffusion coefficient as a function of the denaturant concentration), Nettels *et al*. were able to describe a more quantitative free energy surface of the collapse of Csp.

Lipman *et al.* [63] initiated the use of microfluidic devices combined with smFRET to study protein folding kinetics, and observed the same folding rates as those observed in ensemble measurements. Orte *et al.* [54] also implemented a homemade nanomanipulation system to obtain the kinetic parameters for a triangular kinetic scheme (with the presence of intermediate) of unfolding of the β-barrel protein Citrine (a variant of GFP). More recent reports have continued this work, and such non-equilibrium measurements of folding will continue to be important in the future.

Thermodynamic parameters can also be obtained using force methods. For example, the Crooks fluctuation theorem [64,65] was employed to extract the equilibrium $\Delta G°$ from non-equilibrium force vs. distance curves detailed in [38]. Using optical tweezers, Gebhardt *et al.* [38] obtained the kinetic folding and unfolding rates, as well as the intermediates and transition state locations from the distances to generate a full distance-resolved energy landscape for GCN4 leucine zipper. In another AFM study, Rief and co-workers obtained the folding, unfolding rates (and related to k_{on}, k_{off} binding rates), stoichiometry and cooperativity involved in calmodulin binding to a variety of peptide partners [66,67]. It is important to note that thermodynamic parameters from mechanical unfolding by force techniques could be significantly different from smFRET chemical unfolding experiments. Along these lines, Best *et al.* have shown using ϕ–analysis to characterize transition states that different unfolding pathways are explored by mechanical and denaturant unfolding [44].

3.2 Folding intermediates and the physics of folding landscapes

Many smFRET studies to date have been with simple two-state folders, e.g., CI2 [23], CspTm [58], GCN4 [24], protein L [68], and protein G B1 domain [35]. Additional peaks in smFRET histograms of proteins under partially folding conditions signal the existence of folding intermediates. In some cases, different states are difficult to resolve and the presence of broad distributions will require substantial modeling. For example, recent studies on SNase [69] showed two-state folding behavior for protein variants that have been previously demonstrated as three-state folders. The authors indicate that it is most likely that they were not able to resolve the intermediate states. This illustrates the importance of having multiple

probes, as was the case for studies on CspTm and the four-helical Im7 (immunity protein). Data from multiple probes confirmed the two-state nature of CspTm [58,70] and that Im7 folds via an on-pathway intermediate under appropriate conditions [71]. In another study using two-color coincidence detection (TCCD) coupled to a nano-mixer, Orte *et al.* were able to detect an on-pathway intermediate [54]. Recent work has revealed details of a complex, multistate folding-binding landscape for the neuronal protein α-synuclein [55] (see below – section 4.1 and Fig. 3). In another example, the protein dimer Rop was studied using smFRET by Gambin *et al.*[50]. Based on computational analysis and previous ensemble data, Rop was predicted to have a dual-funneled native state. smFRET experiments directly validated this prediction and also provided interesting insight into the mechanism by which the system interconverts between the two minima.

Similarly, with AFM, the presence of additional features in the extension trace suggests the presence of intermediates [49]. Early AFM work on titin showed seemingly simple unfolding in an all-or-none fashion, although subsequent work showed a reversible intermediate that causes an extension of ~7Å [53,72]. For this protein, an unfolding intermediate was suggested to function as a kinetic trap to buffer and prevent the module from completely unfolding [73]. The existence of intermediates in the fibronectin domain FnIII1 is believed to have relevance for the protein's strength and formation of fibrils [74]. In several protein folding scenarios, intermediates have been suggested as obligate precursors in the folding pathway to the native state and are characterized by an ensemble of minimum energy collapsed structures (MECS) [75]. Ceconi *et al.* [52] have shown using optical tweezers that *E.coli* RNase H unfolds in a two-state manner, and refolds through an intermediate that is similar to that observed in ensemble measurements. This on-pathway intermediate was characterized by 'hopping' movements from intermediate to unfolded states, transient non-local contacts and non-specific tertiary interactions. This study also demonstrated how single-molecule methods could directly prove connectivity in different states involved in protein folding studies. With increased spatial resolution for optical tweezers, Gebhardt *et al.* [38] resolved two intermediate states in the GCN4 based leucine zipper unfolding. In the mechanical unfolding of green fluorescent protein, Dietz and Rief [76] showed the presence of intermediate states that are structurally different from denaturation experiments.

The case of barrierless or continuous protein folding is predicted by the folding landscape theory. Here, folding approaches the 'speed limit' or the maximum rate of protein folding [28,77,78], and the observed rates approaches the diffusion coefficient [79]. Several proteins with fast folding kinetics are believed to be within or near a downhill regime [77,79], and there has been significant discussion about this folding regime [80–84] for the case of BBL. Single-molecule experiments can in principle distinguish between barrierless or global downhill folding and barrier-limited folding. However, this requires that the observation time be in the μs regime for most fast folders [84]. In this context, Chung *et al.* [35] have continued to make progress towards measuring transition path times from smFRET equilibrium trajectories. These types of studies point to the need for continued progress towards more rapid and higher signal/noise smFRET measurements.

3.3 Sizes of unfolded states

smFRET has been used for several studies of the unfolded states of proteins. These heterogeneous ensembles have been of substantial interest because of their intrinsic complexity, links to polymer physics and the distinct possibility of residual structure that guides the subsequent folding of proteins. Most proteins studied by smFRET showed that native states are fairly constant in dimensions but the unfolded states expand with increasing denaturant concentration. Ziv *et al.* [85] explained this behavior using polymer theory. The authors noted an interesting correlation between slopes of collapse and folding free energies

vs. denaturant concentration, pointing to the mediatory role played by the denatured state in denaturant effects on folding. Recent data in FynSH3 using multiple pairwise probes showed that the denatured ensemble dimensions are in agreement with a random coil ensemble, with potentially significant deviations that can suggest residual structure, as seen in NMR studies [86]. Studies on Staphylococcal nuclease (SNase) showed that in 4M GdmCl, the protein has an R_g of 42–44 Å, close to what is expected for a random coil (45Å), but significantly larger than the 37Å measured by SAXS in 6M GdmCl [69]. Recently, Nettels *et al.* [70] studied the temperature-induced collapse of CspTm using smFRET and molecular simulations, and concluded that the "hydrophobic collapse" is not the main mechanism for the observed collapse, and that secondary structure formation and hydrogen bonding plays significant roles in the collapse. This was also verified by using an extremely hydrophilic intrinsically disordered protein, prothymosin α, which also showed collapse. The "unfolded states" of intrinsically disordered proteins (IDP; see section on IDPs below) can be directly studied under native conditions as discussed below.

4. Other novel insights into protein biophysics and biology

4.1 Intrinsically disordered proteins

More than 50% of the cellular proteome consists of proteins with long disordered regions [87], and >70% of cancer-related proteins have significant disorder [88]. Uversky *et al.* introduced the 'D^2 concept' to show that there is a widespread occurrence of disordered proteins in diseases [89]. The advantages of intrinsic disorder in cellular processes include increased flexibility, potential for high binding specificity coupled with lower affinity, and interaction with multiple binding partners [90,91]. With IDPs, the native states have a wider conformational space that is accessible, which could be relevant to protein function through formation of transient encounter complexes via a 'flycasting mechanism' [92,93], although the corresponding slower diffusion would also influence the binding kinetics.

In an early study, Mukhopadhyay *et al.* [61] showed that for the amyloid determining region of the yeast prion Sup35, the natively unfolded ensemble is more compact than the denaturant-induced unfolded ensembles of most proteins, and observed a gradual expansion of the natively unfolded state upon increasing denaturant concentration, indicating a collapsed unfolded state. Moreover, the ensemble is rapidly interconverting (on the sub-μs timescale). This behavior may have relevance for the aggregation of this protein, which is thought of as an example of the class of functional amyloids. With another IDP, α-synuclein, a similar collapsed state is observed in the absence of denaturants, and a non-cooperative transition of the natively unfolded protein with denaturant titration was observed (Fig. 3) [26]. A recent combination computation and FCS study provided new insight into the effects of charge content and distribution on IDP conformational ensembles [48]; the role of charge was also explored in a recent smFRET study [94]. Overall, the disordered states of IDPs encode interesting features that differ from those of unfolded globular proteins, and that may play key roles in their function and dysfunction.

Another exciting feature of IDPs is that most undergo disorder-to-order transitions upon binding to partners, which could be key to their cellular function. Using single-molecule FRET, Ferreon *et al.* [55] studied the coupled folding and ligand binding equilibria of the Parkinson's disease-associated IDP α-synuclein. In the presence of the lipid mimetic SDS and small unilamellar phospholipid vesicles, a remarkable conformational plasticity of α-synuclein was revealed as the protein cycles through multiple conformational states as a function of ligand properties (Fig. 3). The observed conformations exhibited different conformational fluctuations in various timescales. The reported single-molecule results could be of relevance to the *in vivo* function of α-synuclein, the protein's natural membrane interactions, and its propensity to misfold and aggregate. smFRET data in a subsequent

paper [56] showed that the mutation A30P associated with early-onset Parkinson's disease has a pronounced effect on the protein's folding-binding landscape. Other smFRET studies have provided similar interesting information about some aspects of wild-type α-synuclein folding-binding properties [95,96]. AFM studies have also provided new information about this protein [97,98], including revealing the presence of significant intermolecular interactions between α-synuclein molecules, which may be important during aggregation, and residual structure that varies between wild-type and mutant proteins.

4.2 Assisted protein folding

4.2.1 Molecular chaperones—Many cellular proteins are prone to misfolding and aggregation during the folding reaction. To counter these adverse pathways, there is a class of protein molecular chaperones whose primary cellular function is to aid the protein folding process. The best-studied example is the bacterial GroEl-GroES system. The mechanisms of GroEl-assisted folding have been extensively investigated in ensemble experiments [99,100]. Several ensemble studies suggest that GroEl-GroES behaves like a passive Anfinsen cage that captures aggregation-prone folding intermediates [99], although other studies have suggested that GroEl-GroES may play a more active role in assisted folding such as in changing the conformation of substrates inside the cage [100].

Recent studies using smFRET indicates that substrates (maltose binding protein [MBP] and rhodanese) inside the GroEl cavities in the early stages prior to GroES binding and ATP hydrolysis are characterized by a broadened conformational distribution [101,102]. More recently, Chakraborty *et al.* [103] presented smFRET data as supportive of GroEl-GroES rescuing a protein from a kinetically trapped state. Also in recent work, Hofmann *et al.* [104] reported smFRET results that show differential effects of this chaperonin system on folding of the different domains of MBP. In a different chaperonin system, Bechtluft *et al.* [105] used optical tweezers to investigate the mechanism of SecB in the translocation of newly synthesized polypeptides across membranes. Also using MBP as the test substrate, they showed that SecB interacts with the molten globule-like state and extended states of MBP, protecting the hydrophobic regions and preventing aggregation. Thus, in these examples, by binding to chaperones, the folding energy landscape is re-shaped, with previously easily accessible conformational states such as the aggregated states now exhibiting higher energy barriers to formation.

4.2.2 Chemical chaperones - osmolytes—Nature employs naturally occurring small molecules collectively known as osmolytes to modulate the conformational properties of proteins in response to a wide variety of environmental stresses [106]. Ensemble studies by Bolen and co-workers have shed light on the mechanism of protein action for a variety of these osmolytes [107–112]. From single-molecule AFM studies, Garcia-Manyes *et al.* [113] showed that ethanol and the osmolyte glycerol affect the activation energy barrier to unfolding of ubiquitin, and both molecules have varying effects on the protein native, transition and unfolded states. While glycerol has an effect on the native and extended states, it had no effect on the ensemble of minimum energy collapsed structures that are necessary intermediates prior to the native state form.

4.3 Co-translational folding

Co-translational folding incorporates additional interesting elements into the folding process [114]. Folding occurs before the entire protein chain has been synthesized. Additionally, the ribosome plays a significant role in protein folding through a co-translational folding process. Co-translational folding narrows the available configurations that a protein can access. Recently, Katranidis *et al.* [115] used single-molecule fluorescence microscopy to follow the co-translational folding of a fast folding and maturing variant of green fluorescent

protein (GFP), GFP Emerald. They found that the entire process of translation and folding occurs with a time constant of 5.3 min. This time also reflects the maturation of the chromophore, which is the limiting step to the actual detection of the process. They also observed appearance of the fastest GFP molecules within one min for a significant fraction of proteins.

4.4 Bio-mechanical forces

Mechanical forces govern many cellular processes. Studies in AFM and force manipulation methods have given valuable insights into these biological processes [42]. The protein titin acts as an efficient entropic spring, and the purpose of the force hysteresis is to keep efficiency high due to the repetitive mechanical loading in muscles [52]. In a detailed AFM study by Li *et al.* [73] on the different force-extension properties of the sub-domains of the elastic region of cardiac titin, the investigators were able to reconstitute the function of the whole from the sum of its parts. In particular, the unfolding of the proximal Ig modules was shown to protect cardiac sarcomeres from being damaged at forces beyond the physiological range. However, recent studies by Williams *et al.* [116] argue against this conclusion because I27 was found to withstand higher forces and under physiological forces, the partially unfolded intermediate does not contribute to mechanical strength. However, titin may also have other biological functions, e.g., as a mechanical signal transducer [42,117]. Stacked helical ankyrin repeats were found to behave like a mechanical nanospring [118]. The amazing reversible force generated by the ankyrin repeats has relevance in mechanotransduction signaling in cells such as the mechanoreceptors in hair cells. Studies on the extracellular matrix protein tenascin, which consists of fibronectin type III domains, suggest that their elasticity is similar to titin and that they aid in mechanical interactions necessary in cell rolling and migration [74].

An interesting aspect of mechanical unfolding of proteins was observed in studies of variably linked ubiquitin chain either in N-C or Lys48 position, demonstrating a difference in mechanical stability with respect to the direction of the pulling geometry. The lower force necessary to unfold the Lys48-Ub provides a possible mechanism for which proteins will be targeted for proteosomal degradation, where proteins attached to the Lys48-polyubiquitin chain is required to unfold before the polyubiquitin-proteosome interaction breaks [39].

5. Emerging areas and future directions

Several questions in the area of protein folding were posed in the introductory sections of this article. We hope the reader has obtained a feel for how single-molecule methods are contributing to answering several of these key questions. Continued progress in the field will however necessitate improvements in multiple areas including instrumentation and physical, chemical and *in silico* analyses tools, such as by use of microfluidics [63,119–122], improved dyes and sample conditions, smaller cantilevers, enhanced imaging modes [123–125], improved measurements of biomolecular thermodynamics [126], multicolor FRET [127], and extension to shorter single-molecule distance rulers [128–130]. In addition, the theoretical understanding of single-molecule data can build upon excellent existing work in the area [45,131–140]. This is important to permit improved means to uncover thermodynamic and time-dependent information from complex single-molecule data. While most single-molecule folding studies have been on relatively simple systems, the majority of the cellular proteome is composed of large, multi-domain proteins that have complex folding behavior and sometimes a significant degree of irreversibility [141]. Single-molecule measurements will continue to target these types of systems, affording substantial advantages in measuring details of the complexity and also minimizing the side-reaction of aggregation. In addition to probing systems with direct relevance to biology, single-molecule methods will also increasingly target protein systems encoding novel physics, such

as downhill folders, knotted proteins and systems of interacting proteins. More complex protein systems will particularly benefit from the development of new chemical biology methods of sample preparation, for example for site-specific labeling or attachment [142]. In addition to folding, studies are also beginning to target misfolding and aggregation [55,143,144], the latter of which has been closely linked to disease but also has potential functional implications. Single-molecule methods should prove even more valuable for mechanistic studies of this extremely heterogeneous reaction. Another key point is that most folded proteins fold in the unique environment of the cell, presenting additional features such as macromolecular crowding and a huge number of possible binding partners. Understanding the influence of such parameters will also continue to be at the forefront by use of both *in vitro* and cell-based experiments. As the field matures further, the strengths of single-molecule analyses will also be increasingly combined with results of other experimental, computational and theoretical analyses to probe more complex systems with better resolution in many dimensions, and to directly relate folding and dynamics with function at various levels of organization.

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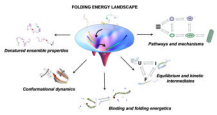

Fig. 1.
Several areas of interest in the protein folding field linked to the concept of the protein folding funnel.

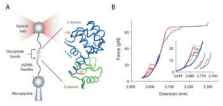

Fig. 2.

T4 lysozyme folding cooperativity investigated using optical tweezers. A. The experimental setup, with the protein attached to beads via long DNA handles, and using optical trap for manipulation. B. Unfolding (red) and refolding (blue) force-extension curves, with the inset showing data at higher resolution, showing variability in folding-unfolding behavior. Adapted by permission from Macmillan Publishers Ltd: Nature, Shank *et al.* [36] 465:637 (2010), copyright 2010.

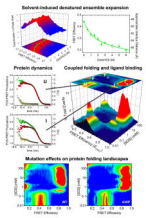

Fig. 3.

Equilibrium, dynamic and binding-folding behavior of an intrinsically disordered protein studied using single-molecule fluorescence. α-Synuclein denaturation demonstrates non-cooperative expansion (top, adapted from [26], Ferreon *et al.* Methods Enzymol. (2010) 472:179). SDS binding reveals multistate folding and varied dynamics (middle, adapted from [55], Ferreon *et al.* Proc. Natl. Acad. Sci. USA, (2009) 106:5645). The Parkinson's disease-linked mutation A30P substantially alters α-synuclein binding-folding landscape (bottom, adapted from [56], Ferreon *et al.* Angew. Chem. Int. Ed. (2010) 49:3469).