HHS Public Access

Author manuscript

J Mol Biol. Author manuscript; available in PMC 2018 March 19.

Published in final edited form as:

J Mol Biol. 2018 February 16; 430(4): 405–408. doi:10.1016/j.jmb.2017.12.019.

Editorial Overview: Single-Molecule Approaches up to Difficult Challenges in Folding and Dynamics

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Single-molecule approaches have gained increasing acceptance in biomedical research and industry in the past two decades and have led to wide-ranging applications due to their unique abilities to study complex dynamics of macromolecules [1–4]. A variety of methods have become available, including single-molecule fluorescence microscopy [5, 6], single-molecule force spectroscopy [4], and nanopore technologies [7–9]. Single-molecule fluorescence detection has become the basis of many super-resolution imaging [1] and high-throughput DNA sequencing technologies [10]. In particular, single-molecule fluorescence resonance energy transfer (smFRET) [11] has been widely used to study conformational transitions of biomolecules. Single-molecule force spectroscopy includes the optical tweezers, magnetic tweezers, and atomic force spectroscopy (AFM) [3]. These tools have made it possible to apply tiny mechanical forces to single molecules and detect their conformational changes in response. Finally, nanopore technologies measure the current change across a nanopore as a single macromolecule passes through the pore, allowing the composition and conformation of the macromolecule to be detected [8, 9]. A notable application of the nanopore technology is nanopore DNA sequencing [7, 9].

In traditional ensemble kinetic studies, macromolecules are first synchronized in specific states and then triggered to react with other molecules or change conformations, such as protein folding or unfolding, through a change in the solution conditions. The observed signal is averaged over different molecules in different states. As the reaction approaches equilibrium or a steady state, the observed signal relaxes over time and eventually stops changing. It is often challenging to deconvolute state transitions from the generally monotonical signal, especially when the reaction involves multiple states or time scales. Single-molecule approaches can overcome these issues, because the signals from different states are often directly observed due to lack of averaging. Furthermore, reaction kinetics can be derived under equilibrium conditions, bypassing the synchronization step required for traditional kinetic studies. Consequently, complex reaction networks have been dissected using single-molecule approaches [12, 13].

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In this special issue, we focus on single-molecule approaches to address challenging problems in folding dynamics of proteins and RNA. The issue contains two reviews and nine research articles, which covers not only single-molecule approaches, but also molecular dynamic simulation and ensemble experimental approaches widely used in folding studies. Two major single-molecule methods, smFRET and optical tweezers are described in the research articles and results from magnetic tweezers and AFM are reviewed. These methods detect changes in distances between two specific sites on proteins or nucleic acids with spatial resolution from subnanometer to nanometers and temporal resolution from microseconds to milliseconds [14–16], depending upon the method. These distance changes are then used to deduce conformational changes of macromolecules. Because one-dimensional distance measurement is often insufficient to define the conformational changes, high-resolution structures of the macromolecules in specific states are often used to help design the sites for fluorescent labeling or mechanical pulling and to interpret the signal in terms of the associated conformational changes.

Protein or RNA folding and unfolding is often described as diffusion across an energy barrier between two potential wells in an energy landscape corresponding to the folded and unfolded states. Whereas the conformations of folded states are often known, the conformations and dynamics of proteins in unfolded states remain controversial. To address this question, Guinn and Marqusee unfolded the acyl-coenzyme A binding protein by mechanical force, temperature, and urea and measured the solvent accessible surface area of the unfolded states generated by the three methods [17]. Here the force-induced protein unfolding was conducted using optical tweezers in the presence of urea in different concentrations. They found that the unfolded states generated by force and urea have the same solvent accessible surface areas within experimental error, whereas the unfolded state at high temperature has a smaller solvent accessible surface area, indicating that the unfolded state is more compact at higher temperatures. On the other hand, Maity and Reddy found that the unfolded ensemble of monellin becomes more compact as guanidinium chloride concentration decreases [18]. Their conclusion was derived from a coarse-grained protein model and molecular dynamics simulation, essentially a single-molecule experiment in silico. Thus, experimental results from both groups demonstrate the complexity of unfolded proteins, which is proving to be an exciting area of current research. In general, protein folding is dominated by a relatively long dwell time of the protein in the unfolded state, whereas the time that a protein actually takes to cross the energy barrier, or the transition path time, is often too short to be detected. Chung reviewed recent progresses in understanding the transition path time of protein and DNA hairpin folding [14]. smFRET and optical tweezers have been used to measure the transition path time, which is 2–16 μs for FBP28 WW domain, depending upon glycerol concentration [19], and 28 μs for the DNA hairpin [15]. Interestingly, the transition path time only weakly depends on the energy barrier height.

Three papers examined single proteins or protein complexes near the folded states. Using high-resolution optical tweezers, Rebane *et al* investigated coupled folding and assembly of three soluble N-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) [20]. Unlike most proteins whose folding precedes their biological functions, SNARE folding per se serves as their major function, which is coupled to synaptic vesicle fusion and

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neurotransmitter release [21]. As a reaction product, the fully assembly SNARE complex needs to be disassembled and recycled for next round of membrane fusion. Numerous SNARE mutations have been identified in patients with epilepsy, intellectual disability, ataxia and other diseases or disorders. Rebane *et al* found that two disease mutations in the SNARE protein SNAP-25B significantly destabilize the C-terminal domain of the SNARE complex, providing an attractive explanation for its loss of function in membrane fusion. Wruck *et al* reviewed recent single-molecule optical tweezers studies of two protein chaperones, Hsp70 and the bacterial trigger factor and [22]. Hsp70 mediates folding of numerous proteins in an ATP-dependent manner whereas the trigger factor modulates protein folding in an ATP-independent manner. However, both chaperones bind client proteins in conformations close to their corresponding folded states, in contrast to previous findings that both chaperones mainly target the unfolded proteins. Thus, both chaperones appear to play an active role in protein folding instead of just shielding unfolded protein from aggregating. Gambin and coworkers used single-molecule fluorescence microscopy to investigate polymerization of ASC, a protein involved in inflammation signaling [23]. They observed that ASC alone can polymerize into a fibril-like structure in a high ASC concentration. smFRET measurements of a small fraction of labeled ASC in the presence of 100 fold excess of unlabeled ASC showed that ASC monomers undergo a major conformational change upon polymerization to bring two subdomains in close proximity. In low ASC concertation, fragmented ASC fibrils can serve as seeds to facilitate fast fibril extension. Thus, ASC can polymerize in a prion-like manner, which may help propagate inflammatory signals from cell to cell.

Two research articles report interesting RNA dynamics detected by smFRET. Daher and coworkers investigated the effect of crowding agents on the docking-undocking equilibrium of the hairpin ribozyme [24]. Two crowding agents are examined, polyethylene glycol and yeast extract. They found that polyethylene glycol stabilizes the ribozyme in the docked and folded state, due to effects of excluded volume and a nonspecific weak interaction between polyethylene glycol and the ribozyme. However, in the presence of yeast extract, the hairpin ribozyme is stabilized in the folded state mainly by specific interactions between molecules in the cell extract and the ribozyme. Van der Feltz *et al* elucidated the effects of uridine modifications on the conformational switching of stem II in the U2 small nuclear ribonucleic acid (snRNA) [25]. U2 stem II can fluctuate between two conformations where either IIa or IIc region is base-paired in a mutually exclusive manner. The authors discovered that uridine 56 pseudouridylation stabilizes stem IIc and slows down its transition to stem IIa. In contrast, uridine 93 pseudouridylation facilitates the transition from stem IIc to stem IIa.

Hammond *et al* studied the role of DDX21, a DEAD-box helicase, in Rev/RRE complex formation, using both ensemble and single-molecule fluorescence approaches [26]. The HIV-1 protein Rev binds its cognate Rev response element (RRE) in the viral RNA transcript, which is required for exporting the transcript to the cytoplasm. The authors found that DDX21 can bind to the RRE and such binding stimulates DDX21's ATPase activity. DDX21 also promotes Rev binding to the RRE. However, the ATP-dependent helicase activity is inhibited by Rev.

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One of the greatest challenges in the protein-folding field is to quantify the stability and folding dynamics of membrane proteins. Recent developments in single molecule studies have allowed determination of the stability of membrane protein *in vitro*. Using smFRET, Krainer *et al* determined the stabilities of a model membrane protein Mistic in micelles formed by different detergents [27]. Mistic is interesting because it is a helical protein yet is soluble in aqueous solution. They found that Mistic is more stable in a hydrophobic environment, but folds faster in micelles containing detergents with zwitterionic headgroups. Fefferson *et al* reviewed applications of single-molecule approaches, including AFM, single-molecule fluorescence, and magnetic tweezers to membrane protein folding [28]. The authors highlighted a key advantage of single-molecule methods, which avoid protein aggregation typically encountered in ensemble studies of membrane protein folding, and demonstrated the great potential of these approaches to addressing changeling biological problems.

The reviews and research articles in this special issue exemplify state-of-the-art applications of single-molecule approaches and associated methods in studies of macromolecular folding and dynamics. Further research is required to better understand membrane protein folding and stabilities, protein misfolding and prevention, chaperoned protein folding, and protein folding in the cell. To this end, different single-molecule methods may be combined to simultaneous detect three-dimensional protein folding and protein-ligand or protein-protein interactions [29].

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