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Review Article

From folding to function: complex macromolecular reactions unraveled one-by-one with optical tweezers

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Single-molecule manipulation with optical tweezers has uncovered macromolecular behaviour hidden to other experimental techniques. Recent instrumental improvements have made it possible to expand the range of systems accessible to optical tweezers. Beyond focusing on the folding and structural changes of isolated single molecules, optical tweezers studies have evolved into unraveling the basic principles of complex molecular processes such as co-translational folding on the ribosome, kinase activation dynamics, ligand–receptor binding, chaperone-assisted protein folding, and even dynamics of intrinsically disordered proteins (IDPs). In this mini-review, we illustrate the methodological principles of optical tweezers before highlighting recent advances in studying complex protein conformational dynamics – from protein synthesis to physiological function – as well as emerging future issues that are beginning to be addressed with novel approaches.

Introduction

Understanding how proteins spontaneously fold into their specific, three-dimensional structures has been the objective of intense research for decades. Proteins can fold via different pathways and populate intermediate states along the way, each with their unique lifetimes and stabilities [1]. Multi-domain proteins may have sophisticated intramolecular interactions that lead to complex and multi-pathway folding [2]. Albeit governed by the same general principles as *in vitro*, inside the cell the folding process is further complicated by various molecules and intracellular factors such as chaperones [3], ionic strength [4], molecular crowding [5], and liquid–liquid phase separation [6]. Once properly folded, the protein can perform its functional role, often interacting with specific partners that can affect its native structure and stability. Conversely, if folding goes wrong, the protein can become useless and toxic, often leading to oligomerization and fibrillar aggregates that have devastating effects on the organism [7]. Understanding the molecular principles of protein folding is thus a prerequisite for tackling diverse functional and dysfunctional aspects of these fascinating macromolecules.

The intricacies of molecular events are often invisible to classical biochemical methods as they probe simultaneously the behaviour of enormous collections of non-synchronized molecules. This is especially important for proteins: each individual protein can fluctuate between different structural conformations through stochastic processes that are governed by probabilities and thermal fluctuations. To go beyond the averaged information provided by these bulk techniques and detect transient and non-cumulative molecular events, methods should be employed that allow the characterization of the structural dynamics of individual molecules. Transient or rare events often contain important mechanistic information about the functions of biomolecules, and can represent the starting points for misfolding trajectories. The last

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two decades have witnessed the advent of impressive technical advances in single-molecule detection techniques [8], such as optical tweezers [9–11] and fluorescence spectroscopy [12], which allow the visualization and modulation of the conformational equilibria of individual molecules with ever greater resolution. These highly advanced techniques have provided a fresh view on the protein folding problem by allowing researchers to visualize, even in real-time, the pathways followed by individual molecules. Moreover, these tools have lately been used by a growing number of laboratories to study other complex biological problems as several advanced instruments are now available commercially (JPK Nano Tracker™ and LUMICKS C-Trap™) and data analysis has become more streamlined and user-friendly for non-physicists.

Here we review the recent scientific literature where optical tweezers were used to study the mechanical properties and conformational dynamics of complex molecular systems. We begin with a short summary of what has been learned on protein folding using simple systems before delving into more complex molecular assemblies and various effects on folding pathways. The purpose of this review is to give the reader a sense of the information and the molecular framework that can be probed with optical tweezers on protein conformational changes. We restrict ourselves to protein conformational dynamics and therefore do not include the extensive work done on the mechanical properties of nucleic acids or the mechanism of molecular motors (see, for example, [13,14]). For information on other force spectroscopy tools for protein folding, such as atomic force microscopy (AFM) and magnetic tweezers, we refer the reader to excellent reviews on those subjects [15–17].

Optical tweezers use gentle force to manipulate molecules

Since the pioneering work of the Nobel prize winner Arthur Ashkin in the late 80s [18–20], optical tweezers have evolved into a behemoth in the field of single molecule biophysics, helping scientists understand the molecular mechanisms underlying the most fundamental biological processes [21–27]. Optical tweezers use light to trap small dielectric objects in diffraction limited spots and to measure the tiny forces acting on them. Over the years, many instrumental and theoretical advances have dramatically refined the optical trapping technique, allowing the development of increasingly sophisticated setups. High-resolution dual-trap optical tweezers with differential detection are currently capable of measuring sub-picoNewton forces and detecting Ångstrom scale displacements on the second time scale [28,29]. These optical tweezers use a single laser to generate two orthogonally polarized beams that are tightly focused via high numerical aperture objectives to form two distinct optical traps (Figure 1). To manipulate individual molecules, each trap is typically used to hold a micron-sized plastic bead that can be biochemically linked to the molecule of interest to function as tethering points. During the experiment, the molecule can be stretched or relaxed by moving one trap relative to the other, while its extension, as it loses or gains structure, is measured with high resolution as a function of the applied tension. Alternatively, the two traps can be either held still at a certain distance, to watch the molecule fluctuating at equilibrium between different molecular conformations at constant average force, or moved relative to each as the molecule unfolds or refolds to keep the applied tension constant [30]. Alternative setups include using only one movable optical trap to hold a bead and attaching a second bead to a fixed pipette tip [31,32].

Single proteins are typically manipulated using DNA molecular handles, as depicted in Figure 1. This experimental strategy presents several advantages: (i) DNA molecules of different lengths and chemical reactivity can be easily produced through standard biochemical methods [11,33]; (ii) the behaviour of DNA under mechanical tension has been characterized in great detail, both experimentally and theoretically [34], allowing for a relatively simple interpretation of the recorded traces when manipulating protein–DNA chimeras [35–38]; (iii) the effects of the handles on proteins' structures and dynamics have been assessed through different methods. For example, DNA-modified RNase H has been shown to retain enzymatic activity and when attached to small handles the protein retains its secondary structure [39]. Additionally, the results of several studies have shown that DNA tethering typically does not change the thermodynamic stability of a protein [40,41], further suggesting that DNA linkers do not affect significantly the structural dynamics of a protein.

In parallel to the advent of high-resolution optical tweezers, the field has also seen the development of hybrid instruments where mechanical manipulation is combined with other techniques, providing the opportunity for additional readouts [42–44]. In particular, hybrid systems integrating optical trapping with fluorescence techniques offer the possibility of transcending the one-dimensional nature of the data obtained through mechanical manipulation, thus providing the opportunity of exploring the energy landscape of biomolecules along different reaction coordinates (Figure 1E) [45–50]. These instruments have given researchers a unique glimpse into the microscopic world of individual biomolecules, as we outline below.

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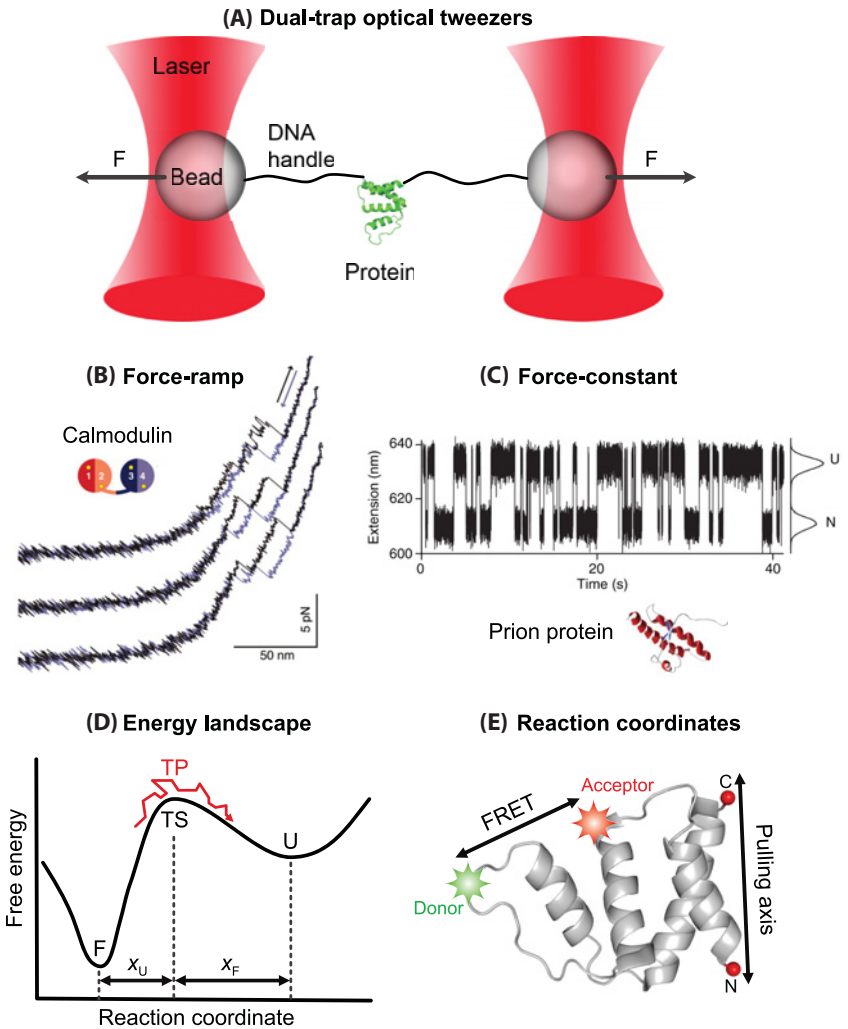

Figure 1. An overview of the optical tweezers methodology and concepts

(**A**) Schematic representation of the experimental strategy typically used to manipulate a single protein using dual-trap optical tweezers. Each optical trap is used to hold a plastic bead that is connected to the protein through DNA molecular handles that function as spacers between the two beads to avoid unwanted interactions between the two tethering surfaces during the experiment. Diverse strategies have been developed to attach the DNA handles to the protein and to the beads, allowing different pulling geometries and experimental conditions [10,11,33,51–53]. During the experiment, the position of the two traps can be changed to vary the distance between the two beads and thus the tension applied on the molecule. In this way the molecule can be stretched and relaxed, and force-ramp data can be acquired (**B**). The force-ramp trajectories show different folding and unfolding routes of single calmodulin molecules (adapted from [54] with permission). Alternatively, the distance between the two traps can be kept fixed to monitor the molecule fluctuate at equilibrium between different structural conformations at a constant average force, or they can be moved through a force-feedback mechanism that keeps the applied tension constant during unfolding and refolding transitions, allowing the acquisition of force-constant data (**C**). The trajectory shows fluctuations between the unfolded and native state of prion protein (adapted from [55] with permission). (**D**) A simplified energy landscape for a two-state protein system (U: unfolded, F: folded) is shown where the free energy of the molecule is reported along a well-defined reaction coordinate, that is the molecular extension. The distances from the folded state (x_U) and the unfolded state (x_F) to the transition state (TS) are indicated and can be determined from either transition rates or (un)folding force distributions [56]. The transition path (TP, red line) is the short region in the single-molecule trajectory when the transitions state barrier is crossed by conformational excursions that contain mechanistic information on the folding process (see discussion on transition paths below). To harness even more information from optical tweezers experiments, hybrid approaches have been developed where mechanical pulling is combined with Förster resonance energy transfer (FRET) measurements between two fluorophores, enabling the monitoring of molecular extension along two different reaction coordinates simultaneously [57] (**E**). Protein structure used in panels (A) and (E) are for illustration purposes only (acyl-CoA binding protein, PDB 1NTI).

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Folding and misfolding of single protein molecules

How proteins fold into their native three-dimensional structure following translation or after spontaneous unfolding, has been the subject of intense investigation for decades. An astonishing body of information has accumulated on every facet of protein folding using diverse conventional bulk methods [1,58–60]. However, the first real-time observation of an on-pathway intermediate state during the folding of a protein was observed by Cecconi et al. [39] using optical tweezers in 2005. The folding of ribonuclease H was observed to proceed through an obligatory intermediate state which resembled a molten-globule that had previously been indirectly detected for the protein using bulk experiments [61]. Optical tweezers allowed the authors to peer into the elusive molten-globule state, which revealed an unusually deformable and weak structure, in line with a lack of consistent tertiary structure formation [62].

After the pioneering work presented in [39], a growing number of proteins have been studied with optical tweezers. For example, the calcium-binding proteins calmodulin and neuronal calcium sensor-1 (NCS-1) have been extensively studied by optical tweezers and found to have exceedingly complex energy landscapes with multiple on- and off-pathway intermediates [21,40,54,63–65] on route to the native structure. Both proteins have similar architecture with two pairs of calcium-binding EF-hands organized into two discrete domains. By carefully dissecting the protein into fragments, Stigler et al. mapped the energy landscape for calmodulin folding and could pinpoint off-pathway misfolded intermediates that consisted of incorrectly paired EF-hands [54]. Interestingly for NCS-1, a protein involved in an array of cognitive dysfunctions, similar incorrect EF-hand pairing was dependent on calcium concentration, implying a link between misfolding and neurodegeneration [21].

The Woodside group has been particularly successful in describing the folding of both nucleic acids and proteins with optical tweezers [22,66], amongst which is the infamous prion protein (PrP). Yu et al. demonstrated the presence of multiple misfolding pathways for PrP and showed that a mutant protein associated with higher aggregation propensity visited some of the misfolded states more frequently [55]. The same PrP protein was also used to follow transition paths: the microscopic route that a protein molecule takes to cross the barrier separating the unfolded and folded states (Figure 1D). The transition path is inherently a high-energy, short-lived and unstable state which makes it particularly challenging to study. The transit times were recently measured by single-molecule fluorescence experiments [67], but a direct visualization of the transition path has not yet been revealed beyond simulations [68]. Neupane et al. used high-resolution optical tweezers to glimpse into the transition paths taken by PrP [69] and found that a single PrP molecule has a broad distribution of transit times, indicative of a rugged and multidimensional energy landscape [70–72]. The mean transit time of 0.5 ms agreed well with expectations from previous measurements on the proteins folding energy landscape [73].

The unfolded state has also gained much attention recently in folding studies, in attempts to pinpoint the early events that lead to successful folding or the non-native interactions that lead to alternative states. The heterogeneous nature of the unfolded state raises the question of whether different experimental methods probe the same ensemble properties. Guinn and Marqusee explored the ensemble of structures in the denatured state of acyl-CoA binding protein (ACBP) through chemical, thermal and forced unfolding experiments [74]. The authors found that urea and force lead to a more extended unfolded state than temperature does and that temperature changes the denatured state ensemble, underscoring the need to proceed with caution when comparing unfolding data obtained with different denaturants.

Clearly, a wealth of information on protein folding has transpired from optical tweezers, which have provided a fresh perspective on this decades-old problem. Now, in the post-maturation years of the method, optical tweezers are being used to peer into ever more complex biological systems including co-translational folding on the ribosome, molecular chaperones, transcriptional regulation, nucleosome dynamics, and ligand binding. We begin our journey by describing protein folding on the ribosome.

Co-translational folding on the ribosome

Proteins can already start to fold as they are being translated and elongated from the ribosome [75]. Translation and folding on the ribosome can induce significant mechanical forces, rendering co-translational folding on the ribosome the subject of many single-molecule studies. Alexander et al. [76] used the calcium binding protein calerythrin to test a hypothesis stating that proteins fold co-translationally at equilbrium due to the translation process being slow compared with common folding transition rates. They used an elegant approach where a ribosome–nascent chain (RNC) complex was tethered in the optical tweezers setup. Calerythrin forms a misfolded (off-pathway) state where non-native pairing of EF-hands occurs amongst EF-hands 1, 2 and 3. Even though the formation of the misfolded intermediate is slowed on the ribosome compared with an isolated construct, it is still populated within the timeframe of *in vivo* elongation of a similarly sized protein chain [77]. To test whether the misfolding occurs during active

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translation, the authors devised a stalled ribosome system that could be activated by addition of a translation mixture. Surprisingly, they found that once translated, the unfolded state was not at equilibrium since in the vast majority of trajectories there was a much larger delay than expected (from the lifetime of the isolated unfolded state) until folding or misfolding was observed. The authors suggested that after translation on the ribosome the unfolded state is transiently in a folding-incompetent state that may be due to interactions with the ribosome. The delay may serve to inhibit misfolding until full elongation is achieved, after which folding can take place efficiently off the ribosome.

Misfolding was also detected during co-translational folding of elongation factor G [78], a multidomain GTPase protein involved in elongation of polypeptide chains. The ribosome was shown to reduce excursions into the misfolded state and when a trigger factor chaperone was included, it allowed the protein to escape from misfolded conformational traps even more successfully, seemingly by compacting the unfolded state to induce more efficient folding (Figure 2) [79]. These studies clearly illustrate the power of single-molecule techniques; such non-equilibrium conformational modes may be difficult or even impossible to detect using ensemble techniques.

A fundamental question is whether proteins fold by the same pathways on and off the ribosome. The data published so far reveal different behaviours from different proteins. Kaiser et al. used optical tweezers to show that the ribosome modulates the folding of the multi-domain protein T4 lysozyme by slowing it ∼100-fold compared with off the ribosome [80]. What about smaller proteins? From a combination of mechanical and chemical unfolding, the src SH3 domain was indeed determined to fold by the same pathway on the ribosome and off [25]. Thus, the fast and two-state folding of small proteins may leave insufficient time for the ribosome to affect the folding pathway, and in some cases folding is even achieved within the ribosome exit tunnel [81].

Chaperone-assisted protein folding

After protein translation, amongst the most important cellular components to maintain protein homoeostasis are chaperones that guide proteins to their correct functional native state and prevent the accumulation of misfolded oligomers [82]. Optical tweezers offer an attractive approach to study chaperone-mediated effects on folding pathways and many recent studies have elucidated chaperone mechanisms [83–87]. Disaggregases are proteins that reverse aggregation and they are therefore integral to correct folding *in vivo* and cell integrity. Avellaneda et al. used a force-clamp experiment to keep maltose-binding protein (MBP) unfolded and monitored the effects of adding ClpB, a disaggregase member of the Hsp100 chaperone family [88]. While holding MBP at a force where folding was prohibited, ClpB was able to induce folding in an ATP-dependent manner. Several models have been suggested to explain how ClpB achieves translocation of substrates through its pore involving different orientations of the polypeptide. To distinguish between these models, the authors integrated single-molecule fluorescence imaging into their setup (Figure 3) which allowed them to simultaneously monitor the binding of fluorescently labeled ClpB and the extension changes in each 'arm' of the polypeptide (essentially the right or left attachment points to the optical traps). This clever approach showed that ClpB is capable of dual strand insertion, yielding fast and processive translocation from either one or both arms.

Neurotransmitter release is the neurons way of communicating signals and this process is in part enabled by the SNARE proteins [89]. SNARE proteins have intrinsically disordered SNARE motifs of ∼60 amino acids and in three key SNARE proteins (syntaxin 1 and SNAP-25 attached to the plasma membrane, VAMP2 on the vesicle membrane), these motifs undergo coupled folding and assembly into a four-helix bundle which drives synaptic vesicle fusion. Shu et al. investigated how the MUN domain of the regulatory factors Munc13-1 and Munc18-1 affected the assembly of the SNARE proteins [24]. Munc18-1 binds both syntaxin 1 and VAMP2 in a ternary template complex and by subsequent formation of a weak tetrameric complex, Munc13-1 and Munc18-1 cooperatively promote assembly of the SNARE proteins. The authors tested the effects of mutations in the MUN domain of Munc13-1 known to perturb interactions with SNARE, SNARE assembly, and neurotransmitter release. The mutations significantly decreased the binding capabilities of the MUN domain to the template complex, providing experimental validation for the critical role of MUN binding for SNARE assembly and vesicle fusion.

Ligand-binding effects on the folding pathway

Within the cellular milieu exists a plethora of molecules that form a vast potential interactome. The presence of a natural, or non-specific, binding ligand can significantly affect the folding process and stability of a protein molecule [90,91]. Consequently, an understanding of how ligands modulate the mechanical stability of proteins is important and can be used to guide the rational design of pharmaceutical compounds or materials.

Many of the early studies on protein–ligand interactions with force spectroscopy were performed using AFM [92–96]. Recently, impressive work using optical tweezers has emerged on the effects of ligands on protein folding

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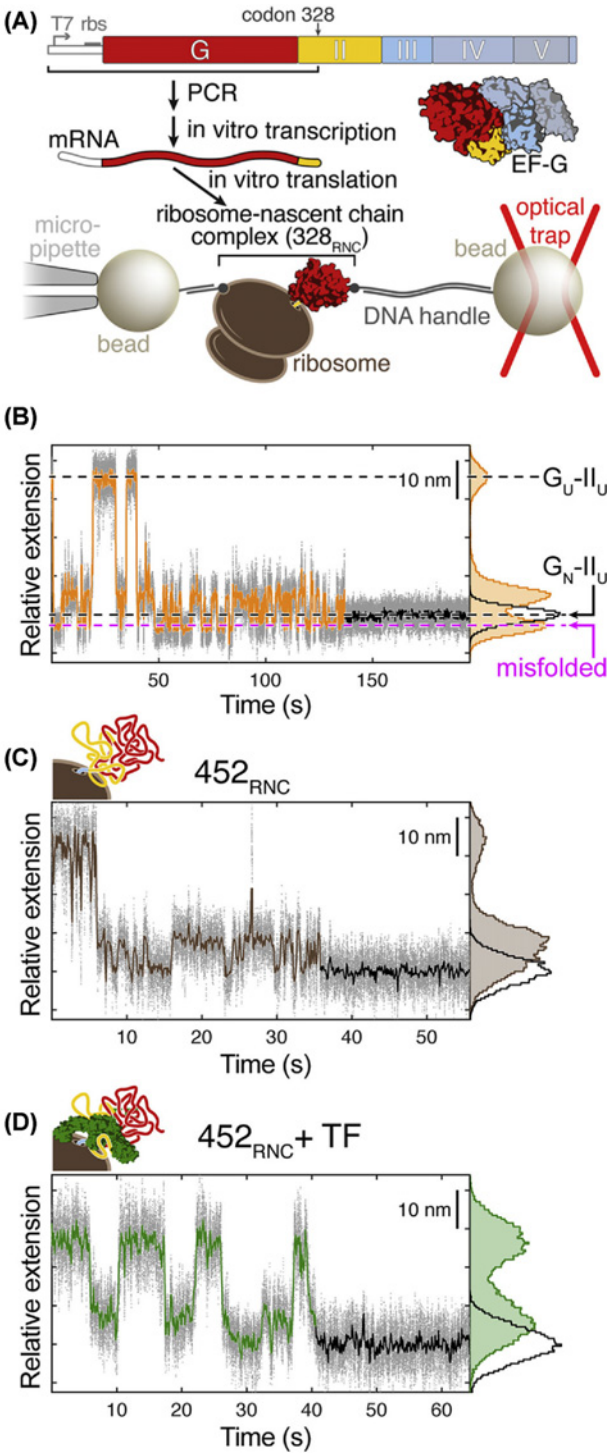

Figure 2. Monitoring co-translational folding of a single protein molecule

(**A**) The molecular system consisting of stalled ribosome-nascent chain complexes (RNC; ribosome and EF-G-II domain) is tethered to two beads; one attached to a pipette, the other manipulated in an optical trap which could be moved at constant velocity and used to determine force and position. (**B**–**D**) Extension vs. time trajectories under different experimental conditions at constant force. (B) Isolated EF-G domain (without ribosome) frequently visits a misfolded state from an intermediate state, before committing to the native fold (shown in black). (C) When the same domain folds on the ribosome, the frequency of misfolding is reduced and the native folding rate increases. (D) The trigger factor (TF) chaperone shifts the population even further from the misfolded state, increasing the efficiency of folding. Raw data acquired with 1 kHz resolution is shown in grey, whereas the solid line represents time-averaging to 10 Hz. Figure adapted from [79] with permission.

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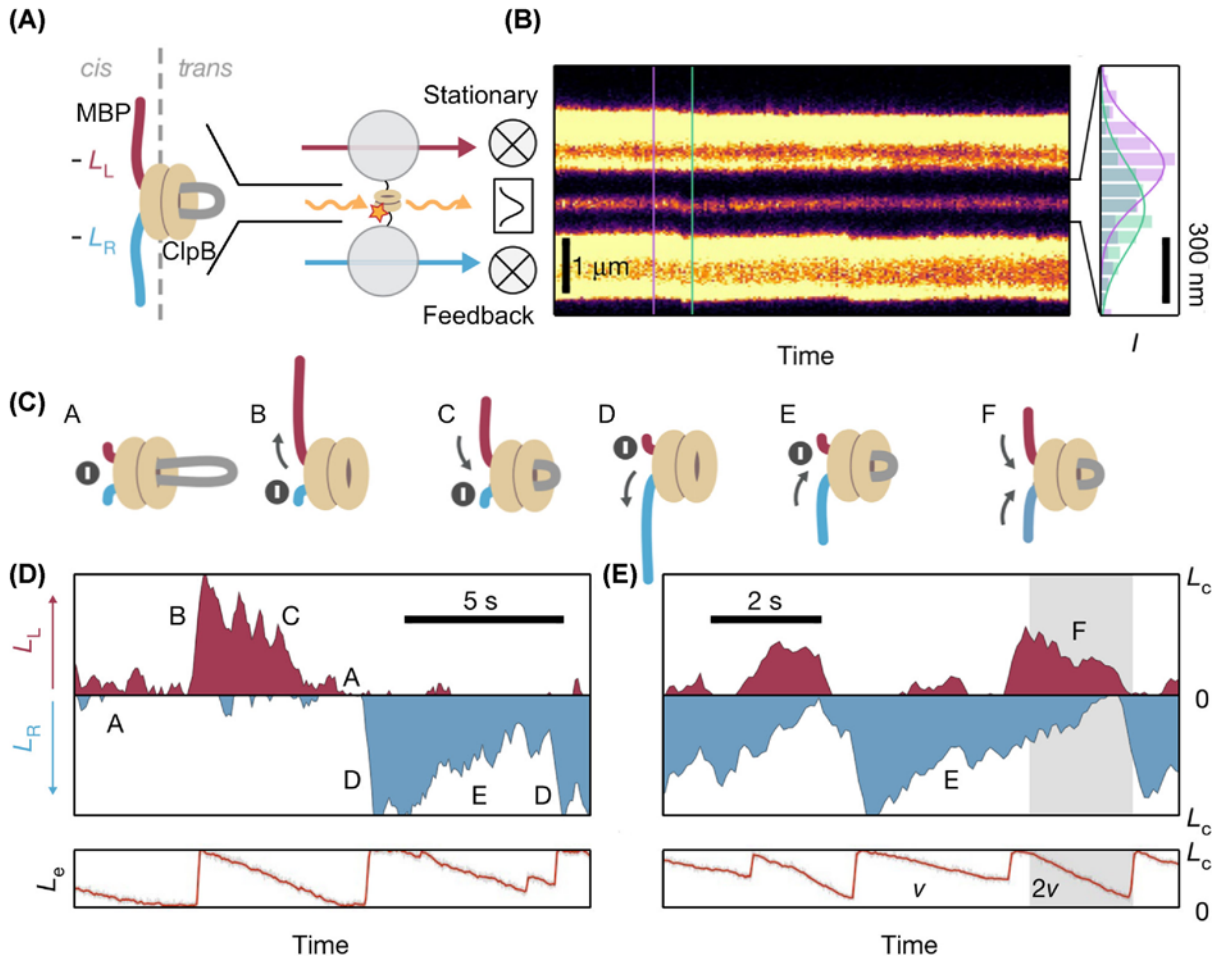

Figure 3. Combining force and fluorescence to decipher disaggregase mechanism

(**A**) ClpB (doughnut shape) is a translocase that reverses protein aggregation. MBP was tethered between two optical traps through DNA handles and kept at a force that prevented folding. When fluorescently labeled ClpB is added, it binds MBP and contracts the polypeptide through its pore. (**B**) The fluorescence kymograph from scanning the beads and tether shows translocation after ClpB binding. The combination of monitoring ClpB and bead positions allowed discriminating translocation of the two ends of the polypeptide. (**C**) Cartoons indicating positions and events from plots in (**D**,**E**). (D,E) Lengths of non-translocated (cis) polypeptide arms, deduced from kymographs, and total polypeptide contour length (L_e, bottom graphs). The grey-shaded area in (E) shows where ClpB translocates simultaneously both arms. Figure adapted from [88] with permission.

[63–65]. Suren et al. looked into the glucocorticoid receptor (GR) and how it binds a steroid hormone ligand [97] (Figure 4). An exciting hypothesis is that the known dependence of the ligand-binding domain of GR (LBD-GR) on molecular chaperones is due to the receptor being prone to incorrect folding, which rendered it inactive. Furthermore, the apo-LBD-GR is thought to be only marginally stable and highly dynamic as the hormone binding site is buried deep within the structure, implying that substantial movements are needed to accommodate it. Using optical tweezers, the authors found multiple intermediate states on route to the native structure. Careful kinetic analysis revealed that dexamethasone, a corticosteroid, binds only to a state involving a lid-domain in the open position which closes upon binding. Once the apo-state unfolded, it populated a broad ensemble of misfolded states that slowed correct folding, providing a rationale for why chaperones are needed *in vivo* to maintain hormone-binding competency.

Sonar et al. used the well-studied ACBP protein and its interaction with octanoyl-CoA as a system to explore the mechanical properties of the protein–ligand complex [98]. The 86-residue all-helical protein was unfolded and refolded by pulling its termini, in the absence [99] or presence of octanoyl-CoA [98]. Similar to the case above with GR and its hormone, ACBP was found to bind octanoyl-CoA only after formation of the native state, leading to significant stabilization. Surprisingly, the distance from the native state to the transition state along the reaction coordinate

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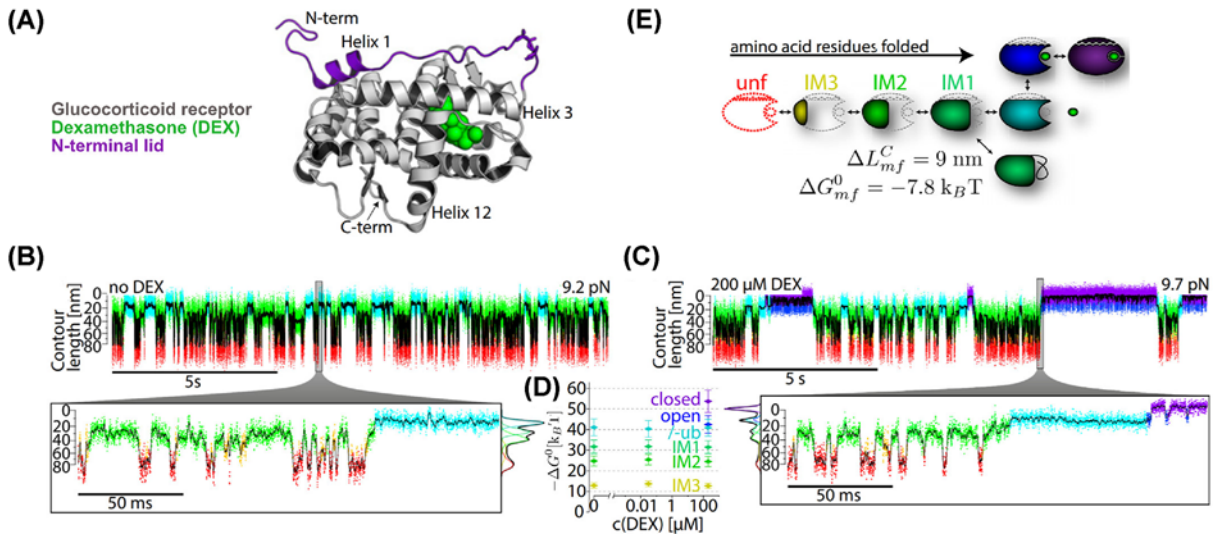

Figure 4. Studying the folding and binding mechanism of the GR and its hormone ligand dexamethasone (DEX)

(**A**) GR with bound DEX shown with green spheres (PDB 1M2Z). The N-terminal lid-structure is shown in purple. Force was applied to the N- and C-termini. (**B**,**C**) Contour length vs. time trace for the GR in the absence and presence of 200 µM DEX. Each conformational state (identified using a hidden Markov model assignment) is coloured differently. Below each trace is a zoom into a ∼200 ms wide region. (**D**) Free energy difference between unfolded state and other populated states as a function of DEX concentration. (**E**) Model for the folding of GR and binding of DEX. Figure adapted from [97] with permission.

(end-to-end distance, see Figure 1D), which is a measure of the deformability of the native structure, was unchanged upon binding of the ligand, with the native state of ACBP holo-form being able to deform a great amount before crossing the unfolding transition state barrier. Steered molecular dynamics (SMD) simulations of the unfolding process furthermore showed that the unfolding pathway was unaffected by the presence of the ligand. The authors speculated that this property is important for ACBP's function as a lipid transporter [100–102]: the protein can still undergo significant deforming forces – e.g. due to translocation, membrane barrier crossing, or interactions with other proteins – without losing its lipid cargo.

A similar combinatorial approach with optical tweezers and simulations was used for studying how binding of cAMP to the two cyclic nucleotide binding (CNB) domains of protein kinase A (PKA) leads to allosteric activation of the kinase [103]. A complex network of allosteric interactions was observed between the CNB domains with asymmetric stabilization effects between the two domains. One of the powerful advantages of optical tweezers is to be able to apply the force vector on well-defined regions of the polypeptide chain, something which is impossible to do by chemical or thermal denaturation. Using exactly that approach, a dynamic switch was uncovered upon cAMP binding, involving a destabilization of a short helical region in one of the CNB domains. SMD simulations showed that the cAMP-bound state had significantly increased interdomain contacts that caused the helical switch motif to unfold before the rest of the CNB domains. But was there a functional importance to this region? To find out, the authors used a mutation that abolished the interdomain contacts between the two CNB domains and monitored folding/unfolding of the switch motif. For the mutant, a decoupling of the switch motif from one of the CNB domains was apparent, leading the authors to suggest that the switch amplifies cAMP-binding signals.

These studies add to the ensemble of research that has been done on the mechanical effects of ligand binding with AFM, where ligand binding has been observed to have varying effects. Correspondingly, predicting the effects of ligands is not straightforward, which emphasizes the importance of integrating molecular simulations with single-molecule experiments to understand the microscopic principles of ligand-dependent folding.

Conclusions and future perspectives

Optical tweezers have enabled the study of biomolecular systems in extraordinary detail, where the single-molecule nature of the technique has provided in many cases a view of biological mechanisms that would be entirely unattainable with other approaches. For example, it is difficult to imagine how the exquisitely complex kinetic trajectories

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from individual GRs (Figure 4) would be deciphered by ensemble methods. Many such fundamental aspects of protein folding, that have been challenging to expose with classical biophysical experiments, have been addressed recently with optical tweezers. We hope to have emphasized that the possibilities are far-reaching for protein folding and interactions. Nonetheless, there are some technical limitations yet to be overcome. Optical tweezers have mostly been restricted to 'soft' α-helical proteins due to the higher forces required to unfold β-sheet proteins. β-sheet proteins have been shown to be more brittle, where the unfolding of β-strands involve the breakage of several hydrogen bonds arranged in parallel [104,105]. On the other hand, α-helical proteins have less ordered interhelical hydrogen bonds and can locally unfold without committing to full unfolding. This behaviour also correlates well with recent experiments showing that domain swapping in β-sheet proteins requires global unfolding contrary to α-helices that can freely swap in the native state [106]. The limiting factor for optical tweezers originates from the elastic properties of the DNA-handles which cannot withstand forces over ∼65 pN without going through a poorly understood unfolding-type reaction [39]. Another important aspect is the reaction coordinate; proteins are usually probed through a linear force vector defined by the attachment points. As a consequence, structural changes of the protein can be detected only if they give rise to measurable variations in its end-to-end distance along the pulling direction. Some authors have tried to circumvent this limitation by applying force through different attachment points on the protein to probe other regions of the energy landscape [31,92,99] or by using hybrid approaches using fluorescence to probe regions outside the force vector [46,57]. This approach is inherently low-throughput and time-consuming. Technical advances are thus needed to improve sample preparations and novel designs of molecular handles will hopefully soon allow more versatility in system choice.

What does the future hold for macromolecular research using optical tweezers? Probing the mechanical properties of well-folded proteins has become straightforward but it is now apparent that a large fraction of the human proteome lacks a stable, three-dimensional structure in their functional state. These intrinsically disordered proteins (IDPs), which play important roles in signaling and regulation [107], are highly dynamic and consist of an ensemble of different energetically similar states. Many IDPs fold upon binding their targets [108,109] whereas others can form tight complexes without persistent secondary or tertiary structure [110,111]. Is the current sensitivity of optical tweezers sufficient to probe the conformational dynamics of IDPs? Recent studies have yielded promising results and indicate that studying IDPs with optical tweezers is indeed possible. The high temporal resolution and low stiffness of optical tweezers allowed Neupane et al. to study the conformational equilibria of α-synuclein, a key driver of Parkinson's disease [112]. Multiple metastable states were detected at low forces and a reconstruction of the energy landscape revealed it to be flat but rough, representing a direct quantification of a proposed feature of IDP landscapes [112,113]. Interlinked with IDPs is the formation of membraneless intracellular compartments through liquid–liquid phase separation [114]. The interactions between disordered regions and nucleic acids have been found to be a critical driver of phase separation [115,116] and deciphering the sequence-structure code underlying the phase separating properties of IDPs remains a key goal. Understanding how droplet formation affects the mechanical properties of nucleic acids and associated proteins is thus a highly interesting avenue for optical tweezers research [117].

Finally, the holy grail of biochemical single-molecule studies is to examine a single protein molecule as it functions in its natural environment – inside the confines of a cell. Although the direct mechanical manipulation of proteins within a cell represents a formidable technical challenge, there have already been promising developments towards this goal [118–120]. The future of optical tweezers is bright as they continue to evolve into an increasingly powerful tool for research stretching across a wide range of biological disciplines.

Summary

- Optical tweezers is a powerful tool to study protein conformational equilibria on the single-molecule level.
- Piconewton forces and subnanometer extensions are accessible with modern instruments.
- Protein folding can now be followed from protein synthesis on the ribosome to functional events such as ligand binding.
- These and more studies are revealing the fascinating yet complex principles that shape the folding process.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

P.O.H. and C.C. conceived and wrote the paper.

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Abbreviations

ACBP, acyl-CoA binding protein; AFM, atomic force microscopy; CNB, cyclic nucleotide binding; GR, glucocorticoid receptor; IDP, intrinsically disordered protein; LBD-GR, ligand-binding domain of GR; MBP, maltose-binding protein; NCS-1, neuronal calcium sensor-1; PrP, prion protein; SMD, steered molecular dynamics.

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