Mechanical forces regulate the function of numerous proteins relevant to physiology. The functions and folding of proteins have been under scrutiny for decades, but it was not until recently that mechanical forces have been considered. Here, we review different techniques for studying protein folding, highlighting their physiological significance.

Mechanical Biochemistry

Mechanical force is a natural perturbation that commonly occurs in biological systems. From the wings of a hummingbird in flight to the bending response of plant organs to light, molecular biomechanics are at play. Mechanical forces also dominate numerous perturbations relevant to human physiology and disease (FIGURE 1). For example, muscles, blood vessels, ligaments, and skin all stem from complexes that are formed with viscoelastic proteins such as elastin and collagen that resist considerable mechanical forces (35, 47, 58, 63, 79). Furthermore, human cardiac and skeletal muscles are composed of long, fibrous proteins that form the sarcomere unit (47) and function in response to mechanical force. Cellular processes such as protein degradation, apparent in each and every cell, employ ATP-powered proteases that work mechanically to unfold and translocate proteins (2, 55). Even the movement of motor proteins that carry cargo such as chromosomes along microtubule tracks is mechanically driven (8). Therefore, mechanical forces are crucial in the function of a plethora of proteins that play central roles in the physiology of all living organisms. Prominent examples include muscle proteins, extracellular matrix proteins, and cell adhesion proteins, such as titin, fibronectin, tenascin, and several collagens. These proteins are characterized by their elastic properties and their ability to function as extensible shock absorbers (23, 60, 76). Present in vivo as tandem repeats, these proteins stretch and unfold reversibly and exhibit mechanical resilience, allowing for a diverse range of structural conformations to be explored and hence full biological function.

Whether mechanically driven or not, proteins are involved in vital biological functions in every single cell. To carry out their functions, however, they must fold into complex three-dimensional structures. In vitro, understanding how one-dimensional primary sequences of amino acids acquire these complex structures is studied in the context of protein folding. Traditional protein folding experiments rely on harsh changes to the protein solution that induce the protein to unfold. By large, methods of perturbation include the use of chemical denaturants, such as urea or guanidine hydrochloride, pH, and temperature (34, 72). Protein unfolding requires the protein to overcome one or more energy barriers that result from the breaking of hydrogen bonds. Only this energy barrier is probed in biochemical studies. However, an important property of protein folding is overlooked by these methods, that of polymer elasticity. Once proteins are extended by force, they behave like elastic polymers and are best described by the worm-like chain model of polymer elasticity (56). Force spectroscopy techniques such as single-molecule atomic force microscopy (AFM) not only access energy barrier(s) but also directly measure the elastic properties of polypeptides, collectively used to investigate protein mechanics (23, 71). Here, force is applied to a single protein, such that it overcomes its folding energy barrier and extends. The mechanical properties of individual protein domains are then determined, revealing insight into the mechanical stability, energy landscapes, and folding pathways of single proteins.

Whether the energy barrier(s) sampled are the same or differ dependent on the method of perturbation used is an important question in understanding the physiology of proteins. In general, there are two different conceptual frameworks to describe protein folding. A hallmark of biochemical experiments is the two-state model that can be described by barrier-limited cooperative folding. Contradictory to this is the more complex rough, funnel-like energy landscape that single-molecule force spectroscopy techniques as well as computational methods readily describe. This raises important questions into which techniques and proposed models are best positioned to describe the behavior of these mechanical proteins in vivo. In the following sections, we review the protein folding field in resolving the on-going conflicts that surround the effect of perturbation on the protein folding landscape, the models used in describing the data, and their relevance to human physiology.
Protein Folding Problem

The early 1960s saw Christian Anfinsen describe one of the most important results in protein folding by determining that all of the information required to fold a protein into its thermodynamically favored native state was embedded within the primary sequence of amino acids (1). The experiment was conducted on the protein ribonuclease and protein unfolding, and subsequent folding was achieved through the addition and dilution of a chemical denaturant, respectively. However, for proteins to fold within biologically relevant time scales, they cannot be purely under thermodynamic control. In the late 1960s, Cyrus Levinthal determined that proteins must fold within defined pathways so that they attain native structure within biologically applicable time scales (49). Probing these folding pathways has since given rise to a number of biochemical techniques that perturb the native protein. Chemicals such as guanidine hydrochloride (GuHCl) and urea, however, remain the most established. With a plethora of both thermodynamic and kinetic denaturation experiments conducted on hundreds of proteins and their mutants, are we any closer in resolving the protein-folding problem?

One of the major revelations by such denaturation techniques was the two-state model to describe protein folding (40). In this instance, the protein exists as either entirely denatured or entirely native with a briefly populated transition state in between. Tryptophan and/or tyrosine residues if buried within the native structure will become exposed upon denaturation and the fluorescence signal recorded (FIGURE 2A). At equilibrium, the relative change in fluorescence is plotted as a function of denaturant concentration (FIGURE 2B). Out of equilibrium, the relative fluorescence can monitor protein unfolding and refolding kinetics (FIGURE 2C) with rate constants plotted as a function of denaturant concentrations. A V-shaped chevron plot signifies two-state kinetics, whereby the linear relationship of the unfolding and refolding limbs with respect to denaturant concentration can be extrapolated to reveal their respective rate constants in the absence of denaturant, \( k_1 \) and \( k_2 \) in FIGURE 2C (40). The relative free energies can be determined both thermodynamically and kinetically to map the folding energy landscape by illustrating the depth of the native well and the height of the transition state barrier, respectively (FIGURE 2D). Two-state folding proteins are verified by equal thermodynamic and kinetic free energies (16, 40, 66).

This inherently simple model has both strengths and weaknesses in its assumptions. On one hand, the model is widespread in its application and has been used to describe the folding of many single-domain model proteins (39, 67, 78). By its very definition, crucial intermediate steps for folding are all high in energy and thus unresolved or kinetically “silent” in experiments. Experimental tuning, however, can sufficiently lower the energy barrier to (or from) the intermediate state to characterize such states. Therefore, one could envisage a more intricate rough funnel-like energy landscape such that smaller conformational ensembles of structures are a prerequisite to guide the protein to the native structure. This view, although conflicting with the two-state description, is in direct accord with the statistical theoreticians that view protein folding as a more complex system (61).

To resolve such an apparent contradiction, recent years have seen advances in experimental and computational techniques. One such experimental development is the ability to probe protein folding by applying mechanical forces at the single-molecule level. The application of force on single proteins provides accuracy in characterizing protein dynamics in two main ways: 1) by providing a defined reaction coordinate and 2) by offering low-populated states that are masked by ensemble measurements to become apparent. Arguably, the results derived could be better represented to understand the roles of proteins in human physiology compared with standard bulk techniques, owing to the direct application of mechanical strain. Indeed, model proteins such as ubiquitin and immunoglobulin domain of titin, single molecule force-clamp spectroscopy have
been applied successfully to characterize protein-folding pathways in response to force (26, 29).

Force-clamp spectroscopy allows the protein’s folding landscape to be dissected in detail, encompassing all the different stages from the highly extended state to the natively folded form. In force-clamp experiments, a polyprotein is stretched, and an electronic feedback mechanism adjusts the length of the protein to keep it at a constant stretching force. The method allows direct force dependencies of reactions, such as protein unfolding to be measured, leading to key parameters (Δx, ΔG) describing the energy landscape to be quantified (30, 73). A further advantage of the force-clamp technique extends to the ability to apply complex force pulses to a single protein. In the simplest case, a constant stretching force is applied for a given time to initiate protein unfolding. To induce protein folding, a low force pulse is applied (≤10 pN). Whether native structure has been formed depends entirely on the quench time and is only revealed when subject to a second unfolding pulse. On ubiquitin, the experiments revealed that no single ubiquitin protein followed the exact same pathway, with stochastic heterogeneity observed in the folding trajectories. The protein was shown to collapse and refold through mechanically labile, structurally heterogeneous, and necessary precursors to the native state (30). From such an ensemble of collapsed conformations, also known as molten globules (3), folding occurs in a two-state manner, whereby native contacts are formed (26).

By contrast, the results obtained from standard denaturation studies are confined to the context of the experiment. Chemical denaturants or high temperatures can neither replicate the extended state nor characterize the elastic properties associated with these proteins and as such question the applicability of the two-state model in describing folding in physiological settings.

**Perturbation of Protein-Folding Landscape**

How does varying protein perturbation method affect the energy landscape (FIGURE 3)? To address this, we must consider whether we are sampling the same denatured state. For chemical denaturants alone, GuHCl vs. urea, there have been discrepancies reported between the unfolding pathways of the same protein depending on the denaturant used (52). The dispute arises from the fundamental differences by which GuHCl and urea denature proteins: in other words, whether the chemical denaturants directly interact with the protein or whether indirect interactions with the denaturant and the water molecules cause the destabilizing effect. In an attempt to resolve such confliction, experiments revealed that, when using different denaturants such as GuHCl or urea, proteins denature via alternative mechanisms. Urea was shown to interact directly with the peptide groups, whereas GuHCl engages in stacking interactions with itself and thus destabilizes planar side chains (4, 19, 50). Often GuHCl is rendered a more efficient denaturant compared with urea (17, 19, 33, 34). It seems fair to assume that the parameters derived are perhaps applicable in the context they are attained (i.e., high molar concentrations of denaturant), yet may not translate in terms of physiological relevance. In any case, certain standardized protocols for characterizing folding landscapes by GuHCl and urea alone would benefit these studies greatly.
Initial force spectroscopy experiments on the 27th immunoglobulin domain of cardiac titin protein revealed that the unfolding rates determined using force were similar to those determined using GuHCl (25). Thus the same unfolding pathway for each method was implied. This would mark the acceptance of force spectroscopy within the protein-folding community. However, further investigations on the unfolding pathway of the same protein would reveal that comparing mechanical unfolding directly to bulk techniques was inaccurate, owing to the differences in the unfolded state sampled by the various techniques (28).

Beta-sandwich proteins such as immunoglobulin domains and fibronectin type III, both of which appear in the muscle protein titin, show important discrepancies when unfolded by force, GuHCl, or temperature. Differences are interpreted in terms of the residual structures within the denatured protein, the nature of the secondary and tertiary interactions, and the relative stabilities of the various structural elements (62). Recent single-molecule investigations into the folding landscape of the alpha-helical calcium-sensing protein calmodulin have also shown similar deviations to previously reported bulk behavior (75). By utilizing optical tweezers to unfold and fold calmodulin domains, the results reveal a complex network of on- and off-pathway intermediates that deviate from the simple two-state kinetics previously used to describe the folding of bulk calmodulin (69). Another example is that of the model protein ubiquitin. Initially described by three-state kinetics in 1996, ubiquitin was later shown in 2000 to fold in a two-state manner (46). Folding rate constants using traditional biochemical mixing techniques were determined at \( \mu = 1,000 \text{ s}^{-1} \) (46), marking ubiquitin as a fast folding protein. However, the folding behavior of ubiquitin when determined from single-molecule force spectroscopy all together tell a very different story. As described in the previous section, ubiquitin was shown to collapse and rapidly sample a subset of structures that are mechanically labile and necessary precursors from which folding occurs to the native state in a two-state manner (30). The folding rate constant determined at quench forces of 10 pN was 0.3 s\(^{-1}\), which is vastly different from those attained from bulk.

**FIGURE 3. Importance of perturbation on the energy landscape**
Can denaturants (D), such as urea, guanidine hydrochloride (top), or temperature (T) (middle), reveal the same unfolded state that is sampled with the application of force (F), irrespective of the direction applied (bottom)? If such a hypothesis stands, then the same energy landscape (right) for all methods of perturbation would be assumed.
Perturbation method affects the unfolded protein conformation and thus folding mechanism as such; proteins that unfold with chemical denaturants appear to adopt a more collapsed structure; temperature induces a structure better described as a random coil, whereas force extends proteins according to the direction applied (FIGURE 3).

Interestingly, experiments thus far have almost exclusively been limited to the application of force in the NH$_2$- to COOH-terminal direction. Recent studies on non-NH$_2$- to COOH-terminal-linked polyproteins have indicated that the mechanics of protein structures depend heavily on the loading geometry (11, 13). Thus mechanical resistance is not dictated solely by amino acid sequence, topology, or unfolding rate constant but depends critically on the direction of the applied extension. Theoretical studies describe protein structures to contain “soft” and “stiff” regions. Therefore, the protein, even on a single-residue basis, will respond to force differently according to pulling geometry. Cysteine engineering has been employed as an approach to gain precise control over the points of force application of single proteins (21). Rather than being limited to one spatial direction, this technique offers multiple reaction coordinates to be probed by force. Such methodology has revealed how the energy landscapes of proteins are affected by changing the direction of the force applied by demonstrating not only a large distribution of unfolding forces (i.e., mechanical stability) but also various directional spring constants (i.e., stiffness). Furthermore, changes in the geometry of the force applied can alter energy landscapes by introducing mechanical intermediates as depicted for green fluorescent protein and enhanced yellow fluorescent protein (7, 20, 22, 64). Hence, if the same protein can populate more than just two states depending on how it is subject to force, simple two-state models cannot be best suited to describe their folding behavior. This being said, there remains the motivation to validate single molecule force spectroscopy results by directly comparing them to rate constants and free energies obtained from “bulk” studies (9, 12, 14). Evidently, the method of perturbation affects the reaction coordinate and the unfolded state and as such the folding energy landscape (24). Thus a direct comparison for validation seems counterintuitive.

**The Effect of Force on a Single Protein**

Atomic force microscopy (AFM) has emerged as a powerful tool to investigate the forces and motions associated with biological molecules. The setup allows for a controlled and calibrated force to be utilized in either 1) the force extension, 2) the force-clamp, or 3) the force-quench mode to examine the dynamics and mechanics of single proteins. At the atomic scale, it is imperative that the parameters derived are associated directly with the protein rather than from any nonspecific interaction. The molecular engineering of polyproteins that are composed of multiple repeating domains of a specified protein serve as fingerprints for single-molecule experiments and allow the mechanical properties of the desired protein to be probed unambiguously (FIGURE 4A).

Immunoglobulin and ubiquitin modules have been subject to such vigorous analysis (23, 28, 30, 31, 74). Force extension experiments extend the protein at constant velocity, revealing the characteristic saw-tooth pattern traces of unfolding (FIGURE 4B). The unfolding events can be fitted to the worm-like-chain (WLC) model to describe polymer elasticity and reveal the end-to-end distance (or contour length) of the protein domains. Furthermore, the force at which the protein unfolds, which is indicative of its mechanical stability, can be characterized. This approach, while allowing observations that are not available in traditional bulk experiments to be seen, yields only qualitative results, owing to the vast changes in force, length, and loading rates that occur simultaneously. Evolving from this marked the birth of pioneering force-clamp spectroscopy (59). In the force-clamp mode, a polyprotein is pulled using a constant stretching force held constant through the feedback by a proportional-integral-derivative (PID) controller. By this mechanism, the length of the protein is adjusted to keep the stretching force constant. Unfolding events are characterized by a step increase in the polyprotein length, revealing the characteristic staircase trajectory (FIGURE 4D). This would allow a direct exploration into the energy landscapes of single proteins. Specifically, force-dependent unfolding kinetics (FIGURE 4D) could extract key features such as the distance to the transition state ($\Delta x$) and unfolding rate constants ($k_u$).

Force-quench experiments have been applied to study protein folding and uncovered novel behaviors that had not been observed in standard bulk experiments, which have developed our understanding of collapsing polypeptides and their search for the native state (FIGURE 4C) (6, 26, 30). In the force-quench mode, first an initial force is applied that promotes the sequential unfolding of each polypeptide module. The force is then quenched to a low force, triggering the collapse of the extended protein. This collapse is characterized by initial elastic recoil of the unfolded polymer followed by a constant and cooperative collapse trajectory, leading to an ensemble of structures that are necessary precursors to the
native state. The recovery of the native protein’s mechanical stability serves as an unambiguous signature of the completion of the folding process (26, 30). A key discovery was that proteins would begin refolding from a highly extended state that is rare or nonexistent in solution studies and, hence,

**FIGURE 4.** Single-molecule atomic force microscopy

A: schematic representation of a single-molecule AFM setup. A polyprotein is adhered onto a gold surface and pulled from the tip of a cantilever by a piezoelectric positioner. A laser that is focused onto the tip of the cantilever is reflected onto a photodiode detector, and the resulting force can be measured. B: typical force extension experiments that are conducted under constant velocity reveal the distinct saw-tooth pattern trajectory. The end-to-end distance [contour length ($L_c$)] of each module of the polyprotein is measured from fitting the data to the worm-like chain model. Unfolding forces that characterize the mechanical stability of the protein are also determined. C: a typical staircase trajectory emerges when high forces are applied to a single protein. Protein refolding can be initiated by quenching to low forces. Refolding rates can be attained from varying the quench time and monitoring the recovery of mechanical stability of the protein domains as a function of time. D: in force-clamp experiments, where the force is kept constant, force-dependent unfolding kinetics can be plotted and extrapolated to reveal the unfolding kinetics in the absence of force. The slope of the fit reveals the distance to the transition state (as shown in E). E: proposed folding energy landscape as a function of force. A clear reaction coordinate (end-to-end distance) can be assigned in the absence of (black lines) and with the application of force. Notice that the end-to-end distance is represented in log scale, and, therefore, the distance between the native (N) and unfolded states (U) is very short. At forces of $-25$ pN (blue line), the application of force introduces an entropic energy barrier. High forces (red line) allow for extended states (E) to be sampled.
traversing regions of the energy landscape untouched by bulk methods. For example, proteins, such as ubiquitin could be extended to ~20 nm. By contrast, in high molar denaturant concentrations, the same protein has a radius of gyration of only ~2.6 nm (41, 44). Rather than envisioning protein folding in simplistic two-state terms, the field is moving to accept that the application of force affects the energy landscape very differently than traditional bulk probes. This does not come without its fair share of major controversy. Foremost, force methods that probe molecular mechanics with such techniques as optical tweezers, which infer relatively low forces, continue to describe protein folding in two-state terms. To dissect this issue, let us first describe how the energy landscape of a molecule is affected by force.

The Bell model that states force perturbation (F) linearly tilts the two-state energy of a molecule along the pulling coordinate (x) by an amount F·x is frequently used to analyze force spectroscopy measurements on single RNA, DNA, and protein molecules, which extend by up to tens of nanometers (15, 51, 82). In 2001, the application of force on single RNA hairpin would be the first of many that would demonstrate a two-state hopping behavior between unfolded and folded states, which has since been observed in many proteins (42, 43, 51). Hallmarks in these experiments are the highly force-dependent observed kinetics with changes in force of only a few piconewtons enough to tilt the propensity for unfolding to completion (15, 32, 51, 82). The remarkable caveat in these studies was the agreement of the free energies when extrapolated to zero force to those calculated from bulk studies. This revelation suggests that both types of experiments sample the same energy barrier. To probe this further, Langevin dynamics simulations revealed that, across the forces that these experiments ranged from (5–20 pN), hopping behavior was readily observed (5), owing to an entropic energy barrier created by force. This artifact, created by introducing force onto the energy landscape, is frequently interpreted as a manifestation of two-state folding/unfolding pathways that is readily seen in bulk experiments. Such a barrier, encountered in the low force region, is therefore a generic property of tethered molecules placed under force. The role played by this barrier may be relevant in physiological settings in vivo, although it has yet to be determined.

Such barriers do not exist in the absence of force; therefore, it is fair to assume that, in bulk settings, these reactions very likely do not take place. And thus the extrapolations to verify free energies to those attained from bulk experiments are yet again seemingly irrelevant. One way of viewing the energy landscape is with a first barrier between the native and the collapsed state, which occurs over very short distances. The second entropic barrier is created by force originating an extended unfolded state (FIGURE 4E). Of the most interest is to study the inner barriers that are typically probed by force spectroscopy at high forces using AFM.

Collectively, the recent developments in single-molecule experiments have allowed characterization of biomolecules in response to force at the Ångström scale. To dissect the underlying mechanical mechanisms further in detail, computational simulations are needed. In recent years, steered molecular dynamics (SMD) simulations have been implemented to reenact in silico what atomic force microscopy, optical tweezers, or indeed the cell itself does to biomolecules. SMD simulations apply an external force to one or more atoms, thereby mimicking force spectroscopy methods. Key simulations in the last decade corroborate and verify many in vitro AFM experiments (36–38, 53, 54, 57). The present consensus within the field is clear: combining SMD simulations with AFM experiments yields detailed and valuable results that are not necessarily available from laboratory experiments alone.

Single-molecule force spectroscopy has also been utilized in investigating the effect of mechanical force on the free-energy surface that governs chemical reactions in human physiology. Force can regulate protein function and enzymatic activity, as well as transform the stimuli into a chemical response (65, 70, 80, 81). Mechanical strain has been shown to open ion channels, activate phosphorylation, and cause catch bond formation, and the mechanisms by which they do this have been gradually emerging, owing to developments in single-molecule force assays (45, 68, 77). In recent years, both optical tweezers and AFM have excelled in describing the role of force in chemical reactions and mechanotransduction in many complexes that include disulfide bond reduction events, the interactions of cytoskeleton proteins, binding to actin networks, and cell response mechanisms (10, 18, 27, 48). Force has a huge role in physiology, and the ability to explore to nanometer resolution the molecular mechanisms of these events in key proteins is of crucial importance.

**Conclusions**

In physiology, force plays a crucial role in countless biological processes, and understanding how muscles, cells, and proteins respond to force is an important driving force in modern day research. The development of single-molecule force spectroscopy has emerged as a prevailing tool to investigate the forces and motions associated with such
processes. Most importantly, the techniques are best positioned to obtain important mechanical properties that are unmet in traditional biochemical assays. However, there seems to remain the propensity to “validate” force spectroscopy results with those set in place from previously attained biochemical experiments. Serious assumptions are made here, namely, that the unfolded state of a protein is the same regardless of the method of perturbation. In this review, we have brought to light important studies that clearly demonstrate how force accesses parts of the protein energy landscape that are inaccessible in bulk studies. Therefore, one cannot assume the same energy landscape is being sampled.

AFM has the capability to uncover the intricate mechanical details of protein-based reactions, allowing conformational changes down to the sub-Ångström scale to be characterized. These developments can be readily combined with simulations and theoretical studies and work in synergy to provide a well rounded view of how proteins respond to force at the single-molecule level.

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