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Review

Single-Molecule Optical Tweezers As a Tool for Delineating the Mechanisms of Protein-Processing Mechanoenzymes

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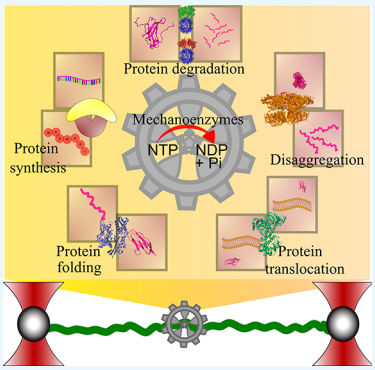
ABSTRACT: Mechanoenzymes convert chemical energy from the hydrolysis of nucleotide triphosphates to mechanical energy for carrying out cellular functions ranging from DNA unwinding to protein degradation. Protein-processing mechanoenzymes either remodel the protein structures or translocate them across cellular compartments in an energy-dependent manner. Optical-tweezer-based single-molecule force spectroscopy assays have divulged information on details of chemo-mechanical coupling, directed motion, as well as mechanical forces these enzymes are capable of generating. In this review, we introduce the working principles of optical tweezers as a single-molecule force spectroscopy tool and assays developed to decipher the properties such as unfolding kinetics, translocation velocities, and step sizes by protein remodeling mechanoenzymes. We focus on molecular motors involved in protein degradation and disaggregation, i.e., ClpXP, ClpAP, and ClpB, and insights provided by single-molecule assays on kinetics and stepping dynamics during protein unfolding and translocation. Cellular activities such as protein synthesis, folding, and translocation across membranes are also energy dependent, and the recent single-molecule studies decoding the role of mechanical forces on these processes have been discussed.



1. INTRODUCTION

Mechanoenzymes are biomolecular motors that convert chemical energy from nucleotide triphosphate (NTP) hydrolysis into mechanical energy for cellular activities. These machines work similarly to engines fueled by NTP and produce directional motion.[1] They can be described as nature's best solution for harnessing chemical energy and transducing it into mechanical motion to perform many cellular activities such as cargo transport, DNA unwinding, nucleic acid/protein synthesis, chromatin remodeling, protein unfolding, translocation, or disaggregation. Enzymes performing these processes regularly require the precise movement of some of their components to generate force, displacement, or angular movement. Some well-studied mechano-enzymes that act as molecular motors include kinesin, myosin, helicases, nucleic acid polymerases, and ATP-dependent proteases. These molecular machines can be classified based on the type of fuel they use (GTP/ATP), the motion they generate (linear/rotary), and the polymeric tracks they move on (chemical nature of substrates they process). Although properties such as functional forms, oligomeric states, active site catalytic residues, sequence of catalytic events, and substrate-interacting regions of these molecular motors have been studied extensively through traditional structural, biochemical, and biophysical approaches, a gap in the understanding of the mechanochemical cycle involving force generation, transduction, and quantitation as well as motor properties of these enzymes was filled only with the advent of single-molecule techniques. Motor properties of these enzymes, such as stepping size and kinetics, velocity, directionality, stalling force, processivity, and energetic costs of individual processes, were elucidated through single-molecule measurements championed by optical-tweezer-based force spectroscopy.[2] Based on the chemical nature of the polymeric tracks they move on, mechanoenzymes can be classified as cytoskeletal motors, nucleic-acid-processing motors, and protein-processing motors. The cytoskeletal motors, such as kinesin or myosin, and nucleic acid motors, such as helicases, polymerases, or DNA packaging motors, have been studied extensively and reviewed elsewhere.[3,4] The current review focuses on describing single-molecule studies on protein-processing molecular motors that are relatively less studied plausibly due to the complexity of the chemical nature of the tracks. We review the insights from single-molecule optical tweezer experiments on the working mechanisms of protein unfoldases, translocases, disaggregases,

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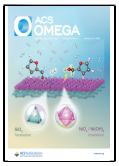

and chaperones and some initial studies on ribosomes (Figure 1).

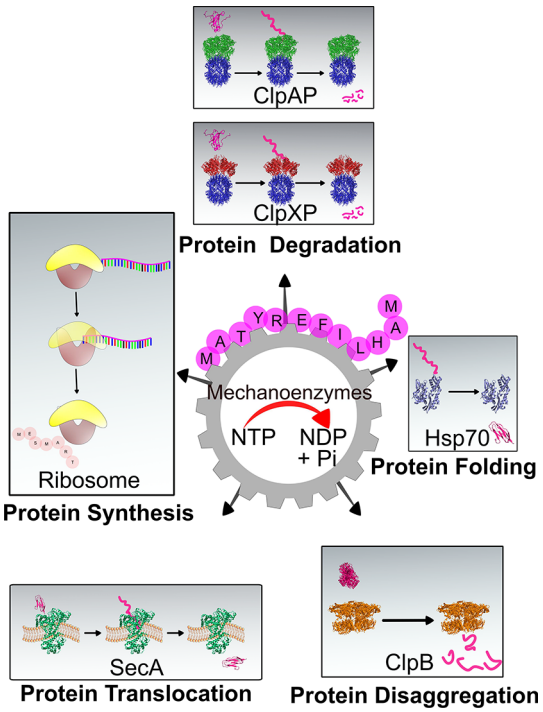

Figure 1. Protein-processing mechanoenzymes. Schematic of various protein-processing cellular processes in which enzymes act as molecular motors performing the mechanical actions using the energy obtained from the hydrolysis of nucleotide triphosphates (mostly ATP/GTP). Mechanical actions and motor properties of enzymes applying force during protein degradation by bacterial proteases, ClpXP and ClpAP; protein folding by DnaK, an Hsp70 chaperone; protein disaggregation by *E. coli* disaggregase ClpB; protein translocation across the membrane by SecA motor; and protein synthesis by ribosomes are discussed in this review. The gray gear-like structure indicates a molecular motor, and the amino acid sequence indicates a protein. Protein structures were obtained from the PDB and visualized using visual molecular dynamics (VMD).[60]

1.1. Protein-Processing Mechanoenzymes. Mechanical forces are ubiquitous during a protein's life cycle, starting from synthesis, folding, unfolding, translocation across various compartments, disaggregation, and finally degradation. Mechanoenzymes act as motors, applying mechanical forces on proteins that are imperative during the protein's life cycle. For example, many proteins have to be transported across various cell compartments, and the openings of these compartments are so narrow that they do not allow the passage of folded proteins. It is now well established that the mechanical translocation of these proteins is carried out by molecular motors present at the interfaces. Proteins at the mitochondrial import channel like mtHsp70 have been shown to pull the polypeptide precursor of the target protein by an ATP-induced conformational change, thereby denaturing the protein mechanically and forcing its entry into the import channels.[5,6]

Similarly, processes like protein degradation also require the mechanical unfolding of substrate proteins, as folded protein sizes are larger than the width of the peptidase entry channels.[7] Bacterial protease ClpXP recognizes the substrate through a peptide tag, mechanically unfolds it, and translocates it to the peptidase chamber for degradation.[8] The structure and assembly state of these enzymes vary widely; most use ATP as their fuel to perform their mechanical actions. Over the past decade, there has been considerable interest in the working mechanisms of these protein-processing molecular motors and the complementary insights provided by single-molecule experiments. Unlike cargo-carrying or DNA-processing motors, these motors interact with substrates that differ widely in structure and sequence. The single-molecule experiments complemented, validated, and resolved long-standing debates and have even led to paradigm shifts in understanding the mechanisms of various mechanoenzymes. This review will discuss different classes of protein-processing molecular motors and how optical-tweezer-based single-molecule assays have provided a more profound and complementary understanding of these enzymes.

2. SINGLE-MOLECULE MEASUREMENTS

An understanding of biological processes at the molecular level has significantly expanded over the past two decades owing to the development and application of single-molecule methods.[9] Single-molecule measurements give complementary information to the ensemble measurements because they can measure anisotropic properties such as force, track molecular displacements, and highlight the stochasticity of various properties, resulting in the visualization of the heterogeneity in molecular properties of different biological motors.[10] In addition, single-molecule assays can give us information on individual steps during a multistep mechanism and the trajectories of individual molecules during a time course that is usually masked in bulk ensemble measurements.[9,10] These methods could be divided into either monitoring methods, such as single-molecule fluorescence imaging, or manipulating methods, known as single-molecule force-spectroscopy (SMFS). SMFS involves the study of the properties of individual molecules by probing them with force in the form of tension and includes techniques such as atomic force microscopy (AFM), optical tweezers (OT), and magnetic tweezers (MT) that have been widely used to divulge information on the motor properties of enzymes, mechanical unfolding pathways of proteins, as well as stabilizing effects of ligands on proteins.[2,11] Among these, optical-tweezer-based assays have championed over others in understanding the mechanisms of molecular motors, with their ability to precisely measure subnanometer displacements with microsecond temporal resolution, applying stretching forces in the range from subpiconewtons to hundreds of piconewtons.[12,13] In addition to the technical advantage of spatiotemporal resolution and range of forces the optical tweezers can measure (Table 1),

Table 1. Comparison of Spatiotemporal and Mechanical Parameters of SMFS Methods

SMFS Technique	Spatial Resolution (nm)	Temporal Resolution (s)	Minimum Stiffness (pN/nm)	Force Range (pN)	Force resolution (pN)	Displacement Range (nm)	Probe Size (μm)
OT	0.1	10^{-4}	0.05	0.1−250	0.02	0.1−10^5	0.25−5
AFM	0.5	10^{-3}	>10	10−10^4	10	5−10^4	100−250
MT	2	10^{-2}	10^{-6}	10^{-3}−200	0.001	0.5−10^4	0.5−5

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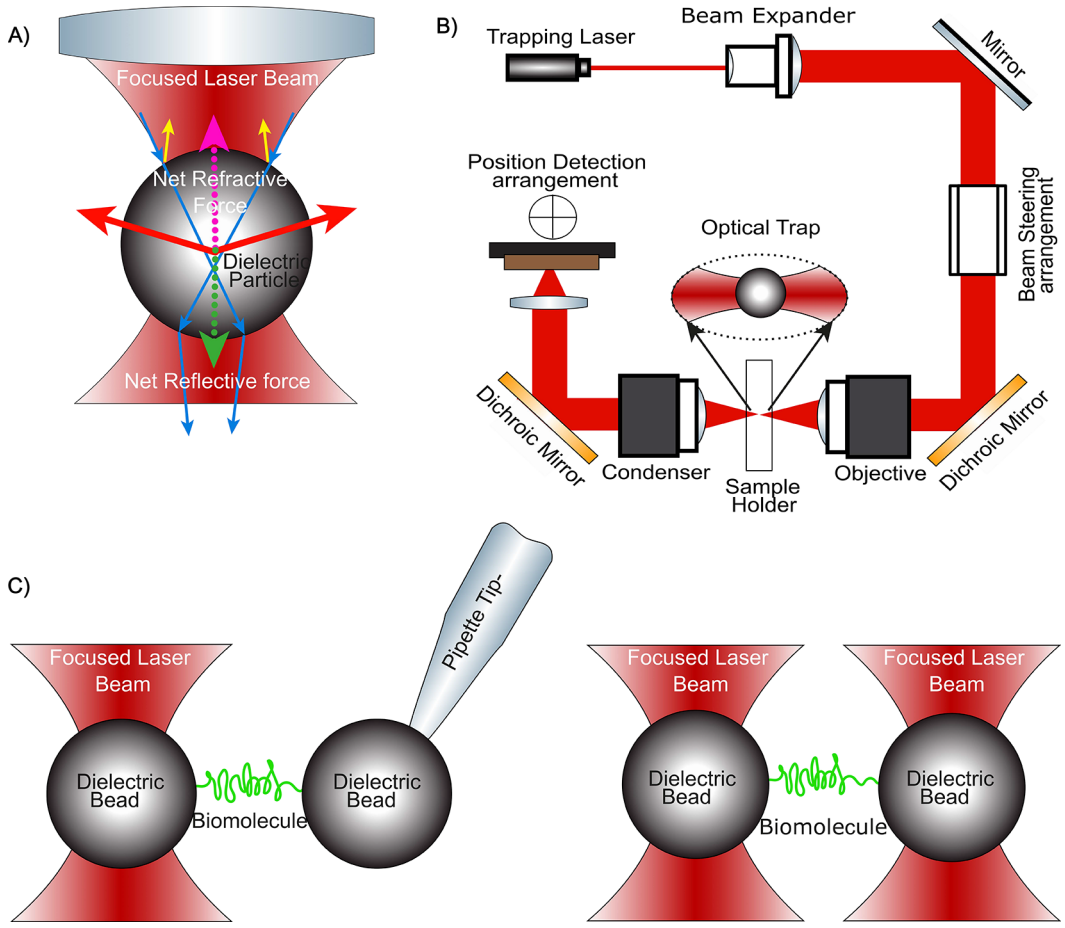

Figure 2. Single-molecule optical tweezers. (A) A dielectric particle is trapped at the center of a focused laser beam as it experiences opposing refractive and reflective forces. (B) Schematic of the laser beam path and optics necessary for creating an optical trap. (C) (Left) Representation of a single-trap optical tweezer in which one bead is trapped in a laser beam and the other is held using a micropipette tip and (Right) a dual-trap optical tweezer in which both the beads are trapped in laser beams. A biomolecule under investigation is attached to the beads from both ends using a specific chemical linkage such as Streptavidin/Biotin or Digoxigenin/Anti-Digoxigenin molecules.

it gives an advantage over other methods due to its ability to steer the beads onto which molecules are attached, facilitating increased interactions between molecular motors and their substrates in the solution for faster data collection. Additionally, the capability of integrating single-molecule fluorescence detection methods such as FRET or confocal microscopy and fluorescence optical tweezers, commonly called "fleezers", with optical tweezers as compared to other force spectroscopy methods such as AFM or magnetic tweezers gives an added advantage in employing it to monitor and manipulate these molecular motors.[12,13] Motor properties such as directionality, velocities, stepping kinetics and dynamics, processivity, energetic costs, and stall forces have been studied in greater detail.[13] Optical tweezers have contributed to detailed investigations of the mechanochemistry of molecular motors, starting from initial studies on myosin's linear movement on actin filaments to the rotational motion of DNA gyrases.[4] They have demonstrated that molecular motors can move processively over long distances with hundreds of steps and varied sizes with nanometer accuracy. Additionally, detailed information such as dwells between the steps, pauses during movement, resisting forces, and other motor properties of nucleic-acid-processing enzymes such as DNA helicases and RNA polymerases and, more recently, preliminary information on protein unfolding and disaggregating enzymes as well as protein folding chaperones have all been possible by employing optical-tweezer-based single-molecule assays.[4,14]

Spatiotemporal resolutions and measurable force ranges of commonly used SMFS techniques are given in Table 1.[9,10,15−17]

2.1. Optical Tweezers: Principles, Instrumentation, and Assays. Optical tweezers are optomechanical devices in which light from a laser beam traps and manipulates microscopic dielectric particles. It works on the principle that light carries momentum and can exert force on particles.[18] Highlighting the invention of optical tweezers and their application to biological systems, its inventor, Arthur Ashkin, won the Nobel prize for Physics in 2018. For a dielectric particle kept in the focused beam of a laser source, the force exerted due to the change in momentum of laser light due to refraction pulls the particle toward the focusing spot, whereas the force due to reflection pushes the bead along the propagation direction of the beam, trapping the particle stably at a point near the focusing spot[19] (Figure 2A). This principle led to the invention of optical traps and further tweezers that were extensively applied to understand the behavior of single molecules.[13,20]

A laser beam from a high-power laser source acts as the trapping beam, and usually, a near-IR laser with a wavelength of 800−1200 nm and power >1 W is used as the trapping laser. The trapping laser's power ensures the trap's stiffness, and the NIR wavelength range ensures good transmittance in the enzyme's

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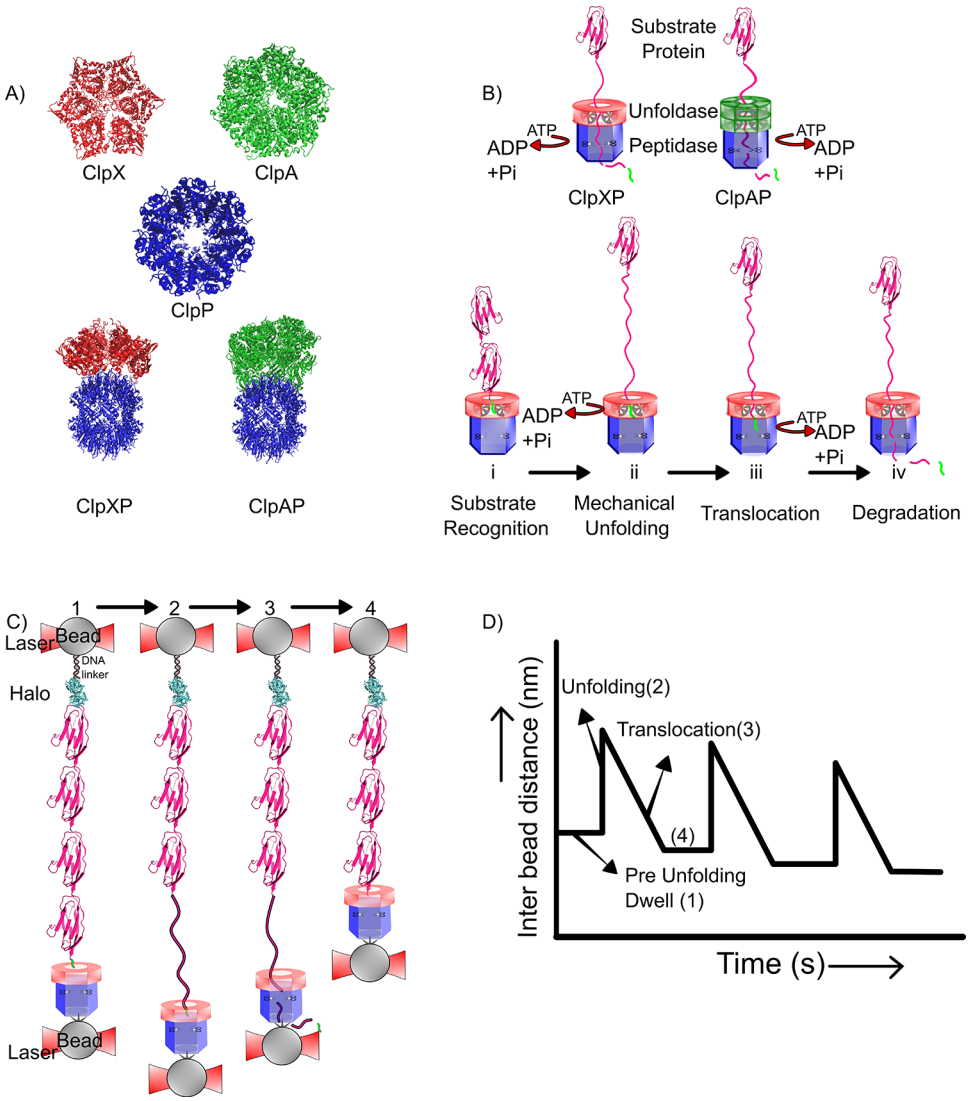

Figure 3. Protein degradation by ATP-dependent proteases: (A) cryo-EM structures of ATP-dependent unfoldases *E. coli* ClpX (6PP7), ClpA (6W22), and the peptidase ClpP (6PPE) in red, green, and blue, respectively. PDB ids for the structures are mentioned in the brackets. A model structure of *E. coli* ClpXP and ClpAP in which hexameric ClpX (Red)/ClpA (Green) are aligned on top of tetradecameric ClpP (Blue). ClpX is a single-ring unfoldase, whereas ClpA is a double-ring unfoldase. (B) ClpX/ClpA recognizes the degron tag on the protein substrate and mechanically unfolds and translocates the protein in an ATP-dependent manner to the protease compartment, ClpP. The protein gets hydrolyzed to small peptide fragments in ClpP in an ATP-independent manner. (C) Optical-tweezer-based single-molecule assay to monitor the events of protein unfolding and translocation during degradation. The enzyme and the substrate are immobilized on two different beads that are held in two laser beams, and interbead distance is monitored, revealing the process of substrate protein unfolding and translocation. (D) A cartoon trace showing the variation of interbead distance with time during protein degradation, highlighting the changes during preunfolding, unfolding, and translocation of protein substrates. Protein structures were visualized using visual molecular dynamics (VMD).[60]

physiologically relevant buffers with low absorbance. The beam is expanded using optics, passed through a beam-steering arrangement, and focused by a high-power objective lens with a high numerical aperture. The focusing spot of the laser beam forms the optical trap where the sample chamber is situated. A condenser then collects the light, and using suitable optics, it is directed to a quadrant photodetector array used as a position/force detection (Figure 2B). The beam-steering mechanism is usually an acoustic−optic modulator which can be used to manipulate the laser beam perpendicular to the direction of propagation which in turn provides the planar movement of the optical trap at the focal plane of the objective lens. Usually, a bright-field microscopic arrangement is incorporated with the optical tweezer setup, which allows the direct visualization of the trapped dielectric spheres. The force per unit distance at which the bead is trapped is called trap stiffness, and it mainly depends on the power of the laser, the numerical aperture of the objective lens, the refractive index and size of the bead, and the temperature.[21] Force calibrations are done prior to the experiment to determine the stiffness of the trap.[18]

Optical tweezers are very versatile for single-molecule studies due to their capability to apply or measure a wide range of forces that can be imparted on the molecules, spatiotemporal resolution, and stability. SMFS can be performed by optical tweezers by attaching optically trapped dielectric particles to the molecule of interest, and manipulating the laser beam can impart tension on the molecule as the other end of the molecule is fixed, usually chemically on a glass slide, mechanically on the tip of a pipet, or another optical trap in the case of a dual-trap optical tweezer[13] (Figure 2C). Biomolecules under study are attached

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to the beads (dielectric particles) using a specific chemical linkage, such as a streptavidin−biotin bond or digoxigenin−antidigoxigenin bond, and will be described for individual assays. Single-molecule assays vary for each enzyme in terms of whether they are single-trap or dual-trap assays or interaction specificities between the bead and the enzyme, which have been described in the next section.

2.2. Biochemistry of Single-Molecule Assays. In addition to complex instrumentation and rigorous data analysis, single-molecule experiments involve designing specific biochemical assays for attaching the substrates and enzymes of interest to the dielectric beads to accurately monitor various single-molecule events. In general, the nature of the interaction between the dielectric beads and the substrates/enzymes of interest is noncovalent antibody−antigen interactions. The polystyrene beads are covalently coated with either streptavidin (neutravidin) or antidigoxygenin and attached to a DNA linker via functionalization with biotin/digoxygenin. The DNA linker is covalently attached to the protein substrate or enzyme of interest using maleimide−thiol chemistry or halotag protein−halo ligand interaction. The substrates under observation are generally multidomain proteins cloned using the iterative cloning method. The linkage chemistry employed by different groups is different based on the class of molecular motors being studied. Experimental design specifics are described briefly for individual enzymes.[22]

Briefly, the sample preparation involves immobilizing substrate-attached beads to the surface of the glass coverslip, trapping the freely floating bead attached to the enzyme with one of the laser traps and bringing it close to the immobilized bead for the formation of tether or contact between the enzyme and the substrate. Alternatively, a microfluidics-based multichannel glass slide has been used. Free-floating beads linked to the substrate and antibody-coated beads from different channels are brought together for tether formation. Generally, the buffer contains a nucleotide regeneration system such as pyruvate kinase/phosphoenol pyruvate in the case of ATP, so that the nucleotide concentration does not deplete drastically during the experiment duration. Over the years, various groups have optimized their assays and sample preparation methods to increase the probability of observing single-molecule events.

3. MOTORS OF PROTEIN DEGRADATION

Protein degradation is an essential cellular process that not only maintains the protein levels in the cell but also removes the damaged and abnormal proteins from the cells. Proteasomes and ATP-dependent proteases carry out part of this protein homeostasis and belong to the class of enzymes called AAA+ enzymes.[23,24] These enzymes generally have two compartments, the upper compartment that binds, unfolds, and translocates the substrate and the lower compartment that hydrolyzes the peptide bonds. Primarily, these enzymes recognize a degron tag, either a peptide tag or polyubiquitin (in the case of proteasomes), that is present on many of the substrate proteins that are to be degraded and mechanically unfold the folded substrates during cycles of ATP hydrolysis and translocate the unfolded protein, also in an ATP-dependent reaction, into the proteolytic chamber where the unfolded proteins are degraded into smaller peptides in an ATP-independent reaction.[23−25] Two of *E. coli*'s ATP-dependent proteases, ClpXP and ClpAP, have been well studied using single-molecule optical tweezer assays that gave us information on the inner workings of these protein destructors.[26,27] ClpX and ClpA are homohexameric enzymes and recognize a degron tag called ssrA peptide. These enzymes differ in architecture; i.e., ClpX is a single-ring hexameric enzyme with one "motor" with 6 ATP binding sites, whereas ClpA is a double-ring hexameric enzyme with two "motors" and has 12 ATP binding sites. The ClpP is a tetradecameric peptidase compartment that catalyzes the peptide bond hydrolysis in an ATP-independent manner (Figure 3A). The enzymes ClpXP and ClpAP have ClpX and ClpA as their ATPase units, which recognize, unfold, and translocate the proteins to ClpP, an ATP-independent proteolytic compartment (Figure 3B). Prior biochemical and biophysical studies have shown us extensive details of the oligomeric nature, structure, and effect of substrate tertiary structure on degradation kinetics and the key residues responsible for ATPase and motor activities.[8,28] With the development of optical tweezers based on the single-molecule assay, in addition to providing information on stalling forces, step sizes, and velocities of these enzymes, it was possible to delineate the individual processes of unfolding and translocation that were difficult to understand independently through ensemble experiments and hence also revealed more detailed information on the mechanism of protein unfolding and translocation.[14]

Initial single-molecule experiments were performed mainly by two groups independently on the ClpX enzyme on a single-chain ClpX (sc-ClpX) variant, where all six protomers were linked genetically, making it easier to study the effect of individual protomers on the overall process of protein unfolding and translocation.[29,30] Generally, during these assays, the biotinylated enzyme is attached to one of the streptavidin-coated beads, and a polyprotein attached to a DNA linker is bound to the other bead using either a biotin/streptavidin or digoxigenin/antidigoxigenin linkage. Although initial studies were performed only using the ATPase ClpX without the peptidase ClpP on Filamin A domains as the substrates, most of these detailed studies on ClpXP involved the degradation of model proteins titin-I27, the 27th domain of the immunoglobulin region of muscle protein titin, and the green fluorescence protein (GFP) for their robust mechanical properties, and unfolding has been well-studied by biochemical and SMFS experiments by AFM.[26,30,31]

3.1. Single-Molecule Assay for Monitoring Protein Unfolding and Translocation. For the single-molecule assay monitoring of the independent processes of protein unfolding and translocation, the biotinylated enzymes (either sc-ClpX or ClpP for ClpXP assays and ClpP for ClpAP) were attached to a streptavidin-coated bead, and the multidomain protein substrate (homo/hetero) genetically linked to the HaloTag protein is attached to the other streptavidin-coated bead using the biotin-DNA-Haloligand linker (Figure 3C). The substrate protein is attached to the degron tag either genetically or chemically. Beads attached to the enzyme and substrate and the necessary components for ATP regeneration are all added to a small channel, and the lasers trap the beads. When beads are brought closer, the enzyme recognizes the degron tag on the substrate, called tethering. During the cycles of ATP hydrolysis, the substrate is unfolded and translocated by the enzyme. The repetitive signal from multidomain protein increases the number of data points from a single tethering event and helps provide accurate information on unfolding rates. Upon unfolding, there is a sudden increase in the interbead distance due to conformational change in the protein, and during translocation, the distance decreases gradually (Figure 3D). Hence, monitor-

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ing the interbead distance changes reveals detailed information on kinetics and pathways of unfolding and translocation. The time between the translocation's end and the subsequent protein unfolding event gives the preunfolding dwell. The translocation traces can be deconvoluted using step-finding algorithms to determine the stepping kinetics and dynamics.[14,30]

3.2. Protein Unfolding. Single-molecule assays mentioned here measure the interbead distance with time, and unfolding is captured by an increase in length due to the conformational transition from a folded state to an unfolded state. These studies highlighted that the protein unfolding process by these ATPases is stochastic, and multiple ATP hydrolysis cycles are needed for unfolding a protein depending on the mechanical stability of the protein. Although multiple ATPase cycles power the unfolding stokes, most of these cycles are futile before successful unfolding, and a single power stoke finally results in unfolding due to transient destabilization of the protein structure, safeguarding it against unfolding.[26] The preunfolding dwell corresponds to the time taken by the enzyme to unfold the protein and reveals the kinetics of unfolding. The distribution of this kinetics is either single or multiexponential depending on the unfolding pathway, resulting in a measurable parameter, the lifetime (τ).

Lifetime depends on the protein's mechanical stability and unfolding power of the enzyme, as observed during the unfolding of various mutants of the titin-I27 by ClpXP and ClpAP.[26,27] Titin-I27 is a mechanically robust protein with H-bonds in the terminal antiparallel beta strands guarding it against the unfolding.[32] Mutants disrupting these H-bonds result in easier unfolding by both ClpXP and ClpAP.[26,27] In addition, the rate of unfolding by the ATPase depends on the local stability of protein structure at the pulling end and not the global stability, as evident from the direction-dependent unfolding studies. In the case of the unfolding of titin-I27, it was observed that the unfolding from the N-terminus was ∼50 times faster than that from the C-terminus. Protein could be unfolded with just one power stroke from the N-terminus.[33] It was proposed that this vast difference in the unfolding times depending on the structurally weaker end might have a plausible role in the evolutionary selection of the placement of degron tags to minimize the energy consumption during unfolding. With ClpXP, the enzymatic protein unfolding was spontaneous and followed the same pathway as mechanical unfolding studies by atomic force microscopy (AFM) or biochemical studies where titin-I27 unfolds in a cooperative two-state pathway, and the GFP unfolds by a three-state pathway.[26,31]

Interestingly, studies by ClpAP showed that the titin-I27 could unfold either by a two-state pathway or a three-state pathway with an intermediate. The three-state pathway was observed by neither AFM unfolding studies nor biochemical studies, indicating that the enzyme could alter the unfolding pathway of the substrate.[34] For efficient motor activity by these unfoldases, the enzyme has to grip the substrate and apply pulling force. This gripping is done by pore loops, i.e., the aromatic/hydrophobic residues that are conserved across various ATP-dependent enzymes that unfold or remodel the substrate. Pore-loop mutational studies on ClpX highlighted the importance of pore loops and the bulkiness of the residues in the pore for gripping during unfolding. These studies showed that the enzyme with tyrosine to alanine mutation in pore loops shows reduced unfolding efficiency. Increasing the number of such mutated protomers in the ClpX hexamer increases the unfolding times or reduces the unfolding probability. These mutants further show unfolding defects and often release the substrate during unfolding, termed slipping.[35,36] The importance of gripping efficiency is also evident from studies on the double-ring ATPase ClpA, which is a faster unfoldase than the single-ring ClpX. In ClpA, both rings have pore loops, doubling the grip strength due to increased surface interactions of ClpA with the degron tag.[27] In the case of the double-ring ATPase ClpA, earlier ensemble studies concluded that the bottom ring plays a significant role in ATP-fueled protein degradation, particularly for highly stable proteins. The top ring contributes to binding, and inactivating it has only a modest effect on protein degradation[37] Single-molecule assays performed on a variant of ClpA that was defective in ATP hydrolysis of the top ring highlighted its importance with a profound impact on unfolding rates.[34]

The single-molecule assays described here provide information on the kinetics and unfolding pathways of the proteins. As the protein unfolds during multiple power strokes, the force that the enzyme generates during each power stroke has not been calculated directly. The model proteins used in these studies, titin-I27 and GFP, are mechanically strong in the N−C direction, as observed during AFM-based unfolding studies. The unfolding forces are ∼200 pN and ∼100 pN at ∼300 nm/s pulling rate.[38] The enzyme pulling rate of ∼4 nm/s and an estimated force of $\sim 5k_BT$ (∼20 pN) from the translocation velocities, as described in the next section, indicate that the enzymes apply a force of ∼20 pN during each power stroke, and depending on the local stability of the protein at the point of pulling, the enzyme will have to apply a higher or lower number of power strokes that can be calculated from the kinetics of unfolding.

3.3. Protein Translocation. The translocation of a protein is a key process in many cellular activities involving moving a protein from one cell compartment to another or transferring substrates from one enzyme to another. It could be a diffusive process or a directed process. During protein degradation by ClpXP and ClpAP, the unfolded polypeptide is translocated by ClpX/ClpA in an ATP-dependent manner into the peptidase chamber, ClpP, for proteolysis. Single-molecule optical tweezer assays played a crucial role in the understanding of protein translocation by these enzymes, as it was almost impossible to visualize this in ensemble assays. In these single-molecule assays, this translocation is captured by decreasing interbead distance with time. Translocation trajectories display a stepwise behavior, implying a power-stroke model and negating diffusion during energy-dependent translocation.[8] This stepwise behavior can be compared to the walking of cargo-carrying molecular motors such as myosin or kinesin on cytoskeletal tracks or nucleic-acid-processing motors on DNA/RNA. Translocation of the unfolded polypeptide through the narrow channels is mediated by its pore loops, which are arranged in a spiral staircase architecture as determined by recent cryo-EM studies.[39,40] Unlike cytoskeletal tracks with a chemically homogeneous framework or nucleic acid tracks composed of only four different units, "walking" on these polypeptide tracks by the molecular motors is a more complex process due to a more heterogeneous framework. It is not clearly understood whether the chemical nature, i.e., the nature of the amino acid, plays a role during the translocation. Unlike stochastic unfolding, translocation was observed to be monotonous to a large extent, and ClpXP and ClpAP walk on the polypeptides with an average velocity of ∼4.5 nm/s and ∼3 nm/s, respectively, in the C-to-N direction.[26,41] These velocities are independent of the applied opposing force under loads <12 pN.[26] The reason for the relatively lower

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velocity of the double-ring ATPase ClpAP is unclear. Still, it could be attributed to a tighter grip or a smaller step size. As discussed, protein translocation occurs in a stepwise manner with a brief waiting time between each step, termed step-dwell. This step-dwell has an exponential distribution with an average lifetime of ∼0.3−0.5 s, roughly corresponding to the time constant for the hydrolysis of ATP to ADP by ClpXP. This closeness in the values of time constants between step-dwell and ATP hydrolysis strengthens the hypothesis that each step is driven by a power stroke generated upon ATP hydrolysis by the enzyme. In the field, there is ambiguity in arriving at the average value of translocation velocity across various groups due to differences in defining a pause. Some step-dwells are longer compared to the average dwell time and are defined as a pause. Two different approaches were used for considering this dwell as a pause. The Baker and Sauer laboratories consider any dwell greater than five times the average lifetime of the dwell as a pause, and the Bustamante group considers a dwell >1 s as a pause and an off-pathway process,[29,33] hence the inconsistent reporting of the translocation velocities, particularly for the ClpXP. Surprisingly, the direction of translocation has a notable effect on the velocity, and both enzymes slow down by ∼25% in the N-to-C direction. This decrease in translocation velocity is due to increased pausing in this direction and not an actual decrease during pause-free movement.[33] A possible explanation for this direction-dependent decrease in velocities is the role played by stereochemical interactions of the polypeptide's certain amino acid side chains with the axial pore loops, temporarily stalling the translocation resulting in a pause. This theory could be tested by performing experiments with D-amino acid polypeptides to observe the effect of inversion of stereochemistry on the translocation velocities. This pausing is higher in ClpAP than ClpXP, and it is not uniformly distributed along the polypeptide, indicating a sequence-specific pausing behavior.[33] The stepwise translocation occurred with a distribution of step sizes between 1 and 4 nm for ClpXP, and conversion of these steps into amino acids is not straightforward as one has to consider the force at which these polypeptides are stretched as well as chemical nature to determine the persistence length of these polymers and further conversion into amino acids using the worm-like chain model of polymer physics.[42] The smallest step is ∼1 nm corresponding to ∼4−6 amino acids; the largest step is ∼20 amino acids, indicating that the enzyme can pull ∼4−6 amino acids per powerstroke with a work output of $\sim 5k_BT$.[26] Interestingly, ClpAP takes steps of only ∼1 nm, and it is unclear if this difference is due to the double-ring ClpA structure or usage of the nonlinked hexamer, unlike ClpX.[41] It is worth mentioning that the ClpXP takes only ∼1 nm step in the N-to-C direction where noncovalently linked ClpX was used.[33] For ClpAP, single-molecule studies revealed previously unknown functions of individual motors of the double-ring ATPase ClpAP. These experiments concluded that the non-dominant top ring, D1, coordinates with the dominant bottom ring, D2, to facilitate translocation. Experiments on mutant ClpA defective in ATP hydrolysis showed that both frequency and duration of the pauses increase during translocation and reduce the velocity significantly, ∼60% and 30% in N-to-C and C-to-N directions, respectively.[34] In addition, the studies showed that the polypeptide slips from the grip of this mutant enzyme under subsaturating conditions of ATP, resulting in increased degradation times. Overall, single-molecule studies have confirmed the processive translocation by the ATP-dependent unfoldases, quantified translocation by providing velocities, step sizes, and step dwell, and revealed novel behavior such as pausing and slipping during translocation.

4. PROTEIN-DISAGGREGATING MOTOR

ClpB disaggregase belongs to the Hsp100 family of proteins, extracts misfolded proteins from protein aggregates, and solubilizes and reactivates them with the help of Hsp70 class chaperones.[43] They are hexameric, and each protomer has two ATP-hydrolyzing rings similar to ClpA (Figure 4A). Bio-

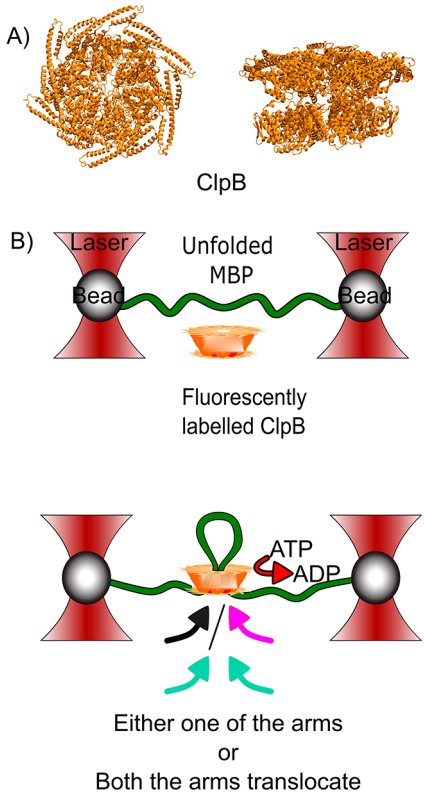

Figure 4. Translocation of a polypeptide through disaggregase ClpB. (A) Top view and side view of the cryo-EM structure of *E. coli* ClpB disaggregase (PDB ID: 5OFO). (B) Schematic of the optical trap experimental setup used by Tans group to study the motor properties of ClpB disaggregase. In the assay, a single domain of maltose binding protein (MBP) was mechanically unfolded and relaxed to a slightly lower force for the disaggregase to interact with the unfolded protein (top). Extrusion of polypeptide loops could occur through either of the pore-loop arms (black/purple arrows) or both arms simultaneously (turquoise arrows). Protein structures were visualized using visual molecular dynamics (VMD).[60]

chemical and structural characterization of ClpB has been carried out extensively, determining the hydrolysis rates, substrate preference, and spiral arrangement of pore loops in the hexamer.[43] Contradicting mechanisms of translocation/threading-based models, such as processive translocation by a power-stroke model or a nonprocessive translocation by entropic pulling or Brownian ratcheting, have been proposed for disaggregation, with ensemble kinetic experiments supporting either of these models.[44−46] Single-molecule force spectroscopy studies on these enzymes are in their nascent stage. The first experiments were on the disaggregase ClpB, from *E. coli*, by the group of Sander Tans at AMOLF, using optical tweezers combined with confocal microscopy.[47]

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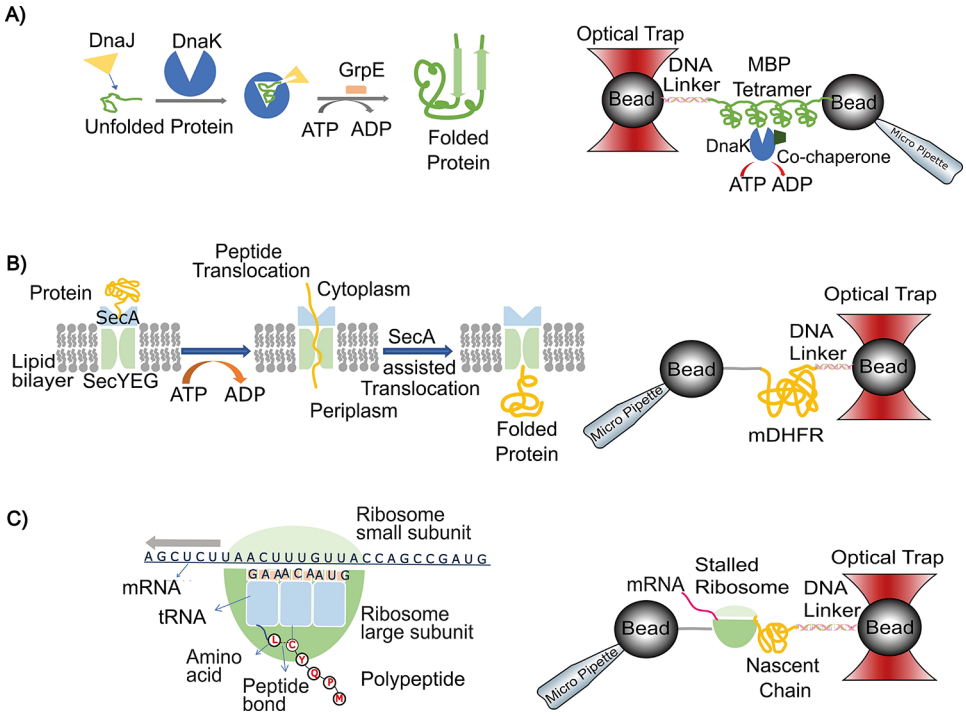

Figure 5. Molecular motors of protein folding, translocation, and synthesis: (A) Cartoon representation of the sequence of events during protein folding by the Hsp70 chaperone, DnaK, and cochaperones DnaJ and GrpE. In the single-molecule assay, poly-MBP is attached to two beads held in an optical trap and stretched to an unfolded state. The presence of the DnaK and cochaperone systems reduced the formation of misfolded states as observed by differences in contour length changes during extension. (B) Protein translocation by the SecA/SecYEG complex. A comparative study on a model protein substrate DHFR, ensemble translocation rates by SecA, and mechanical unfolding by single-molecule studies indicate a Powerstroke model by SecA during unfolding and translocation of proteins across the membrane. (C) A cartoon picture representing the process of translation by ribosomes. Single-molecule assays on the stalled ribosome nascent chain complex (RNC) showed the effect of ribosomes on the kinetics of protein folding without altering the pathway.

Single-molecule assays employed dual-trap optical tweezers in which the maltose binding protein (MBP), attached to the DNA linkers, was bound via digoxygenin−antidigoxygenin interaction on one bead and biotin−neutravidin on the other bead (Figure 4B).[47] The enzyme ClpB was introduced into the channel and was not directly bound to any bead. The protein was mechanically unfolded, and monitoring the interbead distance revealed the conformational changes in the protein, hinting at the mechanism of action of ClpB. Combined with confocal fluorescence measurements, these optical tweezer experiments gave more accurate information for predicting the translocation models by ClpB. These studies partially understood ClpB's motor properties and the model for the processive pulling of polypeptides for disaggregation. They showed extrusion of the loops by ClpB in an ATP-dependent manner, providing unambiguous evidence for processive translocation. Remarkably, these studies have demonstrated that disaggregases are fast and processive motors that can apply forces as large as ∼50 pN. They translocate the polypeptides an order of magnitude faster than protein unfoldases, with former translocation velocities at ∼500 amino acids/second and the latter just around ∼30 amino acids/second.[47] One of the primary reasons for this increased speed is the larger distance ClpB advances with each step, i.e., ∼30 amino acids, compared to ∼10 amino acids for the protein unfoldases. All these studies were performed on mechanically unfolded maltose binding protein (MBP) domains. In addition, using optical tweezers integrated with fluorescent particle tracking, they identified that a polypeptide is inserted into the ClpB's channel as a loop, and the enzyme can translocate the polypeptide using either two arms or one arm of the loop (Figure 4B). The translocation velocities and step sizes scaled appropriately, and the velocities were double when two arms were being translocated simultaneously compared to one arm. The translocation of ClpB by extrusion of loops suggests that the enzyme could target the internal segments of the aggregate and not just the free ends.[47] In addition, their results infer that the folding of polypeptide loops at the exit channel is similar to cotranslational folding during protein synthesis by ribosomes. Although single-molecule optical tweezers on disaggregases are limited to date, other techniques such as SM-FRET have elucidated the ultrafast dynamics of these enzymes various components, particularly the dynamics of the substrate binding domain, middle domain and the pore loops of ClpB.[48]

5. OTHER PROTEIN-PROCESSING MOTORS

5.1. Hsp70. The ATP-dependent ∼70 kDa heat shock proteins (Hsp70) are highly versatile chaperones that play a significant role in protein quality control of a cell and are preserved across all domains of life. Hsp70 assists in properly folding a protein and prevents the formation of misfolded states and aggregates[49] (Figure 5A, left). Optical-tweezer-based single-molecule assays on mechanically unfolded polyMBP protein (tetramer) that are prone to aggregation revealed that the DnaK, a bacterial homologue of Hsp70 and its cochaperones DnaJ and GrpE, favors the formation of the native state as well as prevents the formation of misfolded states (Figure 5A, right).[50] Hsp70 prevents aggregation by binding to small peptide segments of the exposed protein within a groove with an ATP-driven lid. However, the interaction of Hsp70 with protein substrates at

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different stages of folding has not been studied in detail by bulk studies due to many limitations, including conformation dynamics of both the chaperone and the substrate protein as well as the transient nature of the interaction. Due to the ability of the optical tweezer assays to detect the near-native or partially folded state structures based on the changes in contour length as well as maintain these structures by force modulation, these single-molecule studies reported that the Hsp70 complex could bind and stabilize, unfolded and near-native proteins. The Hsp70 did not allow refolding of the mechanically unfolded and relaxed MBP, consistent with the interaction of the complex with the extended state of the protein. In addition, these studies highlighted a previously unobserved role of this Hsp70 lid: it can also interact with partially folded proteins and near-native structures, stabilize them mechanically, and prevent them from completely unfolding, even at forces >40 pN, well above the native unfolding force of 22 pN. These results revealed the hidden interactions of the Hsp70 system with near-native structures and highlighted the role of the lid domain of DnaK that was not observed in earlier bulk studies.[50] In addition to understanding the effect these chaperones impart on the mechanical stabilities of the substrates, there has been an effort to understand the nanomechanics and unfolding pathways of the Hsp70 chaperone.[51]

5.2. Protein-Translocating Motors. Sec translocons move proteins across membranes driven by cytosolic ATPase SecA (Figure 5B, left). Although most of the transported protein substrates are either unfolded or molten globules due to the presence of chaperones, many structured proteins need to be translocated across the membrane.[52] Whether the translocation could happen by a "power-stroke" model where the SecA grips the substrates and pulls them during the cycles of ATP hydrolysis or by a "Brownian−Ratchet" model by passive diffusion of the substrate during the conformational opening and closing of the SecY channel has been a subject of debate.[53,54] Recent studies from the Kaiser group compare unfolding kinetics of DHFR, a standard substrate of SecA, using a combination of single-molecule optical tweezers (Figure 5B, right) and biochemical translocation assays, suggesting that SecA acts as a motor and unfolds and translocates by the power-stroke model, applying forces over 10 pN.[55]

5.3. Ribosomes. Protein synthesis in the cells is carried out by ribosomes, macromolecules that translate mRNA to amino acids in an energy-dependent manner (Figure 5C, left).[56] Single-molecule optical tweezer experiments investigated the folding of nascent polypeptide bound to the ribosome and showed that the kinetics of folding of the T4 lysozyme varies depending on how far it is from the ribosomes (Figure 5C, right).[57] The folding rate decreases by 100-fold when the ribosome is close to the mechanically unfolded T4 lysozyme and increases as the distance between the substrate and ribosome increases. This indicates that the ribosome reduces the formation of non-native contacts during cotranslational folding and prevents misfolding. These experiments suggested a novel role for ribosomes; i.e., in addition to decoding the genetic information, they also make sure that the translated proteins do not fall into energy traps of misfolded states and aggregates.[57]

6. SUMMARY AND FUTURE OUTLOOK

Protein-processing mechanoenzymes are involved in various processes ranging from protein synthesis and remodeling to degradation. An understanding of the motor properties of these enzymes is in its nascent stage, plausibly due to complex interactions between the motor and the substrate. The past decade has seen an emergence of the application of single-molecule optical tweezers to understand the intricate details of the mechanism of action of these motors. Major bacterial proteases have been studied in particular for their unfolding and translocation properties, and studies on disaggregases are in their infancy. These single-molecule assays have played a pivotal role in deciphering enzyme properties such as processivity, translocation velocities, kinetics, and dynamics of translocation steps and the relative power of unfoldases during protein unfolding. In addition, single-molecule studies revealed new features of these motors that were inaccessible to ensemble studies. Observations such as pausing and slipping during translocation indicate preferential and nonpreferential interactions of the ATPase motor pore loops with specific amino acids of the polypeptide, a hypothesis that needs rigorous experimental validation. Additionally, these studies determined that the rate-limiting step during protein degradation could be either unfolding or translocation and is mostly determined by the local structure of the protein substrate. Studies on disaggregases have resolved a long-standing debate on the processive vs nonprocessive nature of these motors and have illustrated that they can work in double gear mode, where they can pull a looped polypeptide through a single arm or double arm and can switch between them during a translocation. An unanticipated result from the studies on disaggregases is that there is an order of difference in the translocation velocities and significant variation in the step sizes between disaggregases and proteases that have similar structural architecture and belong to the same Hsp100 class of AAA+ enzymes. Although the other classes of processing motors, such as Hsp70, ribosomes, or SecA, have not been directly studied for their motor properties, comparing ensemble and single-molecule studies indicates that SecA applies forces >10 pN for unfolding and translocating proteins across the membranes. The effects of molecular machines, Hsp70, and ribosomes have been studied for their role in altering the kinetics pathways of folding and preventing the formation of misfolded states. In addition, more recently, single-molecule studies on protein folding of the chaperone complex GroEL-ES revealed that they accelerate protein folding by enhancing unfolded polypeptide collapse through the induction of contractile force on the substrate chains.[58]

Single-molecule studies, combined with structural data, can provide detailed translocation mechanisms and dynamics by these protein-processing machines. The spiral arrangement of pore loops, as observed from the recent cryo-EM structures of proteases and disaggregases, and the predicted distance that these loops translocate a polypeptide during an ATPase cycle validate well the step sizes observed by the single-molecule studies. Future studies should be directed at understanding the specific interaction of these motors with the structure and amino acid sequence of the proteins. In the future, this information could play a pivotal role in the application of motors such as ATP-dependent proteases as unfoldases and translocases for protein sequencing.[59] Single-molecule studies on disaggregases are in their infancy. The reported study on an unfolded polypeptide provides information on the processive nature of ClpB, and future studies should aim to probe aggregates themselves to characterize the disaggregating power of these enzymes. The development of hybrid methods such as integrating force and fluorescence will provide a deeper understanding of the interactions among various components

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of these enzymes that generate, transduce, and apply mechanical forces as well as create a directed motion.

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