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Review

Force spectroscopy studies on protein–ligand interactions: A single protein mechanics perspective

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A B S T R A C T

Protein–ligand interactions are ubiquitous and play important roles in almost every biological process. The direct elucidation of the thermodynamic, structural and functional consequences of protein–ligand interactions is thus of critical importance to decipher the mechanism underlying these biological processes. A toolbox containing a variety of powerful techniques has been developed to quantitatively study protein–ligand interactions in vitro as well as in living systems. The development of atomic force microscopy-based single molecule force spectroscopy techniques has expanded this toolbox and made it possible to directly probe the mechanical consequence of ligand binding on proteins. Many recent experiments have revealed how ligand binding affects the mechanical stability and mechanical unfolding dynamics of proteins, and provided mechanistic understanding on these effects. The enhancement effect of mechanical stability by ligand binding has been used to help tune the mechanical stability of proteins in a rational manner and develop novel functional binding assays for protein–ligand interactions. Single molecule force spectroscopy studies have started to shed new lights on the structural and functional consequence of ligand binding on proteins that bear force under their biological settings.
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1. Introduction

Over the last decade, the scientific community has witnessed enormous progress in the field of single molecule force spectroscopy. Thanks to the development of instrumentation and protein engineering techniques, force spectroscopy has evolved into an indispensible tool towards examining the mechanical properties and force-induced unfolding-folding dynamics of proteins with unprecedented details at the single molecule level [1–9]; this has led towards a burgeoning and promising field of inquiry: protein mechanics. Among the force spectroscopy techniques, atomic force microscope (AFM) and optical tweezers are the most widely used.

Many proteins and protein complexes are subject to mechanical forces in their native biological settings. Understanding how mechanical forces modulate the conformation, stability and functions of such proteins and the biological systems they constitute is an important goal in protein mechanics. Detailed force spectroscopy studies have revealed new insights into the design principles of elastomeric proteins, specifically how proteins respond and adapt to mechanical stretching forces and how protein unfolding and refolding dynamics are modulated by force [3–6,10]. These studies have brought us a step closer towards understanding how force-bearing proteins respond to mechanical loads under physiological conditions.

Various aspects of protein mechanics have been discussed within several review articles [1–8]. In this review, we will focus on AFM-based single molecule force spectroscopy studies of protein–ligand interactions and their implications in protein mechanics.

Protein–ligand interactions, including protein–protein interactions, play essential biological roles in every aspect of living systems, and have found important applications in medicine and biotechnology [11]. The binding of a ligand to a protein will change the conformation of the protein to a form that is structurally and functionally distinct from that of the ligand-free state via binding-induced conformational changes [12,13]. These ligand-binding events may then initiate a cascade of biological reactions critical towards accomplishing specific biological functions. Accompanying these structural and functional outcomes, ligand binding will also lead to the enhanced thermodynamic (and sometimes kinetic) stability of proteins [14,15]. Moreover, ligand binding may also

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modulate protein folding and unfolding mechanisms and dynamics. In turn, these structural, functional and thermodynamic consequences have been used to quantitatively study protein–ligand interactions in vitro as well as in living systems [16–18]. The development of single molecule force spectroscopy tools has made it possible to investigate protein–ligand interactions that occur in proteins subject to mechanical loads under physiological conditions. In this paper, we offer an overview of protein–ligand interactions as studied using single molecule force spectroscopy techniques, and provide recent examples about how force spectroscopy has yielded insights into how mechanical stability and protein folding/unfolding dynamics are modulated by protein–ligand interactions.

2. AFM: a powerful tool for observing protein mechanics

AFM-based single molecule force spectroscopy has been used as a powerful tool towards probing the mechanical properties and unfolding/folding dynamics of single protein molecules [4,6,10]. By stretching a protein between a sharp AFM tip and a solid substrate, one can directly measure the force-extension behavior of proteins (Fig. 1A). To identify single molecule stretching events, polyproteins made of identical tandem repeats of the protein of interest are typically constructed. Stretching these engineered polyproteins results in force-extension curves that demonstrate a characteristic sawtooth-like pattern in which each sawtooth peak corresponds to the mechanical unfolding of a domain in the polyprotein chain (Fig. 1B). The last force peak often corresponds to the detachment of the polyprotein chain from either the AFM tip or solid substrate. From these experiments, one can readily determine the force required to unfold the protein (Fig. 1C), which is often defined as the protein's mechanical stability. By repeatedly stretching and relaxing the same protein molecule, it is possible to directly measure folding kinetics at zero force. Due to the limited force resolution and poor long-term stability of the AFM, it has been difficult to directly observe protein refolding under a stretching force. In addition, only a few specific cases of equilibrium refolding-unfolding (including ankyrin [19] and calmodulin [20]), have been observed. The force at which a protein unfolds is determined by both the difference in free energy between the folded and mechanical unfolding transition state and the distance between these two states [21]. Ligand binding will enhance the thermodynamic stability of proteins, which relates to the free energy difference between the native and unfolded state. If a ligand binds to the native state with higher affinity than the transition state, ligand binding will preferentially stabilize the native state over the transition state, thus enhancing the mechanical stability of the protein.

3. The effect of ligand binding on protein mechanical stability

It is well known that ligand binding can stabilize the native state of proteins relative to their unfolded state, where the ligand-bound form of proteins exhibit a corresponding increase in thermodynamic stability [14,15]. This property has been extensively exploited to stabilize proteins, both in nature and in laboratory. Many molecular machines in the cell perfectly exploit these properties. These molecular machines are often large protein complexes such as the F0/F1 ATP synthase and molecular chaperon GroEL and GroES, whose thermodynamic stability originates from strong protein–protein interactions. Despite the general correlation between ligand binding and enhanced thermodynamic stability, how ligand-binding affects mechanical stability and mechanical unfolding pathways of proteins was not clear. This is

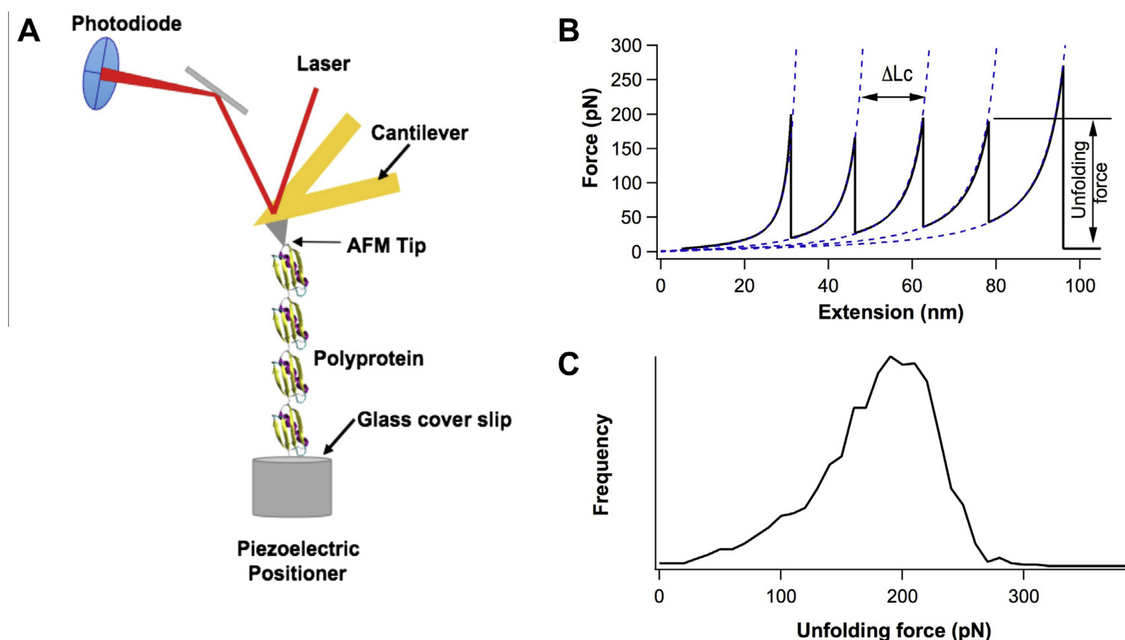

Fig. 1. Schematics of the AFM-based single molecule force spectroscopy. (A) Schematics of the AFM used to study single protein mechanics. A polyprotein made of tandem repeats of the protein of interest is deposited onto a glass coverslip and picked up by the AFM tip via non-specific physisorption. Then the molecule is stretched between the AFM tip and glass coverslip by moving the piezoelectric positioner in the Z-direction. The force acting on the polyprotein can be measured via the flexible AFM cantilever, which serves as a force sensor. The displacement of the AFM cantilever can be measured by bouncing a laser beam to a position-sensitive photodiode and the spring constant of the cantilever can be measured based on Equi-partition theorem. (B) A schematic force-extension curve from the stretching of a polyprotein made of identical tandem repeats. The unfolding of the polyprotein results in force-extension curves of the characteristic sawtooth appearance, where each individual sawtooth peak corresponds to the unfolding of one of the domains while the last peak typically corresponds to the detachment of the molecule from either the glass coverslip or AFM tip. The amplitude of the sawtooth unfolding force peak measures the force at which the protein domain unfolds. Dotted lines correspond to fits of Worm-like chain model of polymer elasticity to the experimental data. Contour length increment ΔLc measures the length increment upon protein unfolding. (C) A sample unfolding force histogram of the unfolding of a polyprotein.

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