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Modulating the Mechanical Stability of Extracellular Matrix Protein Tenascin-C in a Controlled and Reversible Fashion

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Stretching force can induce conformational changes of proteins and is believed to be an important biological signal in the mechanotransduction network. Tenascin-C is a large extracellular matrix protein and is subject to stretching force under its physiological condition. Regulating the mechanical properties of the fibronectin type III domains of tenascin-C will alter its response to mechanical stretching force and thus may provide the possibility of regulating the biological activities of tenascin-C in living cells. However, tuning the mechanical stability of proteins in a rational and systematic fashion remains challenging. Using the third fibronectin type III domain (TNfn3) of tenascin-C as a model system, here we report a successful engineering of a mechanically stronger extracellular matrix protein via engineered metal chelation. Combining steered molecular dynamics simulations, protein engineering and single-molecule atomic force microscopy, we have rationally engineered a bihistidine-based metal chelation site into TNfn3. We used its metal chelation capability to selectively increase the unfolding energy barrier for the rate-limiting step during the mechanical unfolding of TNfn3. The resultant TNfn3 mutant exhibits enhanced mechanical stability. Using a stronger metal chelator, one can convert TNfn3 back to a state of lower mechanical stability. This is the first step toward engineering extracellular matrix proteins with defined mechanical properties, which can be modulated reversibly by external stimuli, and will provide the possibility of using external stimuli to regulate the biological functions of extracellular matrix proteins.

Keywords: mechanical unfolding; tenascin; single-molecule force spectroscopy; mechanical stability; rational design

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Introduction

Stretching force is believed to be an important biological signal in the mechanotransduction network and can induce conformational changes of proteins, including force-induced unfolding, to regulate a broad range of cellular processes.[1–5]

Tenascin-C is a large extracellular matrix protein that plays important roles in regulating cell–matrix interactions.[6] Tenascin-C is subject to significant stretching forces under physiological conditions and is mainly expressed in regions that are subject to heavy tensile load[7] or in tissues that undergo extensive structural remodeling processes.[8–10] The mechanical properties of tenascin-C have been investigated in detail with the use of single-molecule atomic force microscopy (AFM).[11–13] It was revealed that the fibronectin type III (FnIII) domains of tenascin-C can unfold under a stretching force to extend the contour length of tenascin-C to several times its resting length when tenascin-C is fully folded. It was proposed that force-induced unfolding of FnIII domains of tenascin-C can effectively prolong the lifetime of a ligand–receptor bond mediated by tenascin-C.[11] If one can rationally

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Abbreviations used: FnIII, fibronectin type III; TNfn3, third fibronectin type III domain; AFM, atomic force microscopy; SMD, steered molecular dynamics; biHis, bihistidine; FNfn10, 10th FnIII domain from fibronectin; GdmCl, guanidinium chloride; wt, wild type; EDTA, ethylenediaminetetraacetic acid.

regulate the mechanical unfolding behaviors of FnIII domains by controlling their mechanical stability, it may become possible to regulate the response of tenascin-C to stretching force and thus to regulate the biological activities of the extracellular matrix proteins *in vivo*.[1] As the first step, here we report our endeavor to engineer a mechanically stronger mutant of the third FnIII domain of tenascin-C (TNfn3), which can be modulated by metal ions between two mechanical states that exhibit distinct high and low mechanical stability.

The mechanical properties of TNfn3 have been studied in detail with the use of single-molecule AFM and steered molecular dynamics (SMD) simulations.[14,15] These studies revealed that TNfn3 unfolds in a complex fashion involving multiple intermediate states and several energy barriers.[4,14] Such complex unfolding behaviors are in sharp contrast with the simple two-state unfolding behaviors exhibited by many proteins, such as ubiquitin,[16] protein L[17] and protein G,[18] and represent a technical challenge for rational modulation of the mechanical stability of TNfn3.

Mechanical unfolding force is a measure of the mechanical stability of a given protein and is directly related to the height of the mechanical unfolding energy barrier and the distance to the transition state.[19–21] Increasing the mechanical unfolding energy barrier will generally lead to enhanced mechanical stability. However, tuning the mechanical stability of proteins in a rational and systematic fashion remains challenging[22–24] despite the significant progress in the field of single protein mechanics and engineering over the last decade. Only a few examples of successfully enhancing the mechanical stability of proteins were reported on a few isolated cases.[25–30] Recently, using a small nonmechanical protein, GB1, we have developed an engineered metal-chelation-based method to rationally enhance the mechanical stability of proteins.[28] Since bihistidine (biHis)-based metal chelation can be engineered into a wide variety of proteins,[31,32] this method may be applicable to a broad range of elastomeric proteins. As the first step in the application of this method to mechanical proteins that are biologically relevant to mechanotransduction, here we apply this method to TNfn3 with the aim of engineering a mechanically stronger extracellular matrix protein and developing methods to modulate its mechanical stability in a controlled and reversible fashion.

Results

Rational design of the engineered metal chelation site in TNfn3

The key to the use of biHis-based metal chelation approach to enhance the mechanical stability of proteins is to preferentially stabilize the native state over the mechanical unfolding transition state.[28] For proteins that are simple two-state unfolders, such as GB1,[18,28] the design of a metal chelation site to realize preferential stabilization of the native state seems relatively straightforward.[28] However, for proteins such as TNfn3 that involve complex unfolding behaviors, use of engineered metal chelation to enhance their mechanical stability is much more challenging because it requires selective and preferential stabilization of a particular intermediate state over the transition state during the rate-limiting step.

The TNfn3 domain has a typical β-sandwich structure[33] and contains an integrin-binding Arg-Gly-Asp (RGD) loop. The mechanical unfolding of TNfn3 and its structural homologous FnIII domains from fibronectin have been investigated in detail using single-molecule AFM and SMD simulations.[4,14,15,34,35] Although the sequence homology between TNfn3 and its homologous FnIII domains from fibronectin is low, SMD simulations predicted that the mechanical unfolding of both FnIII domains follows similar molecular events.[4] Figure 1 shows our constant force SMD simulation results on both TNfn3 and the 10th FnIII domain from fibronectin (FNfn10). Similar to previous SMD simulation results,[4] our SMD simulations showed that the native state of both FNfn10 and TNfn3 domains are not stable upon stretching. When subjected to stretching force, both domains enter into a stable intermediate state, I1 (referred to as twist intermediate state I1), in which the tertiary structure of both domains remains largely intact, by slightly straightening the disordered N-terminal end of both domains. After this pre-elongation event, the subsequent mechanical unfolding of TNfn3 and FNfn10 is characterized by two distinct barriers: the first barrier corresponds to the transition from the twist intermediate state I1 to an aligned intermediate state I2, and the second barrier corresponds to the transition from the aligned intermediate state I2 to a partially unfolded intermediate I3.[4,14,35,36] Both SMD simulations and single-molecule AFM experiments suggest that barrier I (the transition from I1 to I2) is the rate-limiting step, and the mechanical unfolding force peaks observed in single-molecule AFM experiments on TNfn3 and FNfn10 correspond to the barrier crossing in this rate-limiting step.[14,15,34] During this transition, the N-terminal end of strand A is straightened by breaking key backbone hydrogen bonds between strands A and B and the two β sheets rotate and align relative to each other. For FNfn10, the breaking of the backbone hydrogen bonds between residues 6 and 23 was observed to be a key event coinciding with the transition from I1 to I2 for FNfn10.[36] In contrast, the backbone hydrogen bonds between Ser6 and Phe23 in TNfn3 are unstable. They break and reform during the intermediate state I1 (as evidenced by the fluctuation in interaction energy between $Ser6^{HN}$–$Phe23^{O}$ in Fig. 1d) and break before crossing barrier I in most trajectories.[15] Therefore, the interactions between Ser6 and Phe23 do not make a significant contribution to