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Torturing Proteins–Useful Science or Just a Clever Experiment?

Philip Micheal Williams*

Professor, School of Pharmacy, University of Nottingham, UK

Unfolding individual proteins by mechanical force has occupied biophysicists for the past 15 years or so. From the initial studies of the muscle protein titin, researchers have studied the effect of force on biomolecules ranging from small enzymes to large ribosomes. The majority of experiments use the atomic force microscope (AFM) as a means to stress proteins tethered between a surface and the probe, and elegant molecular biology approaches are used to produce constructs of repeating protein units which, when stretched and unfolded, produce a characteristic saw-toothed pattern that can be used to confirm complete or partial unfolding. In 2005 my colleague Jane Clarke and I noted that much of the work that had been done by then was to demonstrate the power of the AFM as a tool and that forced unfolding was beginning to move beyond a descriptive science towards a quantitative analysis of protein structure, stability and unfolding landscapes. We noted that the study of proteins that experience force in vivo was obvious but necessitating the use of new techniques such as optical tweezers (OT), and in discussing experiments to study the effects of solvent conditions, the direction of force, the marrying with computational simulations and the like, we were implying that the theoretical knowledge underpinning such quantification and necessary technological developments were in place. Some seven years later, it is interesting to see how the field has advanced and whether such quantitative analysis of our 'obvious' proteins has occurred.

The use of force as a denaturant when applied using a device such as the AFM or OT has the property that the denaturation is local. This has the advantage that the manner in which the force is applied to the protein can be varied and the effect studied. But this quality is also a disadvantage as it is difficult to compare directly the results of the force-induced unfolding with those from studies using non-localized denaturants such as chaotropes, heat and pH. A more subtle difficulty with force measurements is that there is no independent measurement of the state of the protein: force is used to unfold the protein and unfolding is measured by a change in force. This contrasts with a solution bulk measurement where, for example, a chaotrope is used to unfold the proteins and their state is measured spectrometrically. The coupling of the force denaturant with the measurement complicates attempts to measure folding under force, in particular when the stiffness of the force transducer is high such as in the AFM, and folding can only be differentiated from chain collapse by attempting to unfold the protein again. Combining single molecule spectroscopic and force measurements is a challenge yet to be met. Despite these limitations many force studies have indeed progressed into studying proteins that experience force in vivo. Have these told us something new and something that could not have been found otherwise, or is force spectroscopy of protein unfolding just a clever experiment?

A biological process in which force-induced protein unfolding is believed to be a central part is that of hemostasis. Von Willebrand factor (VWF) is a large glycoprotein which binds platelets and collagen to form plugs at the site of vasculature injury. The protein exists as large disulfide-linked repeats regulated in size by the circulating enzyme ADAMTS13. This enzyme cleaves the A2 domain within the VWF protein at a site that is exposed on unfolding caused by the sheer forces on the VWF multimer from the flowing blood. Both under-activity (through its reduced expression) and over-activity (through excessive VWF unfolding) of the enzyme lead to life-threatening diseases. So VWF has been and continues to be a target for study using force techniques.

Using optical tweezers and DNA handles, Zhang et al. [1] studied the unfolding of the single A2 domain of VWF under forces of between 5 pN and 15 pN. Using several hundred measurements the dynamic force data revealed a single transition state to unfold under these forces corresponding to an extrapolated force-free unfolding rate of 0.0007 s^{-1}. There was also evidence of an unfolding intermediate; a finding that would not have been noticed in bulk solution measurements. The folding rate was also estimated by studying the fraction of unfolding events recorded after varying zero force dwell times. Using these values, the calculated thermodynamic stability was close to that estimated from urea denaturation measurements. So by using optical tweezers the authors were able to postulate the protein unfolds rapidly and refolds slowly under forces of a few tens of pN, and does so via an intermediate separated by a transition state nearly 4 nm away from the folded state. The experiments recorded an increase in length on unfolding of the domain which when combined with knowledge of the N to C distance measured in the crystal structure gave an average chain length 161 residues at 0.36 nm each; 16 residues short of the 177 in the full A2 domain. Was all the protein unfolded, was the protein partially unfolded at the start, or is a 10% error acceptable?

Recently, the A2 domain attached using the same DNA handles has been studied using a different OT at slightly lower force loading rates and forces from 1 pN to 9 pN. Here, Xu and Springer [2] made some 1881 individual measurements. These data revealed again a single unfolding transition state across the force range but now with an extrapolated force-free unfolding rate five times faster than previously reported. The transition state measured here was located at 2.5 nm from the native state. No intermediate was noted in the recordings and its absence attributed to the lower sample rate of the different instrument. In these measurements the length on unfolding of the domain was 75.6 nm equating to a domain chain length of 189 residues at 0.4 nm per residue, or 213 residues using the 0.36 nm per residue of Zhang. The folding rate reported equates to a stability of 2.2 kcal mol^{-1} that is nearly half that found previously. The study did show that the folding rate could be increased five-fold by the addition of calcium, increasing the apparent stability to 3.1 kcal mol^{-1}. However, the solution measurements and previous force spectroscopy results were recorded in the absence of calcium ions. So which results are correct? To compare let's turn to a third experiment studying the A2 domain again using optical tweezers.

***Corresponding author:** Philip Michael Williams, PhD, Professor, School of Pharmacy, University of Nottingham, UK, Tel: 01-15-95-15-025; Fax: 01-15-95-15-110; E-mail: Phil.Williams@nottingham.ac.uk

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In 2011, Jakobi et al. [3] published a paper that showed the results of unfolding the A2 domain using optical tweezers in the presence and absence of calcium. The A2 domain appeared to be more stable with respect to unfolding when calcium was present, but as only one loading rate was used no extrapolated unfolding rate and transition state location could be measured. Calcium also appeared to increase the propensity of measuring an unfolding intermediate. Again, this contradicts that reported by Xu a few months later. The measured unfolded length of 79 nm is also larger. The boundaries for the domain were different and here the protein was 196 residues long. However, for the 78 nm extension recorded to be due to the complete unfolding of the protein then all these residues must have formed the folded native state. This is counter to what the authors found in their crystallographic studies that indicated the first 16 residues were not structured. In addition to here using a different OT instrument, the method of attachment was also different, as was number of measurements taken.

Three OT studies give conflicting views of the nature and behaviour of the A2 domain under force. Taken individually, each study provides valuable and unique knowledge: The Zhang paper definitively established that the A2 domain has to be in an unfolded form for cleavage by ADAMTS13, the Xu paper shows that calcium at physiological concentrations stabilizes the domain, and the Jakobi paper shows how calcium binds. The conclusions of each are justified by the results of that study: The Zhang paper shows a dominant transition state at 3.9 nm characterized by an unfolding rate of 0.0007 s^{-1} and the presence of an unfolding intermediate, the Xu paper shows single unfolding transition state at 2.5 nm characterized by an unfolding rate of 0.0035 s-1 that is unaffected by calcium, the Jakobi paper shows unfolding via an intermediate that depends on the presence of calcium. However, little consensus is found between the conclusions of these studies.

Force spectroscopy is making new discoveries about the nature and behavior of biological systems in which force is a component. Yet I think we are still missing fundamental theoretical knowledge of the role and effect of force and require more and careful developments in the technology and execution of the force unfolding experiments. At the start of this editorial I mentioned the use of molecular biology techniques to produce constructs of repeating domains and these have been advocated as both a way to help solubilize proteins and provide an internal standard to the measurement. In our studies of the VWF, for example, we are incorporating titin domains so each measurement can be compared directly to that of this 'standard'. However, the different time scales for unfolding between titin and VWF-A2 frustrate these measurements. We require a number of standard samples which, ideally, can be incorporated into each measurement and that have known and appropriate properties. The use of DNA handles with its characteristic B-to-S transition is attractive, and indeed is used in the OT papers discussed here a means of force calibration. An independent measurement of the folded nature of the protein during the force measurement would have an enormous impact on our research and understanding. As a single molecule technique such measurements are inherently difficult, but an achievable half-way solution is to continue efforts in the marriage of molecular simulations with dynamic force measurements. The ability to perform the single molecule measurement in the computer with accurate atomistic detail on experimental and relevant timescales will provide the important links between the chemistry, physics and biology of the role, use and impact of force in nature. And hopefully will keep many biophysicists, me included, occupied for the next 15 years.

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