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DNA stretching as a probe for nucleic acid interactions:

Reply to Comments on “Biophysical characterization of DNA binding from single molecule force measurements” by Kathy R. Chaurasiya, Thayaparan Paramanathan, Micah J.

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We would like to thank the authors of the thoughtful commentaries on various aspects of our review. These commentaries represent a diverse set of opinions on the field of single molecule force spectroscopy. We are unable to address all of the detailed issues brought up in the commentaries due to space constraints. Therefore what follows is a more general view of the most commonly addressed issues.

An isolated DNA molecule yields a lattice of binding sites, representing a microlab for the study of DNA-ligand interactions. Increasing tension along the double-stranded DNA (dsDNA) molecule disrupts tertiary DNA-ligand structures, then dislocates base pairing and stacking and ultimately separates the opposite strands to form single-stranded DNA (ssDNA). Varying the applied force alters the free energy to favor ligands that bind preferentially to DNA in one of these forms. These biophysical experiments not only discern the mode of DNA binding, but may also quantify the biochemistry of protein-DNA interactions [1,2]. These types of experiments offer particular advantages over bulk solution measurements, though important caveats remain. Specific issues surrounding DNA characterization and modeling, protein-DNA interactions under force and the ongoing discussion about overstretching highlight important questions that merit further comment and will hopefully propel additional study [2–8].

In any review, some subjects may only be dealt with briefly, while others must regrettably be omitted altogether. The section on DNA models treats well known descriptions of polymer elasticity; the Worm-Like Chain and Freely-Jointed Chain models. These models are used specifically to quantify DNA-protein interactions in the stretching experiments described further in the review [1], but not as a review of the much larger and interesting field of molecular dynamics simulations. The sophistication of computational techniques has increased rapidly in the last few years, enabling fruitful comparisons between theory and experiment [4,9,10]. Further advances in theoretical treatments of DNA have shed light upon even finer structures

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and dynamics of DNA-protein interactions. Experiments described in the current review don't rely upon the level of detail provided by these results. However, it is clear that future studies must confront key questions of DNA elasticity, especially at extremes of length and applied force [6]. Experiments of greater precision will require models that incorporate torsional flexibility and include discrete protein binding [5,11].

The studies in this review have probed DNA binding for several proteins including the bacterial H-NS and eukaryotic HMG. Either protein illustrates the general technique that quantifies binding for the ligands described throughout this review [1]. Force extension curves are altered in the presence of the protein. Quantifying this change with increasing protein concentrations reveals the binding affinity, and can also provide insight into the specific binding mode. In the case of HMG, average induced bending angles were determined from changes in the persistence length [12], and were in good agreement with other results, including direct visualization methods of AFM [13], as well as cyclization measurements that clearly demonstrate an increase in apparent DNA flexibility induced by these proteins [14]. Distributions of the bending angles seen in AFM experiments further reveal that this change in the persistence length is driven by a combination of discrete bends (static kinks), and increased flexibility at the binding site (flexible hinges) [13]. The *E. coli* bacterial protein HU, by contrast, shows a flat distribution of induced bending angles, indicating that protein binding increases flexibility without creating fixed bending angles [15]. Finally, the observed binding modes may change with increasing protein concentration or with solution conditions. Typically, increasing protein and decreasing salt concentration leads to a stiffening mode corresponding to cooperative binding not discussed specifically in this review, but seen in recent studies on H-NS [16], HMG [12] and HU [15].

Perhaps the most controversial topic in DNA force spectroscopy is the dramatic increase in length that occurs at a nearly constant force of ~65 pN, referred to as the DNA overstretching transition. Appearing as a plateau in the force-extension curve, this phenomenon is preceded and followed by regions of considerably greater apparent stiffness [17]. Two interesting pictures emerged to explain this plateau, with a critical distinction. A thermodynamic argument stressed that bases separate at the unlabeled ends and at regions of low stability (low GC content). Overstretching is thus DNA melting, where force is analogous to the role of temperature in thermal melting experiments [18–20]. Alternatively, it has been proposed that overstretching represents a transition in which base stacking is disrupted but base pairing is preserved in a conversion of B-form dsDNA to a new form of dsDNA, referred to as S-DNA [21–24]. Experimental evidence and theoretical models have been interpreted to favor each model. Yet at this point there is clear evidence that in the presence of binding ligands (especially those that preferentially bind to ssDNA), base pairing must be broken. Recent assays utilize fluorescent binding ligands to give a convincing picture of DNA melting during overstretching [25,26]. However, as noted by Krichevsky [3], the intrinsic flaw of all studies aimed at probing the nature of the overstretched state of DNA by adding ssDNA binding ligands or proteins is that they all may bias this transition away from S-DNA and towards melting, simply by stabilizing the ssDNA form. However, the further counterargument is that most of these ligands or proteins that bind ssDNA fairly slowly are unable to affect the DNA force-induced melting equilibrium, but rather capture the equilibrium propensity for melting in the absence of ligands [27–29]. In this respect, we completely agree with Vologodskii that the observation that DNA overstretching proceeds exclusively from free DNA ends or nicks [26] makes the strongest argument in favor of the melting nature of this transition [6], as there is no physical reason for such extreme phase separation between two forms of dsDNA.

Specific arguments in favor of S-DNA persist, including an important observation stressing that observed DNA stretching curves continue to very high forces, well above the overstretching force plateau. Before the DNA strands finally separate at ~140 pN or higher

forces, a short plateau often appears, with a length typically 10% of the total DNA elongation. If overstretching represents DNA melting, this seems to imply that approximately this fraction of bases must remain paired until the strands separate. Because it is difficult to imagine how such a few base pairs can support such high forces, it is easier to assume that the second high force transition observed corresponds to melting double stranded S-DNA that is formed during overstretching. Furthermore, the observed elasticity of DNA beyond the overstretching region deviates from the elasticity expected from either a single or two parallel ssDNA strands [3, 30,31]. Others dismiss the melting explanation for DNA overstretching based on the results of modeling studies, predicting that force-induced dsDNA melting should be strongly pulling rate dependent [32–34], as observed only for the strand separation transition [22]. Moreover, as mentioned by Krichevsky [3], in contrast to melting, the B-to-S transition is predicted by modeling to depend on the direction of pulling (3'3' vs. 5'5') [21], and some recent experiments seem to corroborate this prediction [31,35].

All of these observations, presently considered as pro S-DNA [3,7,23,36], can be simply explained as kinetic features of force-induced DNA melting. This statement is based on our yet unpublished extensive experimental and theoretical study of the pulling rate dependence of DNA overstretching. In particular, we have discovered that the overstretching force plateau does indeed weakly depend on the pulling rate, and this dependence becomes stronger with faster pulling and in lower salt. This result is analogous to the well-known weak dependence of the DNA melting temperature on heating rate [37], and is a consequence of the highly cooperative melting of long (~100 bp) regions of heterogeneous dsDNA, leading to large sequence-determined peaks in the differential melting profiles [38]. While many direct analogies between thermal and force-induced DNA melting exist, our experiments and theory point out their major differences. In particular, the force strongly favors dsDNA melting from the free ends or nicks vs. internal bubble formation, such that the latter only becomes possible in high salt and for low pulling rates. In fact, internal force-induced dsDNA melting is only possible due to the strong sequence heterogeneity of dsDNA stability [39,40]. Interestingly, we find that the force of strand separation is much more rate-dependent even at moderate pulling rates of ~10 $\mu\text{m/s}$, explaining the pronounced differences between the high-force region of the DNA stretching curves and equilibrium ssDNA stretching curves. Also, the strong dependence of the strand separation force (but not of the overstretching force) on the DNA attachment geometry (3'3' vs. 5'5') [31,35] appears to be a kinetic feature, associated with the non-equilibrium character of the final strand separation. These results do not exclude the possibility that DNA may be stretched with intact base pairing using much higher forces (nN or N) and extremely high pulling rates ($> \sim 1 \text{ mm/s}$) as was recently observed for very short molecules (< 100 base pairs) [35]. However, over the timescales and solution conditions of the experiments in this review, conversion to the S state (if it exists) is not a factor that affects DNA-ligand interactions.

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