Direct Measurements of Base Stacking Interactions in DNA by Single-Molecule Atomic-Force Spectroscopy

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We investigate the elasticity of two types of single-stranded synthetic DNA homopolydeoxynucletides, poly(dA) and poly(dT), by AFM-based single-molecule force spectroscopy. We find that poly(dT) exhibits the expected entropic elasticity behavior, while poly(dA) unexpectedly displays two overstretching transitions in the force-extension relationship. We suggest that these transitions, which occur at ~23 pN and ~113 pN, directly capture, for the first time, the mechanical signature of base-stacking interactions among adenines in DNA, in the absence of base pairing.

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Up to now, the studies of molecular elasticity of nucleic acids primarily focused on double-stranded DNA, dsDNA [1–15], because its mechanics strongly affects its important biological function in transcription, replication, recombination, and repair [1]. Double-stranded DNA undergoes a characteristic overstretching transition that lengthens the molecule by about 70% at nearly a constant force of 65 pN [2,12,14,15]. The mechanics of single-stranded nucleic acids, ssNA, attracted somewhat less attention, even though ssNA are important intermediates in the above-mentioned mechanochemical reactions. Relevant studies have focused on electrostatics and folding properties of ssNA at low stretching forces [15–20]. However, single-stranded nucleic acids offer a unique opportunity to study fundamental base-stacking interactions between nucleobases within the same strand, in the absence of base paring interactions that occur between the bases located in the complementary strands [21–23]. It is known that base-stacking is strongest among adenines (A) and weakest among thymines (T) and uridines (U) [21,24]. Thus, base-stacking is expected to be the most pronounced in polydeoxyadenylate, poly(dA), and weakest in polydeoxythymidylate, poly(dT), or polyuridylate, poly(U). Base-stacking interactions in poly(dA) favor the parallel orientation of consecutive bases and therefore they are expected to generate quasiregular helical structures [24]. Conversely, in poly(dT), the thymines are turned ''out'' and do not stack [25]. Thus, poly(dT) is expected to be in a random-coil form [26]. The base-stacking interactions are expected to introduce significant enthalpic components into the molecular elasticity of ssNA. However, how the base stacking affects the elasticity of single-stranded DNA, ssDNA, is still unclear. Although some progress in the theoretical modeling of base stacking has been made [22,27] and some qualitative experimental results reported [26,28,29] as well as the very recent result concerning the base-stacking in RNA [30], direct measurements of base-stacking in DNA are still missing. In this Letter, we present and discuss the results of single-molecule elasticity measurements of single-stranded poly(dA) that for the first time, to the best of our knowledge, directly capture and quantify the mechanical fingerprint of the base-stacking interaction among adenines, in the forms of a low-force (~23 pN) plateau and a high-force (~113 pN) plateau in the force-extension relationship, demonstrating that the elasticity of ssDNA may be much more complex than previously thought.

Measurements of the elasticity of ssDNA were carried out in solution, at room temperature on our homemade AFM instrument designed and equipped specifically for force spectroscopy measurements [31,32], as schematically shown in Fig. 1. This instrument was built with a high precision piezoelectric *XYZ* stage (P-517.3CL,

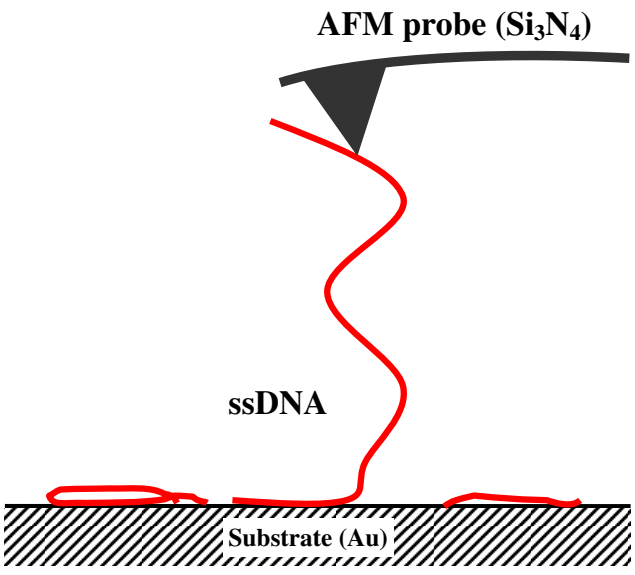

FIG. 1 (color online). Schematic of single-molecule atomic-force microscopy measurements of ssDNA molecules (the figure is not to scale).

Physik Instrumente) equipped with three capacitive sensors, which provide an open-loop resolution of 0.1 nm in the Z axis and 1 nm in the X and Y axes. We equipped this instrument with a low noise MultiMode AFM head from Veeco, Inc (Woodbury, NY). When using Microlever cantilevers (Veeco) with spring constants of 15–20 pN/nm, the AFM head produced a rms force noise of about 8.2 pN in the 1–500 Hz bandwidth. Single-stranded poly(dA) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO); Single-stranded poly(dT) was purchased from MP Biomedicals, Inc. (Solon, OH). Eighty μl of ssDNA solution ($\sim$60 ng/μl) in Tris-EDTA buffer (10 mM Tris+HCl, 1 mM EDTA, pH 8; Sigma-Aldrich, Inc), supplemented with 150 mM NaCl, was deposited onto a freshly-evaporated gold surface. After the sample was incubated for 2–3 hours, it was gently rinsed 3–5 times with the buffer solution. Untreated silicon nitride AFM cantilevers (Microlever, Veeco) were employed for picking up molecules and pulling measurements. The spring constant of each cantilever was calibrated in solution, using the energy equipartion theorem approach [33]. This measurement relied on attaching ssDNA molecules to the gold substrate and AFM tip by nonspecific adsorption, which proved to be a simple, but robust method used in previous DNA force spectroscopy studies [20,34]. In this method, the AFM tip picks up ssDNA fragments at random positions. Since in this study we use homopolynucleotides, which contain only one type of nucleotides in the whole chain, the randomness of the fragments picked up for measurements does not affect the result.

Three typical force-extension curves of poly(dA) out of 15 similar sets of recordings obtained on different molecules are shown in Fig. 2(a). Figure 2(b) shows one of the curves at higher magnification and compares the stretching trace with the relaxing trace obtained on the same molecule. No hysteresis was observed between the stretching and relaxing parts of the cycle, even after the same molecule was repeatedly stretched and relaxed more than 50 times. It is striking that the force spectrograms in Figs. 2(a) and 2(b) (which overlap well after extension normalization, see Fig. 2(b) inset) reveal two pronounced plateau features. The first plateau occurs at a force of 23 $\pm$ 1 pN and overstretches the polynucleotide by $\sim$80%. This low-force plateau is very similar to the plateau theoretically predicted by Buhot and Halperin [24] based on their model of base-stacking interactions in poly(dA). The plateau likely represents the unwinding of the poly(dA) helix, and its force directly indicates the strength of base-stacking interactions among the adenines. The second plateau occurs at a force of 113 $\pm$ 2 pN and overstretches poly(dA) by an additional $\sim$16% [Figs. 2(a) and 2(b)]. It is noted that this second plateau was not predicted by Buhot and Halperin [24]. We speculate that during this phase of base-unstacking, poly(dA) is still in the helical form, albeit this helix must be extended, and the high-force plateau

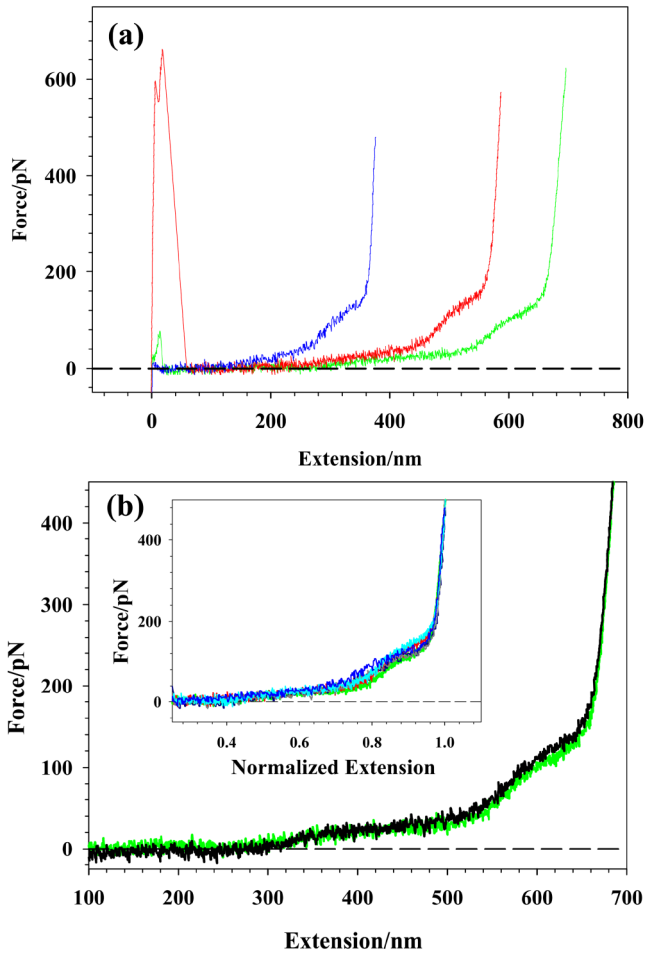

FIG. 2 (color online). (a) three typical force-extension measurement curves for poly(dA), (b) magnified view of the force curve in (a) corresponding to the longest molecule and the comparison between the stretching trace (green, online; gray, in print) and the relaxing trace (black). The inset plot shows the overlapping of six recorded force-extension curves of poly(dA) on a normalized extension basis. These recordings were obtained in different experiments on different poly(dA) molecules.

represents the reorientation of bases, which is accompanied by the flip of the backbone bonds to new torsional states that increase the distance between the consecutive phosphates in a semidiscontinuous fashion. The reorientation of bases may, at higher forces, be also accompanied by a forced conformational transition in the deoxyribofuranose rings from their C3' endo pucker (5.9 Å spacing between the neighboring phosphates) to a C2' endo pucker (7 Å spacing between the neighboring phosphates) [35,36]. Such a transition would produce an additional extension of the backbone chain up to $\sim$19%, which coincides with the width of the second plateau. A similar plateau was already observed in the force-extension relationship of a polysaccharide amylose, which was linked to the forced flipping of the sugar rings from a chair to a boatlike structure [32]. We also note that similar to the plateau in the elasticity profile

of amylose, the high-force plateau in the elasticity profile of poly(dA) displays a finite slope. This observation favors our conjecture about the origin of this plateau as involving a forced conformational transition of the furanose sugar rings. However, high-level *ab initio* quantum mechanical calculations suggest that deoxyribose has a ~1 kcal/mol preference for the C2' endo pucker [37], which does not support this hypothesis. Noticing that the ~1 kcal/mol energy difference between the two puckers is fairly small, it is not clear which conformation a long single-stranded poly(dA) will take on under certain salt and *p*H conditions, in solution. Even though the structural (x-ray, NMR, other spectroscopies) literature on double-stranded nucleic acids is extremely rich [25], there are very few published papers about the puckering of deoxyribose in single-stranded DNA. In fact, the frequently cited work by C. Altona *et al.* [38] predicted an equilibrium between C2' endo and C3' endo puckers of deoxyribose (~70% of C2' endo). However, this NMR study was carried out on very short oligos: dApdApdA in pure D_2O (no salts), and it is likely that low ionic strength conditions may force the sugar ring into a C2' endo pucker that provides a greater separation between phosphates and thus minimizes their electrostatic repulsion. These observations suggest that further NMR studies, including measurements in the presence of salts, will be needed to resolve the furanose pucker in the relaxed poly(dA). To decipher the details of molecular events that occur during the stretching of poly(dA) through both plateau phases and to get an insight into base-unstacking transformations, long-time steered molecular dynamics simulations will be required [39].

By integrating the area under the plateaus in the normalized force-extension curve [Fig. 3(b)], the total energy, which is necessary to overstretch poly(dA), is determined to be 3.6 ± 0.2 kcal/mol per base ($n = 15$). It is striking that this value is consistent with the reported value obtained by different methods for the base-stacking energy among adenines, $3.2 \sim 4.0$ kcal/mol [23,28,40–42]. This agreement strongly supports our conjecture that the two plateaus in the force-extension curve of poly(dA), have indeed captured the mechanical fingerprint of base-stacking interactions in ssDNA, in the absence of base pairing.

Figure 3(a) shows three representative force-extension recordings obtained for single-stranded poly(dT), and Fig. 3(b) shows the comparison of the recordings of poly(dA) and poly(dT) on a normalized extension basis. We find that the elasticity of poly(dT), which has been postulated to experience no base-stacking interactions [24,25], follows the simple entropic behavior and does not show any plateau features. This observation reinforces our hypothesis that our force-extension measurements of poly(dA) indeed reveal directly, for the first time, to the best of our knowledge, the effect of base-stacking interactions on the molecular elasticity of a ssDNA.

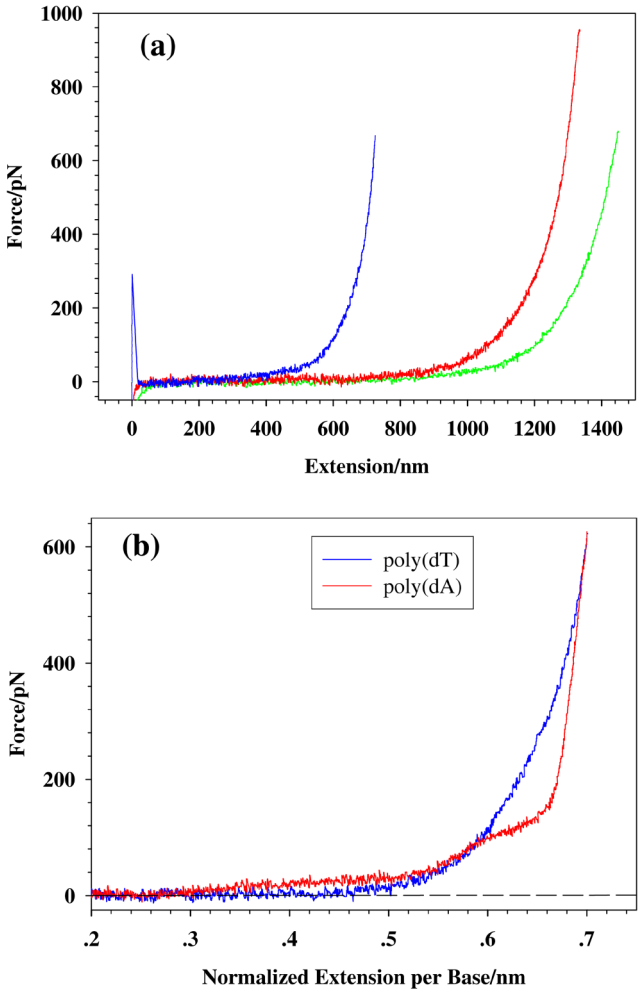

FIG. 3 (color online). (a) three typical force-extension measurement curves for poly(dT), (b) comparison between poly(dA) and poly(dT) on a normalized extension basis. We assume that at a force of 600 pN, ssDNA is fully stretched, and the distance between two neighboring phosphates is 0.7 nm.

In summary, we present and discuss the elasticity profiles of single-stranded poly(dA) and poly(dT) obtained by AFM-based single-molecule force spectroscopy. Our results show that poly(dT) exhibits the expected entropic elasticity behavior, while poly(dA) displays two overstretching transitions, which have not been previously observed. We propose that these two force plateaus are due to breaking the stacking interactions between consecutive adenines. However, the molecular events that occur during the stretching of poly(dA) through the two plateau phases remain unclear and warrant further studies.

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