Origin of Overstretching Transitions in Single-Stranded Nucleic Acids

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(Received 19 July 2011; revised manuscript received 14 August 2013; published 31 October 2013)

We combined single-molecule force spectroscopy with nuclear magnetic resonance measurements and molecular mechanics simulations to examine overstretching transitions in single-stranded nucleic acids. In single-stranded DNA and single-stranded RNA there is a low-force transition that involves unwinding of the helical structure, along with base unstacking. We determined that the high-force transition that occurs in polydeoxyadenylic acid single-stranded DNA is caused by the cooperative forced flipping of the dihedral angle formed between four atoms, O5'-C5'-C4'-C3' (γ torsion), in the nucleic acid backbone within the canonical *B*-type helix. The γ torsion also flips under force in *A*-type helices, where the helix is shorter and wider as compared to the *B*-type helix, but this transition is less cooperative than in the *B* type and does not generate a high-force plateau in the force spectrums of *A*-type helices. We find that a similar high-force transition can be induced in polyadenylic acid single-stranded RNA by urea, presumably due to disrupting the intramolecular hydrogen bonding in the backbone. We hypothesize that a pronounced high-force transition observed for *B*-type helices of double stranded DNA also involves a cooperative flip of the γ torsion. These observations suggest new fundamental relationships between the canonical structures of single-and double-stranded DNA and the mechanism of their molecular elasticity.

DOI: 10.1103/PhysRevLett.111.188302 PACS numbers: 87.15.−v, 82.35.Pq, 82.56.Pp, 87.80.Nj

Over the past decades, the mechanical properties of double-stranded DNA (dsDNA) have been studied extensively due to their importance in fundamental cellular processes [1]. DsDNA was found to undergo two transitions when stretched. The first transition occurs at ~65 pN and the molecule overstretches 70% beyond its contour length [2–6]. The second transition occurs between 100 and 350 pN (see Supplemental Material, Fig. S1 [7]) with the transition force and the amount of overstretching depending on the nucleic acid sequence, ionic conditions, and the loading rate [8,9]. The mechanisms underlying these transitions remain controversial [10,11].

The mechanical properties of single-stranded nucleic acids were studied less extensively, but recently it was shown that certain single-stranded homopolynucleotide sequences of strongly stacked bases that form helical structures [12] have unexpected mechanical properties [13–21]. It was shown that single-helical polynucleotides composed of adenines such as poly(A) and poly(dA), and of cytosines, such as poly(C), exhibit a low-force transition when stretched beyond ~20 pN [17–20]. [Nucleic acid nomenclature: poly(A) = single-stranded RNA composed of repeating adenine; poly(dA) = single-stranded DNA composed of repeating adenine; poly(dT) = single-stranded DNA composed of repeating thymine; poly(dC) = single-stranded DNA composed of repeating cytosine; poly(C) = single-stranded RNA composed of repeating cytosine; poly(dG)poly(dC) = double-stranded DNA composed of repeating guanine (strand A) and repeating cytosine (strand B); poly(dG-dC) = double-stranded DNA composed of repeating guanine-cytosine pair]. In addition, poly(dA) undergoes a second, high-force transition when stretched beyond ~110 pN [19,20], which is absent in poly(A) [22] (Fig. 1, and Supplemental Material, Fig. S2 [7]). While the low-force transition can be tentatively associated with unraveling of the helical structure generated by base stacking [17–21], the origin of the second transition in poly(dA) has remained unknown. We note that the second transition in poly(dA) is strikingly similar to the second, high-force transition that occurs in dsDNA, such as

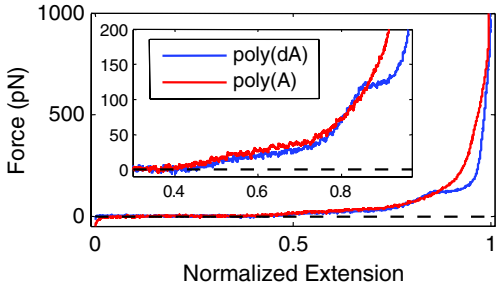

FIG. 1 (color online). Force spectra of poly(A) (red) and poly(dA) (blue). Superimposed force extensions of poly(A) and poly(dA) normalized to the highest force. Poly(A) force spectrum exhibits a force plateau of 19.7 ± 3.9 pN (mean ± s.d., $n = 95$ experiments on 27 molecules) followed by a quasilinear force region to ~200 pN. Poly(dA) force spectrum exhibits two force plateaus of 21.7 ± 5.0 pN and 125.3 ± 25.1 pN (mean ± s.d., $n = 70$ experiments on 23 molecules).

λ-phage DNA and double-stranded poly(dG-dC) (see Supplemental Material, Fig. S1 [7]) [8]. The dsDNA poly(dG)poly(dC) has a similar hydrogen bonding pattern between the nucleobases as ds poly(dG-dC) but does not have a pronounced second transition (see Supplemental Material, Fig. S3 [7]) [23]. The structural difference between these various DNAs is that poly(dA), like both λ-phage DNA and poly(dG-dC) is believed to be in the canonical *B*-type helix, while poly(A) and poly(dG)poly (dC) are in the *A*-type helix [12,13]. These observations suggest that the presence or absence of the second high-force transition is related to the type of the helix structure formed by these nucleic acids.

Here we use AFM-based single-molecule force spectroscopy supplemented with nuclear magnetic resonance (NMR) spectroscopy and molecular mechanics (MM) simulations to further examine the mechanisms of molecular elasticity of single-stranded homopolynucleotides, including poly(C) and poly(dC) (details on materials and methods in the Supplemental Material, [7]). The polymer poly(C) was found to have a low-force plateau at 20 pN, but was not tested at forces greater than 60 pN [16] and poly(dC) that has not been examined by single-molecule force spectroscopy so far. We pinpoint the origin of the high-force transition in polydeoxyadenylic acid, poly(dA), and speculate a fundamental relationship between the structures of single- and double-stranded DNA and the mechanism of their high-force overstretching transitions.

First, we characterized poly(C) (*A*-type helix [12]) by AFM and found that its force spectrum remains similar to poly(A) [Fig. 2(a)] with no presence of the high-force transition. Second, the force-extension curve for poly(dC) does not display a force transition at 20 pN. It is remarkably different than that of poly(C) and, instead, compares well to the force-extension curve of poly(dT) [Fig. 2(b)], which is known to behave as a freely jointed chain (FJC) [19] because it is unstructured and its bases are turned out and tilted, with no stacking interactions [12,24]. This difference between the poly(dC) and poly(C) mechanics can possibly be explained by base stacking. Base stacking is an important stabilizing element in poly(C) and is likely absent in poly(dC) because, based on the similarity in AFM force extension data, poly(dC) must be unstructured like poly(dT). The structural data to confirm this hypothesis are pending an ongoing investigation. The first force plateau at ~20 pN now found in poly(C), poly(A), and poly(dA) is likely related to unwinding of their helical structures [18] since these polymers derive their helical stability through base stacking [24].

The second, high-force plateau of the poly(dA) force spectrum (Fig. 1) is unique among single-stranded polynucleotides. Previously, Ke *et al.* hypothesized that it may be due to the backbone deoxyribose flipping between the C3' and C2' *endo*-pucker forms [19]—where a carbon atom (C3' or C2') in the sugar ring is out of the plane

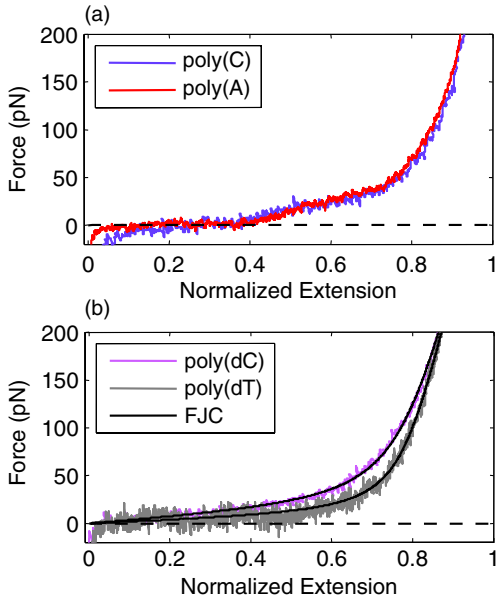

FIG. 2 (color online). Force spectra of different single-stranded nucleic acids. (a) Superimposed normalized force spectra of poly(C) (magenta) and poly(A) (red). Poly(C) force spectrum exhibits a force plateau of 24.3 ± 4.4 pN (mean ± s.d., 53 force curves on 14 molecules). (b) Superimposed normalized force spectra of poly(dT) (gray) and poly(dC) (pink). Both behave as unstructured, freely jointed, polymers.

due to steric reasons (see Supplemental Material, Fig. S4 [7]). The flip from C3' endo to C2' endo would, in principle, increase the distance between consecutive phosphates [25,26] and account for the AFM length measurements of the high-force plateau. We investigated this possibility by performing NMR spectroscopy on a 30-mer of poly(dA), and we concluded that the backbone sugar is already in the extended conformation, C2'-endo (*B*-type helix), in the relaxed state of poly(dA), and thus this force plateau cannot be due to the sugar flipping (see Supplemental Material [7], NMR spectra, Figs. S5–S8).

We further probed the mechanism behind this high-force plateau by stretching poly(dA) in different solvents. The force spectra of poly(dA) stretched in low-ionic-strength solvents do not exhibit any force plateaus (see Supplemental Material, Fig. S9 [7]). Since the DNA stability provided through base stacking is salt dependent [27], we concluded that base stacking is necessary for the force transitions to occur. The difference in the NMR spectra of dA in solution with excess salt (150 mM NaCl) and without salt also indicate a more compact backbone (150 mM NaCl), possibly due to a helical structure (see Supplemental Material, Fig. S8 [7]) supporting our interpretation. NMR spectra of oligo dA in both salt conditions also rules out the possibility of poly(dA) forming some double-stranded structures in the presence of excess salt which potentially could have produced a force plateau [2,6,8,9,28–30].

Base stacking alone is not sufficient to account for the high-force plateau in poly(dA) because both poly(A) and poly(C) also undergo strong base stacking [24] without exhibiting a high-force plateau. We hypothesize that this high-force plateau in poly(dA) may involve some forced conformational transitions within the DNA backbone. The additional O2'H in ribonucleotides is the only difference between the poly(A) and poly(dA) molecules that is responsible for poly(A) generating an *A*-type helix via OH-backbone hydrogen-bonding interactions. To test whether these H bonds affect the mechanics of poly(A), we stretched poly(A) in 1M urea. We hypothesized that urea, which has properties that disrupt hydrogen bond networks [31], would decrease intramolecular interactions in poly(A), making it behave more like poly(dA) which lacks sugar-backbone hydrogen-bonding interactions. We expected this solvent should have no effect on the force spectrum of poly(dA) since there are no intramolecular interactions to disrupt.

The results of 1M urea on poly(A) and poly(dA) are shown overlaid in Fig. 3. Remarkably, force spectrums of poly(A) in 1M urea reveal a second, high-force plateau that is reproducible and exhibits almost zero hysteresis (see Supplemental Material, Fig. S10 [7]), and shows a first plateau that remains unchanged from poly(A) without denaturant (see Supplemental Material, Fig. S11 [7]). The second plateau for poly(A) with 1M urea occurs at a force of ~70 pN and stretches the molecule by 9.7 ± 1.9% per nucleotide (mean ± s.d., measured by FJC fitting and normalized to longest contour length). As expected, the force spectrum of poly(dA) with 1M urea is still similar to poly(dA) in normal TE buffer with salt (see Supplemental Material, Fig. S12 [7]). These observations again suggest that this high-force plateau in the poly(dA) force spectrum is related to the helical structure of poly(dA) organized by base stacking in the absence of intramolecular hydrogen bonding networks in the backbone. We hypothesize that it may be caused by the forced flipping of one of the backbone torsions (the angle between two adjacent planes formed by four atoms in the nucleic acid backbone) across an energy barrier after bases unstack and the helical structure is unwound.

We executed molecular mechanic simulations on 12-mers of poly(A) and poly(dA) to investigate the molecular mechanism behind the high-force plateau. Molecules starting from their canonical forms were stretched in 0.00275 Å/bp steps to a final extension of 8 Å/bp with an energy minimization at each step. We identified the abrupt flipping of the γ torsion (defined by the dihedral angle between backbone atoms O5'-C5'-C4'-C3') to the *trans* position as the most striking change occurring in both poly(A) and poly(dA) simulations, as shown in Fig. 4(a). We also conducted steered molecular dynamic simulations on 12-mer and 24-mers of poly(A) and poly(dA) and identified the same abrupt conformational

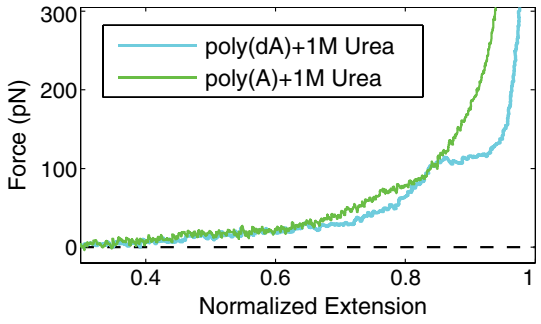

FIG. 3 (color online). The effect of 1M urea on poly(A) elasticity. Superimposed normalized force spectra of poly(dA) with 1M urea and poly(A) with 1M urea. Poly(A) + 1M urea has two force plateaus of 19.9 ± 4.0 pN and 69.6 ± 7.3 pN (mean ± s.d., $n = 65$ experiments on 6 different molecules). The low-force plateau occurs at an identical force as the low-force plateau in poly(A) without urea (see Supplemental Material, Fig. 11 [7]). Poly(dA) with 1M urea does not show any significant difference from poly(dA) without urea (see Supplemental Material, Fig. 12 [7]).

change of the γ torsion (see Supplemental Material, Fig. S13 [7]); however, steered molecular dynamics simulations were unable to reproduce either the poly(A) or the poly(dA) force-extension curve with enough detail to be directly compared to the AFM force-extension data. We suspect that molecular dynamic time scales, force field, and water models were unable to appropriately address the mechanism behind this high force transition. The probability of γ torsion flipping at a given extension is shown in Fig. 4(b) for each molecule, obtained from molecular mechanic simulations. The γ torsions of the poly(dA) molecule have a high likelihood of being flipped at the highest extensions, at 6 to 8 Å per nucleotide, which indicates a cooperative transition. In contrast, the γ torsions of poly(A) and poly(C) (see Supplemental Material, Fig. S14 [7]) have low probability of flipping at any extension. The flipping of the γ torsion to the *trans* position in the backbone allows a nucleotide to extend from 7.06 to 7.74 Å in poly(dA) and allows a nucleotide to extend from 6.88 to 7.47 Å in poly(A) as shown in Fig. 5(b) (average extension measured from P_n to P_{n+1} from molecular mechanics trajectories before and after γ flipping). Given a maximum extension of DNA of ~8.0 Å per nucleotide (determined from SMD simulations at high force and MM simulations at high energies), the cooperative flipping of γ torsions to the *trans* position could potentially allow stretching by ~8.5% of the final length [(7.74 Å − 7.06 Å)/8 Å = 8.5%]. This is consistent with experimental data for poly(dA) which indicates that the second high-force transition contributes 12.1 ± 2.6% to the final length (mean ± s.d., measured by FJC fits and normalized to longest contour length). The hypothetical cooperative flipping of γ torsions in poly(A) would allow stretching by ~8%, but such additional stretching is not

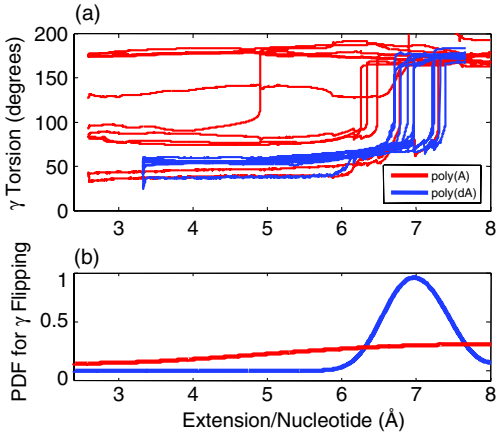

FIG. 4 (color online). Results of molecular mechanic simulations of poly(A) and poly(dA) 12mers. Poly(A) starting configurations were from the *A*-type single-stranded RNA helix and poly(dA) starting configurations were from the *B*-type single-stranded DNA helix. (a) Changes in the γ torsion during the extension of nucleic acids which shows an abrupt change from *cis* to *trans* conformation. (b) Kernel density estimated probability density function (PDF) for the extension at which a γ torsion flips to *trans* conformation.

observed in regular buffer. AFM measurements of poly(A) in urea capture a high-force transition whose contribution to the final length is $9.7 \pm 1.9\%$ (mean $\pm$ s.d.), which is consistent with the predicted length gain from cooperative γ flips in poly(A). The coordination of the flipping of the γ torsion during the molecular trajectories for poly(A) and poly(dA) were remarkably different. This shows that in both cases the γ torsion provides sufficient changes in length to induce a plateau, but only in poly(dA) does this occur in a cooperative manner.

Why does the γ torsion flip cooperatively in poly(dA) but not poly(A)? Molecular trajectories of poly(dA) [see Supplemental Material, Fig. S15 [7]] suggest that γ flipping first occurs at the separation of a base-stacking pair of adenines. The disruption of base stacking in the trajectories

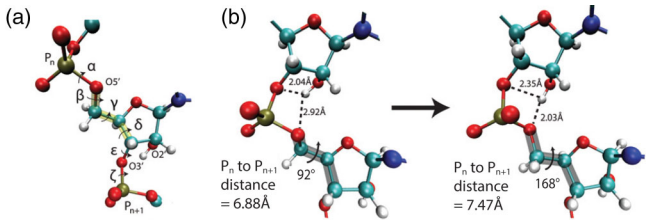

FIG. 5 (color online). The atomic representation of the flipping of a γ torsion in poly(A) [similar for poly(dA)]. (a) Shows the locations of the torsion angles in the nucleic acid backbone labeled by Greek letters. Relevant atoms are also labeled. (b) Shows a representative snapshot of the γ torsion (gray highlight) before (left) and after flipping (right) and the consequential increase in the phosphate distances for poly(A) [averaged from trajectories, similar for poly(dA)], as described in main text.

is quickly followed by consecutive γ flips in all the other bases—a cooperative event. The molecular trajectories for poly(A) [see Supplemental Material, Fig. S15(b) [7]] and poly(C) differ from poly(dA) for three main reasons: (i) some γ torsions are already in the *trans* position near the starting configuration (Fig. 4(a) and Supplemental Material, Fig. S14 [7]); (ii) throughout the extension the remaining γ torsions in the *cis* state flip gradually; (iii) the flipping of γ torsions in poly(A) and poly(C) do not require disrupting the base stacking. Unlike poly(dA) which has no hydroxyl group, γ torsions in poly(A) and poly(C) are individually flipped during interactions between the hydroxyl group, O2'H, and neighboring phosphate-bound oxygen atoms, O3' and O5' [see Supplemental Material, Fig. S15(b) [7]]. This is illustrated in Fig. 5(b), where the O2′H of the poly(A) backbone is closer to the O3′ of the $n+1$ nucleotide unit ($\sim$2.0 Å) than to the O5′ atom ($\sim$2.9 Å) [Fig. 5(b), left]; however, during the stretching of poly(A), a lower energy configuration becomes available where the O2′H atom shifts away from the O3′ atom (now $\sim$2.4 Å) toward the O5′ atom (now $\sim$2.0 Å) [Fig. 5(b), right]. The shift in the O2′H atom requires flipping the γ torsion to the *trans* state; this local conformational change prevents γ torsions from flipping in a global cooperative manner because it sequesters the γ torsions from the *cis* state. These considerations are consistent with our experiments that adding a hydrogen bonding network disrupting solvent (e.g., urea) to the buffer can disrupt this backbone interaction, which presumably allows the γ torsions to stay in the *cis* state until the helix is unwound.

In summary, we analyzed atomic force spectroscopy measurements with help of MM simulations and NMR spectroscopy to understand the mechanism of overstretching transitions in single-stranded nucleic acids. All the transitions we identified were completely reversible, which suggests that these transitions are intrinsic to the configuration of polynucleotides. The low-force transitions of poly(A), poly(C) and poly(dA) likely involve straightening their initial helical structure as well as disruption of the base stacking. The high-force transition in poly(dA) is likely caused by the cooperative flipping of the γ torsion to the *trans* position from the canonical position that is enabled by base stacking and a backbone that is not conducive to intramolecular hydrogen bonding. A similar high-force transition can be induced in poly(A) using a hydrogen bond-disrupting solvent that eliminates the interaction between O2'H and O3' and O5'. We speculate that the γ torsion flipping may be a general mechanism of base-stacked DNA and may contribute to the high-force plateau in the dsDNA B-type helix upon its melting (see Supplemental Material, Fig. S1 [7]). These observations provide a simple unified model for the mechanism of overstretching transitions in DNA.

We thank Dr. Xueqing Zou and Professor Klaus Schulten for insightful discussions regarding this

manuscript and we thank Dr. Igor Kuznetsov and Dr. Minkyu Kim for a critical reading of this manuscript. We would like to acknowledge support from the NSF GRFP 1106401 to Z. N. S. and the NSF MCB-0717770 to P. E. M. Z. N. S. and M. R. contributed equally to this work.

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