Two distinct overstretched DNA structures revealed by single-molecule thermodynamics measurements

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Two distinct overstretched DNA structures revealed by single-molecule thermodynamics measurements

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Double-stranded DNA is a dynamic molecule whose structure can change depending on conditions. While there is consensus in the literature about many structures DNA can have, the state of highly-stretched DNA is still not clear. Several groups have shown that DNA in the torsion-unconstrained B-form undergoes an “overstretching” transition at a stretching force of around 65 pN, which leads to approximately 1.7-fold elongation of the DNA contour length. Recent experiments have revealed that two distinct structural transitions are involved in the overstretching process: (i) a hysteretic “peeling” off one strand from its complementary strand, and (ii) a nonhysteretic transition that leads to an undetermined DNA structure. We report the first simultaneous determination of the entropy (ΔS) and enthalpy changes (ΔH) pertaining to these respective transitions. For the hysteretic peeling transition, we determined ΔS \sim 20 \text{cal} / \text{mol} / \text{K} and ΔH \sim 7 \text{ kcal/mol}. In the case of the nonhysteretic transition, ΔS \sim 3 \text{ cal} / \text{mol} / \text{K} and ΔH \sim 1 \text{ kcal/mol}. Furthermore, the response of the transition force to salt concentration implies that the two DNA strands are spatially separated after the hysteretic peeling transition. In contrast, the corresponding response after the nonhysteretic transition indicated that the strands remained in close proximity. The selection between the two transitions depends on DNA base-pair stability, and it can be illustrated by a multidimensional phase diagram. Our results provide important insights into the thermodynamics of DNA overstretched and conformational structures of overstretched DNA that may play an important role in vivo.

DNA can exist as a single-stranded polymer or a double-stranded helical structures. In cells, DNA primarily exists in the stable B-form (B-DNA), which contains two strands that are associated by Watson-Crick base-pairing interactions, and are stabilized by stacking interaction between adjacent base pairs. The transition from B-DNA to single-stranded DNA (ssDNA) is called DNA melting, and it is necessary for many fundamental processes such as DNA replication, gene transcription, and DNA damage repair. In vivo, DNA melting can occur with assistance from DNA helicases or ssDNA binding proteins (1, 2). In vitro, DNA melting can occur by directly heating or pulling the two complementary strands apart in a single-molecule unzipping experiment (3).

Double-stranded DNA can exist in several different structures from the B-form, such as A-DNA and Z-DNA. These alternative structures can be promoted under certain conditions (4, 5). ssDNA can also exist in elongated forms in the presence of DNA damage repair proteins, such as RecA and Rad51 (6, 7), or DNA intercalating ligands, such as the dyes YOYO-1 and ethidium bromide (7). Mechanical stretching of DNA may produce a similar transition.

A structural transition, referred to as the DNA overstretching transition, occurs at a force of around 65 pN. After this transition, DNA is stretched to about 1.7 times the contour length pertaining to the B-form (8, 9). Since its discovery in 1996, there has been a debate about the mechanism of this transition and the nature of overstretched DNA. The central question is whether overstretched DNA is (i) ssDNA due to force-induced melting of the duplex, or (ii) a unique elongated form of dsDNA (S-DNA) resulting from a hypothetical B-to-S transition (8, 9).

Both models have strengths and weaknesses in the interpretation of experimental data. A series of experiments support force-induced melting that leads to one ssDNA strand under tension through peeling from nicks or open ends of DNA or two separated single strands under tension through melting inside the DNA (internal melting) (10–15). Particularly, studies of the dependence of the transition force on temperature \( F_{ov} (T) \) have determined ΔS and ΔH during the transition in dye-free conditions (16). The values are in good agreement with the thermal melting transition (16), and they disfavor a nonmelting mechanism. Whether the overstretched DNA has only one strand or two strands under tension can be studied by the dependence of the transition force on the ionic strength (15). Two such experiments have been reported. One study supports one strand (15), and the other study supports two strands under tension (10). Thus, based on these experiments, peeling and melting have been proposed to explain the DNA overstretching transition. Furthermore, force-induced DNA melting was also reported in full-atom molecular dynamics simulations (17).

In contrast, observations in a different series of experiments imply a nonmelting mechanism. In Experiment 1, the force-response of overstretched DNA is inconsistent with that of one ssDNA strand or two noninteracting ssDNA strands (18, 19). In Experiment 2, a second transition at an even higher force has frequently been observed that leads to final strand separation after the 65-pN overstretching transition (19–22). The existence of this second transition, which is definitely a melting transition, supports the notion that the first transition (at approximately 65 pN) is not a melting transition (19–21). In Experiment 3, Paik, et al., and some of us, showed that end-blocked, torsion-unconstrained DNA (which prevents peeling) still undergoes a nonhysteretic DNA overstretching transition at approximately 65 pN (19, 23). A DNA melting mechanism, however, may also explain these experimental results. For example, the unique force-response in Experiment 1 may represent an internally melted DNA whose two strands are interacting with each other. The secondary transition in Experiment 2 may be explained by breaking the last base pairs holding the strands together due to the heterogeneity in the DNA sequence (24). The nonhysteretic transition

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in Experiment 3 on the end-blocked, torsion-unconstrained DNA may be an internal DNA melting transition. In addition, simulations and theoretical modeling studies suggest the existence of nonmelted elongated dsDNA (25, 26).

Theoretically, these two conflicting mechanisms can be reconciled by the existence of two modes of DNA overstretches transitions at approximately the same force and elongation (18), which may be the origin of confusion in the field. Consistent with this view, a series of experiments by our lab have revealed a hysteretic transition and a nonhysteretic transition at approximately 65 pN, which can be selected or coexist via small changes in factors that affect DNA base pair stability (20). The hysteretic transition has been shown to be a peeling transition. Whether the nonhysteretic transition leads to a previously proposed nonmelted “S-DNA” (8, 9, 18) or an internally melted DNA (10, 15) remains unclear. Due to this uncertainty, hereafter we refer the DNA after the nonhysteretic transition as “nonhysteretic overstretched DNA.”

This research aims to provide new insights to the understanding of the following two major questions about DNA overstretches transitions: (i) whether “nonhysteretic overstretched DNA” is internally melted DNA, and (ii) how the selection between the hysteretic peeling transition and the nonhysteretic overstretches transition depends on experimental conditions. Crucial to the success of this research, an unambiguous experimental indicator is needed to judge whether the transitions are related to DNA melting. One possible approach is to stain the overstretched DNA with fluorescence dyes specific to ssDNA or dsDNA in order to visualize the DNA structural compositions directly (12). This approach, however, has a disadvantage of perturbing the stability of DNA structures, and it may influence the experimental outcomes. Therefore, to eliminate possible effects of DNA binding agents, it is important to study overstretches based on the intrinsic properties of the transitions and resulting structures of naked DNA. One intrinsic property is the thermodynamics of the transitions. $\Delta S$ and $\Delta H$ during DNA melting have been studied extensively and are well characterized (16). If the nonhysteretic overstretches transition is not a DNA melting transition, these values are expected to differ from the values pertaining to DNA melting. In addition, intrinsic structural properties of an overstretched DNA, such as the number (one or two) of strands under tension. In the case of two strands, their spatial proximity may provide further important insights. In this contribution, these intrinsic properties are carefully examined for the hysteretic peeling transition and the nonhysteretic overstretches transition without using any DNA binding dyes.

As pointed out by Rouzina, et al. (15, 27), $\Delta S$ and $\Delta H$ during DNA overstretches transition can be directly determined by measurements of $F_{ov}(T)$ using the following equations: $\Delta S = -(\partial F_{ov}/\partial T)\Delta b$ and $\Delta G = \Delta \Phi + \Delta H - T\Delta S = 0$. Here $\Delta b$ is the DNA extension change per base pair during the transition (SI Appendix, Extension changes during transition), $\Delta \Phi$ is the force dependent free energy change that can be calculated with force-responses of B-DNA and overstretches DNA (SI Appendix, Entropy and enthalpy changes). According to recent studies from our lab, there exist two distinct transitions based on whether hysteresis exists (19, 20). Previous measurements of $\Delta S$ and $\Delta H$, however, do not demonstrate any apparent distinct values (14). One possibility is that the hysteretic and nonhysteretic transitions are DNA melting transition giving similar values of $\Delta S$ and $\Delta H$. An alternative possibility is that the hysteretic and the nonhysteretic transitions have distinct values of $\Delta S$ and $\Delta H$. Under those experimental conditions, however, DNA only underwent the peeling transition; thus, the other transition type was not observed. To test these possibilities, we remeasured $F_{ov}(T)$ them over a wider temperature range and determined $\Delta S$ and $\Delta H$ during respective transitions.
In addition to the measurements of $F_{ov}(T)$, important hints to possible structures of an overstretched DNA can be obtained by studying $F_{ov}$ as a function of ionic strength $F_{ov}(1/I_0)$ (15). Here $I$ is ionic strength, which is also the concentration of NaCl in this research, and $I_0 = 1$ M, the standard ionic strength. From $F_{ov}(1/I_0)$, a linear relation as a function of $\ln(I/I_0)$ exists with a slope $\partial F_{ov}/\partial \ln(I/I_0) = \nu k_B T/I_0$ for $I < I_0$. $I_0$ $-0.71$ nm is the Bjerrum length in water at room temperature. The structural coefficient $\nu$ is approximately 1.2 if the transition leads to one strand under tension while the other recoils (i.e., peeling), and $\nu$ is approximately 0.5 if the two strands are tightly associated with an interstrand distance considerably less than the Debye screening length. In this research, we also remeasured $F_{ov}(1/I_0)$ to see whether there exist distinct values of $\nu$ during the respective two transitions. Finally, phase diagrams for the selection of transitions are constructed from these results.

Results
Our results were based on measurements of $F_{ov}(T)$ and $F_{ov}(1/I_0)$ using a transverse magnetic tweezers setup (28) (Fig. 1A, SI Appendix, Magnetic tweezers measurements, and SI Appendix, Temperature control and measurement). In our experiment, $F_{ov}$ is determined at the onset of the transition (Fig. 1B and C) in order to attribute the force to a specific transition (SI Appendix, Determination of transition types). Analogous to other phase transitions, a clear signature at the onset of the overstretching transition is a dramatic increase in extension fluctuations. In our experiments, $F_{ov}$ is defined as the force where the variance of the DNA extension increased to $500$ nm$^2$.

To find the onset transition, cycling between a force below the transition force and a series of increasing higher forces are performed (SI Appendix, Fig. S4). At each of the higher forces, the DNA is held for 10 s (Figs. 1B–4), during which the DNA extension and variance are measured. The force-extension and force-variance curves in Fig. 1B and C were obtained by this method. If a variance of greater than $500$ nm$^2$ is found, the corresponding force is identified as $F_{ov}$. In addition to determining $F_{ov}$, force cycling allows us to determine the transition types. In the peeling transition, hysteresis in extension change can be observed due to the slow reannealing process that occurs at the lower forces; whereas, in the nonhysteretic transition, no hysteresis in extension change will be observed due to much faster transition kinetics (19, 20, 29).

Fig. 1C inset shows that the variance monotonically increases as force increases in the nonhysteretic transition but not in the hysteretic peeling transition. This difference is caused by the slow stochastic nature of the peeling transition (19, 20, 29). Thus, determination of the transition force will have a larger variation in the hysteretic peeling transition than it will have in the nonhysteretic transition (SI Appendix, Fig. S6).

Using the above method, $F_{ov}$ was determined at different temperatures from which $\Delta S$ and $\Delta H$ could be calculated. Fig. 2A shows $F_{ov}(T)$ measured in $150$ mM NaCl and pH 7.5. A piecewise linear temperature measurement was revealed with two distinct slopes: $\partial F_{ov}/\partial T \sim 0.12$ pN/K from $11^\circ$C to $18^\circ$C, where the nonhysteretic transition was determined, and $\partial F_{ov}/\partial T \sim -0.77$ pN/K at $T$ greater than $18^\circ$C, where the hysteretic peeling transition was determined. Switching from nonhysteretic transition to hysteretic peeling transition as temperature increases is consistent with an earlier observation that the level of hysteresis can be suppressed by lowering temperature (30). We emphasize that the approximately $18^\circ$C switching temperature (temperature at which the transition switches from the nonhysteretic transition to the hysteretic peeling transition) observed here is likely a response of the less stable AT-rich DNA region (SI Appendix, Determination of the transition force).

Fig. 2B shows two independent experiments performed in $10$ mM (blue) and $500$ mM (red) NaCl. In $10$ mM...
NaCl, the transition was entirely the hysteretic peeling transition in the experimental temperature range with a slope of approximately $-0.92 \text{ pN/K}$; whereas, in 500 mM NaCl, a piecewise linear temperature response similar to that in Fig. 2A was observed with a slope of approximately 0.10 pN/K in the nonhysteretic transition and approximately $-0.44 \text{ pN/K}$ in the hysteretic peeling transition. To better extrapolate these results to in vivo conditions, where magnesium exists in a mM concentration range, we also studied the effects of magnesium. In 150 mM NaCl, Fig. 2C shows that the piecewise linear temperature response still exists in 5 mM MgCl$_2$, with similar slopes to the experimental data obtained in the absence of magnesium for the same DNA. The apparent effects of magnesium are that: it increases $F_{ov}(T)$ by approximately 1.5 pN, which is in agreement with previous studies (31), and it increases the switching temperature by less than 1 °C.

Multiple independent experiments (SI Appendix, Fig. S8) have yielded the average and standard deviation of the slopes of 0.10 ± 0.02 pN/K in 500 mM NaCl (ten experiments) and 0.12 ± 0.02 pN/K in 150 mM NaCl (six experiments) in the nonhysteretic transition; approximately $-0.45 \pm 0.05 \text{ pN/K}$ in 500 mM NaCl (three experiments), and approximately $-0.67 \pm 0.11 \text{ pN/K}$ in 150 mM NaCl (five experiments) in the hysteretic peeling transition. These slopes allowed us to calculate $\Delta S$ and $\Delta H$ per base pair during the nonhysteretic transition and the hysteretic peeling transition (SI Appendix, Entropy and enthalpy changes) and compare them with that determined in the thermal melting transition (averaged over a sequence with 50% GC content) (16) shown in Table 1.

It is of interest to know whether the two strands of DNA are in close proximity to each other after the two respective transitions. Therefore, we studied $F_{ov}(T/I_{B})$. Fig. 3 shows two independent experiments at 11 °C (black) and 23 °C (red). At 11 °C, $F_{ov}$ was found to be a piecewise linear function of ln($I/I_{0}$), with two distinct slopes: 2.9 ± 0.1 pN in greater than 20 mM NaCl, where the nonhysteretic transition was determined, and 5.7 ± 0.1 pN in less than 20 mM NaCl where the hysteretic peeling transition was determined. These slopes correspond to $\nu = 0.53 \pm 0.02$ in the former and $\nu = 1.03 \pm 0.02$ in the latter. According to the predictions by Rouzina, et al. (15), $\nu = 0.53 \pm 0.02$ infers that the interdistance between the strands of the overstretched DNA is less than one Deybye length (approximately 1 nm at 100 mM NaCl). This result suggests that the two strands of the “nonhysteretic overstretched DNA” are likely in close proximity. Moreover, $\nu = 1.03 \pm 0.02$ observed in the hysteretic peeling transition is close to the theoretically predicted value of 1.2 for the hysteretic peeling transition (15). In another experiment at 23 °C, the transition was determined to be the hysteretic peeling transition for 1 mM $< I < 500$ mM. The corresponding slope is $6.6 \pm 0.16 \text{ pN}$ and $\nu = 1.15 \pm 0.02$. As shown in Fig. 3, the linear range is only up to 100 mM; therefore, our fittings are up to 100 mM NaCl—the same range as that used by Wenner, et al. (10).

Based on the experimentally determined force responses of the respective DNA states (B-DNA, ssDNA, or “nonhysteretic overstretched DNA”) (SI Appendix, Extension changes during transition), $\Delta S$ and $\Delta H$ during the DNA melting transition obtained in previous DNA thermal melting transition (16), and $\Delta S$ and $\Delta H$ during the nonhysteretic transition measured in this research (Table 1), as well as $F_{ov}(T/I_{0})$ measured in this research (Fig. 3), we can construct phase diagrams to predict the states of a DNA molecule with open ends or nicks and the selection of the transitions as a function of external force $F$, temperature $T$ and ionic strength $I$ (details in SI Appendix, Phase diagrams).

For clarity, we first consider the phase diagram projected onto the $F$-$T$ plane for a fixed ionic strength of 150 mM. The boundary between the B-DNA and ssDNA (solid colored lines) where the free energy change $\Delta G^{B-ss}(F, T) = 0$ can be calculated from existing entropy and enthalpy data obtained from DNA thermal melting experiments (16). This boundary will vary with GC content. Because there is no existing free energy data of the nonhysteretic transition, and our prior studies have shown that the nonhysteretic transition is insensitive to GC content (19, 20), we used the entropy and enthalpy changes in Table 1 to calculate the boundary by $\Delta G^{B-ss}(F, T) = 0$, where NHO refers to “nonhysteretic overstretched” for short. The boundary between “nonhysteretic overstretched DNA” and ssDNA (dashed line) is calculated by $\Delta G^{NHO-ss}(F, T) = \Delta G^{B-ss}(F, T) - \Delta G^{B-NHO}(F, T) = 0$. These three lines then determine the phase boundaries of the system and meet at a triple point that corresponds to the switching temperature that was previously introduced. The data obtained from studies of $F_{ov}(T)$ in Fig. 2A and C are replotted in Fig. 4A for comparison.

We have mentioned that the selection of the transitions depends on factors that affect DNA base pair stability. Analogous to the phase diagram, we can construct a phase diagram for the selection of the transitions. For a given GC content, the point at which the change from a nonhysteretic transition to a hysteretic peeling transition is given by the switching temperature or triple point. Using similar calculations as above, we can calculate a line in the $I$-$T$ plane that divides these two transitions. Each line in

### Table 1. Comparison of $\Delta S$ and $\Delta H$ between our results and that reported in thermal melting experiments

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<tr>
<th>Quantities</th>
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<th>Our data, hysteretic transition</th>
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<td>$I$ in mM</td>
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<td>10</td>
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<td>$\Delta S$ cal/(K mol)</td>
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<td>$\Delta H$ kcal/mol</td>
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<tr>
<td>$\nu$</td>
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<td>$1.03 \pm 0.02$</td>
<td>$5.7 \pm 1.6$</td>
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</table>
DNA molecules (open circles) are also plotted for comparison. Strength from the same DNA molecule (filled circles) and from other ten mental data of the dependence of the switching temperature on ionic transition, and the region below, a nonhysteretic transition will occur. Experimental data of the dependence of the switching temperature on ionic strength, temperature, and sequence. Predicted phase boundaries for different GC contents are shown in different colors. The gray dashed line to the left of the triple point is the boundary between B-DNA and the nonhysteretic overstretched DNA. The data from Fig. 2 are plotted together in the same symbols for comparison. The filled circles obtained on different DNA molecules. The triple points obtained from different experiments are also plotted for comparison.

Fig. 4B corresponds to a different GC percentage, and it divides the I-T plane into the hysteretic peeling transition region (above the line) and the nonhysteretic transition region (below the line). The triple points obtain from different experiments are plotted in Fig. 4B for comparison. The filled circles obtained on the same DNA, while open circles are obtained from other ten different DNA molecules. Fig. 4 helps to emphasize that the experimentally observed transition is sensitive to temperature, ionic strength and GC content.

Discussion
We have shown that \( F_{ov}(T) \) and \( F_{ov}(I/I_0) \) have distinct trends in the nonhysteretic transition and the hysteretic peeling transition. \( \Delta S \) and \( \Delta H \) determined in the hysteretic peeling transition are consistent with those measured in DNA thermal melting transition experiments (16). The slight difference between our data and those from DNA thermal melting could be explained by a finite heat capacity change during DNA melting (SI Appendix, Effects of heat capacity change) (14, 15).

Striking results were found in the nonhysteretic transition. \( \Delta S \) is a small negative value, which may suggest an ordered “non-hysteretic overstretched DNA” structure that has slightly lower entropy than B-DNA together with surrounding water and ion distributions. The small \( \Delta H \) value of approximately 1 kcal/mol is about one order of magnitude smaller than that measured in thermal melting or hysteretic peeling transition. In addition, our study of \( F_{ov}(I/I_0) \) was consistent with a picture that the two strands in the nonhysteretic overstretched DNA are close to each other at an interstrand distance within the Debye screening length (15).

One important question remains regarding the exact structure of the nonhysteretic overstretched DNA. We considered two possibilities: (i) the structure could be some new regular double-stranded structure with regular short-ranged bonds and residual helicity (i.e., the previously proposed “S-DNA”), or (ii) the structure could be the two separated strands with broken hydrogen bonds. These two melted strands, however, can still interact with each other strongly via electrostatic and steric interactions. We cannot draw a firm conclusion between these two possibilities because \( \Delta S \) and \( \Delta H \) during the force-induced DNA internal melting transition were not directly measured.

Providing the final answer regarding the structure of the nonhysteretic overstretched DNA is not the purpose of this research. The main point of this research was to show that there exist two transitions that have distinct entropy and enthalpy changes during overstretching of DNA with open ends or nicks; however, it is interesting to note these results can be explained by the existence of a nonmelting DNA overstretching transition, which warrants further study. It is also worthwhile to mention a few previous experiments that may be related to this research. It has been found that torsion-constrained DNA did not undergo overstretching transition at approximately 65 pN unless the DNA is underwound (12). This result is also consistent with results obtained from another single-DNA stretching experiment by Bryant, et al. (32), and it is consistent with the high resolution atomic force microscopy imaging of DNA overstretched by molecular combing method (33).

These results raise interesting questions regarding the physiological relevance of the DNA overstretching transition. The hysteretic peeling transition is sensitive to factors that affect DNA base pair stability, and the transition force can be as low as 40 pN in 150 mM NaCl for AT-rich DNA at 37 °C (Fig. 4B). This force is close to the force range that can be generated by a single RNA polymerase (34) or DNA polymerase (35) in the force range of 20–40 pN. In comparison, the nonhysteretic transition is much less sensitive to factors that affect base pair stability. According to the predictions in Fig. 4B, the nonhysteretic transition may occur at greater than 25 °C for GC-rich DNA. The approximately 60 pN transition force is about 30 pN greater than the reported force range that can be generated by RNA polymerase (34) or DNA polymerase (35). In the presence of DNA intercalators, however, it is known that elongation of double DNA requires less force. For example, recent experiments showed that the presence of a YOYO-1 force of a few picoNewtons could elongate DNA contour by approximately 1.5-fold (36). Although the structure of the nonhysteretic overstretched DNA remains unknown, we imagine that DNA bound with YOYO-1 may resemble the DNA structure because it is only 10% shorter. In cells, DNA-distorting proteins play important roles in processing information in DNA and in organizing chromosome DNA. Among these proteins, many of them use side chain intercalation to distort the DNA backbone (37). Therefore, binding of these proteins may also be susceptible to DNA tension.

Materials and Methods
Refer to SI Appendix for details of the DNA construct, magnetic tweezers measurements, temperature control and measurement, determination of transition types, determination of the transition force, extension changes during transition, entropy and enthalpy changes, phase diagrams, elimination of thermal expansion effects, convection in the flow channel, and effects of heat capacity change.

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