Effect of pH on the Overstretching Transition of Double-Stranded DNA: Evidence of Force-Induced DNA Melting

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ABSTRACT When a single molecule of double-stranded DNA is stretched beyond its B-form contour length, the measured force shows a highly cooperative overstretching transition. We have investigated the source of this transition by altering helix stability with solution pH. As solution pH was increased from pH 6.0 to pH 10.6 in 250 mM NaCl, the overstretching transition force decreased from 67.0 ± 0.8 pN to 56.2 ± 0.8 pN, whereas the transition width remained nearly constant. As the pH was lowered from pH 6.0 to pH 3.1, the overstretching force decreased from 67.0 ± 0.8 pN to 47.0 ± 1.0 pN, but the transition width increased from 3.0 ± 0.6 pN to 16.0 ± 3 pN. These results quantitatively agree with a model that asserts that DNA strand dissociation, or melting, occurs during the overstretching transition.

INTRODUCTION

By stretching single molecules of DNA using optical tweezers or atomic force microscopy (AFM), a number of investigators have shown that DNA exhibits an unusual elastic behavior (Cluzel et al., 1996; Rief et al., 1999; Smith et al., 1996). These experiments move one end of a DNA molecule while measuring the force on the opposite end through means of an optical trap or AFM tip. The resulting force-extension curve is then used to describe molecular behavior under various solution conditions. At ~60–70 pN, the force-extension curve for double-stranded DNA (dsDNA) exhibits a plateau, indicating that the DNA can be elongated with very little additional force. This cooperative overstretch transition continues until the molecule is stretched to 1.7 times its B-form contour length, where the force increases rapidly and matches the force-extension curve of single-stranded DNA (ssDNA).

The overstretching transition has been attributed to a secondary structure transition from B-form to S-form DNA (Cluzel et al., 1996). The structure of S-form DNA is not well understood, but molecular models have been proposed (Cluzel et al., 1996; Konrad and Bolonick, 1996; Kosikov et al., 1999; Lebrun and Lavery, 1996) in which DNA unwinds to form a ladder-like structure or in which the DNA molecule forms a reduced-radius fiber. These models assume that the bases remain paired during the overstretching transition and that the transition is reversible. Although they are able to reproduce the observed plateau in the force-extension curve, the models are not able to predict the transition force or its width. The plateau is attributed to a transition in which only the base-stacking interactions are lost. A good fit to the observed data can be obtained using a two-state transition model (Ahsan et al., 1998; Cizeau and Viovy, 1997) or a simple elastic model (Haijun et al., 1999). However, because they are unable to predict the overstretching transition force, it is not clear how the models are related to the actual structural changes that occur as the DNA is stretched.

Rouzina and Bloomfield (2000a,b) have developed a theory that predicts that strand dissociation, or melting, occurs during the overstretch transition. The theory quantitatively accounts for the observed overstretching force and the slope of the overstretching force as a function of extension. It also predicts that the overstretching force will decrease if conditions are changed to destabilize the helix.

To test this theory, we have used an optical tweezers instrument to measure the overstretching force and the width of the overstretching transition as a function of pH. Because extremes of high or low pH destabilize the helix and lower the thermal melting point of DNA (Lando et al., 1994), the theory predicts that the overstretching force will decrease in both low and high pH. Our data show that the overstretching force and thermal melting point as a function of pH exhibit similar trends and that the model of force-induced melting accurately describes the dependence of the overstretching force on pH.

MATERIALS AND METHODS

The dual-beam optical tweezers instrument used in this study consists of two counter-propagating 150-mW, 850-nm diode lasers focused to a small spot inside a liquid flow cell with 1.0-NA Nikon water-immersion microscope objectives. The force measurement was calibrated by applying a known external force to a bead in the optical trap and measuring the resulting change in bead position using position-sensitive photodiode detectors. After trapping a bead, the liquid cell surrounding the bead was oscillated at a known frequency and amplitude. The amplitude of the observed oscillating force due to viscous drag on the bead (Mehta et al., 1998) was measured as a function of frequency, giving the detector signal as a function of applied force. The amplitude of this signal was linear in applied force, and this linear relation determined the calibration factor for the two detectors. Based on the standard deviation of multiple calibration measurements, the force measurement error of the instrument is ±0.5 pN.

To tether single molecules of DNA, one 4.1-μm-diameter streptavidin-coated bead (Spherotech, Libertyville, IL) was trapped in the optical

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tweezers and then attached by suction to a pipette with a 1–2-μm tip. Another such bead was captured and held in the optical trap while a dilute solution of biotinylated DNA was run through the cell. Once a DNA molecule was attached to the trapped bead, the bead on the pipette was moved toward the trapped bead until the opposite end of the molecule was bound. The procedure is identical to that described in Fig. 3 of Bennik et al. (1999). Biotinylated DNA was obtained by incubating bacteriophage λ DNA with biotin-11-dCTP (Sigma Chemical Co., St. Louis, MO), dATP, dTTP, dGTP, and Klenow *exo*$^-$ DNA polymerase (New England Biolabs, Beverly, MA). T4 DNA ligase (New England Biolabs) was added to repair single-strand nicks.

When a single molecule was tethered between the two beads, force-extension measurements were made by measuring the force on the bead in the trap while moving the pipette a known distance. A schematic diagram of the experiment is shown in Fig. 1. The absolute extension of the molecule was estimated by measuring the distance between the centers of the two beads using an image captured with a CCD camera. The change in position of the pipette was measured using a feedback-compensated piezoelectric translation stage that is accurate to 5 nm (Melles Griot 17PCZ013, Irvine, CA.) The position measurement was converted to a measurement of the molecule extension by correcting for the trap stiffness, which was 83 pN/μm. For the measurements reported here, the pipette was moved in 100-nm steps and after each step the force was measured 100 times and averaged. Each step took ~0.5 s. The force-extension curves did not change significantly when the pulling rate was varied by changing the step size from 10 to 500 nm.

The tethering buffer was 1 mM cacodylate with 250 mM NaCl, pH 6.01. After tethering, at least 4 ml of experimental buffer was run through the cell. The cell volume was less than 400 μl, and we found this amount was sufficient to completely exchange buffers. The experimental buffers at pH $>$ 7 were 10 mM Na_2CO_3 and 230 mM NaCl, which was titrated with HCl to obtain pH 9.71, 10.13, and 10.61. The buffers at pH $<$ 5 were 10 mM formic acid and 247.5, 245.0 or 242.5 mM NaCl, which was titrated with 2.5, 5.0, and 7.5 mM NaOH to obtain pH 3.14, 3.49, and 3.93, respectively.

Thermal melting curves were obtained by increasing temperature 0.2°C/min and following the absorbance increase at 260 nm using a GBC UV 918 spectrometer with a Peltier thermocell attachment. Melting temperature (T_m) at each pH was determined by averaging the midpoint of two to three curves, and pH values were corrected for temperature dependence using published $d(pK_a)/dT$ values (Stoll and Blanchard, 1990). Melting data have been obtained with nonbiotinylated λ DNA at concentrations of 25.0 ± 4.5 μg/ml.

RESULTS

A typical overstretching curve in 1 mM cacodylate buffer with 250 mM NaCl is shown in Fig. 2. The force versus extension curve begins to rise as the DNA helix is extended to near its normal contour length of 16.5 μm. The curvature of this rise depends on the persistence length, contour length, and elastic modulus because entropy, in the form of bends or kinks, is removed from the DNA during the initial extension.

After a steep rise in force, reflecting the resistance of DNA to stretching, the force plateaus. The change in force over this plateau is a few piconewtons as the DNA molecule is pulled to ~1.7 times its B-form contour length. After the plateau, the force again increases rapidly, and the force-extension curve matches that of ssDNA (data not shown) (Smith et al., 1996). We define the overstretching force as the force required to stretch a DNA molecule halfway through the overstretching transition, or to 1.35 times its contour length. There is usually some hysteresis when the DNA is relaxed, which we attribute to the re-annealing of locally melted DNA, but under these conditions, the relaxation curve usually matches the stretching curve exactly at forces below 40 pN.

The data in Fig. 2 have been fit to the extensible worm-like chain model. In this model, the extension of the worm-like chain in response to an applied force F is described in the limit of high force ($FP/k_BT > 1$) by Odijk (1995):

$$\frac{x}{L_0} = 1 - \frac{1}{2}\left(\frac{k_BT}{FP}\right)^{1/2} + \frac{F}{K} \qquad (1)$$

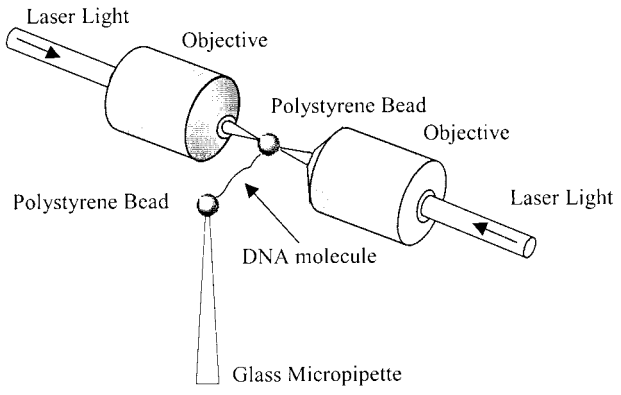

FIGURE 1 Schematic drawing of an optical tweezers experiment in which a single DNA molecule is stretched between two polystyrene beads. (Drawing is not to scale.) One bead is held on the end of a glass micropipette by suction, while another bead is held in an optical trap. Two counter-propagating laser beams focused to a common point form the optical trap.

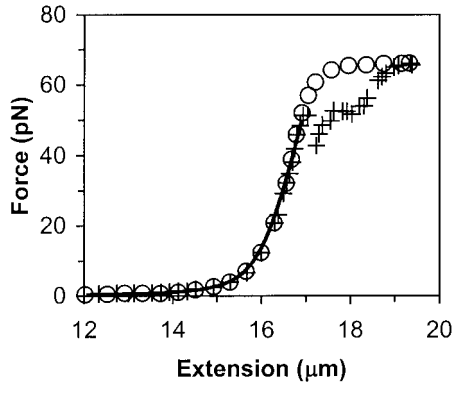

FIGURE 2 Representative data for overstretching a single dsDNA molecule in pH 6.0 buffer with 250 mM added NaCl. (○), data obtained when stretching the DNA molecule; +, data obtained when the DNA is relaxed. The solid line is a fit to the extensible worm-like chain model (Eq. 1), with a length of 16.5 μm, a persistence length of 45 nm, and an elastic stretch modulus of 1010 pN. The stretch data are reproducible within ±0.8 pN, whereas the relaxation data vary for different molecules.

More accurate models have been proposed (Bouchiat et al., 1999), but our contour length measurements are not accurate enough to distinguish between the models. For the fit shown in the figure, we obtain a contour length of $L_0 = 16.5$ μm, a persistence length of $p = 45$ nm, and an elastic stretch modulus of $K = 1010$ pN. These values are in good agreement with those that have been reported previously using other optical tweezers instruments (Baumann et al., 1997; Wang et al., 1997).

As pH is raised (Fig. 3), the overstretch transition occurs at a progressively lower force, and the transition width is constant within experimental error. Three separate stretches were averaged for each pH value to obtain the data shown in Fig. 3. Only the stretching portion of these data is shown, although each set of data displayed significant hysteresis upon relaxation similar to that shown for pH 6.0 in Fig. 2. The inset to Fig. 3 shows a single stretch in pH 10.6, illustrating the roughness of the transition at high pH as well as the large hysteresis seen in these data.

As pH is lowered (Fig. 4), the overstretching transition again occurs at lower force, but in contrast to the high pH data, the change in force over the course of the overstretch transition increases dramatically. Here the stretching part of the force-extension curve is shown for each case except pH 3.1, for which we show the relaxation curve. The stretching curve for pH 3.1 shows a few abrupt jumps (Fig. 4, inset.) We attribute the jumps to nonspecific sticking of the DNA to the bead because the relaxation data did not show this feature. This is expected because the pK_a of streptavidin is between 5 and 6 (Green, 1990), so at low pH the streptavidin-coated beads become protonated and may attract the negatively charged DNA molecule. The data for pH 3.5, pH 3.9, and pH 6.0 did not show any hysteresis under these conditions. After obtaining curves at low pH, the pH 6.0 curve could be reproduced, indicating that the changes to the DNA molecule at low pH are reversible.

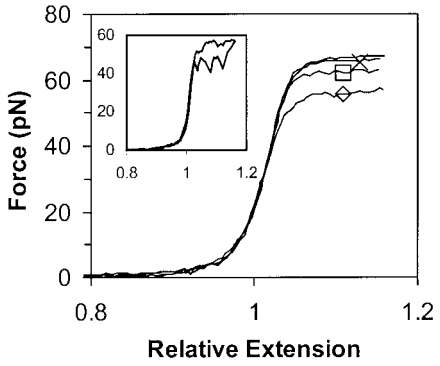

FIGURE 3 Force-extension curves for dsDNA in buffer at pH 6.0 (–), pH 9.7 (×), pH 10.1 (□), and pH 10.6 (◇) with 250 mM added NaCl. The relative extension is the extension divided by the B-form contour length of the molecule. The shape of the curve changes very little as the pH is increased from 6.0 to 10.6, but the value of the overstretching force decreases from 67.0 ± 0.8 pN to 56.2 ± 0.8 pN. These data are shown as the average of at least three stretches at each pH. The resulting average overstretching force and standard error from these measurements is reported in Table 1. The inset shows stretching and relaxation curves for a single molecule in pH 10.6 with no averaging, illustrating the increased hysteresis and the roughness of the overstretching transition. The stretching curve has an average force of 56 ± 1 pN and the relaxation curve has an average force of 45 ± 5 pN.

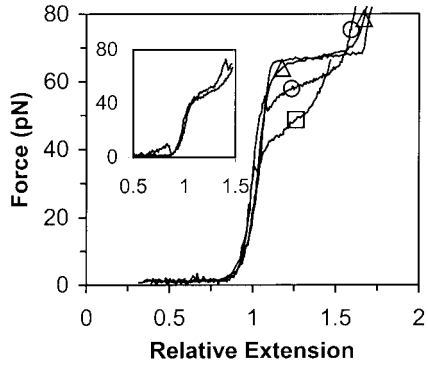

FIGURE 4 Force-extension curves for dsDNA in buffer at pH 6.0 (–), pH 3.9 (△), pH 3.5 (○), and pH 3.1 (□) with 250 mM added NaCl. The shape of the curve at low force changes very little as the pH decreases from 6.0 to 3.1. The width of the overstretching transition changes from 3.0 ± 0.6 to 16.0 ± 3 pN and the overstretching force decreases from 67.0 ± 0.8 to 47.0 ± 1.0 pN. These data shown are representative data from single stretches of two different molecules. The average overstretching force for at least three stretches and resulting standard error from these measurements is reported in Table 1. Except for pH 3.1, the data shown for each case are from stretching the DNA strand. None of the curves showed significant hysteresis except for pH 3.1, which shows sudden changes in force most likely due to sticking of the DNA to the bead, as shown in the inset. For this pH the relaxation curve is shown on the main graph. The inset is both the stretch and relax curve for pH 3.1.

We have parameterized the overstretching transition into an overstretching force and a transition width. The overstretching force is related to the stability of the DNA helix, whereas the transition width describes the cooperativity of the transition. Our method for calculating the transition width will be described in the next section, where we will introduce a model to describe the pH dependence of the width. Table 1 summarizes the overstretch parameters and shows that $F_{\text{overstretch}}$ decreases as helix stability is decreased by either raising or lowering pH. At high pH, the transition width matches the pH 6.0 values, but at low pH it increases by a factor of 5. Additionally, the apparent length of ssDNA decreases at low pH.

To compare these data with the predictions of force-induced melting theory, we have also measured the melting temperatures of λ DNA by UV spectrophotometry. The results are shown in Fig. 5.

DISCUSSION

We have demonstrated that lowering the melting point of dsDNA by changing the pH lowers the overstretching force, as predicted by theory (Rouzina and Bloomfield 2000b).

TABLE 1 Parameters describing the overstretching transition of dsDNA in buffer of various pH with 250 mM added NaCl

pH	$F_{overstretch}$ (pN)	$\Delta b(F_{overstretch})$ (nm)	Δf expt. (pN)	Δf fit (pN)	P_{ss} (FJC) (nm)
3.15	47.0 ± 1.0	0.09	16.0 ± 3	14	0.2
3.49	56.4 ± 0.4	0.14	10.5 ± 2	9	0.3
3.93	66.3 ± 0.4	0.17	4.3 ± 0.9	4	0.55
6.01	67.0 ± 0.8	0.19	3.0 ± 0.6	3	0.7
8	65	0.22	2.5	3	0.75
9.7	65.5 ± 0.6	~0.22	2.5 ± 1.0	3	
10.1	62.7 ± 0.6	~0.22	3.0 ± 1.0	3	
10.6	56.2 ± 0.8	~0.22	3.5 ± 1.5	3	

$F_{overstretch}$ is the force required to stretch the DNA halfway through the overstretching transition, whereas Δf is the transition width, as described in the text. Δf expt. represents the measured transition width, whereas Δf fit represents the transition width obtained from fits based on the assumption that the apparent persistence length of ssDNA decreases at low pH. The last column gives the persistence length used for the fit. Representative fits are shown in Fig. 7. The data for pH 8 were taken from Smith et al. (1996) and was measured in 150 mM NaCl buffer, so $F_{overstretch}$ is expected to be slightly lower than it would be in 250 mM NaCl. Results are reported as mean ± SE for at least three measurements.

Although this theory predicts an approximate overstretching force of between 60 and 80 pN in high salt at neutral pH, a much more accurate prediction is made for the change in overstretching force with a change in melting temperature. The relation

$$\delta F = \delta T \frac{\Delta S}{\Delta b} \tag{2}$$

is analogous to the Clausius-Clapeyron equation, where δF is the change in overstretching force, δT is the change in melting temperature, ΔS is the difference in entropy between the single- and double-stranded forms of DNA, and Δb is the difference in length per base pair between the single- and double-stranded forms. δT is taken from Fig. 5, whereas δF and Δb are taken from Figs. 3 and 4. In calorimetric studies ΔS is obtained at the melting temperature T_m.

If the transition entropy were independent of force, temperature, and pH, Eq. 2 would be exact. If the transition entropy is affected by solution conditions, Eq. 2 is approximate, with ΔS representing an average value between the two sets of conditions corresponding to the two different transitions: one at zero force and the melting temperature and another at the overstretching force and room temperature (Rouzina and Bloomfield, 2000b). It has been shown (Rouzina and Bloomfield, 2000a) that ΔS is insensitive to the force in the studied range of $F < 100$ pN. However, ΔS is known to strongly depend on temperature due to the large positive heat capacity change of DNA upon melting (Chalikian et al., 1999; Holbrook et al., 1999; Rouzina and Bloomfield, 1999). At an ionic strength of 250 mM and pH 6, λ-DNA melts at 93°C, with an entropy change of $\Delta S =$ 25 cal/mol *K* bp. In our experiments DNA force-induced melting occurs at room temperature, which implies a much lower transition entropy of ~12 cal/mol *K* bp, assuming a change in heat capacity of 65 cal/mol *K* bp (Chalikian et al., 1999).

Another factor that may lower the apparent ΔS in Eq. 2 is the change in solution pH. It has been shown (Privalov and Ptitsyn, 1969) that ΔS decreases at low and high pH faster than would be expected based solely on the lowering of the melting temperature. Thus ΔS itself should vary with pH. However, there are not enough experimental data to determine expected changes in ΔS. Therefore, in fitting our experimental data on $F_{overstretch}$(pH) to the dependence predicted by Eq. 2 with the measured function T_m(pH) (see Fig. 5), we use a constant average transition entropy ΔS^* for all pH values. The results of our fit are presented in Fig. 6, with the best fit value $\Delta S^* = 9.5$ cal/mol *K*. The latter quantity seems reasonable in the light of the above discussion. Even

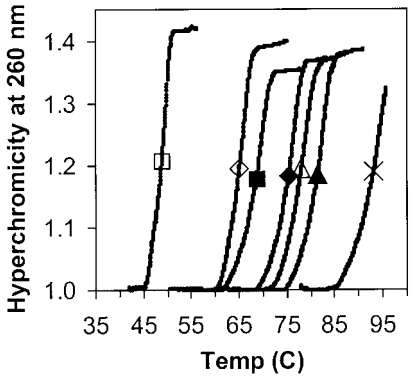

FIGURE 5 Thermal denaturation curves as a function of pH. Melting curves of λ DNA have been obtained by UV spectrophotometry at 260 nm. T_m values follow pH values that have been corrected for temperature dependence: □, pH 3.1 (48.8°C); ◇, pH 3.5 (64.7°C); △, pH 3.9 (78.0°C); ×, pH 6.0 (93.0°C); ▲, pH 9.2 (81.4°C); ◆, pH 9.7 (75.3°C); ■, pH 10.2 (68.2°C).

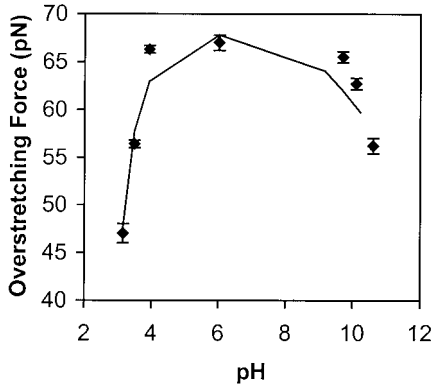

FIGURE 6 Overstretching force as a function of pH. The diamonds show the measured overstretching force as a function of pH. The error bars are taken from the data in Table 1. The line represents the predicted overstretching force from eq. 2 with $\Delta S^* = 9.5$ cal/mol *K* and δT taken from Fig. 5.

though we have neglected the possible dependence of ΔS^* on pH and therefore were unable to fit all of the data points within their experimental error, Eq. 2 is clearly dominated by the strong variation of T_m with pH, which produces similar changes in $F_{overstretch}$ as a function of pH.

A surprising result of our experiments is the apparent change in the length of the single-stranded form at low pH. The slope of the overstretching transition, and therefore the width of the transition, also increases greatly at low pH. It appears that the transition becomes less cooperative, but this is not observed in thermal melting (Lando et al., 1994). The change in length suggests a change in the properties of the single-stranded form. The protonation sites on the nitrogen bases are N3 cytosine (pK_a 4.6) and N1 adenine (pK_a 3.8). Minor protonation of N7 guanine has also been observed (Lando et al., 1994). Because N3 cytosine and N1 adenine participate in hydrogen bonding, they will be protonated only if the DNA strands have dissociated. Thus, force-induced melting causes protonation of the DNA strands, which in turn leads to a much different single-stranded stretching curve. Apparently, the charge reduction of ssDNA makes it harder to stretch. One might suggest that protonation of the bases should have an effect similar to increasing ionic strength, because both factors lead to damping of the electrostatic repulsion in ssDNA.

The increasing flexibility of ssDNA with salt has been shown experimentally using diffusion measurements (Tinland et al., 1997). The authors assumed ssDNA to be a freely jointed chain (FJC) with a rise per base pair of 4.3 Å and obtained its persistence length, which varied from 5 nm at 1 mM ionic strength down to 0.8 nm at 0.1 M ionic strength. The latter number seems to be in quantitative agreement with the result of Smith et al. (1996), who derived a persistence length of 0.75 nm by fitting a ssDNA force-extension curve to a FJC model at 150 mM ionic strength. In this model, the ssDNA extension $b_{ss}(F)$ is given by (Smith et al., 1996)

$$b_{ss}(F) = b_{ss}^{max}\left(\coth(2\tilde{F}) - \frac{1}{2\tilde{F}}\right)\left(1 + \frac{F}{K_{ss}}\right) \qquad (3)$$

where $\tilde{F} = FP_{ss}/k_BT$ is the reduced force, b_{ss}^{max} is the contour length per base in ssDNA, and P_{ss} is the persistence length of ssDNA. The values from their fit were $P_{ss} = 0.75$ nm, $b_{ss}^{max} = 0.56$ nm, and $K_{ss} = 800$ pN.

However, this persistence length is significantly lower than the high-salt persistence length of 1.5–2.0 nm measured by transient electric birefringence (Mills et al., 1999) for unstacked poly(dT) ssDNA and the rise per base $b_{ss}^{max} =$ 0.5 - 0.7 nm. For stacked poly(dA) ssDNA at 4°C these authors measured $b_{ss}^{max} = 0.32$ nm and $P_{ss} \approx 5.2$ nm. Here we scaled the original values reported by the authors by a factor of 2/3, which relates the persistence length in the worm-like chain model to its value in the FJC model (Rouzina and Bloomfield 2000a). The authors argue that the above value of the persistence length represents its lowest limit determined by backbone rigidity and is not influenced by electrostatic interactions.

The much shorter apparent persistence length obtained from the stretching experiments may reflect the formation of secondary structures (hairpins) in the ssDNA, as originally suggested in Smith et al. (1996). This conclusion is supported by a recent study (Maier et al., 2000) in which ssDNA stretching curves for two DNA species with 30% and 50% GC content were measured. With a force measurement resolution of 10^{-2} pN, they saw a significant increase in the force at low extensions in GC-rich ssDNA, which was attributed to more extended secondary structure formation in this case.

In contrast to dsDNA, the force-extension curves for ssDNA do not serve as a good tool for extracting its persistence length. The apparently good fit to the FJC model with three parameters yields an unrealistically short value of P_{ss}. In addition, this fit does not work for the high forces of 100 pN $< F <$ 800 pN recently studied with AFM (Clausen-Schaumann et al., 2000; Rief et al., 1999). Apparently, natural ssDNA polymers do not behave like a FJC or a worm-like chain either at low or high forces.

According to our experimental data, lowering the solution pH below 4 makes ssDNA progressively harder to stretch. We can speculate that strong pH reduction further promotes formation of secondary structures in ssDNA. In fact, formation of a parallel duplex in poly(dA) below pH ~4 is well known (Saenger, 1984). However, one would expect hairpins within ssDNA to be completely pulled out at forces lower than 15 pN (Essevazroulet et al., 1997). The apparent shortening of ssDNA at high forces above $F_{overstretch}$ and at low pH might signify additional stacking in ssDNA.

Despite the fact that ssDNA is not a FJC, we can use the force-extension expression of this model to simply describe the ssDNA stretching curve, such that it can be used for a fit of the complete force-extension curve for dsDNA, including the overstretching transition. We will attempt to quantify the arguments listed above by fitting the ssDNA within the FJC by changing the parameter P_{ss}, keeping in mind that this probably does not represent the true persistence length of the DNA molecule.

The force-dependent contribution to the transition free energy between the double-stranded and single-stranded forms of DNA at high forces typical of the transition can be approximated as a linear function of the force:

$$\Delta G(F, \text{pH}) = -[F - F_{overstretch}(\text{pH})]\Delta b(F_{overstretch}), \qquad (4)$$

where

$$\Delta b(F) = b_{ss}(F) - b_{ds}(F). \qquad (5)$$

The length of the molecule per base pair is b, so Δb is the change in length of the molecule during the transition from

double-stranded to single-stranded form. Therefore, the effect of force on the double-helix stability should become weaker when the elongation Δb becomes smaller. We can use Eq. 4 to calculate the width of the overstretching transition in terms of the force:

$$\delta F = \frac{\partial F}{\partial \Theta}\bigg|_{\Theta=1/2} \times \Delta\Theta = 4\sigma^{1/2}\left(\frac{k_B T}{\Delta b(F_{\text{overstretch}})}\right). \quad (6)$$

Here $\Theta(F)$ is the fraction of base pairs in the helical state (Zimm, 1960):

$$\Theta(s(F), \sigma) = \frac{1}{2} + \frac{s-1}{2[(s-1)^2 + 4s\sigma]^{1/2}}, \quad (7)$$

which is a function of just two parameters: the cooperativity σ, related to the energy of the boundary and the loop factor (Grosberg and Khokhlov, 1994), and the stability $s(F)$ of helix relative to coil:

$$s(F) = \exp\left(\frac{\Delta G(F)}{k_B T}\right) \quad (8)$$

The width of the transition will actually be somewhat larger than given by Eq. 6, because not only Θ but also the molecular elongation in both double- and single-stranded states are functions of the applied force. Note that Eq. 6 states the transition width, δF, can increase due to the decreasing elongation per base pair even if the cooperativity parameter σ remains constant.

We fit our measured DNA stretching curves at low pH to the weighted average between $b_{ds}(F)$ and $b_{ss}(F)$, the stretching curves for the double- and single-stranded forms of DNA.

$$b(F) = \Theta(F) b_{ds}(F) + (1 - \Theta(F)) b_{ss}(F) \quad (9)$$

$\Theta(F)$ is calculated according to Eqs. 4, 7, and 8. We take $b_{ds}(F)$ in the form of the extensible worm-like chain model of Eq. 1, with the following parameters: $P_{ds} = 50$ nm, $b_{ds}^{max} = L_0/N = 0.34$ nm, and $K_{ds} = 1000$ pN. Here N is the number of base pairs in the DNA molecule. These values are assumed to be independent of solution pH. $b_{ss}(F)$ is calculated from Eq. 3.

If we assume that the cooperativity parameter for DNA melting is small, $\sigma = 8 \times 10^{-4}$, we can fit the measured stretching curves for DNA at low pH by adjusting the flexibility of ssDNA only. We also fix the single-stranded contour length and elastic modulus at their measured values at pH 8 and vary only the persistence length P_{ss}, trying to match part of the stretching curve above the transition at $F > F_{\text{overstretch}}$. The fitted values of P_{ss} are presented in Table 1, together with the elongation per base pair upon melting taken at the transition midpoint according to Eq. 5. The fitted curves are then used to calculate the complete DNA stretching profile according to Eq. 8. Two such fits for pH 3.5 and pH 6 are presented in Fig. 7. The overall fit is very good, even though the transition broadening is slightly underestimated. This is most likely due to the linear approximation to the transition free energy as a function of force, which becomes progressively less accurate at lower forces.

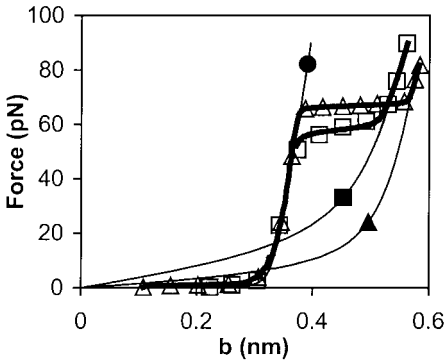

FIGURE 7 Representative fits of the experimental DNA stretching curves at pH 6 and pH 3.5. b is the DNA extension per base pair. The thin lines marked by solid symbols are modeled single-stranded stretching curves in pH 6 (▲) and pH 3.5 (■) and a modeled dsDNA curve (●), whereas the thick solid lines are fits to the stretching curves. The open symbols are measured data points for pH 6 (△) and pH 3.5 (□).

The increasing flexibility of ssDNA can reasonably account for the observed transition broadening at low pH. The fitted P_{ss} value appears to be a quickly decaying function of pH, as seen in Table 1. All of the values of P_{ss} are unrealistically small, but this just means that the FJC is not a good model to describe ssDNA force-extension curves. The values obtained illustrate that lowering the pH significantly changes the elasticity of ssDNA.

CONCLUSIONS

In this work, we have compared the DNA overstretching transition with thermal melting in identical buffer as a function of solution pH. Most importantly, we find that the overstretching force closely follows the melting temperature in its dependence on solution pH. Thus, at pH < 4 and pH > 9.5 the double helix becomes progressively less stable compared with the single strand. This leads to the simultaneous decrease in the overstretching force and melting temperature of DNA. The measured proportionality coefficient between $F_{\text{overstretch}}$(pH) and T_m(pH) provides a reasonable estimate for the entropy of the DNA melting transition.

In addition to the expected lowering of the overstretching force at high and low pH, the width of the overstretching transition shows very interesting behavior. At high pH, the transition width does not change significantly as the overstretching transition force is lowered, whereas at low pH the width increases by a factor of 5. We attribute this transition broadening to the growing flexibility of ssDNA at low pH associated with its charge neutralization, and we are able to fit our data to a model in which only the persistence length of the ssDNA is affected at low pH.

The data reported here support the interpretation of the overstretching transition as a melting transition rather than a transition from B-form to a double-stranded S-form. High and low pH have little effect on dsDNA and would not be expected to change its secondary structure (Costantino and Vitagliano, 1966; Luck et al., 1970; Maiti and Nandi, 1986). It is known from thermal melting studies of DNA (Birshtein and Ptitsyn, 1966; Lando et al., 1994; Record, 1967) that the main protonation and deprotonation sites are accessible only on ssDNA and are the same ones that participate in hydrogen bonding. Single-stranded DNA protonation occurs at the NH_2 amino groups of A, G, and C bases with $pK_a = 3.8$, 2.9, and 4.6, respectively (Lando et al., 1994), whereas the deprotonation sites are the -NH-CO- groups of the bases with pK_a between 9.5 and 10.5 in high salt.

The melting nature of the overstretching transition is also supported by the hysteresis observed in the release part of the DNA stretching cycle. Under the conditions used for Fig. 2, the hysteresis generally does not extend farther than halfway through the overstretching transition, whereas the rest of the overstretching curve is reversible. If the overstretched form of DNA consists of two single strands, then the increasing hysteresis at high pH can be explained by a growing negative charge on the ssDNA as they deprotonate at high pH. Conversely, the absence of hysteresis observed in the low pH buffers can be explained by charge neutralization and reduction of the mutual repulsion between the two strands.

It has been argued (Smith et al., 1996) that the overstretching transition cannot be a melting transition because a single DNA molecule can be stretched all the way through the transition without breaking, even when the molecule is tethered on opposite strands, as is the case in this study. It was later shown (Hegner et al., 1999; Rief et al., 1999) that the force-extension curve of dsDNA at forces above the overstretching transition has a quasi-plateau. The value of the force in this plateau grows rapidly with the DNA pulling rate and reaches values that are up to ~5 times higher than $F_{\text{overstretch}}$ (Rief et al., 1999). The authors suggested this rate-dependent high force regime corresponds to DNA melting, although they attributed the equilibrium overstretching force plateau to a B- to S-DNA transition. This conclusion was in line with all of the previous interpretations of DNA stretching experiments.

We believe, instead, that the rate-dependent part of the dsDNA force extension curves above $F_{\text{overstretch}}$ corresponds to non-equilibrium rupture of the small number of intact inter-strand bonds that are left after the majority of the bonds have melted during an equilibrium process in the course of DNA overstretching. These few bonds survive due to the one-dimensional nature of the DNA melting transition (Rouzina and Bloomfield, 2000a) and appear under conditions in which the strand unbinding is irreversible, such that no thermodynamic equilibrium between the double- and single-stranded forms of DNA required for conventional melting is possible. Under these conditions it is the enthalpy of the strand separation of ~15 kT per base pair, rather than its free energy of ~2 kT per base pair that should determine the force of strand separation. As for any non-equilibrium rupture force it should be rate dependent (Evans and Ritchie, 1997). The statistics of the events of the last bond ruptures should account for the longevity of the DNA inter-strand bonds at forces above $F_{\text{overstretch}}$. This theory is also consistent with early observations that the strand separation temperature for DNA in melting experiments is actually much higher than the melting temperature (Geiduschek, 1962).

In three upcoming papers (Rouzina and Bloomfield, 2000a,b; Williams et al., 2001) we compare the force-induced DNA melting hypothesis to the B- to S-DNA transition hypothesis. There (Rouzina and Bloomfield, 2000a) we calculate the DNA force-extension curve assuming the melting nature of the transition and using experimental stretching curves of ds- and ssDNA. The calculated DNA stretching curve superimposes on the experimental one with a highly reasonable choice of cooperativity parameter. This does not exclude the possibility of a transition to S-form but suggests that B-DNA should be melted by forces on the order of $F_{\text{overstretch}}$. We also use all available data on S-DNA modeling as well as thermodynamic arguments to show that the B to S transition in dsDNA should occur at forces almost an order of magnitude higher than the observed overstretching force.

Recent experiments (Clausen-Schaumann et al., 2000) have demonstrated that the overstretching force decreases with increasing temperature. This is also consistent with the theory of force-induced melting presented here. However, in the work of Clausen-Schaumann et al. (2000), the data were interpreted as a melting transition only when the DNA was stretched at temperatures greater than room temperature. It was argued that a transition to S-DNA always occurs at 65 pN. Thus, if the DNA molecule is destabilized, as we have done in our study, it will melt during the overstretching transition, but very stable DNA in high salt or at low temperatures will undergo a transition to S-DNA and then melt at the end of the transition during the rate-dependent part of the stretch. A reviewer has suggested that the three central points in Fig. 6 form a plateau due to the occurrence of the S-DNA transition. However, there is no special reason to expect the S-DNA transition to occur at 65 pN. In addition, it would be a remarkable coincidence if the S-DNA transition and the melting transition had such similar force-extension curves.

In other new work (Rouzina and Bloomfield, 2000b; Williams et al., 2001) we show that, in addition to the pH dependence of $F_{\text{overstretch}}$, the temperature dependence and the sequence dependence of $F_{\text{overstretch}}$, the hysteresis of the stretching curves, and effect of cross-linking on the salt dependence of $F_{\text{overstretch}}$ all argue in favor of the universal melting nature of the overstretching transition. Although we

cannot completely rule out the S-DNA interpretation, we believe that our melting interpretation provides a more complete, unified, and predictive account of the available data. Of course, complete confirmation of this theory will still require more experimental and theoretical work.

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