Critical bending torque of DNA is a materials parameter independent of local base sequence

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Short double-stranded DNA molecules exhibit a softening transition under large bending which is quantitatively described by a critical bending torque τ_c at which the molecule develops a kink. Through equilibrium measurements of the elastic energy of short (∼10 nm), highly stressed DNA molecules with a nick at the center we determine τ_c for different sequences around the nick. We find that τ_c is a robust materials parameter essentially independent of sequence. The measurements also show that, at least for nicked DNA, the local structure at the origin of the softening transition is not a single-stranded "bubble."

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I. INTRODUCTION

The bending elasticity of double-stranded (ds) DNA is completely described, at long length scales $L \gg \ell_p$ (where $\ell_p \approx 50$ nm is the persistence length), by one materials parameter, the bending modulus $B \approx 200$ pN × nm^2 (related to ℓ_p by $B = kT\,\ell_p$). The bending modulus B describes only linear elasticity, but for a long molecule $L \gg \ell_p$ (L is the contour length) nonlinear bending elasticity is irrelevant, in the sense that there is no way of manipulating the ends of the molecule which causes it to bend sharply for a substantial fraction of the time. Throughout this paper, all statements (such as the one above) refer only to boundary conditions where the ds molecule is held only at the ends, and through torque-free connections. This regime is described by the worm-like-chain (WLC) model [1], where the bending elastic energy for a molecule of contour length $2L$, schematized as a rod, is written

$$E = \int_0^{2L} ds\, \frac{1}{2}\frac{B}{R(s)^2}, \tag{1}$$

where R is the radius of curvature and s the arc length along the rod. This form corresponds to the linear elasticity regime of a thin rod [2]. This regime has been studied extensively, both in single molecule and in ensemble experiments [3–8]. Summarizing this considerable amount of work for our purposes, we may say that the bending modulus B (or equivalently ℓ_p) is indeed a robust materials parameter for ds DNA, which depends only weakly on the conditions of the experiment. For instance, ℓ_p varies no more than 15% with base pair (bp) composition [7], no more than 30% with temperature in the range 5 to 60 °C [8] (the corresponding decrease of B in this temperature range is less than 20%), and is essentially independent of ionic strength above ∼10 mM for monovalent ions [6] (the case of polyvalent ions is different).

At short length scales, $L < \ell_p$, the bending elasticity of DNA is similarly completely described by two materials parameters: the same bending modulus B, which describes the linear elasticity regime, and the recently introduced critical bending torque $\tau_c \approx 30$ pN × nm, which describes the nonlinear elasticity regime [9–11]. For such short molecules, highly stressed states are easily realized, for example, through the DNA minicircles [12], or the construction of Fig. 1 [9], or the chimeras of Refs. [13–16], not to mention the DNA beacons [17] and their technological applications [18]. Our purpose here is to show that the critical bending torque τ_c is, like B, a robust materials parameter, specifically, that it depends only weakly on base pair composition.

II. MATERIALS AND METHODS

A. DNA samples

Single-stranded (ss) DNA concentration was 2 μM, in 10 mM Tris buffer, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, pH 7.9. The samples were annealed and equilibrated by the following procedure: 30 min at 95 °C and brought back to room temperature over 12 h, then stored at room temperature (24 °C) for at least 2 d (50 h). We have confirmed that the monomer and dimer populations thus obtained are indeed equilibrium populations, by examining electrophoresis patterns 4, 11, 25, and 50 h after annealing. Initially (after annealing) the samples are mostly in monomer form, while the dimer band develops over a timescale of a few hours. The conclusion is that the finite (50 h) equilibration time used for the experiments does not introduce a significant systematic error.

The DNA sequences used in the experiments are listed in Ref. [19].

B. Gel electrophoresis

Monomers and dimers were separated on 5% polyacrylamide gel in TBE buffer. Samples were loaded every 10 min [Fig. 2(a)], so the total running time for the different lanes was 65, 55, 45, 35, 25, 15, and 5 min. The purpose is to extrapolate the band intensities backwards in time to the gel loading time. This is because as the sample runs through the gel, a certain amount of monomer dimer interconversion occurs, visible as interband smear in Fig. 2(a). To extract the equilibrium (i.e., initial) monomer and dimer amounts we use a simple reaction-diffusion model where monomers and dimers have different mobilities in the gel and given rates of interconversion; we adjust the model parameters to fit, with fixed parameters, the gel profiles at the different times and

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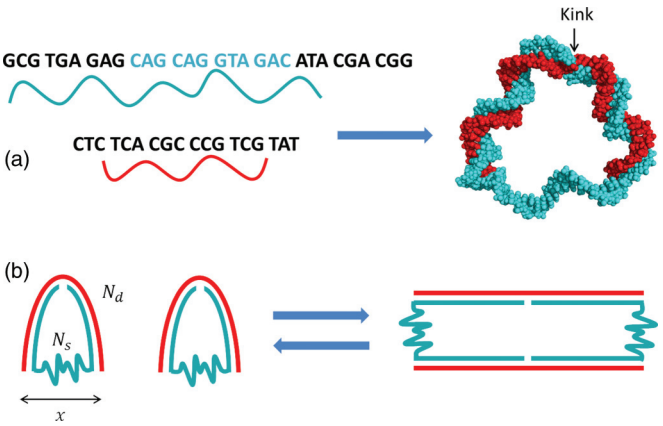

FIG. 1. (Color online) (a) Cartoon of the stressed DNA molecules used in the experiments. The ds DNA is from the MD simulations in Ref. [36]: we have used part of their structure L8sa, which contains a kink (note that there is no nick in those simulations). The ss DNA is from PDB 1BNA. Our molecules are formed by partially hybridizing two linear strands, so there is a nick at the center of the ds part. In the example sequence given, the black bases are complementary to each other, while the blue or light gray bases form the ss part of the hybridized molecule. The elastic energy of this molecule consists of the bending energy of the ds part and the stretching energy of the ss part. (b) Cartoon of the dimerization equilibrium.

can thus extrapolate the monomer and dimer concentrations at zero time, i.e., the equilibrium concentrations. We use the data for 55, 45, and 35 min; the reaction-diffusion model is

$$\frac{\partial C_m}{\partial t} = D_1 \frac{\partial^2 C_m}{\partial x^2} - v_1 \frac{\partial C_m}{\partial x} - 2k_1 C_m^2 + 2k_2 C_d, \quad (2)$$

$$\frac{\partial C_d}{\partial t} = D_2 \frac{\partial^2 C_d}{\partial x^2} - v_2 \frac{\partial C_d}{\partial x} + k_1 C_m^2 - k_2 C_d, \quad (3)$$

where C_m and C_d are the concentrations of monomers and dimers in the solution, K_1 is the rate of monomers to dimers conversion, and K_2 the rate of dimers to monomers conversion. An example of these fits is shown in Fig. 2. The mobilities are measured from the time-lapse gels (Fig. 2); the interconversion rates are essentially fixed by the requirement of reproducing the interband intensity at the different times. We numerically solve the above equations; varying the initial concentrations of monomers and dimers, we find the values which best fit the three selected profiles in a gel and thus obtain the initial concentrations of monomers and dimers. These values are used in the calculation of the energy of the stressed DNA molecules.

C. Electrostatic correction to the measured energy

We apply a correction to the energy given by (4), reflecting the electrostatic and strain energy in the dimer (Fig. 1) due to the electrostatic repulsion between the ds DNA parts, which stretches the ss parts [9]. Specifically, we minimized the energy $E_{\text{correction}} = (E_{\text{electro}} + E_{\text{strain}})/2$, which is the sum of the screened electrostatic interaction between discrete charges distributed on the two ds DNA backbones using a Debye length of 1 nm and the elastic energy of two stretched ss DNA strands each of n_k Kuhn lengths $l_k = 1.5$ nm, treating each as a Hookean spring with spring constant $(3k_BT)/(2n_k l_k^2)$. We assume the simple planar geometry of Fig. 1, so the EED determines all the distances. The minimum of this energy (i.e., for the equilibrium value of the EED) is used to correct the measured energy; this correction is $<1\ k_BT$.

III. RESULTS

We measure the critical bending torque τ_c using the method described in Ref. [10]. Two linear DNA strands are hybridized to form the ring molecule of Fig. 1. This molecule has built in internal stress, as the ds part (N_d base pairs long) is bent, while the ss part (N_s bases long) is stretched. The ds part is, by construction, nicked in the middle. This molecule can relax its internal elastic energy by forming dimers as in Fig. 1(b). Since base pairing in one dimer is identical to base pairing in two monomers, the equilibrium concentrations of monomers and dimers result from a balance between the elastic energy of the monomer, which we call E_{tot}, and the dissociation entropy of the dimer, according to Refs. [9–11,13,20]:

$$E_{\text{tot}} = \frac{1}{2} kT \ln \frac{X_D}{X_M^2}, \quad (4)$$

where X_M, X_D are the mole fractions of monomers and dimers. The concentrations of monomers and dimers are measured by gel electrophoresis of the equilibrated samples (see Methods and Fig. 2). The elastic energy of the monomer [Fig. 1(a)] is then obtained from (2). A small (<1 kT) correction is applied to this formula, to take into account some residual electrostatic energy in the dimer [9]; this is described in Methods. We also note that in our geometry (no torsional constraints), the bending elasticity behavior is essentially the same for nicked and non-nicked DNA [11] (the only difference being a slightly lower value for τ_c in the nicked case). Therefore we perform this study on nicked molecules, where we can use the simple equilibrium method above.

The molecule of Fig. 1(a) is essentially a system of two coupled springs: the ds part, which is bent, and the ss part, which is stretched. Evidently one can generate a series of molecules with different bending states for the ds part, by keeping this part fixed while varying the number of bases N_s in the ss part. The elastic energy of the molecule, which is the measured quantity, is the sum: $E_{\text{tot}} = E_d + E_s$, where E_d and E_s are the elastic energies in the ds and ss parts, respectively. To obtain the critical bending torque τ_c we proceed as follows.

We use the analytic expression [10] for the bending energy E_d of the ds part versus end-to-end-distance (EED) x:

$$E_d(x) = \begin{cases} \tau_c \arccos\left(\frac{x}{2R}\right) & \text{for} \quad 0 < x < x_c \\ \frac{5B}{L}\frac{x_0 - x}{2L} - T \ln\left(\frac{2L-x}{2L-x_0}\right) & \text{for} \quad x_c < x < x_0 \end{cases}, \quad (5)$$

where $R = L(1 - \frac{2}{45}\gamma^2)$ and $x_0 = 2L(1 - \frac{LT}{5B})$; $\gamma = L\tau_c/(2B)$. This formula describes the bending energy versus EED of a rod of contour length 2L, bending modulus B, which develops a (constant torque) kink at a critical value τ_c of the internal torque. The upper form in (3) corresponds to the kinked solution, the lower to the smoothly bent (WLC) one. The critical EED x_c at which the molecule develops a kink is found by equating the upper and lower expressions [or, to order γ^2, from $x_c = 2L(1 - 4\gamma^2/15)$]. The contour length of the DNA is $2L = 0.33\text{nm} \times N_d$. The expression (3) depends

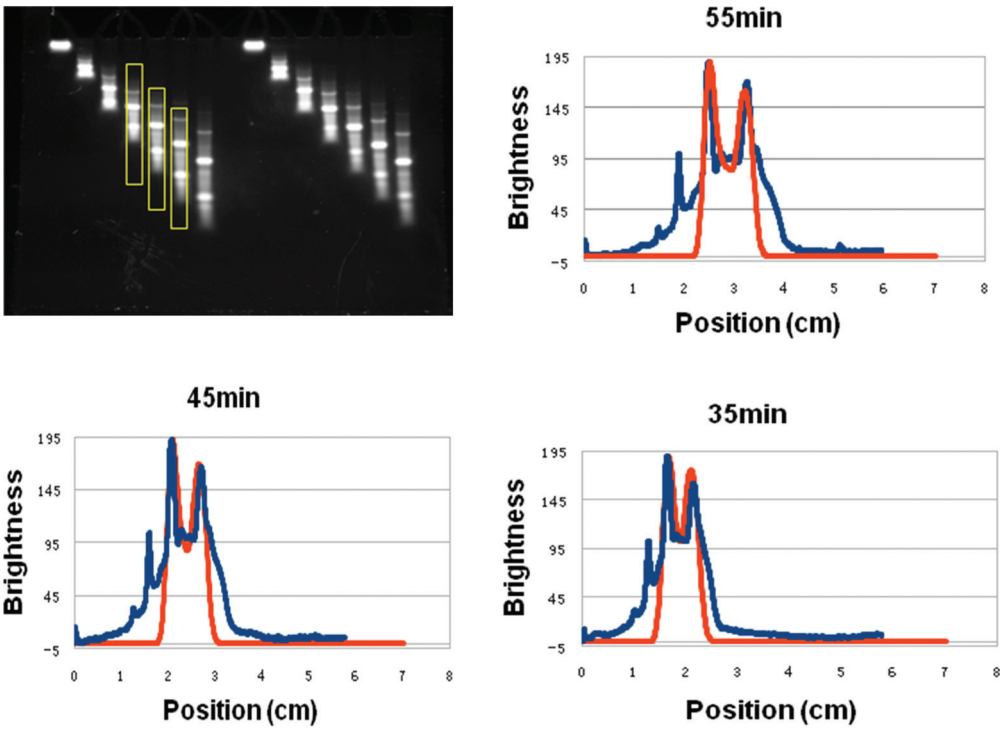

FIG. 2. (Color online) Example of a gel used to determine the concentrations of monomers and dimers in the dimerization equilibrium experiments. All lanes in the left half of the gel were loaded with the same sample, at successive (10 min) intervals (the right half of the gel shows the same procedure but a different sample). The two brightest bands correspond to monomers (in front) and dimers; the trimer band is also visible. Some monomer-dimer interconversion takes place as the sample runs through the gel, visible as interband "smear." The purpose of this "time delayed" procedure is to extrapolate back in time the initial (equilibrium) concentrations of monomers and dimers at "zero" time. The intensity profiles for the three lanes encased in the box (yellow) are shown in blue or dark gray in the graphs, while the fit with the reaction-diffusion model used to extract the equilibrium concentrations of monomers and dimers is shown in red or light gray.

on three parameters: the critical bending torque τ_c, the bending modulus B, and the contour length of the molecule $2L$.

For the stretching energy of the ss part $E_s(x)$ we use a polynomial expansion of the Marko-Siggia expression [4]:

$$E_s(x) = \frac{9kT}{4N_s\ell_s^2}\left[x^2 + \frac{x^3}{N_s\ell_s} + \frac{3x^4}{(N_s\ell_s)^2}\right], \tag{6}$$

where ℓ_s is the persistence length of ss DNA.

The elastic energy $E_{\rm tot}$ is calculated from

$$E_{\rm tot} = E_d(x_{\rm eq}) + E_s(x_{\rm eq}), \tag{7}$$

where $x_{\rm eq}$ is determined from the mechanical equilibrium condition:

$$(\partial E_d/\partial x)_{x_{\rm eq}} + (\partial E_s/\partial x)_{x_{\rm eq}} = 0. \tag{8}$$

Finally, τ_c in (5) is adjusted to fit the calculated value of $E_{\rm tot}$ to the measured value. It is useful to perform the measurements for a series of molecules with increasing N_s and fixed N_d, as this corresponds, in effect, to varying the EED x (see Fig. 1). In Fig. 3 we show measurements of the elastic energy $E_{\rm tot}$ for such a series of molecules, with $N_d = 18$, and N_s varying between 12 and 33. The two different regimes are obvious; for $N_s < 24$ the ds part of the molecule is kinked, while for $N_s > 24$ it is smoothly bent (on average). The line in the figure represents the model (5)–(8), giving a value $\tau_c = 26.9$ pN × nm (we used $B = 200$ pN × nm^2, $\ell_s = 0.764$ nm, consistent with literature values). These data are from Ref. [10], reproduced here to make the paper self-contained.

Now we concentrate on the kinked states and explore the sequence dependence of the critical bending torque τ_c. Figure 4(a) shows how the critical bending torque τ_c is influenced by the identity of the bases on each side of the nick. Note that τ_c determines both the slope and the magnitude of the

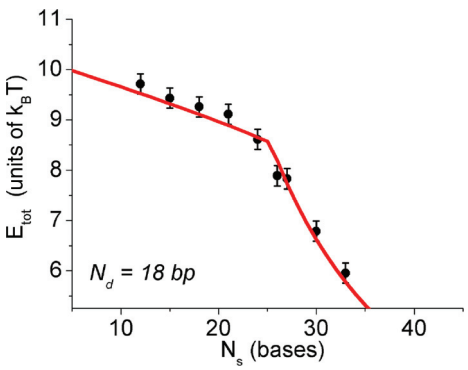

FIG. 3. (Color online) The elastic energy $E_{\rm tot}$ [Eq. (4)] measured for a series of molecules as in Fig. 1, with fixed $N_d = 18$ (the number of bp in the ds part) and varying N_s (from Ref. [10]. For $N_s < 24$ the ds part of the molecule is kinked, for $N_s > 24$ it is smoothly bent; the solid line is calculated as explained in the text, giving the value $\tau_c = 26.9$ pN × nm for the critical bending torque.

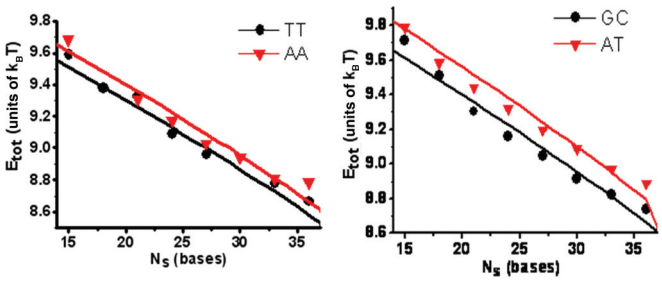

FIG. 4. (Color online) The influence of base sequence on the critical bending torque τ_c. (a) The elastic energy $E_{\rm tot}$ in the kinked regime for two series of molecules (fixed $N_d = 24$) differing only in the two base pairs flanking the nick. For the circles, the base sequence around the nick is TT, for the triangles AA. The corresponding values of τ_c are essentially indistinguishable: $\tau_c = 26.4$ pN × nm and $\tau_c = 26.7$ pN × nm. (b) The elastic energy $E_{\rm tot}$ in the kinked regime for two series of molecules (fixed $N_d = 24$) differing only in a 6 bp tract around the nick. The circles correspond to a GC tract, the triangles to an AT tract. The corresponding values of τ_c are once again essentially the same: $\tau_c = 26.7$ pN × nm for the GC tract and $\tau_c = 27.2$ pN × nm for the AT tract.

energy in this representation, so these are quite constrained one parameter fits. We measured the elastic energy for three series of molecules, respectively, with bases TT, AA, and GG at the nick and otherwise identical. For clarity, we show only two sets of experimental data and fitting curves in Fig. 4(a). The critical bending torque τ_c obtained from each set of measurements is listed below:

DNA with sequence AA around the nick: $\tau_c = 26.7$ pN × nm;

DNA with sequence TT around the nick: $\tau_c = 26.4$ pN × nm;

DNA with sequence GG around the nick: $\tau_c = 26.7$ pN × nm.

The above results show that there is essentially no dependence of the critical bending torque τ_c on the identity of the bases flanking the nick. Given this null (but interesting) result, we did not think it useful to examine all possible combinations of nearest neighbors, or to study the effect of next-nearest neighbors. We infer from our null result that the bending elasticity in the nonlinear (kinked) regime is not determined by a single base stacking interaction at the nick, but is a more collective property of the system, even though the kink itself may be a localized defect. This is also the meaning of the statement in the title that τ_c is a materials, or thermodynamic, parameter.

We next investigated whether base pairing energies influence τ_c. To this end, we compared two ds sequences, one with a 6 bp GC tract around the nick, the other with a 6 bp AT tract; the rest of the sequence was identical in the two cases. Figure 4(b) shows the measured elastic energies; the values for τ_c extracted from these measurements are

DNA with 6 bp GC tract around the nick: $\tau_c = 26.7$ pN × nm;

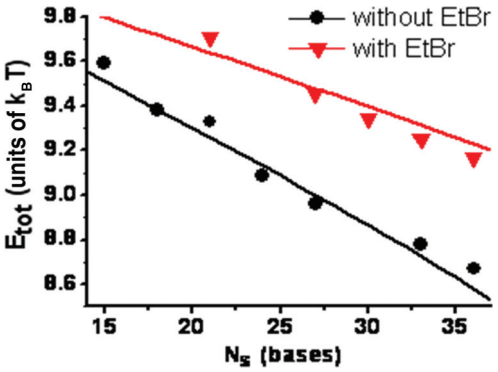

FIG. 5. (Color online) The influence of EtBr binding on the elastic energy of a series of molecules with $N_d = 24$; triangles are in the presence of 10 μM EtBr, circles in the absence of EtBr. The total ds DNA concentration is 2 μM; i.e., the molar ratio is 1 EtBr molecule every 4–5 DNA bp. The solid lines are fits to the model [Eq. (5)–(8)], using a 20% longer contour length for the triangles, i.e., $2L = 0.33$ nm × N_d for the circles but $2L = 0.40$ nm × N_d for the triangles. The extracted values of τ_c are, however, essentially the same in the two cases: $\tau_c = 26.8$ pN × nm with EtBr and $\tau_c = 26.4$ pN × nm without. In short, the increase in contour length of the DNA with EtBr bound accounts entirely for the difference in measured elastic energy in the two cases; the critical bending torque τ_c does not change.

DNA with 6 bp AT tract around the nick: $\tau_c = 27.2$ pN × nm.

Again τ_c is essentially identical in the two cases, in fact, slightly (but measurably) larger for the molecules with the AT stretch around the nick, although this tract is of course thermally more unstable than the GC tract. This result proves that for these molecules there is no ss "bubble" formed at the kink. This agrees with the conclusion of our recently published study of the temperature dependence of the elastic energy of these molecules [21].

Having established to our satisfaction that the bending torque τ_c is essentially independent of base sequence, we turned to small molecule binding and examined the effect of ethidium bromide (EtBr: $C_{21}H_{20}BrN_3$), a well-known DNA fluorescent dye, on this elastic parameter. EtBr binds to ds DNA intercalating between the bases; in the presence of 10 mM EtBr, we do find a measurable increase in the elastic energy of the molecules (Fig. 5), though this increase is small (∼6%). However, this change can be attributed entirely to the increase in contour length $2L$ of ds DNA with EtBr bound [22]. We fit the energy graphs of Fig. 5 implementing in the model a change in contour length $2L$ with EtBr (27% increase) which is appropriate to our conditions according to literature values; then we find essentially no change in τ_c:

DNA in the presence of 10 μM ethidium bromide: $\tau_c = 26.6$ pN × nm;

DNA in the absence of ethidium bromide: $\tau_c = 26.4$ pN × nm.

IV. DISCUSSION

DNA flexibility has been studied through cyclization experiments for more than 30 years [7,23–28], the recent

focus mostly being whether the WLC energy (1) is a good representation of this flexibility, and its limits of validity [12]. The study by Cloutier and Widom [25] reporting enhanced flexibility, i.e., softening, of DNA for large curvatures compared to the WLC (check) sparked the development of models to address this softening [29–32], more experiments [26,33,34], and controversy. The last arises because cyclization measurements are nonequilibrium experiments in which one measures rates: the interpretation of the measurements and model building from the data is necessarily not straightforward.

However, instead of studying *the process* of bending, as a first step it seems desirable to study the simpler problem of the equilibrium elastic energy of DNA *constrained* into a bent conformation. This is the strategy of the present experiment, which was first reported in Ref. [9]. The simplicity of the experiment, where the elastic energy is measured directly, is rewarded by the appearance of an unambiguous signature of the softening transition, in the form of a kink in the energy curve of Fig. 3. It also suggests a minimal model, where the WLC solution (1) breaks down for a critical value τ_c of the internal bending torque of the molecule [9,10]. We have shown that this one-parameter model quantitatively describes the elastic energy of whole series of differently stressed molecules [10]. This emboldens us to propose τ_c as a materials parameter of DNA mechanics on the same standing as the bending modulus B (or equivalently the persistence length l_p). It is then interesting to ask how this materials parameter depends on base sequence and conditions, which we address partially in this paper (the temperature dependence of τ_c is explored in Ref. [21]). If τ_c had large variations with sequence, it would be of limited utility as a "materials" parameter; the same could be said of B. However, we find that τ_c is, on the contrary, remarkably robust. Because we associate the critical bending torque τ_c with a localized kink in the middle of the molecule (where the bending torque is maximum in our geometry, and which is also the position of the nick), it is natural to assume that if there is a sequence dependence to τ_c, the most critical bases would be the ones around the nick. Correspondingly we explored different combinations of bases nearest neighbors (nn) to the nick [Fig. 4(a)] but found no measurable variations in τ_c. In our description this says that τ_c is a thermodynamic parameter determined microscopically not by one single stacking interaction at the nick but rather by a more collective effect. A different but related consideration is that the kink in our constructions may not necessarily occur at the nick. Given sequence-dependent changes in twist, and uncertainty about the junction between the single and double stranded segments of the molecule, it is not obvious where the nick will be positioned, on average, with respect to the plane of the molecule. If the nick is positioned on the inner face of the double stranded segment, it is under compression, and a kink opening the opposite side may form independently of the nick, and not necessarily exactly at the bp step corresponding to the nick. In this scenario, the identity of the bases flanking the nick may make no difference. Still the fact remains that even changing a 6 bp tract around the nick from AT to GC makes no difference to the measured τ_c. We note in this regard that Zhang and Crothers [24] find, through cyclization experiments, 28% lower bending rigidity for a long (30 bp) AT repeat compared to random DNA. We further note that specific sequence motifs may be more bendable than others, and that such effects may contribute to nucleosome positioning [35].

In summary, the microscopic conformation at the kink cannot be determined by these measurements alone, and several scenarios are possible, which are probably best investigated through MD simulations [36]. A simple kind of kink is formed by unstacking one bp step, opening the major groove side of the bp step and bending the DNA towards the minor groove. This conformation, suggested in Ref. [37], was observed in MD all-atoms simulations of DNA minicircles [36]. They also observed a second kind of kink in their simulation ("type II kink"), where one base pairing and one base stacking is broken. Both these defects are compatible with our measurements, we believe. In particular, our result that τ_c does not depend on the identity of the bases flanking the nick does not exclude the unstacked bp step (or the unpaired bp) scenario. Namely, breaking one base stacking has an energetic cost of $\sim$1 kcal/mol $\approx$ 1.5 kT with differences of order $\sim$0.5 kcal/mol for the different base combinations used in this study [38]. However, the elastic energy for our molecules, the work one has to do to bend a DNA oligomer sharply, is of order $\sim$10 kT. Therefore, the energy scale of one stacking interaction cannot be determining the elastic energy of the sharply bent molecule, whereas the energy scale of the critical bending torque $\tau_c \approx 30$ pN $\times$ nm ≈ 7 kT does. So it is perhaps not surprising that τ_c is not sensitive to single bp x substitutions.

A second possibility for the conformation of the kink is a ss "bubble," suggested by Ref. [29]. We believe we can exclude this possibility for our nicked molecules, because of the very slight temperature dependence of the elastic energy of these molecules [21], and because substituting a 6 bp AT tract around the nick for a GC tract does not affect the elastic energy [Fig. 4(b)].

A third possibility for the kink may be a short region where DNA transitions to the S form [39,40]. S DNA has some of the required properties in view of the present results and the results in Ref. [21], including bending softness (persistence length $\sim$12 nm [41,42], negligible entropy change for the B to S transition [24,43], and insensitivity of the B to S transition to bp composition [41,44]. However, the microscopic conformation of S DNA is not yet clear [45]. On the other hand, for our molecules, the microscopic conformation of the kink could conceivably be obtained by direct structure determination.

Finally, we address again the concern expressed in some quarters that the softening transition shown in Fig. 3 may be due to the DNA in our construct peeling off at the ends. This scenario is contradicted by experimental controls published in Ref. [21], namely: (a) experimental measurements with sequences of high or low GC content at the ends yield the same value of τ_c, with the softening transition occurring at the place predicted by the critical torque theory, specifically, the sequence ($N_d = 18$): 5′-CTC TCA CGT TCG TCG TAT, which has a low binding energy TAT triplet at one end and

a high binding energy triplet CTC at the other end, and the sequence ($N_d = 30$) 5′-CTG CTC TCA CGT GTG GAG TCG TCG TAT GTC, which has high binding energy triplets at both ends, exhibit the same τ_c; and (b) experimental measurements at different temperatures between 20 and 70 °C show that the observed softening transition is essentially unaffected by temperature in this range. Both controls are incompatible with melting at the ends being at the origin of the softening transition in our nicked molecules. Also incompatible with this scenario is the range of the "kinked" regime versus N_s for the larger N_d molecules (Fig. 5 in Ref. [21]).

Last but not least, the physics goes against the peeling at the ends scenario. Namely, to the extent that we can schematize the ds DNA in our constructs as a semi-flexible rod (the view of the WLC model) pulled in at the ends, there is no force trying to separate the strands at the ends, because the curvature at the ends is zero since the torque is zero. There is, however, a force trying to separate the strands in the middle of the construct, where the torque and the curvature are maximum. Thus with the nicked constructs of the present study, if the observed softening transition was due to local melting of the helix, the melting would be in the middle, not the ends. This is the "bubble at the kink" scenario of Ref. [29] (itself ruled out, for our nicked constructs, by the measurements versus temperature in Ref. [21] and also by the measurements in the present paper where we substitute an AT tract for a GC tract around the nick and observe no change in τ_c).

Without nick, the argument above may not apply, since peeling off at the ends might be a lower free energy solution than peeling off in the middle, because of the bubble entropy term. While this is irrelevant for the present experiments which concern nicked DNA only, what is really the situation for non-nicked DNA? It has become apparent that there are two schools of thought on this point. One believes that the bending mechanics of nicked DNA is very different from that of intact DNA. We subscribe to the opposite position, that the two are quite similar, differing only, in our description, in the precise value of the critical bending torque τ_c. We came to this conclusion through a series of measurements of the elastic energy of the same constructs as we use here but without nick [11]. The caveat is that those experiments use a rather more indirect method to measure the elastic energy, based on melting curve analysis. On the other hand, we also find that Zhang and Crothers [24], using their high-throughput cyclization experiments, come to the conclusion that "the nicks hardly alter the bending flexibility" of DNA. Then we see the cryo-EM study [46], where the authors find that 94 bp long DNA minicircles have the same shape (with no kinks) whether nicked or not nicked. So we keep our opinion that nicked and intact DNA have similar bending mechanics and encourage our readers to form their own.

In summary, we find that the critical bending torque τ_c is indeed a robust materials parameter essentially independent of local changes in the sequence, with the previously determined value $\tau_c \approx 27$ pN × nm for nicked DNA [10]. We then examined the effect of ethidium bromide (EtBr), an intercalator which substantially modifies the structure and thermal stability of ds DNA. Here we find that the elastic energy of stressed molecules as in Fig. 1 is indeed different (though the difference is small) in the presence and absence of EtBr (Fig. 5), but this difference is accounted for entirely by the change in contour length of ds DNA with EtBr bound [22], i.e., by a geometrical effect. The critical bending torque τ_c is unaffected. More in detail, the experimental conditions of Fig. 5 were 10 μM EtBr and 2 μM ds DNA of length 24 bp, that is, five molecules of EtBr per ds DNA 24mer. If the saturation binding is 1 EtBr every 2 bp [47], and if this corresponds to ∼40% elongation [22], we expect ∼20% elongation in our case. In the fit shown in Fig. 5 we used a value of L 20% larger ($2L = 0.40$ nm × N_d instead of $2L = 0.33$ nm × N_d); this returns the value $\tau_c = 26.8$ pN × nm, essentially the same as without EtBr. The Bustamante group, in their first report on the manipulation of single DNA molecules [3], also considered the effect of EtBr on the mechanics of this molecule; they similarly found that the change in the force-extension curve could be interpreted by a change in contour length of the molecule without any change in the bending modulus B. With the present measurement, we conclude that both the linear (B) and the nonlinear (τ_c) bending elasticity parameters of DNA are unaffected by the presence of EtBr. We may view this result as a remarkable control of the soundness of the critical bending torque model: it accounts exactly for the two energy curves (with and without EtBr) in Fig. 5 by changing in the model the one parameter that is known to be affected by EtBr, namely, increasing the contour length $2L$ of the molecule.

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