Measurements of the hysteresis in unzipping and rezipping double-stranded DNA

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Complete unzipping and rezipping of λ-phage double-stranded DNA is achieved by applying a constant force. A strong hysteresis is observed at all tested time scales and temperatures. Hysteresis also occurs for partial unzipping, indicating stability for the partially open state over a force range of 2–5 pN. Results are compared to nearest-neighbor model simulations, and reasonable agreement is found.

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I. INTRODUCTION

The separation and recombination of the two strands that compose a double-stranded DNA (dsDNA) molecule is essential for biological processes such as transcription and replication. Not only is the complete opening and closing of DNA important, but the maintenance of partially open dsDNA is necessary as well. *In vivo*, several biological elements, such as single-stranded binding proteins and helicases, are involved in maintaining partially open DNA; however, determining the stability of partially open DNA as a function of force alone may provide useful information of conditions in which additional stabilizing factors are required.

Much theoretical work has studied the unzipping and rezipping of homopolymers as a function of applied force [1–4]. At equilibrium, a homopolymer can be described by a two-state system, either completely zipped or completely unzipped. There are no stable states in which a homopolymer is partially open. However, natural DNA is a heteropolymer, which is not adequately described by homopolymer theories in all regimes [5–9]. In particular, heteropolymer theories predict metastable partially open states and these have been found in natural DNA [10].

DNA unzipping has been studied experimentally using single-molecule techniques such as atomic force microscopy [11], optical traps [12], magnetic tweezers [10], and soft microneedles [13]. The unzipping is usually carried out either at constant force [10] or at constant displacement [12,14]. λ-phage dsDNA has been unzipped in both regimes. At constant rates, sequence-dependent fluctuations in force are observed in both unzipping and rezipping [12,13]. At constant force, unzipping is characterized by sequence-dependent jumps and pauses in the extension [10]. The pauses indicate a partially open state consisting of both single-stranded DNA (ssDNA) and dsDNA, where the applied force prevents the molecule from unzipping or rezipping. The existence of these metastable states in natural DNA and RNA has been discussed in the literature [5,9,10,15,16] and attributed to the presence of energy barriers that prevent the molecule from unzipping or rezipping to a more energetically favorable state. The duration of the metastable states has been investigated [5]; however, the range of forces over which these metastable states persist has not yet been explored.

One method for probing the stability of partially open states is unzipping and *rezipping* DNA by application of a constant force. Determining both the unzipping force F_u and the rezipping force F_z allows one to measure the range of forces over which open dsDNA is stable for a given time.

This work presents the complete unzipping and rezipping of λ-phage DNA by constant force. The measurements show a strong hysteresis, resulting in $F_z \sim 0.7F_u$, which indicates that partially open states can be very robust as a function of force. This hysteresis is shown to persist over a broad temperature range including 35–40 °C, the temperature range characteristic of most mammalian cells.

II. MATERIALS AND METHODS

A. Experimental setup

The DNA construct used for unzipping measurements has been described previously [10]. Briefly, it consists of a linker λ-DNA (New England Biolabs) which is hybridized and ligated to one end of the λ-DNA strand which is to be opened. The second strand of the λ-DNA to be opened is hybridized and ligated to a biotinylated oligonucleotide. The other end is closed with a hairpin loop to prevent complete separation of the construct in an unzipping event. The linker λ-DNA is tagged with a digoxigenin-labeled oligonucleotide. It is attached to a glass capillary coated with an antidixogenin antibody. The λ-DNA strand to be opened is bound to a 2.8-μm streptavidin-coated magnetic bead (Dynabeads) via the biotinylated oligonucleotide. The λ-DNA and beads are stored at 4 °C after preparation and incubated at room temperature for 15 min prior to the experiment. The unzipping experiments are done in a PBS buffer (*p*H 7.4, 10 mM phosphate, 137 mM NaCl, 2.3 mM KCl).

Mechanical unzipping of dsDNA is carried out by a magnetic tweezer apparatus [17]. A stack of magnets exerts a force F on the magnetic beads by $F=m\nabla B$, where m is the magnetization of the bead and B is the magnetic field. The force on the beads is controlled by the distance of the magnet from the beads, with a force range of 1–30 pN. The spread in the magnetization of the beads leads to a standard deviation of ~30% in the force measurements. The experimental temperature is controlled by placing a thermoelectric cooler on top of the aluminum sample holder, with an uncertainty of ±1 °C.

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TABLE I. Thermodynamic parameters for helix propagation [17] used in Monte Carlo simulations. Only two nucleotides of the base quartet are shown. The other two are obtained from the usual complementarity *A-T* and *G-C*.

Base quartet	ΔH (kcal/mol)	ΔS (e.u.)
5′-*GC*-3′	−11.1	−28.4
CG	−10.1	−25.5
GG	−6.7	−15.6
GA	−7.7	−20.3
GT	−8.6	−23.0
CA	−7.4	−19.3
CT	−6.1	−16.1
AA	−8.4	−23.6
AT	−6.5	−18.8
TA	−6.3	−18.5

B. Simulations

Monte Carlo simulations are carried out using experimentally determined nearest-neighbor energy parameters for the sequence (48 502 bps) of λ-phage [15,18]. The energy difference ΔG between an open base pair and a closed base pair is determined by the free energy of ssDNA under an applied force $g_u(F)$ and the enthalpic difference, ΔH, and entropic difference, ΔS, between dsDNA and ssDNA:

$$\Delta G = \Delta H - T\Delta S - 2g_u(F). \tag{1}$$

The free energy term $g_u(F)$ can be derived analytically by integrating the extension $x(F)$ of the ssDNA, modeled by a modified freely jointed chain (mFJC),

$$x(F) = NL\left[\coth\left(\frac{Fb}{k_bT}\right) - \frac{k_bT}{Fb}\right]\left(1 + \frac{F}{S}\right), \tag{2}$$

where N is the number of bases, L is the spacing between bases (5.6 A), b is the Kuhn length (1.5 nm), and S is a characteristic force (800 pN) related to the bond elasticity [5,19]. Values for ΔH and ΔS from Ref. [18] are given in Table I.

III. RESULTS

The complete unzipping of λ-DNA is carried out by incrementally increasing the applied force F by ΔF every time interval Δt; likewise, rezipping is accomplished by incrementally decreasing F by ΔF every Δt. The instantaneous extension of the molecule is measured once a second. Partial unzipping is carried out in a similar fashion and will be discussed later. Figure 1 shows typical extension versus force data for a single molecule that is unzipped and subsequently rezipped at room temperature where $\Delta t = 10$ s and $\Delta F = 0.5$ pN. Thus there are ten extension measurements at each force. Initially F is increased from 14 pN to 19 pN without resulting in any observable unzipping. As F is increased above 19 pN, unzipping occurs in a series of jumps and

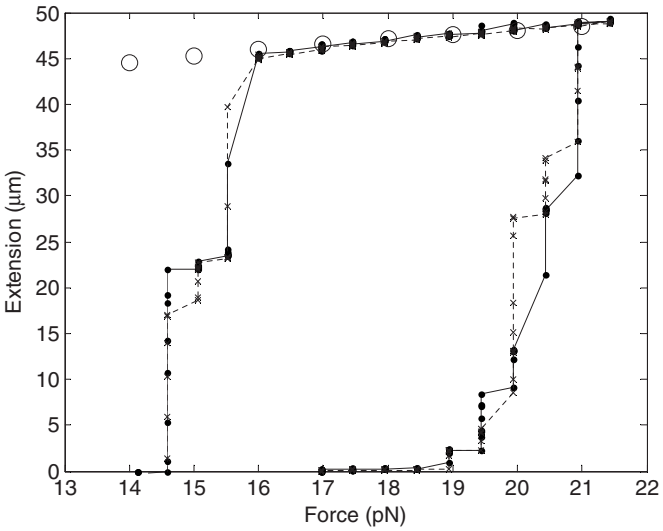

FIG. 1. Complete unzipping and rezipping of λ-phage DNA. The open circles show the mFJC-predicted extension for ssDNA of length 97 kbps. The force is incrementally increased or decreased every 10 s. The measurement is taken twice, the first cycle shown by the solid line, the second cycle shown by the dashed line.

pauses until the entire molecule is unzipped with a total extension of approximately 48 μm at $F = 21$ pN. The solid line in Fig. 1 indicates the extension of the molecule as it is unzipped for the first time. The pauses in unzipping can be seen as plateaus, in which the extension shows little change as force is increased. We observe that the extension at these points is not constant; this results from a combination of ssDNA stretching due to the increased force and from limited unzipping about the pause point. The unzipping rate has a range of up to 8 kbp/s. Once the DNA is fully unzipped at 21 pN, increasing F to 22 pN does not significantly change the extension. At this point, F is then decreased in steps of 0.5 pN every 10 s. The unzipped molecule in Fig. 1 shows a slight decrease in extension as F is lowered from 21 pN to 16 pN due to the elasticity of ssDNA. The open circles in Fig. 1 show the extension as a function of F predicted by Eq. (2) for the case where the DNA remains completely open, and the agreement with the observed extension indicates negligible unzipping in this force range. Thus, the fully unzipped state is quite stable in the force range 16–21 pN. As F is decreased below 16 pN, the extension of the molecule rapidly decreases, indicating the rezipping of the dsDNA at a rate of approximately 10 kbp/s. At 14 pN the DNA is fully rezipped and the cycle is then repeated, shown by the dashed line in Fig. 1, to demonstrate the variation in single-molecule trajectories and the reproducibility of the measurements. A very clear hysteresis is seen in the force versus extension curve for $F = 14$-21 pN. This hysteresis represents a deposition of 2×10^{-18} J of energy into the system.

The hysteresis of the system is due to sequence-dependent energy barriers within the unzipping landscape [5,9,16]. Natural DNA is composed of both *A-T* base pairs and *G-C* base pairs, which have binding energies ranging from roughly $1k_bT$ to $3k_bT$. A homopolymer approximation can be made by determining the average binding energy of the entire sequence. In this approximation, $F_H = F_u = F_z$ can be cal-

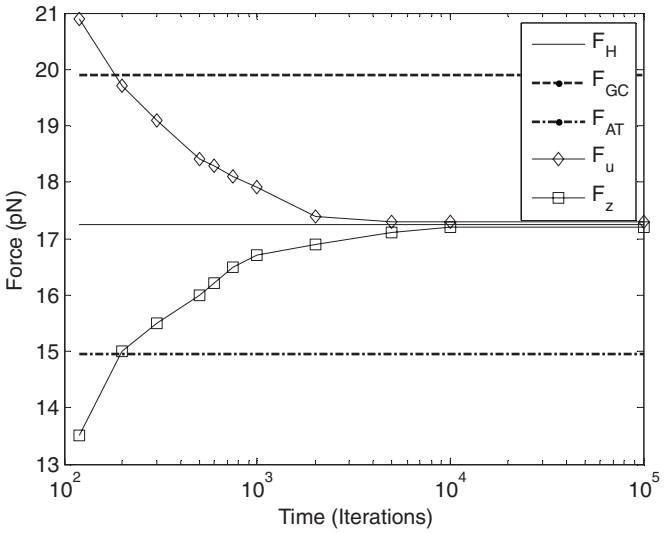

FIG. 2. Simulated F_u (diamonds) and F_z (squares) compared to the homopolymer critical force (F_H, solid line) for the polymer $(A)_6(G)_6$ as a function of iterations. The dashed lines show the calculated force for a pure G polymer and a pure A polymer, 19.9 pN and 14.95 pN, respectively.

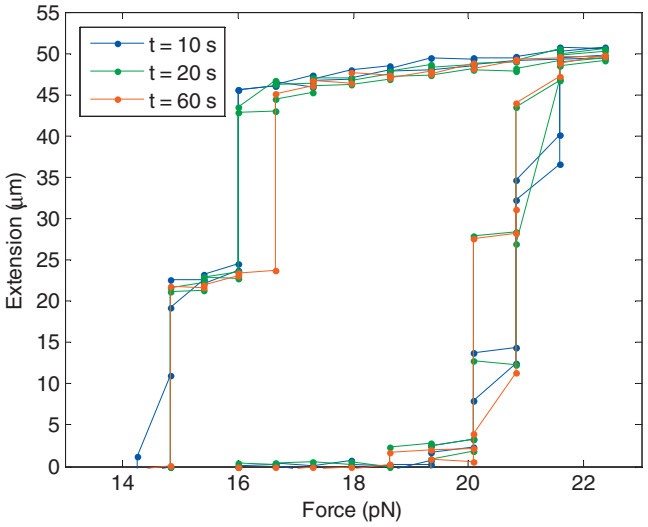

FIG. 3. (Color) DNA extension as a function of applied force. The force is incrementally increased or decreased every Δt seconds for Δt=2, 10, 60. Hysteresis is not significantly altered over this time range.

culated by Eq. (1) where F_H is the force at which the free energy of the completely unzipped molecule equals that of the completely zipped molecule. However, the energy landscape will still contain local energy barriers at this force. Furthermore, the height of the energy barriers will depend on the applied force, and barriers encountered in unzipping at a higher force may not correspond to those seen in rezipping at a lower force. Experimentally, the time required for unzipping or rezipping to occur is determined by the height of these energy barriers at the applied force. Thus it is expected that the amount of hysteresis observed will be dependent on the sequence being unzipping and the time scale of the experiment. A Monte Carlo simulation of a simple heterogeneous sequence composed of five $(A)_6(G)_6$ repeats has been carried out to explore the time dependence on F_u and F_z. The forces required for complete unzipping and rezipping to occur an allotted number of iterations are shown in Fig. 2.

There are three basic regimes for barrier penetration. At very long times, any energy barrier can be overcome due to thermal fluctuations and F_u=F_z=F_H, as shown in Fig. 2. At very short times, F_u will exceed the G-C unzipping force and F_z will be smaller than the A-T rezipping force: not only must the energy barriers be removed, but a downhill slope at all points in the energy landscape is required at these short times. Without a downhill slope, the unzipping fork will exhibit slow diffusive behavior [5]; however, by increasing the force, the diffusion has a preferred direction and unzipping can occur in the short time allotted, as shown in Fig. 2.

Between these two regimes of very long and very short times, a quantitative analytical relationship between the energy barrier height and the time required to overcome it with thermal fluctuations can be derived from basic thermodynamics. The time t required to overcome an energy barrier of height ΔE is determined by

$$t = t_0 e^{\Delta E/kbT}, \tag{3}$$

where t_0 is a characteristic attempt time. Likewise, the height of the barrier which can be overcome in a time t can be found by

$$\Delta E = k_b T \ln(t/t_0). \tag{4}$$

To a first-order approximation, ΔE is proportional to the applied force [5], and thus the force required for unzipping and rezipping within an allotted time t will approach the equilibrium force in a logarithmic fashion [5].

The time dependence of the observed hysteresis in λ-phage is experimentally examined by varying Δt from 10 s

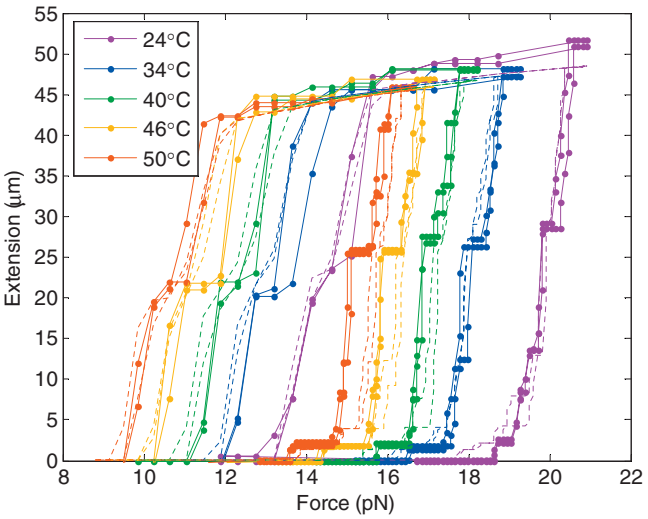

FIG. 4. (Color) DNA extension (solid lines) as a function of applied force for temperatures 24–50 °C. The force is incrementally increased or decreased every 2 s. Simulations at corresponding temperatures and forces are shown in dashed lines.

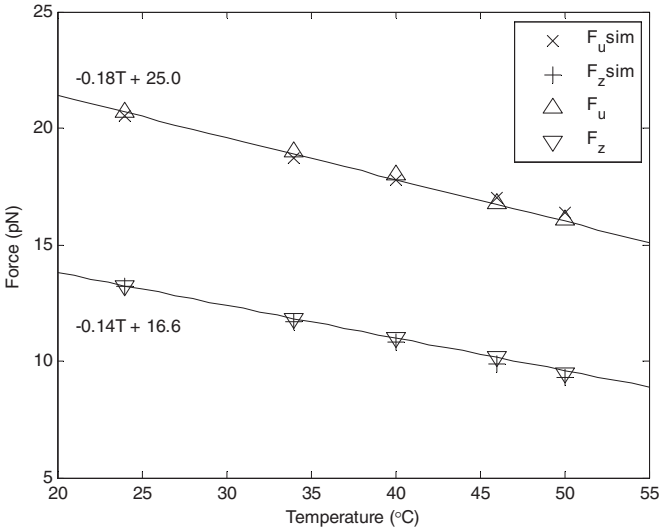

FIG. 5. F_u and F_z, defined as the force at which the molecule is completely unzipped or zipped, as a function of temperature. The triangles represent actual data points; the solid lines are linear best fits to the data. The simulated values for F_u and F_z show reasonable agreement.

to 1 min, shown in Fig. 3. In this regime, the hysteresis is not strongly sensitive to Δt. Finite lifetimes of the molecular construct prevent a study at longer times; however, simulations predict that time scales of days to weeks would be necessary to significantly reduce the amount of hysteresis seen in experiment. Given that DNA replication and transcription must take place in minutes or hours, the hysteresis seen in experiment is expected to correspond to processes occurring *in vivo*.

Many biological processes are known to be temperature sensitive, and DNA *in vivo* is generally maintained above room temperature; consequently, it is useful to determine the temperature dependence of the hysteresis. As temperature is increased, dsDNA becomes less stable and both F_u and F_z should decrease with temperature. The solid lines in Fig. 4 show the measured unzipping and rezipping of a single molecule at multiple temperatures ranging from 24 to 50 °C, where the red curve corresponds to the highest temperature and the violet curve to the lowest. As expected F_u and F_z decrease with increasing temperature, though the hysteresis remains approximately constant. The temperature dependence of F_u and F_z can be more easily distinguished in Fig. 5, which displays F_u and F_z corresponding to the data shown in Fig. 4. In this temperature range, a linear dependence is found with F_u decreasing by 0.18 pN/ °C and F_z decreasing by 0.14 pN/ °C. These best fit lines are shown by the black lines in the figure. This temperature experiment has been carried out for several molecules, and similar results are observed.

Previous work has used Monte Carlo simulations based on the nearest-neighbor model [15] to predict the pause points in the unzipping. We have applied a similar treatment to predict the force versus extension curves as a function of temperature, and the results are shown by the dashed lines in Fig. 4, where the conversion from unzipped base pairs to extension was achieved using Eq. (2). It can be seen from Fig. 4 that reasonable agreement is found. The simulated and experimental values of F_u and F_z agree to within 1 pN, as shown in Fig. 5. The best correspondence between experiment and simulation is obtained for a characteristic attempt frequency of 10^5 s^{-1}, which is fairly consistent with previous estimates of 10^6 s^{-1} [9].

The unzipping force for λ-phage as a function of temperature has been reported previously [20], and the measurements showed deviations from the theoretical model presented in this work. In that report, both the amount of unzipping measured and the time allotted for unzipping, 1500 bps in 15 min, differed from this work, 48.5 kbps in approximately 10 s. It is possible that this experiment is not as sensitive to local conformational changes, as in the experiment presented by Danilowicz *et al.* [20]. Further investigation is required to determine the underlying cause for the apparent discrepancy between these two measurements.

Thus far it has been demonstrated that fully unzipped DNA is very stable as a function of applied force, but one might be concerned that this stability is a unique property of the fully unzipped state, which may not apply to DNA that is only partially open. Thus we will consider the robustness of a pause point at a position in the sequence where the DNA is not fully unzipped.

As a molecule unzips, there is a "fork" or a boundary between ssDNA and dsDNA. The fork moves along the sequence as more ssDNA opens up, until the end of the sequence is reached. Once the DNA has fully unzipped, the fork is lost. In order to rezip, the molecule must reestablish a new fork at the correct point in the sequence. It is possible that a free energy barrier to the formation of a new fork may cause the observed hysteresis in complete unzipping [21]. To explore this possibility, the complete unzipping and rezipping of λ-DNA, which was shown in Fig. 1, was followed by partial unzipping, shown by the solid curve in Fig. 6. The partial unzipping is achieved by increasing F to 20.5 pN. At this force, the extension of the molecule is 28 μm, corresponding to approximately 28 kbps. Unzipping to this point is followed by a decrease in force that eventually results in complete rezipping when F is decreased below 15 pN. The cycle was repeated in which the molecule was unzipped to an extension of 34 μm, corresponding to approximately 34 kbps. This is again followed by a complete rezipping when F is decreased below 15 pN.

While F_z of the partially open molecule is unchanged from F_z of the completely open molecule, a limited amount of rezipping does occur at higher forces. The open circles and squares in Fig. 6 show the expected extension for 28 kbps and 34 kbps, respectively. This corresponds with the measured extension for forces between 18 and 20 pN; however, between 15 and 18 pN the measured extension decreases slightly, corresponding to limited rezipping. Thus the number of open base pairs is stable over a range of 2 pN, while it shows limited rezipping over a range of 5 pN. Hence the large hysteresis is not unique to the *complete* unzipping of the molecule; *partial* unzipping also results in significant hysteresis.

λ-phage has an unusual sequence that contains a very deep energy minimum when unzipped from the *AT*-rich end, as shown in Fig. 7(a). F_H for the *AT*-rich part F_{HAT} of the

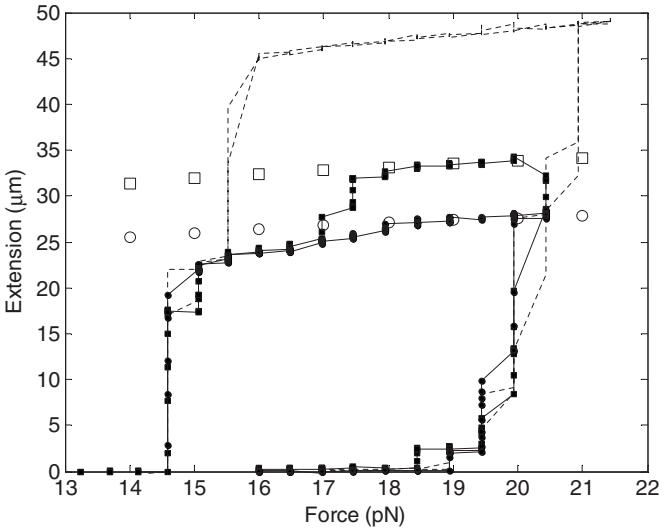

FIG. 6. Complete unzipping and rezipping of λ-phage DNA (dashed line) followed by a partial unzipping and rezipping (solid line). The force is incrementally increased or decreased every 10 s. The measurement is taken twice, the first cycle shown by solid circles, the second cycle shown by solid squares. The open circles and squares show the mFJC-predicted extension for ssDNA of length 56/68 kbps. The partially open DNA is stable over a force range of 2–5 pN.

sequence is significantly lower than the F_H for the entire sequence, and thus the energy landscape, at $F=F_{HAT}$, for unzipping halfway through sequence does not exhibit this large energy minimum. However, the observed hysteresis for partial unzipping is relatively unaltered as shown in Fig. 6. Finally, when λ-phage is unzipped from the *GC*-rich end, the energy landscape exhibits a large maximum rather a deep minimum. We have carried out unzipping and rezipping experiments beginning at the *GC*-rich end and have obtained a similar hysteresis (data not shown). Thus the hysteresis is not dependent upon λ's unique deep energy minimum, but would be expected for any natural sequence.

Alternatively, the robustness of partially open DNA as a function of force can be measured by partially unzipping a molecule by applying a force F_0 and then adding a small oscillating force component. For a robust pause point, the molecule will neither unzip nor rezip if the force is kept constant at F_0. If an oscillating force is added to F_0, the molecule can begin to rezip or unzip locally and one can probe the energy landscape near the pause point by examining the change in extension as a function of the oscillating force. As shown in Fig. 7(b), the deep energy minimum near 27 kbps consists of a series of closely spaced energy minima, so applying a small oscillating force can move the unzipping fork from one energy minimum to another.

For very small amplitudes, the number of base pairs is expected to remain unchanged because energy barriers prevent either unzipping or rezipping. At slightly higher amplitudes, local unzipping or rezipping will begin to occur as the molecule is able to overcome small energy barriers. The symmetry between unzipping and rezipping behavior as well as the stability of the pause points depends on the initial choice of F_0 as well as the details of the energy landscape.

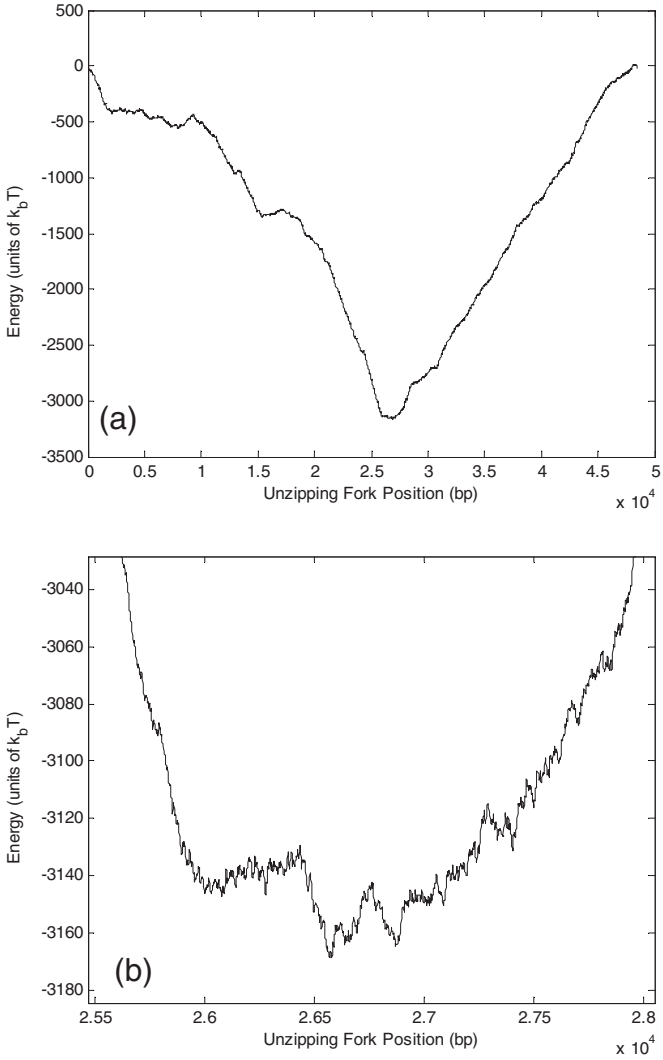

FIG. 7. (a) The energy landscape of λ-phage where unzipping is initiated from the *AT*-rich side. (b) A close-up view of the energy landscape near the deep energy minimum.

Since we are exploring points near the deep potential minimum shown in Fig. 7(b), the increasing amplitude will result in increased unzipping and rezipping, but the molecule will remain partially open. As the time-varying force reaches the critical value required to overcome all energy barriers, complete unzipping and rezipping will occur during each cycle of the oscillating force. Figure 8 shows the response of λ-DNA to a time-varying force. At $F_0=19$ pN the molecule is unzipped to an extension of 26 μm and an oscillating force with a slowly increasing amplitude is added. The oscillating force actually consists of incremental steps of 0.7 pN taken every 2 s. The green curve shows the extension as a function of time, and the blue curve shows the corresponding force as a function of time. As expected, limited unzipping and rezipping occurs within the deep minima while exhibiting pause points which are stable over 1–2 pN. Once a critical force amplitude is reached, complete unzipping and rezipping occur over the course of one cycle.

Many of the pause points seen in Fig. 8 were not seen in the case where the force was increased or decreased mono-

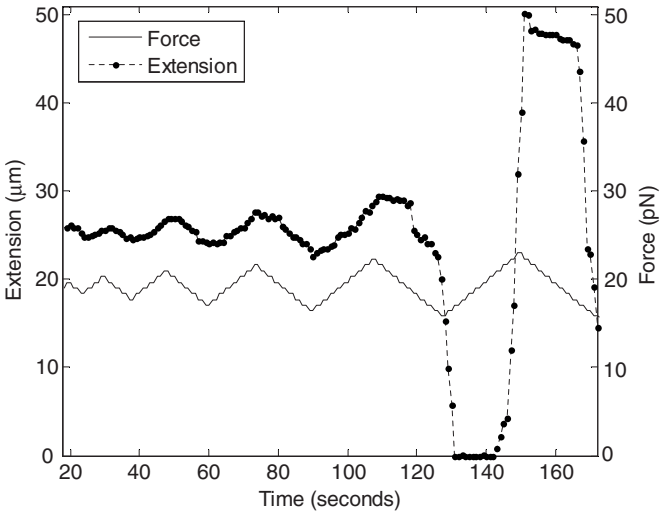

FIG. 8. Probing the pause points with an oscillating force. The solid line represents the force as a function of time. The dashed line represents the extension of the molecule as it responds to the external force.

tonically, so the oscillating force technique not only allows us to probe the stability of the pause points in response to an oscillating force; it allows us to measure the energy landscape in regions of the sequence where no pause points occurred when the molecule was unzipped or rezipped at constant force.

IV. CONCLUSIONS

Complete unzipping and rezipping of λ-phage dsDNA at room temperature has been performed, and a significant hysteresis in the extension versus force curves was observed for forces between 14 and 21 pN, resulting in an energy deposition of 2×10^{-18} J per unzipping-rezipping cycle. Partial unzipping reveals a similar hysteresis in the extension versus force curves with partially open DNA being stable over a range of 2–5 pN. The hysteresis remains almost constant for time steps ranging from 10 s to 1 min, suggesting that the hysteresis will persist even if the time steps were extended to days, exceeding the time required for most *in vivo* DNA processes. The hysteresis is also insensitive to temperature over a range of 24–50 °C, suggesting that changes in temperature do not influence the robustness of the partially or completely open DNA. The stability of the number of open base pairs over a range of forces up to 5 pN may be useful *in vivo* where enzymes must operate in an environment in which available energy may fluctuate.

ACKNOWLEDGMENTS

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