Single-molecule studies of DNA mechanics

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During the past decade, physical techniques such as optical tweezers and atomic force microscopy were used to study the mechanical properties of DNA at the single-molecule level. Knowledge of DNA's stretching and twisting properties now permits these single-molecule techniques to be used in the study of biological processes such as DNA replication and transcription.

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Abbreviations

AFM	atomic force microscopy
bp	base pair
ds	double-stranded
FJC	freely jointed chain
ss	single-stranded
WLC	worm-like chain

Introduction

Until recently, physical and chemical studies of DNA were performed in bulk, whereby large numbers of molecules were sampled simultaneously. Inherent averaging in such measurements makes it difficult to resolve the time-dependent stresses and strains that develop in DNA during the course of its biological reactions. Processes like protein-induced DNA bending, induced-fit molecular recognition between proteins and DNA, and the mechanochemical energy transduction of DNA-binding molecular motors were not directly accessible to study.

In the past decade, this situation has changed dramatically. New methods to manipulate single molecules now offer researchers the opportunity to directly measure the forces generated in biochemical reactions and, even, to exert external forces to alter the fate of these reactions.

Stretching DNA

The elastic behavior of dsDNA has been investigated in various laboratories using a variety of forces, for example, hydrodynamic drag [1–3], magnetic beads [4], glass needles [5] and optical traps [6,7]. Magnetic beads attached to the ends of DNA by biotin–avidin can be pulled by external magnets. These magnetic tweezers are a useful tool, particularly in the range between 0.01 and 10 pN. Slightly higher force regimes can be probed with optical tweezers, which allow one to apply and sense forces on micron-sized dielectric particles, such as plastic microspheres, in an aqueous environment [8,9]. A trap is formed by focusing a laser beam onto a micron-sized spot through a microscope objective. A particle with an index of refraction higher than that of the surrounding medium experiences a force equal to the rate of change of momentum of the refracted trapping beam. For a laser beam with a Gaussian profile, this force attracts the bead and traps it at the center of the beam near the focus. External forces acting on the bead can be measured by observing either the particle position in the trap or the corresponding deflection of the trapping beam. Trapping forces typically range between 0.1 and 100 pN.

In atomic force microscopy (AFM), a tip at the end of a flexible cantilever of known force constant is scanned over the sample. Bending of the cantilever can be monitored by the deflection of a laser beam reflected off its back [10,11]. If a molecule is attached between the tip and a surface, and the tip is lifted upward, a force/extension curve can be obtained. Typical forces range from 10 to 10,000 pN.

Complex behavior has been revealed by elasticity studies of individual dsDNA molecules. In this case, the range of forces applied to the molecule determines the nature and length scale involved in the elastic response, with higher forces probing shorter length units. So far, at least four different force/extension regimes have been characterized for dsDNA.

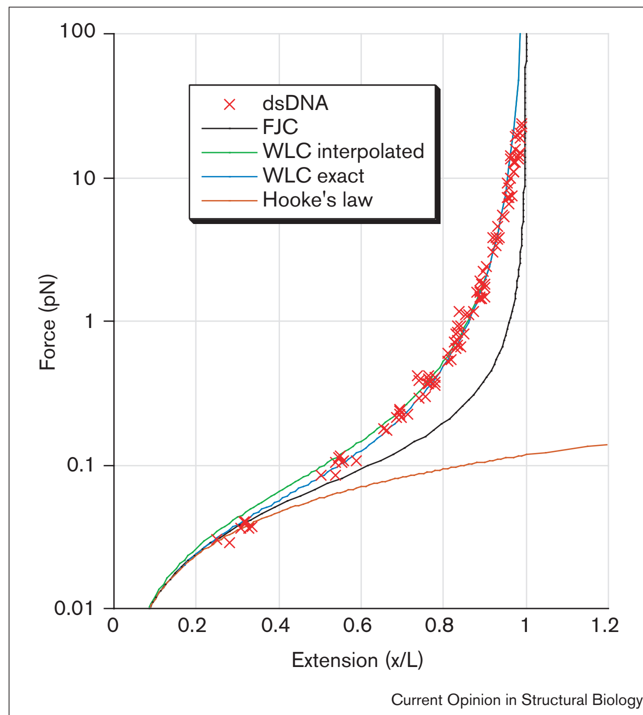
The force/extension regimes of DNA

Entropic elasticity regime

A dsDNA molecule in solution bends and curves locally as a result of thermal fluctuations. Such fluctuations shorten the end-to-end distance of the molecule, even against an applied force. This elastic behavior is thus purely entropic in origin. Two models are often used to describe the entropic elasticity of DNA. In the freely jointed chain (FJC) model, the molecule is made up of rigid, orientationally independent Kuhn segments whose length, b, is a measure of chain stiffness. The alignment of segments by tension is described by the Boltzmann distribution. In the inextensible worm-like chain (WLC) model, the molecule is treated as a flexible rod of length L that curves smoothly as a result of thermal fluctuations. The rod's local direction decorrelates at distance s along the curve according to $e^{-s/P}$, where the decay length, P, is the persistence length of the chain. The stiffer the chain, the longer the persistence length. For dsDNA in physiological salt, the persistence length is approximately 50 nm. Forces of the order $k_BT/P = 0.1$ pN, where k_B is the Boltzmann constant and T is temperature, are required to align polymer segments with these dimensions.

Extension experiments have provided the strictest test to date of these two models [4,6,7,12,13•]. Results, shown in Figure 1, indicate that, even though the FJC model can describe the behavior of dsDNA in the limit of low forces, it

Figure 1



Force versus extension data (red crosses) for λ phage dsDNA (48,502 bp) pulled by magnetic beads in 10 mM Na^+ buffer [4]. The data are fit to a WLC model solved numerically (WLC exact) or using Equation 3 (WLC interpolated), both assuming P = 53 nm. The FJC curve assumes b = 2P = 106 nm. The Hooke's law force curve is from Equation 2.

fails at intermediate and high forces. The WLC model, on the other hand, provides an excellent description of molecular elasticity at low and intermediate forces. The exact force (F) required to induce an end-to-end distance extension of x in a chain of contour length L must be obtained numerically [14–16], but a useful approximation is given by [14]:

$$\frac{FP}{k_BT} = \frac{1}{4\left(1-\frac{x}{L}\right)^2} + \frac{x}{L} - \frac{1}{4}. \tag{1}$$

The inextensible WLC model fits dsDNA data at forces up to 10 pN, as shown in Figure 1. Setting P = 53 nm gave the best fit to the force/extension data of Smith *et al.* [4], which were taken in 10 mM Na^+ buffer.

The Hookian spring: 'Ut tensio, sic vis'

At the lowest forces accessible in these studies, the molecule behaves as a Hookian spring and its extension is proportional to the force applied at its end. The expansion of Equation 1 for small values of x/L gives:

$$F = \frac{3k_BT}{2P}\left(\frac{x}{L}\right) \tag{2}$$

Thus, dsDNA behaves as a linear spring with a Hooke's constant $k_{DNA} = 3k_BT/2PL$, that is, inversely proportional to the length of the molecule and its persistence length. A 10 μm dsDNA molecule, for example, has a spring constant of approximately 10^{-5} pN-nm^{-1}. An identical expression is predicted by the FJC model, within this force limit, if the size of a Kuhn segment is taken to be twice the persistence length of the chain [17].

Intrinsic elasticity regime

Figure 1 shows deviation from the model above 10 pN. Indeed, the end-to-end distance becomes longer than its theoretical B-form contour length L, indicating the existence of a finite stretch modulus. Thus, at these high forces, the chemical structure of DNA is being altered and the elastic response is not merely entropic. Experiments performed with laser tweezers [6,7] give a clearer view of the linear elasticity regime, as shown in Figure 2, in which the molecule behaves as a stretchable solid. Assuming that the contour length of the molecule increases linearly with the applied force [6,18], the following formula can be used between 5 and 50 pN:

$$x = L\left(1 - \frac{1}{2}\left(\frac{k_BT}{FP}\right)^{1/2} + \frac{F}{S}\right) \tag{3}$$

where S is the stretch modulus of the molecule. S equals approximately 1000 pN in 150 mM Na^+. The stretch modulus of a simple elastic rod is related to its intrinsic persistence length, P_i, as:

$$P_i = Sr^2/4k_BT \tag{4}$$

where r is the rod's radius. An intrinsic persistence length of 60 nm is thus obtained for dsDNA, assuming its radius is 1 nm, in fair agreement with the value obtained from the entropic elasticity measurements.

The overstretching transition

When the molecule is subjected to forces of 65 pN or more, it suddenly changes form, stretching up to 70% beyond its canonical B-form contour length [5,6]. Various models for the structure of this so-called S-form DNA have been proposed [19–21] and await experimental verification. The B-to-S overstretching transition occurs within a narrow range of forces (see the flat plateau in Figure 2), suggesting a cooperative process [22,23]. S-form DNA is stable in high salt up to forces of between approximately 150 pN (for random sequence) and 300 pN [for poly(dG–dC)] [24••,25•]. Above these forces, S-DNA melts into single strands that exhibit the characteristic force/extension behavior of ssDNA (Figure 2).

Breaking covalent bonds in DNA

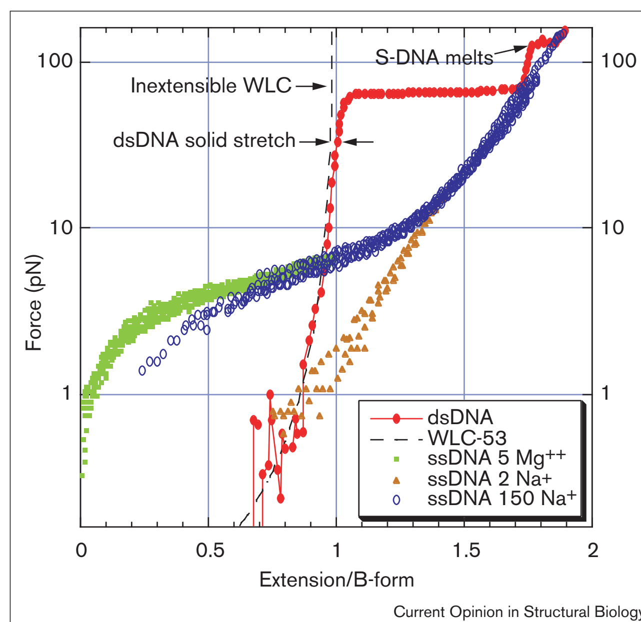
What force is needed to cause bond scission in DNA? Bond potential theory predicts an excess of 5000 pN is needed, but experiments in which bulk DNA was

sheared in flowing buffer have yielded values of only 100–300 pN. Single molecules of dsDNA were broken with a receding water meniscus [26] at forces estimated to be 960 pN (correcting Young's modulus doubles the published scission force of 480 pN). Short dsDNA molecules pulled with an AFM tip [27] sustained forces over 1700 pN. The 'correct' tensile strength is difficult to define because it depends on the rate of stretching, the length of the molecule (number of bonds) and solvent factors, such as bond hydrolysis at sites of DNA depurination. Similarly, covalent bond strength of approximately 1000 pN was determined by AFM experiments on polysaccharide molecules in water [28•].

Stretching single-stranded DNA

By attaching dsDNA between beads and melting off the unlabeled strand with distilled water or formaldehyde, a single strand connecting two beads is obtained [6]. As shown in Figure 2, ssDNA is more contractile than dsDNA because of its high flexibility, but it can be stretched to a greater length because it no longer forms a helix. Although the force/extension curve can be fit with an FJC model modified by including a stretch modulus (*ad hoc*) [6], almost as good a fit can be obtained with a simple WLC model using the correct sugar–phosphate distance without need for a stretch modulus. However, both models fail in high salt (perhaps due to hairpin formation) and in low salt (probably due to excluded volume effects) [6]. Further theoretical and experimental work is clearly needed here.

Figure 2



Force/extension behavior of dsDNA and ssDNA. Different DNA molecules were pulled with force-measuring laser tweezers [6]. Both pulling and relaxing curves are shown, so all force curves were reversible. Dashed line data (WLC-53) are from Equation 1, assuming P = 53 nm. The dsDNA curve was taken using a 10.4 kbp restriction fragment in 50 mM Na^+ and 5 mM Mg^{++} buffer [25•]. The same fragment and buffer were used to make ssDNA (labeled ssDNA 5 Mg^{++}) [40••]. The ssDNA curves in 150 mM Na^+ and 2 mM Na^+ were taken using 48 kbp λ phage DNA [6]. See text for further details.

Unzipping the double helix

Essevaz-Roulet *et al.* [29] performed the mechanical separation of the complementary strands of an individual λ phage dsDNA molecule. They used the bending of a glass microneedle to determine the forces required to pull apart the 3′ and 5′ extremities of the molecule. Strand unzipping occurred abruptly at 12–13 pN and displayed a reproducible 'saw-tooth' force variation pattern with an amplitude of +/– 0.5 pN along the DNA. As shown in Figure 3, this force variation pattern was found to match the corresponding variation in the GC content of DNA averaged over 100 bp. An upper bound for the maximum spatial resolution of such an experiment is set by thermal fluctuations and by the entropic elasticity of the ssDNA portions of the molecule.

Higher resolution can be obtained using shorter, stiffer linker molecules. In our laboratory, we have recently used laser tweezers to monitor the unwinding of a 12 bp poly(GC) DNA hairpin flanked by 600 bp dsDNA handles. At about 16 pN, the rapid increase of force that defines the stretchable solid regime of the handles is interrupted by a short region in which the force remains constant over the approximately 10 nm distance required to unwind the hairpin (Figure 3). Once the hairpin has been fully opened, the behavior of the handles reduces to the conventional double-stranded case. Similar forces were also obtained by Rief *et al.* [24••] using short AFM cantilevers. They unzipped poly(dG–dC) at approximately 20 pN and poly(dA–dT) at approximately 10 pN.

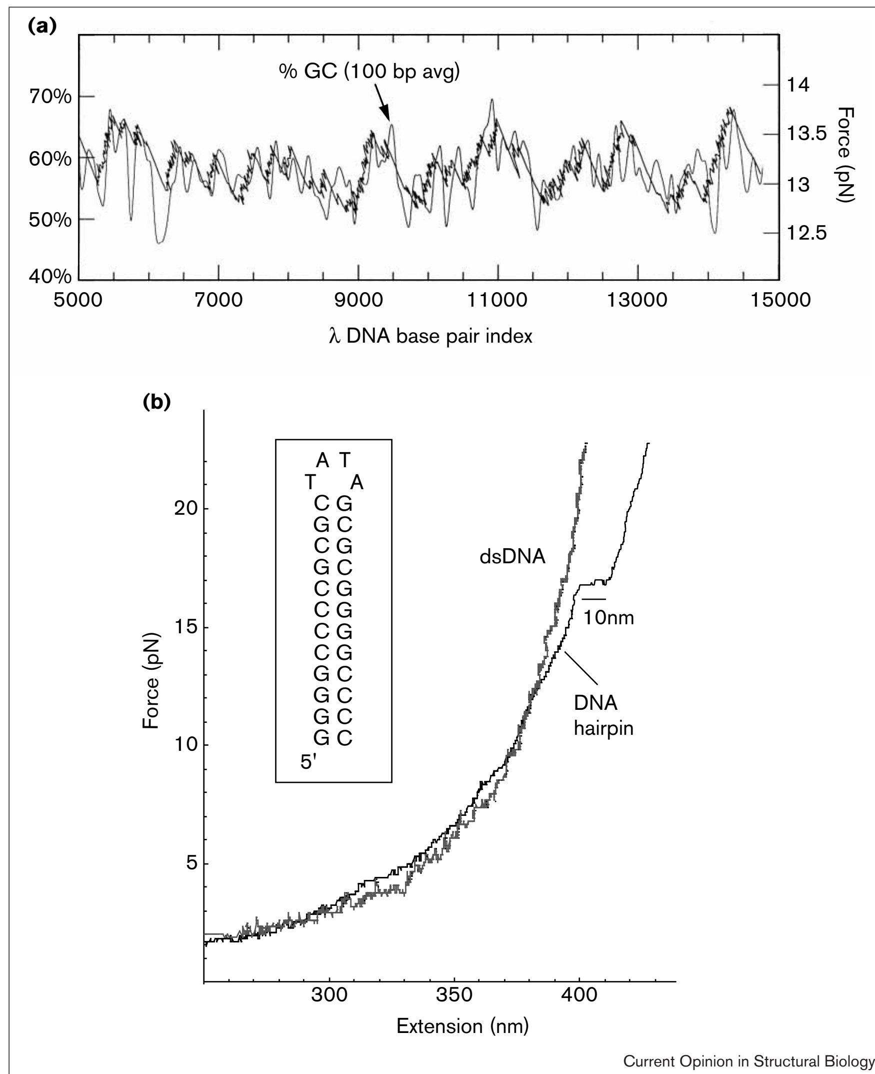
Bockelmann *et al.* [30] used equilibrium statistical mechanics to model the force/extension obtained experimentally. Agreement between simulation and experiment was remarkable, indicating that the force variations reflect DNA sequence composition, specifically the GC content. More importantly, it shows that, unlike macroscopic slip-stick processes, the 'molecular stick-slip' motion observed in this case does not involve instabilities. Instead, each point on the force/extension curves corresponds to an equilibrium position.

These forces may be interpreted, therefore, as being equilibrium forces and thermodynamic state quantities, such as free energy (ΔG), can be obtained by the integration of the force along the distance of the unwinding plateau. For our 12 bp hairpin, integration yields a ΔG per GC base pair of about 9 kJ/mol, which is equal to the value obtained in conventional bulk experiments.

Mechanical supercoiling of DNA

Strick *et al.* [12,13•] devised an elegant experiment in which they attached both strands of a dsDNA molecule to a flat surface at one end and to a magnetic bead at the

Figure 3



Mechanical unzipping of DNA. **(a)** A comparison between the force signal and average GC content along a segment from 5000 to 15,000 bp of the sequence of λ DNA from [29]. The smooth curve is %GC averaged over 100 bases and the jagged curve is the force signal (versus sequence-normalized distance) obtained by mechanical opening. **(b)** Unzipping of a 12 bp poly(GC) hairpin. A single-stranded region able to form a short hairpin (see small panel) was flanked by two dsDNA handles. The handles were end-labeled with biotin (5′ handle, 531 bp) or digoxigenin (3′ handle, 613 bp) and thus attached to appropriately labeled polystyrene beads. Unzipping was fully reversible and was performed in standard force-measuring laser tweezers as described in [6] in a 150 mM Na^+ buffer (pH 7.0).

other. By external rotation of a magnetic field, the DNA molecules became supercoiled. The authors then determined how the elasticity depended on the twist. Small twists (< 1% change in linking number) in either direction cause molecules to increase in contractility relative to relaxed DNA. Such an effect is predicted for a rod that alleviates excess torsional energy by forming plectonemes (i.e. twisted loops that branch laterally from the direct end-to-end path in the rod) [31]. Extending the rod converts writhe into twist, at the expense of extra tension. Unlike the simple rod, the tension in underwound dsDNA reaches a critical value (F_c–) of approximately 0.5 pN and then plateaus as the molecule is stretched. Apparently, the DNA strands separate (melt) to absorb the molecule's linking number deficit and remove plectonemes, consistent with reports [32,33] that negatively supercoiled DNA cannot change its twist by more than approximately 1%. Molecules with large positive linking numbers display a different force plateau at a higher critical force, F_c+, of approximately 3 pN. Apparently, local regions of the DNA adopt a new form, named 'P-DNA', with very short helical pitch (hypercoiled DNA).

Ionic effects

Ions strongly affect the bending behavior of DNA in processes like chromosome formation, viral packaging, replication and transcription. DNA's bending rigidity is described by the worm-like model of a polyelectrolyte as being composed of two parts: the electrostatic persistence length (P_e) due to intrachain repulsion and the intrinsic persistence length (P_i) due to base stacking. Typical estimates of P_i are around 45 nm. The overall persistence length is then $P = P_i + P_e$.

Single-molecule experiments in various buffer conditions are now revealing how ions modify the rigidity of DNA [7,34]. In monovalent salt (e.g. Na^+), the measured

persistence length is consistent with an electrostatic contribution that varies inversely with the ionic strength, as predicted by theory [35,36]. In this case, P_e is related to the Debye–Hückel screening length κ^{-1} and the Bjerrum length l_b (0.7 nm in water/monovalent ions) by $P_e = \kappa^{-2}/4l_b$. The model fails, however, with multivalent ions like $Co(NH_3)_6^{3+}$ and spermidine^{3+}. Measurements with these ions gave P values of 25–30 nm, lower than the 'intrinsic' persistence length P_i. Perhaps DNA locally bends towards the transiently bound multivalent ions, shortening the intrinsic persistence length [34].

The eventual limitations of the 'mechanical' description of dsDNA as a rod are revealed by changes in ionic strength. Lowering the ionic strength increases the measured persistence length, but seems to reduce DNA's elastic stretch modulus, contradicting the elastic rod model (Equation 4).

Reversibility versus hysteresis

The free energy change in a single molecule can be obtained by integration of a reversible force/extension curve. In this case, the molecule passes only through a succession of time-independent equilibrium states [37]. Thus, measurements must be made slower than the slowest relaxation process in the molecule/bead system. Slow processes may include transport of stretch (Rouse–Zimm modes), transport of twist [38•], formation of plectonemes [13•], DNA zipping and rezipping, and protein binding. The hallmark of thermodynamic reversibility is the retracing of the force/extension curve upon contraction, that is, the relaxation curve coincides with the stretching curve. Force curve hysteresis is a sensitive indicator of the kinetics of protein–DNA binding and refolding (see chromatin below).

Protein–DNA interactions

Special mechanical properties of DNA are frequently invoked to explain protein binding and it is now possible to directly test some of those models with single-molecule experiments. Furthermore, forces can be applied to the composite DNA–protein structure that probe its biologically active conformation. Studies of RecA–DNA filaments have helped to quantify the role of DNA's bending rigidity in its biological function.

The mechanics of RecA–DNA filaments

RecA is a small bacterial protein that catalyzes homologous recombination and repair of DNA. When coated with RecA, DNA is 1.5 times longer and less twisted than B-form DNA (20° per base pair versus 35° per base pair). Shivashankar *et al.* [39] anchored λ phage DNA at one end to a glass coverslip and at the other end to an optically trapped polystyrene bead. From force/extension measurements in the presence of RecA and ATP, they obtained the persistence length of the RecA–DNA complex and the detailed kinetics of the polymerization reaction. Using similar techniques, Hegner *et al.* [25•] characterized the stretch modulus and bending rigidity of RecA–DNA filaments in the presence of various cofactors. The stretch modulus for a dsDNA–RecA fiber was a stiff 5 nN fiber in [γ-thio]-ATP, but decreased to a softer 2 nN in ATP. In this case, variation of stretch modulus with cofactor coincides with RecA's function, as ATP hydrolysis reduces the protein's affinity for DNA and, hence, the structural integrity of the filament.

Replication and transcription studies

Single-molecule measurements have been used to investigate mechanochemical properties of polymerases, such as their stall forces [40••,41–46]. Yin *et al.* [41,42] immobilized *Escherichia coli* RNA polymerase on a surface and provided a DNA template attached to a bead. An optical trap was used to pull on the DNA and oppose translocation. Polymerization velocity was fairly insensitive to force until it stalled reversibly at 25 pN [41,42].

A different method was used in the recent study of T7 DNA polymerase by Wuite *et al.* [40••], who pulled on both ends of the template and observed changes in its elastic properties as ssDNA was converted to dsDNA by the enzyme. As ssDNA and dsDNA have different force/extension properties (Figure 2), the progress of polymerases along their ssDNA templates could be monitored in real time by fitting the force/extension data to a combination of ssDNA and dsDNA stretching curves. T7 DNA polymerase velocity was sensitive to template tension: rates increased to a maximum at 6 pN, but then fell linearly with tension until the enzyme reversibly stalled at 34 pN. In contrast to RNA polymerase, the rate-limiting step for the polymerization reaction in DNA polymerase is apparently force-sensitive. Higher template tensions (approximately 40 pN) triggered a fast $3'\rightarrow5'$ exonucleolysis.

Chromatin studies

Little is known about the forces that stabilize the variously compacted forms of chromatin and how these factors control the accessibility of chromosomes to transcription and replication. To explore these issues, Cui and Bustamante [47•] stretched and relaxed chicken erythrocyte chromatin fibers with laser tweezers. In the low force regime (< 8 pN), force/extension curves were reversible and individual fibers could be cycled many times with identical results. From these force/extension measurements, the internucleosomal attractive energy is approximately 3.4 k_BT at physiological ionic strength. This value for the interaction energy suggests a mechanism for the local access of *trans*-acting factors to chromatin: on average, two adjacent nucleosomes should be found in an open (accessible) state about 4% of the time.

In the intermediate force range, the curves are hysteretic, but repeatable, indicating reversible rearrangement of the nucleosomes on a slower timescale than the relaxation of the molecule. At higher forces (> 2 pN), the nucleosomal core particles begin to disassociate from the DNA and the force/extension curve never retraces its path.

Outlook

Started less than a decade ago, single-molecule experiments with DNA and proteins are helping us answer questions about biological function that would be difficult or impossible to address in bulk experiments. Further refinement of the current manipulation methods, including the incorporation of single-chromophore fluorescence detection, is likely to occur in the immediate future. Scientists may soon learn how to use mechanical force to control the dynamics, time evolution and fate of chemical and biochemical reactions. Conversely, it may be possible to understand how chemical reactions lead to the generation of force and movement in molecular machines.

Update

In a very recent study, single-molecule twisting techniques (described above) have been used to study the relaxation of DNA supercoils by individual topoisomerase molecules [48]. Here, DNA plectonemes were observed to relax, two turns at a time, by individual ATP-dependent catalytic turnovers. These relaxation events were recorded as discrete jumps (~90 nm) in the extension of a DNA molecule held under constant force (0.7 pN). These experiments directly measured enzyme turnover as a function of applied force. Unexpectedly, too much torque decreased the turnover rate, an effect never observed with bulk experiments.

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