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Torsional Rigidity of Positively and Negatively Supercoiled DNA

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Time-correlated single-photon counting of intercalated ethidium bromide was used to measure the torsion constants of positively supercoiled, relaxed, and negatively supercoiled pBR322 DNA, which range in superhelix density from +0.042 to −0.123. DNA behaves as coupled, nonlinear torsional pendulums under superhelical stress, and the anharmonic term in the Hamiltonian is approximately 15 percent for root-mean-square fluctuations in twist at room temperature. At the level of secondary structure, positively supercoiled DNA is significantly more flexible than negatively supercoiled DNA. These results exclude certain models that account for differential binding affinity of proteins to positively and negatively supercoiled DNA.

The motivation for studying the torsional rigidity of supercoiled DNA is threefold. First, such study provides a stringent test of current theoretical models of DNA that assume that the twisting (and bending) internal forces are harmonic and isotropic (1, 2). Second, the twisting rigidity of DNA is an important determinant of the energetics of formation of complicated nucleoprotein structures (3). Third, although the torsion constant has been extensively studied for linear DNA (4–8), its dependence on superhelix under physiological conditions has not been systematically measured.

We obtained torsion constants by fitting the fluorescence anisotropy decay of ethidium bromide intercalated in various supercoiled topoisomers of pBR322 to the theory of Barkley and Zimm with the use of a Marquardt-based algorithm (2, 9). The anisotropy decay arises from the reorientation of excited-state ethidium bromide due to the twisting (and bending) of the DNA helix, and it was detected by time-correlated single-photon counting. The Barkley-Zimm theory, which models DNA as a uniform elastic rod that bends and twists in a viscous medium, is a harmonic approximation. The fitted value of the torsion constant depends weakly on the bending rigidity chosen, which we assume is constant and independent of superhelicity. Because of the weak dependence on bending and the fact that DNA is not severely bent in a plectonemic superhelix (10), this assumption is justified.

We chose a bending constant that yields a persistence length of 525 Å. We also analyzed the data with the exclusion of the effect of bending (infinite bending constant), which yields a lower limit to the torsion constant. In this limit, the Allison-Schurr and Barkley-Zimm models of DNA dynam-
ics are mathematically identical. The fractional difference in torsion constants between topoisomer samples is nearly independent of the particular bending rigidity chosen. The torsion constant increases by approximately 1.6 when bending is included in the analysis.

The parallel, perpendicular, and anisotropy curves for a negatively supercoiled sample are shown in Fig. 1. The quality of the data is representative of all samples. A two-exponential fit to magic angle data, not shown, deconvoluted with the instrument response function, yields $x^2 < 1.3$ and shows that >99% of the fluorescent photons arise from intercalated ethidium bromide (lifetime $= 22.5$ ns) with the remaining <1% arising from free dye (lifetime $= 1.6$ ns). This is true for all samples, including positively supercoiled DNA. The anisotropy fit to each sample, derived with the use of just a single torsion constant (rather than a sum) is excellent ($x^2 < 1.3$), which indicates that the harmonic approximation is good for each narrow distribution of topoisomers.

If the torsion constant changes between samples that differ significantly in average superhelicity, then the twisting potential has an anharmonic component. To determine the sensitivity of our system to changes in torsion constant, we measured the anisotropy decay of phage $\lambda$ DNA in sucrose solutions with viscosities from 1.0 to 2.45 cP (ranging from 0 to 24% sucrose final concentration). According to theory, the anisotropy decay depends on the inverse square root of the product of the viscosity and torsional constant ($I_2$). Therefore, varying the viscosity $\eta$ should simulate a change in the torsion constant, and a plot of $(1/\eta)^{1/2}$ versus the anisotropy fit parameter should yield a straight line. Figure 2 shows that fluorescence polarization anisotropy is sensitive to changes in viscosity, and hence torsion constant, of 10% or less (11).

The best fit torsion constants for our topoisomer samples under physiological salt and low-salt conditions are shown in Fig. 3. At 175 mM ionic strength, the torsion constant increases linearly as the superhelical density becomes more negative (Fig. 3). At low ionic strength ($\approx 7.5$ mM) and for nonnegative superhelical densities, the same trend is observed (Fig. 3).

Two other groups have studied the dependence of the torsion constant on superhelicity using fluorescence depolarization. Millar et al. (7) compared pBR322 of “native” superhelical density to linear calf thymus DNA in 0.2 M salt. They found that negatively supercoiled pBR322 is torsionally more stiff than calf thymus DNA. Schurr and co-workers have studied supercoiled DNA under a variety of conditions (12). In one study with 0.1 M salt, they concluded that there is no variation in torsional stiffness between three samples at superhelical densities of $0.048$, and $0.083$ (13). In a later study with low-salt conditions ($\approx 15$ mM), they concluded that the torsion constant increases with negative supercoiling in a complex manner that varied over many weeks (11).

The torsion constant of our relaxed sample, $1.9 \pm 0.1 \times 10^{-19}$ erg-cm, is in good agreement with the fluorescence anisotropy work of others (6, 7, 14), although there is some variability in the literature depending on assumptions of helix radius, persistence length, and contribution of bending to depolarization. Cyclication studies, which measure the probability of ring closure of short $\approx (500$ bp) DNAs (4, 8), generally yield somewhat higher values for the torsion constant, although Taylor and Hagerman have recently measured a value of 2.0 $\pm 0.2 \times 10^{-19}$ erg-cm using this method (5, 15). This method, however, cannot be used to study topoisomers outside the thermally accessible states near the relaxed topoisomer.

One possible complication with our measurement is that highly negative supercoiling can induce structural transitions in DNA. For our physiological salt conditions, we argue that any secondary structures in non-B form that may arise from superhelical stress, such as left-handed Z-DNA or cruciforms, would alter the measured torsion constant only by a small amount. Although cruciforms are extruded in pBR322 at superhelical densities more negative than $\approx 0.08$.

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**Fig. 1.** (A) Parallel and perpendicular and (B) anisotropy decay modes of the fluorescence of intercalated ethidium bromide in negatively supercoiled DNA ($I$, intensity). The quality of the data is indicative of all of the topoisomers measured. Conditions: DNA ($200 \mu$g/mL) and 2 $\mu$M ethidium bromide buffered at pH 8.0 in tris. Physiological salt solution contained 50 mM tris buffer, 150 mM KCl, and 1 mM EDTA; low-salt solution contained 5 mM tris and 0.5 mM EDTA. We prepared positively supercoiled pBR322 in vivo by treating strain AS19 (pBR322) with novobiocin according to the protocol of Lockshon and Morris (23), and the sample is thus a kinetic distribution of topoisomers. Our estimate of $\sigma = +0.042$ is a minimum estimate based on the ability of agarose gel electrophoresis to resolve overwound topoisomers. Negatively supercoiled samples were prepared by the method of Singleton and Wells (26). A reference topoisomer distribution, which was used to define the relaxed linking number $L_{k,r}$, was prepared in a physiological salt buffer identical to that used for the spectroscopy. Gel electrophoresis of samples after exposure to light showed no light-induced nicking of the DNA. Approximately 5% of the DNA was nicked before spectroscopy, presumably because of handling. The solid line in the anisotropy curve is the Barkley-Zimm best fit curve, derived with the use of a nonlinear Marquardt-based fitting algorithm. The dipole moment of the ethidium with respect to the helix axis was assumed to be 70$^2$ (27), and the helix radius was assumed to be 13.4 A (7). The anisotropy function is the standard $(I - I_0)/(I_0 - 2I_2)$, corrected for laser light fluctuations and polarization sensitivity of the detection system. The time resolution of our instrument was 75 ps, and measurements were taken from 0 to 75 ns, the period in which twisting dominates the fluorescence depolarization. The instrument response function (full-width at half maximum $\approx 75$ ps) was deconvoluted only for the magic angle data. Interference filters were used to rigorously exclude Raman and Rayleigh scattered light, and excitation was effected with a mode-locked dye laser set at 550 nm to minimize fluorescence from free dye (28).

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**Fig. 2.** Dependence of anisotropy decay on relative viscosity. Fits were made to the anisotropy decay of phage $\lambda$ DNA by allowing both the initial anisotropy and a fit parameter $A$ to vary. According to theory (1, 2), $A = 2k_BT/\pi B\sqrt{\eta C}$, where $B$ is the hydrated DNA radius (13.4 A) and $\eta$ is the relative viscosity. Therefore, a change in viscosity simulates a change in the torsion constant. Plotting $A$ versus $1/\eta$ should yield a straight line. The data agree with theory. The sucrose concentrations were measured to four significant figures with a Bausch and Lomb refractometer. The viscosity as a function of sucrose concentration and temperature is extensively tabulated in (29).
Fig. 3. Torsion constants of topoisomerase samples in 150 mM KCl, 50 mM tris, 1 mM EDTA (○; ionic strength = 175 mM) or in 5 mM tris and 0.5 mM Na₂EDTA (□, △, and crosshatched square; ionic strength 7.5 mM), all at pH 8.0. Samples were prepared as described in the legend to Fig. 1. Error bars represent the SD when the number of measurements n = 3 or the spread between independent measurements when n = 2. When error bars are absent, the deviation is less than the symbol width. At high ionic strength, the σ = 0 data is the average of measurements made on linear and relaxed plasmid DNA. At high ionic strength the torsion constant increases linearly with negative supercoiling. For the low-salt data, closed squares (■) represent topoisomerase structures that are not likely to exist; the hatched square represents a linearized DNA sample; open squares (□) represent topoisomerase structures under moderate to high superhelical stress where alternative structures may complicate the analysis (see text). Both low- and high-salt data were analyzed with the same persistence length and helix radius, because there are uncertainties in the literature about the exact values at low ionic strength for the helix radius, persistence length, and Poisson’s ratio. Therefore, a direct comparison of the torsion constants between the different ionic strengths is not appropriate. However, it is clear that positively supercoiled DNA is torsionally more flexible than relaxed or negatively supercoiled DNA. The low-salt data were obtained by precipitating the high-salt samples and resuspending in the low-salt buffer. Consequently, the actual superhelical density of the low-salt samples is more negative than that shown (30). (There is no reliable measurement in the literature on the magnitude of the shift at this ionic strength.) The linear DNA sample, which is free to assume an equilibrium twist equivalent to the low-salt reference state, is therefore presented as a positively supercoiled (σ = 0.02) sample with this convention.

(16, 17), they constitute only a small fraction of the DNA, on the order of 25 bp out of the total 4363 bp. Furthermore, pBR322 does not contain extensive alternating purine-pyrimidine tracts, and the formation of Z-tracts is strongly dependent on length. For instance, Singleton et al. (17) find that Z-DNA forms at a superhelicity of ~0.072 in pBR322 at 200 mM ionic strength only if a 26-bp or longer alternating G-C tract is inserted into the plasmid (18).

Under physiological salt conditions, alternative structures would, in the worst case, lead to an underestimation of the true torsion constant. Two possibilities can be considered. (i) If the alternative structure is more stiff than B-DNA, its presence will not alter the dynamics, and hence the torsional rigidity, reported by the ethidium (1), because a localized stiff spot in a flexible rod does not significantly alter the overall dynamics. (ii) If the alternative structure is more flexible, it can reduce the measured torsional rigidity—local weakness can alter the dynamics of an otherwise stiff rod (1). In this case, the increase in torsion constant found with increasing negative superhelicity is a lower bound for the rigidity of the remaining B-form DNA. Our assumption of no alternative structures, therefore, yields a lower limit to the nonlinearity of the DNA.

Our interpretation is a lower limit for another reason: cruciforms and Z-tracts, if present, remove superhelical turns that would make negatively supercoiled DNA less torsionally stressed, and, in effect, they contract the x-axis of Fig. 3. The slope would then be steeper, indicating that the DNA is torsionally even more nonlinear than shown in Fig. 3.

At low ionic strength, positively supercoiled DNA is torsionally more flexible than relaxed DNA. At nonnegative superhelicity densities, the torsion constant also increases more rapidly with the change in linking number (Δlk) than it does under physiological ionic strength (closed squares, Fig. 3). This is in part because a greater fraction of Δlk goes into the change in twist (ΔTw) at low salt concentration—the superhelical radius increases (10, 19) and makes the writhing (Wr) more energetically costly. Hence, the base pairs sample a greater range in equilibrium twist angle and make the nonlinearity in torsion constant more evident. Furthermore, the increased superhelical radius at low salt concentration leads to greater torsional stress than that at high salt concentration. The greater torsional stress under these conditions may lead to significant alternative structures at negative superhelic densities, making the interpretation of these data problematic (open squares, Fig. 3).

On the basis of the above arguments, our data indicate that the torsion constant for DNA linearly increases with superhelicity. If the DNA is modeled as a series of base pairs (flat disks) coupled by torsional springs, this linear increase in torsion constant indicates that a cubic term should be added to the twisting Hamiltonian (20). At physiological ionic strength, the best fit line of torsion constant per base pair versus σ is:

\[ C/\hbar = m\sigma + b \]

where C is the torsion constant, h is the height per base pair (=3.4 × 10⁻⁶ cm), m is the experimentally determined slope, σ is the fractional change in linking number (Δlk/Lk₀), and b is the y-intercept, which is also the torsion constant per base pair in the absence of topological stress. We find that b = 135 and m = 218 (both in units of k₀T per base pair, where k₀T = Boltzmann’s constant times temperature, is the thermal energy). Note that this approximation must break down at sufficiently positive superhelical density, or a zero or negative torsion constant would result.

To understand in molecular terms the change in torsion constant after supercoiling, we must know what fraction F of Δlk goes into ΔTw and into Wr. (F \(\equiv\) ΔTw/Δlk; recall that Δlk = ΔTw + Wr). The change in twist per base pair is simply ΔTw/N, or FΔlk/N, where N is the number of base pairs in the DNA molecule.

Three groups have estimated F, Klenin et al., using Monte Carlo simulations, found that F = 0.3 (0.2 to 0.4) (21). Boles et al., using electron microscopy and topological methods, found that F = 0.28 (22). Hunt and Hearst (19), using analytical mechanics, estimated that F = 0.2 to 0.3 for physiological superhelic densities. In fact, F is most likely not a constant, but instead a function of superhelicity (19). For the purposes of our discussion, we assume F is a constant.

The twisting potential energy per base pair, U, of a supercoiled DNA molecule can now be written:

\[ U = b/2 (\psi' - \phi)² + Fm/3 (\psi' - \phi)³ + b'/2 (\psi - \phi)² + Fm/3 (\psi - \phi)³ \]

where \(\psi\) is the equilibrium angle between base pairs for relaxed DNA (0.601 radians or 34.4°), \(\psi'\) is the equilibrium angle for a particular topoisomer, \(\psi''\) = 2πFΔlk/N + 0.601), b' is the torsion constant at the superhelical density corresponding to equilibrium twist angle \(\psi''\) (equal to C/\hbar in the previous
interaction of p107 with cyclin A independent of complex formation with viral oncoproteins

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The p107 protein and the retinoblastoma protein (RB) both bind specifically to two viral oncoproteins, the SV40 T antigen (T) and adenoviral protein E1A (E1A). Like RB, p107 contains a segment (the pocket) that, alone, can bind specifically to T, E1A, and multiple cellular proteins. Cyclin A bound to the p107 pocket, but not the RB pocket. Although both pockets contain two, related collinear subsegments (A and B), the unique sequence in the p107 pocket that occupies the space between A and B is required for the interaction with cyclin A.

The cellular protein, p107, shares a number of properties with the retinoblastoma product (RB), a known tumor suppressor. Both proteins form complexes with SV40 large T antigen (T) and the adenoviral protein E1A (1–3), and RB interacts with the human papilloma virus E7 protein (4). Complex formation may, in part, underlie the transforming functions of these viral proteins (2–6). RB also interacts specifically with several cellular proteins in vitro (13–15). These interactions may account for the proposed regulation of the cell cycle by RB (7–12). RB and cyclin A, another protein that functions to regulate the cell cycle, both bind specifically to the transcription factor, E2F (16–21). These interactions may also contribute to cell cycle control.

The p107 protein has clear sequence similarity to RB. The RB sequence contains a stretch of ~400 amino acids (the pocket) that can alone bind T, E1A, and the aforementioned cellular proteins (15, 22–24). The pocket is composed of two subsegments (A and B) that are separated by a spacer of ~75 residues (Fig. 1). Foreign sequences can be substituted for the spacer without disturbing binding of T and E1A (22, 23). Similarly, a segment of p107 containing ~600 residues (residues 252 to 816) can also bind T and E1A. The segment composed of two subsegments similar to A and B of RB, parts A and B of p107 are separated by a spacer of ~200 amino acids (Fig. 1) (1). Substituting the RB spacer for the spacer in p107 did not affect binding of T or E1A (1), suggesting that the...