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Effect of ethidium binding and superhelix density on the apparent supercoiling free energy and torsion constant of pBR322 DNA

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Abstract

The value of the twist energy parameter (E_T) of pBR322 is determined near zero superhelix density from topoisomer distributions created under various conditions. The resulting value, $E_T = 1155 \pm 65$, at 37°C is essentially unaffected by adding 10 mM Mg^{2+}, or by changing the kind of Topo I from chicken-red-cell to calf-thymus. This value significantly exceeds that ($E_T = 950 \pm 80$) measured for p30δ DNA under indentical conditions by the same method in the preceding paper. Decreasing the temperature from 37 to 21°C yields a slightly larger value, $E_T = 1340 \pm 130$, but the statistical significance of the increase is marginal. Attempts to determine reliable E_T values for pBR322 at higher superhelix densities by ethidium binding were frustrated by the fact that good fits of the equilibrium dialysis results could not be achieved using a single value of E_T. Moreover, the curves of apparent E_T versus binding ratio r vary considerably from one preparation to another, and for a given preparation vary with time after cell lysis up to about seven weeks, after which they settle in to nearly reproducible behavior. The apparent E_T values obtained from competitive dialysis experiments are typically rather low ($E_T \sim 700$) for small r and nearly native superhelix density, and rise up to 1300 to 1500 with increasing binding ratio (up to $r = 0.055$) and decreasing negative superhelix density. The observed trend of apparent E_T values extrapolates to a still larger value at $r \approx 0.07$, $\sigma = 0$, which significantly exceeds the value obtained from topoisomer distributions near $r = 0$, $\sigma = 0$. These findings cast considerable doubt on the reliability of apparent E_T values obtained for pBR322 by ethidium binding studies. The differences between pBR322 and p30δ are attributed to one or more different secondary conformations that prevails over part of the pBR322 sequence, and to the metastable prevalence of one or more of those as dye is added. In the case of pBR322 DNA, the torsion constant, molar ellipticity at 250 nm, and plateau diffusion coefficient from dynamic light scattering at $K = 4.5 \times 10^5$ cm^{-1} all vary with superhelix density in a similar way, showing dips at $\sigma = -0.010$ and jumps at $\sigma = -0.037$. This behavior, which is similar to that observed previously for pUC8 dimer DNA, suggests that two successive transitions in secondary structure are induced in pBR322 by increasing negative superhelix density. The possible role of such structural transitions in the contrasting behaviors of pBR322 and p30δ is discussed.

Keywords: Ethidium binding; Superhelix density; pBR322 DNA

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1. Introduction

The twist energy parameter (E_T) that governs the supercoiling free energy of pBR322 DNA was measured under various conditions by different groups, but the resulting values are highly disparate. Analyses of topoisomer distributions (in the absence of ethidium) yielded $E_T = 1130$ at 37°C [1], $E_T = 1320$ at 35°C [2] and $E_T = 1610$ at 20°C [3], all near zero superhelix density. Competitive dialysis studies of ethidium binding to two different samples with different *initial* native superhelix densities yielded $E_T = 280 \pm 70$ and $E_T = 347 \pm 50$ at relative low binding ratios and nearly native *final* superhelix densities (as defined in the previous article [4]), namely $\sigma = -0.047$, at 20°C [5]. Chloroquine binding studies on the same samples at much higher binding ratios and nearly vanishing *final* superhelix densities yielded $E_T = 360$ and $E_T = 460$, also at 20°C [5]. Analysis of strand separation transitions of pBR322 suggests that $E_T = 1175 \pm 40$ at relatively low ionic strength at 37°C [6,7]. Not only are these results surprisingly scattered, but the majority conflict rather strongly with those obtained for p30δ DNA in the preceding article.

The torsion constant (α) of native supercoiled pBR322 was previously measured by time-resolved fluorescence polarization anisotropy (FPA) in the presence of various concentrations of chloroquine [5] and ethidium [8]. The torsion constant of the supercoiled form decreased significantly upon binding either dye, in contrast to the linearized species, and differed from that of the linearized DNA, even when the binding ratio sufficed to completely relax the supercoiled form [5,8], that is when $r = r^*$, as defined in the preceding paper [4]. This unexpected behavior was attributed to long-lived metastable secondary structure that persists in the dye-relaxed supercoiled pBR322 [8]. Persistence of long-lived metastable secondary structure was also observed in freshly linearized pBR322 [9].

The torsion constant of pUC8 dimer DNA was measured previously as a function of superhelix density over the range from zero to native ($\sigma = -0.05$) [9]. It was found to exhibit a significant dip in the central region from $\sigma = -0.020$ to -0.035. The circular dichroism (CD) at the trough at 250 nm and the apparent diffusion coefficient from dynamic light scattering (DLS) at large scattering vector ($K = 4.5 \times 10^5$ cm^{-1}), which we refer to as D_{plat}, also exhibited substantial dips in the same region. Those data provide clear evidence for what appear to be at least two extensive transitions in secondary structure of pUC8, as the superhelix density is varied from zero to native ($\sigma = -0.05$). A subsequent study of the torsion constant of pBR322 extended over a much wider range of superhelix density, but reported only one value for the circular species in the range from $\sigma = 0$ to -0.05, so provides no information about variations in α over that range [10]. Moreover, a quantitative comparison of any torsion constant in ref. [10] with those reported here is not meaningful, since those data were analyzed using the incorrect anisotropy expression of Barkley and Zimm [11].

Our primary objective now is to prepare and investigate pBR322 using precisely the same protocols that revealed the ideal behavior of p30δ in the preceding paper [4]. Here we find strong evidence for the existence of metastable secondary structure in ethidium-relaxed pBR322. To investigate the possible origins of that, we investigate several other properties that are sensitive to secondary structure, namely the torsion constant, circular dichroism, and apparent DLS diffusion coefficient at large scattering vector, D_{plat}, as a function of superhelix density. In this case, too, the data provide clear evidence for what appear to be two extensive transitions in secondary structure of pBR322, as the superhelix density is varied from zero to native values.

2. Materials and methods

Supercoiled pBR322 was grown in the same HB101 host employed previously for p30δ, and the plasmid was isolated, purified, and characterized by exactly the same protocols described in the preceding paper [4]. Linking differences of all samples were determined by the band-counting method used previously [4].

Competitive dialysis experiments were per-

formed and the fluorescence intensity ratios were measured using the same apparatus and protocols described before [4]. The data were also analyzed in the same ways using the same theory and algorithms. In some cases, nicked circular DNA was used in place of linear DNA, but that had no apparent effect on the results, as expected. Topoisomer distributions were created at 37°C using the same topoisomerase 1 (Topo I) from chicken erythrocytes and the same procedures that were employed in the preceding paper [4]. One distribution of topoisomers was created in the absence of added Mg^{2+} as before, and one in the presence of 10 mM Mg^{2+}. Two additional topoisomer distributions were created, one using calf-thymus Topo I from Bethesda Research Labs (BRL) at 37°C and one at 21°C, both in the presence of 10 mM Mg^{2+}. These different experiments were intended to examine the effects, if any, of Mg^{2+}, type of Topo I, and temperature on E_T. Each topoisomer distribution was resolved by gel electrophoresis, stained, and photographed as described previously [4]. The film was scanned with the same densitometer, the ratios of peak areas were determined as before, and the data analyses were performed using precisely the same algorithms.

The torsion constant was measured by FPA using the same protocols and conditions described previously [5,8,12], except that the temperature was 21°C and the NaCl concentration of the LSTE buffer (10 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) in these samples was 0.01 M rather than 0.1 M, to correspond to our previous work on pUC8 dimer DNA [9]. As in the preceding work, the values reported here are proportional lower bounds. The circular dichroism and DLS measurements were performed at 21°C in the same LSTE buffer, as done previously for pUC8 DNA [9].

3. Results and discussion

3.1. Results of topoisomer distribution experiments

E_T values obtained from the different topoisomer distribution experiments are listed in Table 1. These values all apply to the same preparation of pBR322 at low superhelix density in the absence of ethidium. Standard errors are taken to be 9.6%, as described in the preceding article. There appears to be no significant effect of 10 mM Mg^{2+} or type of Topo I on E_T at 37°C. The mean of our data for 37°C, $E_T = 1155 \pm 65$, is practically the same as the value (1130) reported by Horowitz and Wang [1], but is significantly lower than that (1320) reported by Duguet at 35°C or that (1610) reported by Shore and Baldwin at 20°C [2,3]. The ligation media of Horowitz and Wang and Shore and Baldwin contain 10 mM Mg^{2+}, while that of Duguet contains 5.5 mM Mg^{2+}. Extrapolating the E_T versus temperature data of Duguet [2] to 20°C yields $E_T \approx$ 1544, which approaches the value (1610) of Shore and Baldwin. After the raw data of Shore and Baldwin for short DNAs are analyzed using a rigorous theory, it is found that all of their E_T values exceed those of Horowitz and Wang by a factor of about 1.4, regardless of DNA length [13]. For reasons that are not yet clear, the E_T values obtained for pBR322 from topoisomer distributions near zero superhelix density appear to fall into two classes, namely those with $E_T \approx 1150$ at 37°C ([1] and the present work) and those with somewhat larger values [2,3].

Table 1
Optimum values of E_T obtained by ligation of pBR322 under various experimental conditions

Exp.	10 mM Mg^{2+}	Enzyme type	T (°C)	pH	E_T [a]
1	yes	chicken erythrocyte	37	8.5	1140
2	no	chicken erythrocyte	37	8.5	1200
3	yes	calf thymus	37	7.5	1130
4	yes	calf thymus	21	7.5	1340

[a] Relative errors are taken to be 9.6% as described in Appendix D of the preceding paper.

Although our data indicate a slightly larger value, $E_T = 1340 \pm 130$, upon decreasing the temperature from 37 to 21°C, the statistical significance of the increase, $\Delta E_T = 185 \pm 145$, is marginal. In any case this increase is not nearly great enough to account for the large discrepancy between the E_T values reported by Horowitz and

Wang [1] and Shore and Baldwin [3]. The low statistical precision of ΔE_T precludes a reliable determination of enthalpies and entropies of supercoiling from our data. The earlier results of Depew and Wang [14] likewise do not afford sufficient precision to determine reliable ΔH and ΔS values. Recent results of Duguet [2] span a sufficiently wide range of temperatures (35–85°C) to estimate an average slope, $\Delta E_T/\Delta T \approx -13.2$/K. Over the range 21–37°C, this predicts a change $\Delta E_T = (16)(13.2) = 211$. On the basis of calorimetric measurements of ΔH, Seidl and Hinz [15] suggest that $\Delta H = (2.4)\Delta G$, whence $\Delta S = (1.4)\Delta G/T$, and $\Delta E_T = (1 - 294/310)(1.4)\ E_T = (0.072)E_T = 83$. Within the substantial experimental error, our measured ΔE_T is consistent with the results of either Duguet or Seidl and Hinz, but lack sufficient precision to conclusively support either of those.

The mean value $E_T = 1155 \pm 65$ obtained here for pBR322 significantly exceeds (by 20%) the corresponding value $E_T = 950 \pm 80$ obtained for p30δ under identical conditions by the same method in the preceding paper [4]. The quoted uncertainty in the former quantity is the standard deviation of the mean, which is calculated from the 9.6% statistical errors in the three individual values for $T = 37$°C by conventional error propagation rules. The quoted uncertainty in the latter quantity is simply the reproducibility error, which is the mean of the squared deviations of the individual values from their mean. We have quoted the larger of the standard deviation of the mean or the reproducibility error in each case. In any event, the significant difference in E_T values already suggests that pBR322 exhibits some significantly different secondary structure than p30δ.

3.2. Results of competitive dialysis experiments

Fluorescence intensity ratios for one preparation of pBR322 are collectively fitted and plotted versus ethidium binding ratio in Fig. 1. The extremely poor fit indicates that a single value of E_T cannot account for all of the competitive dialysis data. The same qualitative conclusion holds for each of the three preparations of pBR322 examined. Hence, the fluorescence intensity ratios must be analyzed individually to obtain the *apparent* E_T value prevailing for each binding ratio. The results of such analyses for three different preparations of pBR322 are displayed in Fig. 2. The data vary greatly from one preparation to another. The only common feature of these data is that the apparent E_T typically increases with increasing ethidium binding ratio, or decreasing superhelix density. At low binding ratios near native superhelix density, the apparent E_T values of pBR322 may be substantially lower than for p30δ, and at high binding ratios may significantly exceed that of p30δ, and even approach that reported by Shore and Baldwin [3].

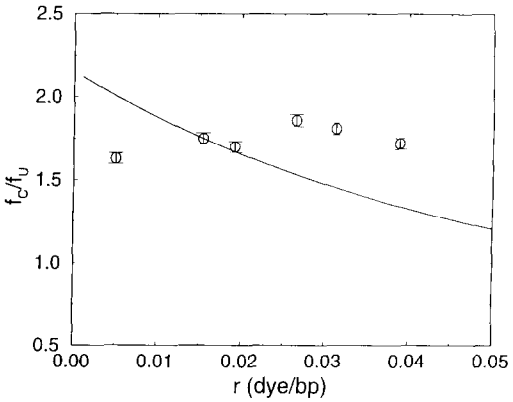

Fig. 1. Fluorescence intensity ratio f_C/f_U versus ethidium binding ratio r (dye/bp) for a single preparation of supercoiled pBR322. f_C and f_U are the fluorescence intensities of, respectively, supercoiled and linearized DNAs from the same competitive dialysis experiment. Open circles are experimental points and the solid curve is the best-fit theoretical curve. The linking difference of the native supercoiled pBR322 is −23 turns. The collective optimum E_T value and the r values for each experiment are determined by the algorithm described in Appendix C of the preceding paper. The optimum twist energy parameter is $E_T = 1040$. SSD = 0.431. Optimum r values lie quite close to the added EB/BP ratios. Experimental conditions are NSTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH = 7.8 at 22°C) at 37°C.

Results of competitive dialysis measurements on a single preparation of pBR322 at different times after cell lysis are displayed in Fig. 3. With increasing time after cell lysis, the apparent E_T evidently decreases and the curves appear to flat-

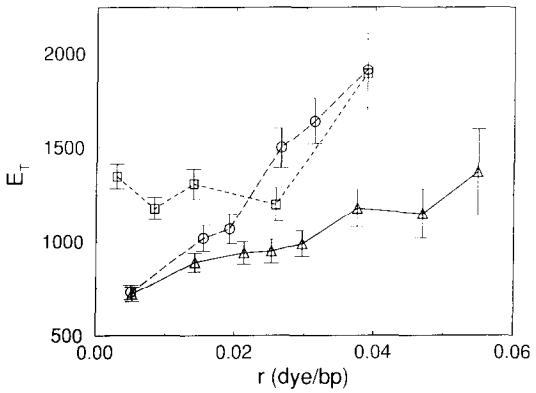

Fig. 2. Twist energy parameter E_T versus ethidium binding ratio r (dye/bp) for three different preparations of supercoiled pBR322. The values of E_T and r are determined by analyzing individually the fluorescence intensity ratio for each competitive dialysis experiment according to the algorithm described in Appendix C of the preceding paper. Optimum r values lie quite close to the added EB/BP ratios. The linking differences of these native supercoiled DNAs are -23 turns (circles), -22 turns (squares), and -26 turns (triangles). Experimental conditions are the same as in Fig. 1.

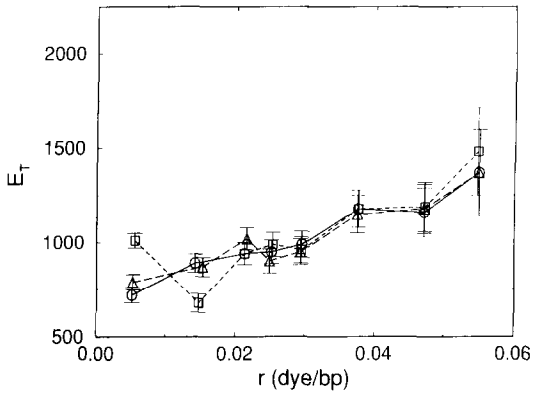

Fig. 4. Curves of twist energy parameter E_T versus ethidium binding ratio r (dye/bp) for a single preparation of supercoiled pBR322 at different times after cell lysis. Times after cell lysis are 1.5 months (triangles), two months (squares), and six months (circles). The values of E_T and r are determined by analyzing individually the fluorescence intensity ratio for each competitive dialysis experiment according to the algorithm described in Appendix C of the preceding paper. Optimum r values lie quite close to the added EB/BP ratios. The linking difference of this native supercoiled DNA is -26 turns. Experimental conditions are the same as in Fig. 3.

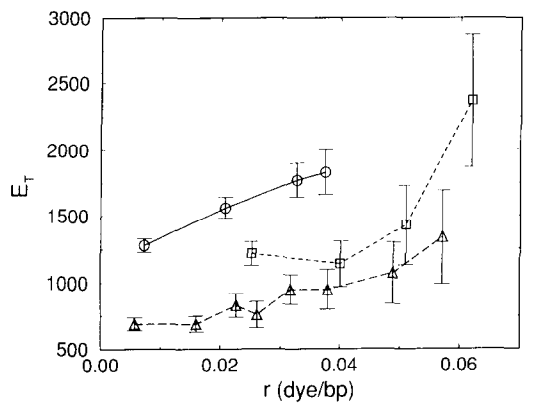

Fig. 3. Curves of twist energy parameter E_T versus ethidium binding ratio r (dye/bp) for a single preparation of supercoiled pBR322 at different times after cell lysis. Times after cell lysis are five weeks (circles), six weeks (squares), and six months (triangles). The values of E_T and r are determined by analyzing individually the fluorescence intensity ratio for each competitive dialysis experiment according to the algorithm described in Appendix C of the preceding paper. Optimum r values lie quite close to the added EB/BP ratios. The linking difference of this native supercoiled DNA is -26 turns. Experimental conditions are NSTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH = 8.5 at 21°C) at 37°C.

ten. Significant changes occur even after six weeks. Results of competitive dialysis measurements on another sample of pBR322 with the same linking difference at various times from 1.5 months (seven weeks) or more after cell lysis are shown in Fig. 4. These data indicate that successive apparent E_T measurements on a *single equilibrated* sample are quite reproducible over most of the observed range of binding ratios, though not at the lowest binding ratios.

Taken together, these data argue strongly for the existence of metastable secondary structure in supercoiled pBR322, which evidently evolves for six weeks or more, before settling in to some more or less reproducible behavior. Even in that settled condition, the apparent E_T values increase from about 700 at low binding ratio and native superhelix density up to 1300 or 1500 with increasing binding ratio (up to $r = 0.055$) and decreasing superhelix density. We suspect that very long-lived metastable secondary structure persists even in the settled condition, in part because the trend of apparent E_T values extrapo-

lates to a value at $\sigma = 0$, $r = 0.07$ that lies well above the measured value at $\sigma = 0$, $r = 0$. Of course, if altered secondary structure prevails over much of the sequence, it is very likely that the intrinsic binding constant and intrinsic twist also differ from that of the equilibrium linearized DNA. In such a case, the meaning of the apparent E_T values obtained from ethidium binding studies is unclear, so they must be interpreted cautiously. For this reason, one cannot conclude confidently that E_T actually decreases with increasing negative superhelix density to values well below 1000 at $\sigma = -0.05$, as the data at first glance suggest. Although the apparent E_T values obtained for pBR322 by ethidium binding studies cannot be regarded as reliable indicators of the actual E_T values that govern the free energy of supercoiling, they nevertheless provide a useful indication of the relative affinity of the supercoiled DNA for the dye.

3.3. Summary of topoisomer distribution and ethidium binding results

A schematic diagram that summarizes the results of these experiments is shown in Fig. 5. The E_T near $\sigma = 0$, $r = 0$ is significantly larger for pBR322 than for p30δ. In the case of pBR322, the *apparent* E_T is considerably lower at $\sigma = -0.05$, $r = 0$ than at $\sigma = 0$, $r = 0$, and increases substantially with increasing bound ethidium and decreasing superhelix density across the σ–r plane. Indeed, the extrapolated apparent E_T near $\sigma = 0$, $r = 0.07$ significantly exceeds that near $\sigma = 0$, $r = 0$. The observed differences in results between pBR322 and p30δ are attributed to differences in secondary structure, specifically to one or more different conformations that prevails at least part of the time over at least part of the sequence in the Topo 1-relaxed pBR322, and to the persistence of one or more different metastable conformations that prevails over domains of variable extent in the dye-relaxed pBR322. This conjecture, which may account also for the observed variability from one preparation of pBR322 to the next (Fig. 2), is supported by the observed variation of the results with time after cell lysis (Fig. 3). We emphasize that the

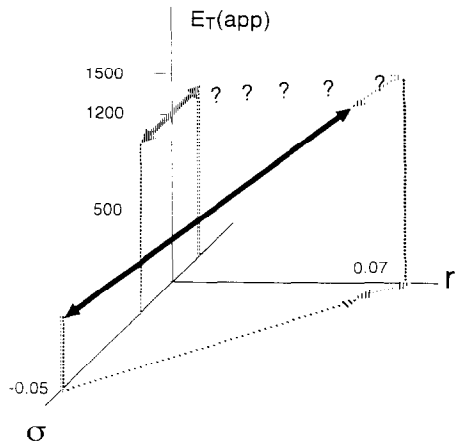

Fig. 5. Schematic illustration of results for pBR322. Analysis of topoisomer distributions yields $E_T^{app} = 1200$ near $\sigma = 0$ in the E_T^{app}–σ plane (stripped double headed arrow in that plane). The ethidium binding method yields E_T^{app} values that are considerably less than 1200 near $r = 0$, $\sigma = -0.05$, but which increase substantially along the diagonal (solid double headed arrow) to values significantly higher than 1200 at $\sigma = 0$, $r = 0.07$. Question marks indicate the region where E_T^{app} has not been measured. E_T^{app} measurements by the ethidium-binding method become extremely unreliable as σ approaches zero.

preparation-to-preparation variability of pBR322 is most unlikely to arise from variations in the experimental protocol for the dialysis experiments, since the same protocols and experimenters produced identical (to each other) results for different preparations of p30δ DNA, and for different runs on the same sample of pBR322, provided the time after cell lysis exceeds six weeks. Strong evidence for the persistence of metastable secondary structure in linearized [9], ethidium-relaxed [8], and chloroquine-relaxed [5,12] pBR322 was presented previously. It was conjectured that such metastable secondary structure in pBR322 arises as a consequence of structural transitions induced by changes in superhelical stress, as observed for pUC8 DNA [9].

3.4. Results of the optical experiments

Direct evidence for transitions in secondary structure induced by superhelical stress over (at

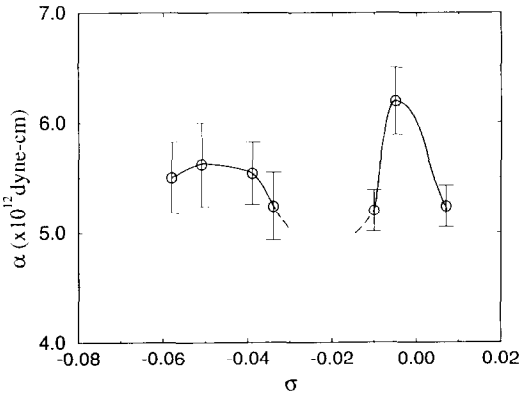

Fig. 6. Torsion constant α of pBR322 versus superhelix density σ. α is the average of FPA results for the two longest time spans (0–70 ns and 0–120 ns). DNA concentration is 40–50 μg/ml. Ethidium was added to a concentration of 1 dye per 300 base pairs. Experimental conditions are LSTE buffer (10 mM NaCl, 10 mM Tris, 1 mM EDTA, pH = 8.0) at $T = 21$°C.

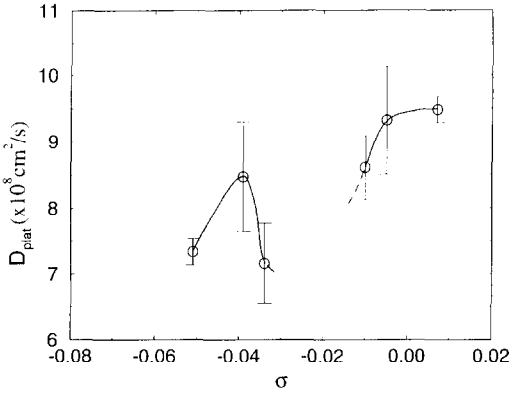

Fig. 8. Plateau diffusion coefficient D_{plat} of pBR322 versus superhelix density σ. D_{plat} was measured by dynamic light scattering at $K = 4.5 \times 10^5$ cm^{-1}. DNA concentration is 40–50 μg/ml. Experimental conditions are the same as in Fig. 6.

least) part of the sequence of pBR322 comes from the measured torsion constant (α), molar ellipticity at the minimum near 250 nm [θ], and D_{plat}, which are plotted versus superhelix density in Fig. 6–8. With increasingly negative superhelix density, the structural transitions are manifested by simultaneous dips in α, [θ], and D_{plat} near $\sigma = -0.010$ and by the simultaneous jumps in α, [θ], and D_{plat} near $\sigma = -0.037$. These results are qualitatively similar to those for pUC8 DNA [9], although the magnitudes of the changes are not as large. In contrast, the α values for p30δ are essentially independent of superhelix density. The apparent absence of structural transitions in p30δ is consistent with the absence of any observed metastable behavior in our samples of that DNA.

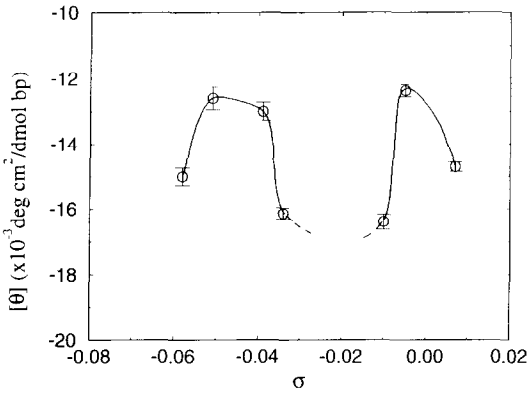

Fig. 7. Molar ellipticity [θ] (at the minimum near 250 nm) of pBR322 versus superhelix density σ. DNA concentration is 40–50 μg/ml. Experimental conditions are the same as in Fig. 6.

3.5. A tentative explanation for the different behaviors of p30δ and pBR322 DNAs

It was recently demonstrated that a change in sequence over a small region ($\lesssim 30$ bp) of a much longer (~ 1100 bp) restriction fragment of pBR322 produces a change in secondary structure that extends over a surprisingly large domain spanning several hundred base-pairs or more [17]. That and related observations on the same fragment suggest that the secondary structure over (at least) part of the pBR322 sequence is not a constant unique structure, but instead fluctuates between two or more different conformations that extend over rather large domains, and whose relative stabilities depend sensitively upon sequence, even over rather small fractions of the total domain. We postulate that the equilibrium between secondary structures of pBR322 shifts with increasingly negative superhelix density to

first one alternate secondary structure near $\sigma = -0.010$ and then to another near $\sigma = -0.37$, as proposed previously [8], and as suggested by the results in Figs. 6–8. We also propose that the change in sequence required to go from pBR322 to p30δ stabilizes one of its co-existing secondary structures, so that it continues to prevail even up to native superhelix density ($\sigma = -0.05$). Consequently, p30δ would be expected to undergo no structural transitions induced by superhelical stress, and to exhibit no metastable secondary structures in either the Topo 1- or dye-relaxed molecules, as observed. The region of the pBR322 sequence that is altered by inserting the yeast sequence lies between the BamHI (375) and SalI (651) sites, and constitutes the right (3′-) end of the ~1100 bp restriction fragment that exhibits the long-range effect of sequence on secondary structure [17], as described above. This region is well separated from the sites of reported strand separation transitions, namely (3180–3305) and (4131–4260) [6], which are observed at lower ionic strength in any case. Thus, the latter do not appear to play a direct role in the different behaviors observed for p30δ and pBR322. Clearly, much further work is required to work out the detailed mechanism of the long-range effects, the secondary structures involved, which might be relatively minor variants of B-helix, their sequence preferences, and the effects of superhelical stress on their relative stabilities. Nevertheless, the available evidence suggests that extensive transitions in secondary structure are induced by superhelical stress in the replicative form of M13mp7 [18] and in pUC8 [9], as well as in pBR322. In such cases one would not expect the supercoiling free energy to remain quadratic up to native superhelix density ($\sigma = -0.05$). The ideal behavior observed for p30δ in the preceding paper may well be exceptional rather than the norm. Consequently, considerable caution is advisable in extrapolating the ideal results for p30δ to other plasmids.

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