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Torsional stress and local denaturation in supercoiled DNA

(DNA superhelicity/AT-rich sequences/regulation/DNA repair)

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ABSTRACT **It is shown that local denaturation can be a natural consequence of supercoiling, even in environments where base pairing of linear DNA is energetically favored. Any change in the molecular total twist from its unstressed value is partitioned between local denaturation and smooth twisting in both the native and coil regions so as to minimize the total conformational free energy involved. Threshold degrees of torsional deformation are found for the existence of stable, locally melted conformations. As these thresholds are surpassed, the number of denatured bases increase smoothly from zero. Existing experimental evidence regarding denaturation in supercoiled DNA is in good agreement with the predictions of this theory. In addition, from existing data one can estimate the partitioning of superhelicity between twisting and writhing. Possible consequences of stress-induced strand separation on the accessibility of the DNA to enzyme attack are discussed. Control of local melting by DNA topoisomerases and DNA gyrases could regulate diverse events involved in transcription, replication, recombination, and repair.**

A covalently closed, circular molecule of DNA has a fixed linking number Lk, measuring the number of times one strand of the duplex links through the closed circle formed by the other strand (1). In the absence of single-strand breaks, Lk remains a fixed integer independent of changes in shape due to bending, twisting, or local denaturation. Lk may be decomposed into terms Wr, the writhing number, and Tw, the total twist of the molecule:

$$Lk = Tw + Wr. \qquad [1]$$

This equation is a mathematical reformulation of a relationship first described by Vinograd *et al.* (2). Tw measures the number of times either strand twists around the central axis of the molecule, whereas Wr measures the shape of this central axis. Both may vary as the molecule bends, even though their sum, Lk, remains constant. In this way the constraint imposed by ring closure, the constancy of Lk, is seen as a coupling between twisting and bending.

A closed circular molecule is in its relaxed state if, when it has $Wr = 0$, the duplex is wound at the precise twist rate of minimum energy, $Tw_o = 10.4$ base pairs per turn (3). The linking number of a ring molecule in this state is Lk_o. A molecule whose linking number differs from Lk_o is said to be supercoiled. If $\Delta Lk = Lk - Lk_o > 0\ (< 0)$, the molecule is positively (negatively) supercoiled. According to Eq. 1 the possible supercoiled conformations must differ from the relaxed ones, either by bending (i.e., changes in Wr) or by twisting or both. However, bending and twisting induce elastic restoring stresses and hence require energy (4). Thus, a supercoiled molecule will distribute ΔLk between Wr and $q = Tw - Tw_o$ in the manner that is energetically most favorable in its local environment. The techniques developed below provide a first method for estimating this distribution. This important information has been extremely difficult to determine by other means. For example, no relationship is known for a closed space curve of fixed length between Wr and the minimum possible total squared curvature consistent with that value of Wr. Certainly, there is no reason to assume these quantities are linearly related. Therefore, attempts to associate a quadratic energy directly to Wr are without theoretical foundation. Hence, one cannot at present estimate the partitioning of ΔLk from supercoiling energies.

Hsieh and Wang (5) have suggested that the existence of unpaired bases could be energetically favored in supercoiled DNA. The present paper quantitatively examines this question by considering how that portion of ΔLk which appears as twist manifests itself. The change q in the total molecular twist from its unstressed value may take two forms. Either the double helical regions may have their twist rate altered by a smooth torsional deformation or portions of the molecule could denature. In the latter case the two locally melted single strands may wind about each other, permitting the remaining helical regions to approximate more closely their unstressed torsional conformation. Depending on the torsional stiffnesses of helical and coiled DNA, the value of q, and the free energy of denaturation in the local environment, one or both of these responses could occur. The balance between melting and twisting that arises will minimize the total free energy involved.

Many experiments suggest that sufficiently supercoiled DNA may be locally denatured. Single-stranded regions have been observed in native PM2 DNA with electron microscopy (6). As many as three regions of denaturation were seen, together containing approximately 3% of the bases and located in the AT-rich, early melting portions of the molecule. When the local environment favored base pairing, otherwise identical nicked molecules lacked single-stranded regions. Both methylmercury and formaldehyde have been used as chemical probes for unpaired bases (7, 8). The response of native PM2 DNA to either probe was consistent with approximately 3.7% of the bases being unpaired under the experimental conditions. Again, nicked molecules gave no evidence of unpaired bases. Supercoiled DNA is susceptible to single-strand-specific endonuclease attack, whereas the relaxed and nicked forms are either totally insensitive or much less so, depending upon the nuclease involved (9–12). Finally, superhelical DNA forms complexes with single-strand-specific DNA-binding proteins (13, 14).

In what follows the imposed torsional stresses are regarded as arising from supercoiling of closed circular DNA, because most experimental results pertain to this situation. However, other constraints can also produce these stresses and, hence, local denaturation. For example, suppose a linear segment of DNA is held rigidly at two points. Then attack by DNA topoisomerase or DNA gyrase in the region between these points can impose or alter torsional stresses there.

Many of the events involved with transcription, replication,

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Abbreviations: nat, native; den, denatured; Lk, linking number; Wr, writhing number; Tw, total twist.

recombination, and repair are known to be sensitive to the degree of superhelicity of the DNA involved (10, 15–21). Some of these changes of activity might result from stress-induced local melting. Certainly, local strand separation provides a unified mechanism by which supercoiling could elicit many diverse responses.

LOCAL DENATURATION AND TWIST

Consider a closed circular molecule of DNA consisting of N base pairs that is supercoiled by an amount $\Delta Lk = Lk - Lk_o$. Let that portion of ΔLk that is partitioned to torsion be q. Then q itself may be divided between the helical and the coil portions of the molecule: $q = \Delta Tw(\text{nat}) + \Delta Tw(\text{den})$. Assume that n sequential base pairs are denatured. If the resulting single strands were unstressed they would have zero average twist rate. However, under the imposed torsional stresses they may twist around each other with average twist rate τ_c. Then

$$\Delta Tw(\text{den}) = -\frac{n}{A} + \frac{n\tau_c}{2\pi}, \qquad [2]$$

where $A = 10.4$ is the number of base pairs per turn of the unstressed duplex. Let the undenatured portions of the molecule experience a smooth torsional deformation consisting of a variation τ_h in their angular twist rate from its unstressed value. At elastic equilibrium τ_h is constant along all base-paired regions of the molecule (4). Therefore, $\Delta Tw(\text{nat}) = \tau_h(N - n) \div 2\pi$, so that

$$\tau_h = [2\pi q + (2\pi n/A) - n\tau_c]/(N - n), \qquad n \neq N. \quad [3]$$

The free energy required to partition q so that n base pairs are denatured is the sum of contributions from twisting in both the coil and the helical regions, and from denaturation. The free energy of denaturation of n sequential bases is taken as $F(\text{den}) = a + bn$, where b is the free energy required to melt one base pair and a is the extra free energy needed to open the first pair. (Note that $b < 0$ corresponds to denaturing conditions in linear, unstressed molecules, whereas $b > 0$ corresponds to conditions in which base pairing is energetically favored.) The free energy of smooth deformation of the helical regions, $F(\text{nat})$, is dominated by the contribution from the elastic energy density. Because this twisting results in no significant change of order, configurational entropic contributions do not arise. Therefore, $F(\text{nat}) = C_h\tau_h^2(N - n)/2$, where C_h is the torsional stiffness of helical DNA (4). The free energy of twisting of the unpaired strands about each other in the coil regions is also quadratic, $F(\text{coil}) = C_c\tau_c^2 n/2$, where C_c is the effective torsional stiffness of the coil (22, 23). It is known that $F(\text{coil})$ is dominated by entropic contributions, with electrostatic repulsion a secondary factor (23). Substituting from Eq. 3, the total free energy is

$$F(\tau_c, n) = \begin{cases} 2\pi^2 q^2 C_h/N, & n = 0 \\ \dfrac{nC_c\tau_c^2}{2} + a + bn + \dfrac{C_h}{2(N-n)}\left[2\pi\left(q + \dfrac{n}{A}\right) - n\tau_c\right]^2, & 0 < n < N \\ \dfrac{NC_c\tau_c^2}{2} + a + bN, & n = N \end{cases} \qquad [4]$$

For a given value of q, $-N/A < q < N/A$, we determine the values of n and τ_c for which the total free energy $F(n, \tau_c)$ is minimized. (Here, n is regarded as a continuous variable whose physically realizable values are integers with $0 \leq n \leq N$.) Necessary conditions for the existence of a minimum in the interval $0 < n < N$ are $F_n = F_{\tau_c} = 0$, where the subscript denotes partial differentiation (24). By evaluating F_{τ_c}, equating the result to zero, and solving for the value of τ_c, one necessary condition of minimization becomes

$$\tau_c = \frac{2\pi C_h\left(q + \dfrac{n}{A}\right)}{C_c(N - n) + C_h n}. \qquad [5]$$

Hence, the condition that $F_n = F_{\tau_c} = 0$ reduces to

$$\alpha n^2 + \beta n + \gamma = 0, \qquad [6a]$$

where

$$\alpha = (C_h - C_c)[b(C_h - C_c) + (2\pi^2 C_h C_c/A^2)] \qquad [6b]$$

$$\beta = 2C_c N\alpha/(C_h - C_c), \qquad [6c]$$

$$\gamma = bC_c^2N^2 - 2\pi^2 C_h C_c q[q(C_h - C_c) - (2C_c N/A)]. \qquad [6d]$$

Three cases occur depending upon the value of α, and hence upon the free energy of denaturation of one base pair, b. First $\alpha = 0$ at the critical value $b = b_c = 2\pi^2 C_h C_c/[A^2(C_c - C_h)] < 0$. (The helix is stiffer than the coil so that $C_h - C_c > 0$.) In this case $F_n = 0$ exactly when $q = C_c N/[A(C_h - C_c)]$. For this value of q (which is positive), $F_n = 0$ for all n so melting is effortless. For all other values of q, $F_n < 0$ for all n, so that the minima of $F(n, \tau_c)$ occur at the endpoints $n = 0, N$. Similarly, if $\alpha < 0$ so that $b < b_c < 0$, then $F_n < 0$ for all n, $0 < n < N$. Again, the minima occur at $n = 0, N$. The most important case is $b > b_c$ so that $\alpha > 0$. Solution of Eq. 6 a–d shows that in this case $F_n = 0$ for a single value $n = n_q$

$$n_q = \frac{C_c N}{C_h - C_c}\left\{-1 + \left[1 - \frac{\gamma(C_h - C_c)^2}{\alpha C_c^2 N^2}\right]^{1/2}\right\}, \qquad [7]$$

which is in the physically significant range $n_q \geq 0$ exactly when $\gamma \leq 0$. One can show that, if $q_1 < q_2$ are the two roots of

$$\left(\frac{q}{N}\right)^2 - \frac{2C_c}{A(C_h - C_c)}\frac{q}{N} - \frac{bC_c}{2\pi^2 C_h(C_h - C_c)} = 0, \qquad [8]$$

then $\gamma \leq 0$ exactly when $q \leq q_1$ or $q \geq q_2$. These roots (which are always real when $\alpha > 0$) are

$$q_1, q_2 = \frac{C_c N}{A(C_h - C_c)}\left(1 \pm \sqrt{1 + \frac{bA^2(C_h - C_c)}{2\pi^2 C_h C_c}}\right). \qquad [9]$$

It follows that q_2 is always positive, whereas q_1 has the opposite sign as b. Finally, one may show that when τ_c and n satisfy the condition expressed by Eqs. 5 and 7, then $F(n, \tau_c)$ has a minimum (24).

To interpret these results physically, note that the free energy $F(\tau_c, n)$ can be minimized for a partially melted state ($0 < n < N$) only when $F_n = F_{\tau_c} = 0$. Under sufficiently denaturing conditions (i.e., where $b < b_c < 0$ so that $\alpha < 0$) the only possible minima of F occur at the endpoints $n = 0, N$ so that these are the only stable melted conformations. Only when $\alpha > 0$ does there exist a partially melted state for which the free energy is minimized. This happens only when the free energy of denaturation per base pair exceeds the critical value $b > b_c$. Then there are two values q_1, q_2 such that a partially melted stable shape exists exactly when $q \leq q_1$ or $q \geq q_2 > 0$. That is, if a molecule is sufficiently positively supercoiled (i.e., $q > q_2$), it becomes energetically favorable to denature so that the excess twist is absorbed in the more flexible coil regions. When $q \leq q_1$ there exists a partially denatured state for which the total free energy is minimized. The sign of q_1 depends upon the value of b, the free energy of denaturation per base pair. When $b < 0$ so that melting is energetically favored in linear, unstressed molecules, then $q_1 > 0$. In this case any negative supercoiling or slight positive supercoiling (i.e., $q < q_1$) produces partial denaturation. Similarly, when base pairing is energetically

favored (i.e., $b > 0$) then $q_1 < 0$. Now the molecule must be sufficiently negatively supercoiled ($q < q_1$) before a denatured state exists for which the free energy is minimized. Finally, when $q = q_1$ or $q = q_2$, substitution into Eq. **6 a–d** shows that $n_q = 0$. Therefore, as q surpasses either threshold, the number n_q of denatured bases increases smoothly from zero.

Under appropriate conditions on b and q the free energy $F(n, \tau_c)$ is minimized for the values τ_c and n_q given in Eqs. **5** and **7**. In addition, the undenatured state $n = 0$ is also a local minimum. A population of identical molecules will be divided between these two states in the Boltzmann ratio

$$p_o/p_n = \exp[\Delta F/kT] \qquad [10]$$

where $\Delta F = F(n_q, \tau_c) - F(0,0)$, as given by Eq. **4**. For a fixed value of $b > b_c$, as $q < q_1$ ($q > q_2$) decreases (increases), the fraction of molecules found in the denatured state becomes larger.

To this point it has been implicitly assumed that all denaturation is concentrated at a single site. Due to the extra free energy a required to denature a first base pair in a region, one expects at most a small number of melted segments to occur due to torsional stress. The precise number of such regions will depend upon base sequence and q.

COMPARISON WITH EXPERIMENT

The division of q between twisting and melting has been shown above to depend on the torsional stiffnesses C_c and C_h of coil and helical DNA and on the free energy of denaturation. These parameters must be estimated before quantitative predictions from this theory can be made. A change τ_h in the twist rate of the double helix from its unstressed value imposes stress energy density $C_h\tau_h^2/2$. At stressed elastic equilibrium τ_h is constant; $\tau_h = \theta/m$, where θ is the deviation from equilibrium twist angle over m base pairs. The energy per base pair required for this deformation is $C_h\theta^2/2m^2$. The magnitude of root mean square fluctuations in θ for one base pair is $\bar{\theta} = (kT/C_h)^{1/2}$; so $C_h = kT/\bar{\theta}^2$. Recent experimental results give $\bar{\theta} = 0.07$ radians (= 4°), from which C_h is found to be 8.5×10^{-12} erg/radian^2 (25).

The torsional stiffness C_c of twisting in the coil regions of DNA has been estimated from unwinding data (22, 23). C_c has been inferred to vary slightly with the length of the coil region and more strongly with the salt concentration. For present purposes a low-salt value of $C_c = 3.6 \times 10^{-13}$ erg/radian^2 is used (D. Crothers, personal communication).

The free energy of denaturation of n sequential base pairs is taken as $F(\text{den}) = a + bn$. The extra free energy a required to open the first base pair is approximately 8 kcal/mol (1 cal = 4.184 J), largely from the extra stacking energy involved (26). The value of a does not affect the shape or minima of $F(n, \tau_c)$, but does influence the population distribution between the states through Eq. **10**. The free energy of denaturation of a base pair is

$$b = \Delta H[1 - (t/t_m)]. \qquad [11]$$

Because $\Delta H = 9$ kcal/mol, knowledge of the transition temperature t_m will determine b at any t (27). t_m varies with base sequence, pH, and the solvent and ionic properties of the molecular environment. Because b is smallest for AT-rich sequences, local melting is expected to occur first in such regions. There is a large literature concerning the variation of t_m with sequence and environment, from which appropriate values of b may be deduced as needed. (See ref. 27 for references.)

As an example, AT-rich sequences in 0.1 M salt have transition temperatures between 60°C and 68°C, depending on details of sequence (28). The helix stabilization free energy per base pair, b, at 25°C in this case is found from Eq. **11** to vary from 0.95 kcal/mol to 1.13 kcal/mol. For subsequent calculations we use the average value of $b = 1.04$ kcal/mol $= 7.22 \times 10^{-14}$ erg per base pair. In this case the thresholds q_1, q_2 for denaturation, expressed in terms of the twist density $\delta = q/Tw_o$, are found from Eq. **9** to be $\delta_1 = -0.018$ and $\delta_2 = +0.107$. That is, the Watson–Crick helix must be either underwound by 1.8% or overwound by 10.7% before stable denaturation can occur in such AT-rich sequences at 25°C. The threshold b_c at which melting becomes total regardless of the degree of supercoiling is $b_c = -0.98$ kcal/mol, which corresponds to a temperature of $t = 1.109t_m \cong 100$°C for AT-rich molecules.

Consider native PM2 viral DNA at 0.1 M salt concentration and 25°C. These molecules contain $N = 10{,}600$ base pairs and are supercoiled an amount $\Delta Lk = -100$, corresponding to a density $\Delta Lk/Tw_o = -0.098$. Table 1 displays the predictions of the present theory concerning the number n_q of denatured base pairs and the fraction p_n of molecules found in the partially melted state under these conditions for various values of the twist density δ. There is a dramatic appearance of denatured molecules at values of δ slightly beyond the (mechanical) thresholds δ_1, δ_2 for the existence of stable melted states. Both microscopic (6) and chemical (7, 8) evidence shows that native PM2 DNA contains between 300 and 400 denatured bases, corresponding to a twist density, δ in Table 1, of −0.06 to −0.072. Therefore, about 65% of ΔLk appears to be partitioned to twist in this case.

The initial nicking rate of DNA by single-strand-specific endonucleases provides a measure of the presence of locally melted regions. By use of several different nucleases (S1, mung bean, *Alteromonas*, and *Neurospora* endonucleases), it was found that, when conditions favor base pairing (i.e., $b > 0$), sufficient negative supercoiling markedly enhances the susceptibility of DNA to attack (9–11). For each nuclease a neg-

Table 1. Predictions of theory

δ	n_q	p_n
0.200	684	1.00
0.150	314	1.00
0.120	92	1.00
0.114	47	0.948
0.113	40	0.150
0.112	33	0.004
0.110	18	0.00009
0.105	0	—
−0.015	0	—
−0.022	21	0.000035
−0.024	36	0.0169
−0.025	43	0.33
−0.026	51	0.995
−0.030	80	1.00
−0.050	228	1.00
−0.060	302	1.00
−0.070	376	1.00
−0.080	450	1.00

The number n_q of base pairs that denature stably at twist density $\delta = q/Tw_o$ (under the conditions described in the text) is shown for a molecule of $N = 10{,}600$ base pairs. The fraction p_n of the molecules appearing in the locally melted state is also given. These quantities are computed from Eqs. **7** and **10**. For all negatively supercoiled cases ($\delta < 0$), the twist of the coiled regions is $\tau_c = -0.27$ radian per base pair (where the negative sign means that the handedness of twist is opposite to that of the double helix). Under conditions of extreme positive supercoiling, the locally melted strands are twisted by $\tau_c = +1.57$ radian per base pair, suggestive of bunching at the melted regions. Critical values of δ are seen at which the population of molecules with unpaired bases becomes important.

ative threshold is observed in the superhelix density which must be passed before significant attack occurs. These thresholds vary with the enzyme system, as would be expected if different enzymes require different amounts of melting to act. Only for *Alteromonas espejiana* nuclease has the susceptibility of both negatively and positively supercoiled DNA been studied (11). There two thresholds were observed. When the superhelix density σ satisfies $\sigma < -0.02$, the initiation rate for nicking increases from essentially zero. Similarly, when $\sigma > 0.14$ the enzyme again produces significant nicking. Provided that the fraction of ΔLk partitioned to twist at these superhelix densities is between about 0.6 and 0.9, these results correspond closely to the threshold twist densities for onset of melting, $\delta_1 = -0.018$ and $\delta_2 = +0.107$, found above. Furthermore, mung bean nuclease attacks specifically in AT-rich regions of supercoiled molecules, precisely where denaturation is expected (29).

REGULATION BY DENATURATION

As described above, the state of base pairing can be controlled by the amount of torsional stress imposed. This in turn is determined by the balance between the activities of DNA gyrases, enzymes that introduce supercoils, and DNA topoisomerases, enzymes that relax them. In this manner these enzymes could regulate diverse biological events. Because the free energy of denaturation depends upon base sequence, the sites of expected local melting under stress would constitute a portion of the inherited information content of the DNA. AT-rich sequences are known to denature most easily, so it is reasonable to consider whether such local regions of the genome could serve as sites of stress-controlled initial enzyme attack.

Many experiments show that activities involved in transcription, replication, recombination, and repair are affected by the superhelicity of the DNA involved (10, 15–21, 30–33). Some of these alterations might occur in response to stress-induced strand separation. For example, the RNA polymerase coded by phage N4 requires denatured DNA as a template (15). *In vivo* transcription by this enzyme is inhibited by coumermycin, which inactivates host DNA gyrase, suggesting that supercoiling is required for N4 transcription. Similarly, Wang (10) has shown that PM2 DNA must be sufficiently supercoiled before significant transcription by *Escherichia coli* RNA polymerase core enzyme can occur. The frequency of initiation of transcription of phage λ DNA appears to be enhanced by increased superhelicity (30).

The integrative recombination of phage λ DNA into the host genome proceeds with any efficiency only when the viral DNA is sufficiently negatively supercoiled (18, 31). Relaxed or slightly positively supercoiled substrate was much less effectively integrated unless DNA gyrase was present. Analysis of the phage λ and host integrative recombination attachment sites demonstrates a homologous region of 15 base pairs, of which 12 are AT (32). It is just such sequences which would be expected to denature under torsional stress. Superhelical φX-174 replicative form I DNA takes up ^{32}P-labeled, homologous single-stranded fragments, although relaxed molecules do not (33). This may be due to annealing of the fragments to single-stranded complementary regions.

The interruption of base pairing at molecular lesions caused by UV-irradiation would strongly favor such sites for further stress-induced local melting, because the extra free energy (a = 8 kcal/mol) required to melt a first base pair would not be needed there. For this reason, when a region of irradiated DNA is torsionally stressed, local denaturation would be expected at these sites of radiation damage. This could provide a mechanism by which the repair system recognizes such damage. Significantly, repair of UV-irradiated DNA is inhibited by antagonists to DNA gyrase (19).

DISCUSSION

The present paper develops an initial model for the local melting of torsionally stressed DNA. As such it contains considerable simplifications of the expected behavior of actual DNA. Most importantly, the detailed effect of base sequence is not considered explicitly. Instead, initial melting is thought to occur at the most AT-rich regions. Details of base sequence will be important when considering multiple regions of melting and also near or above the transition temperature t_m. To this end a statistical-mechanical theory of stress-induced melting that includes effects of base sequence is being developed. The stress-induced formation of hairpin loops at inverted repeat sequences provides an alternative to the twisting of the melted regions considered here (34, 35). The mechanics of this important situation will be described elsewhere.

Other simplifications in the present approach include the following. Entropic effects due to solvation and cation binding have not been explicitly treated. The present view of denaturation as a simple unzipping is probably somewhat simplified. In reality, the melting consequent on torsional stress could involve complicated forms of partial strand separations or bond weakening whose mechanics and energetics are quite intricate. The association of a quadratic energy density to twisting in the coil regions is probably not reasonable at high twist rates τ_c, although one expects it is satisfactory for negatively supercoiled molecules. Both torsional stiffnesses C_c and C_h may depend upon environmental factors such as the concentrations of various cations (36). Finally, possible changes in the partitioning of ΔLk consequent on denaturation are not considered.

In spite of these approximations, this model has been shown to be in good agreement with existing experimental evidence. It also provides a method for estimating the partitioning of superhelicity between twisting and writhing.

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