Temperature dependence of the gel electrophoretic mobility of superhelical DNA

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ABSTRACT

We have determined the gel electrophoretic behavior of closed circular plasmid pSM1 DNA (5420 bp) as a function of both temperature and of linking number (Lk). At temperatures below 37°, the electrophoretic mobility first increases, then becomes constant as Lk is decreased below that of the relaxed closed DNA. As the temperature is increased above 37° the electrophoretic mobility first increases as Lk decreases and then varies in a cyclic manner with further decreases in Lk. As the temperature is increased over the range 37° - 65° the cyclic behavior is manifested at progressively smaller decreases in Lk and the amplitude of the cycles increases. We interpret the results in terms of the early melting of superhelical DNA, in which the free energy associated with superhelix formation is progressively transferred to local denaturation. Using a two state approximation, we estimate the free energy change in the first cyclic transition to be 35 Kcal/mole DNA at 37° and to decrease linearly with temperature. The free energy becomes equal to zero at a temperature of 71.6°, which lies within 3° of the melting temperature for the corresponding nicked circular DNA. From the slope of this relationship we estimate the apparent entropy and enthalpy of the first mobility transition to be 6.0 Kcal/mole base pair and 17.3 cal/mole base pair/degree, values consistent with duplex melting.

INTRODUCTION

The gel electrophoresis behavior of closed circular DNA is greatly influenced by the requirement that the linking number be conserved. This means, in particular, that changes in duplex winding, or twist, be associated with equal and opposite changes in superhelical winding, or writhing (32). Unlike a linear or nicked circular DNA, whose mobility in agarose gels is primarily determined by the molecular weight, the mobility of closed circular DNA varies also with the linking number (14). These characteristics have been used to advantage in the study of the occurrence of localized special duplex structures, such as cruciforms and stretches of Z DNA.

Resolution of topoisomers has been greatly improved with the recent use of two dimensional gel electrophoresis (16, 18, 22). By means of experiments involving the analysis of families of topoisomers with this technique, it has

been demonstrated that the transitions from a palindromic sequence to a cruciform (6, 11, 18) and from an oligo-d(PuPy) B structure to a Z structure (12) can be driven by the free energy of superhelix formation under the appropriate conditions. In addition, evidence has been presented that cruciform formation is a very slow process at room temperature but that extrusion is kinetically favored at temperatures of 50° (11) or of 65° (6). In these experiments cruciform extrusion was allowed to occur by incubation at the appropriate temperature, but gel electrophoresis was conducted at room temperature.

In the present investigation we examine the gel electrophoresis behavior of an extensive family of topoisomers of plasmid pSM1 DNA, of length 5420 bp (21), over a temperature range of 25° to 65°, with the first dimension of electrophoresis at the controlled temperature. We find that a series of mobility transitions occurs as linking number and electrophoresis temperature are systematically varied. These transitions are manifested as cyclical variations in mobility with decreasing ΔLk (the linking number relative to that for a relaxed DNA). It is suggested that the cycles are associated with the sequential melting of local regions of increasing relative stability and that they reveal the fine structure of early melting in closed circular DNA. Similar results, to be reported elsewhere, were obtained in experiments with pBR322 DNA.

Comparable extents of duplex unwinding, as shown by mobility changes, may be achieved by alteration in either temperature or in linking number. This virtual equivalence of T and ΔLk makes possible the construction of thermodynamic cycles for analysis of the free energy associated with these transitions. The formation of cruciforms from near-palindromic sequences remains a possible explanation of the cyclic variation in mobility with linking number. The data are also consistent with a more general model in which, above critical paired values of T and ΔLk , formal changes in writhing are transferred in a cyclic manner into duplex denaturation. We have estimated the apparent changes in free energy and in enthalpy and entropy which accompany the first cycle and find that they are consistent with early denaturation.

MATERIALS AND METHODS

Plasmid DNAs

Plasmid pSM1, derived from R factor R12, was obtained as described previously (20). DNA samples of varying superhelix density were prepared by relaxing 0.2 μ g pSM1 DNA in 25 μ l topoisomerase reaction buffer with 1.0 unit of calf thymus topoisomerase I (Bethesda Research Laboratories) at 2° for 30

min. The topoisomerase reaction buffer was 20 mM Tris-HCl (pH 8.0), 0.2 M KCl, 0.5 mM dithiothreitol (DTT), 0.1 mM EDTA, and 30 μ g/ml BSA. The reaction was terminated by the addition of 6.25 μ l of 5% SDS, 0.15% bromphenol blue, 50% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The solution was preincubated for 10 min at the temperature of the first dimension (tube gel) of the electrophoresis, then layered onto the surface of the gel.

Electrophoresis

The electrophoresis (E) buffer for both first and second dimension was 90 mM Tris-borate (pH 8.4 at 25 $^{\circ}$), 3 mM EDTA. First dimension electrophoresis was carried out in 0.7% agarose (Type 1, Sigma Chemical Co.) tube gels prepared in E buffer. Following solution of the agarose, water was added to correct for that lost during the solubilization procedure. The solubilized agarose was then pipetted into 27 x 0.6 cm (i.d.) glass tubes, partially constricted at the bottom and sealed with Parafilm. After cooling, the Parafilm was removed, and the gel was ejected with a syringe and cut to 22 cm. For first dimension electrophoresis experiments carried out at 50 $^{\circ}$ to 65 $^{\circ}$, cheese cloth was secured to the bottom of the tube by a piece of rubber tubing. Electrophoresis took place at 50 V for 18 hr in a Buchler electrophoresis apparatus maintained at constant temperature ($\pm 0.1^{\circ}$) by a Lauda Model B-2 water incubator/circulator. Foam sheets were placed around the water jacket of the tube gel apparatus to improve thermal insulation. The sample containing plasmid DNAs of different superhelix densities and a control DNA sample containing native (form I) and nicked (form II) pSM1 DNAs were loaded onto separate tube gels. After electrophoresis in the first dimension, the gels were cooled to room temperature. The control gel was stained in E buffer containing 0.5 μ g/ml ethidium bromide, while the experimental gel was soaked in E buffer containing chloroquine for at least 4 hr (25). The chloroquine concentrations were 2.0 μ M for gels used at 25 $^{\circ}$ to 45 $^{\circ}$ and 1.5 μ M for gels used at 50 $^{\circ}$ to 65 $^{\circ}$. Two additional gels at 65 $^{\circ}$ were employed with second dimension electrophoresis carried out in 3.0 and 4.5 μ M chloroquine in E buffer because the single gel at 1.5 μ M chloroquine did not permit sufficient resolution of all topoisomers. Following measurement, under UV light, of the distances travelled by form I and form II plasmid in the control gel, the experimental gel was cut to an appropriate 12.5 cm segment containing the entire range of topoisomers. This segment was placed in an 13 x 0.6 x 0.7 cm (deep) well cut at one end of a 15 x 15 x 0.8 cm horizontal slab gel for second dimension electrophoresis. The gel consisted of 0.7% agarose prepared in E buffer with the specified amount of chloroquine. Second dimension elec-

trophoresis was at 55 V for 18 hr at 25°. The slab gel was then stained in E buffer containing 0.5 µg/ml ethidium bromide for 40 min and destained in E buffer containing 1 mM MgSO₄ for 2 hr. The DNA bands were illuminated with a Spectroline UV lamp and photographed with Kodak Royal Pan film.

Analysis of Topoisomer Distributions

The distances migrated by individual topoisomer bands were calculated by measuring the distances travelled relative to nicked pSM1 DNA and adding the distance travelled by nicked plasmid DNA in the control gel. Mobilities of topoisomer bands in the first dimension were calculated using $\mu = d/tE$, where μ = electrophoretic mobility (cm²/V-hr), d = distance migrated (cm), t = time of electrophoresis (hr), and E = electric field (V/cm). Relative mobilities were calculated by dividing by the mobility of that topoisomer of ΔLk closest to 0.

The linking difference for each band is defined as $\Delta Lk = Lk - \langle Lk^0 \rangle$, and values of ΔLk were measured by the band counting method (14). The linking difference is measured by counting bands from the peak of the gaussian distribution of topoisomers for the relaxed circular DNA (9, 23) in the electrophoresis buffer at the temperature of the first dimension of electrophoresis, $\langle Lk^0 \rangle$. Fractional values of $|\Delta Lk|$, which we label δ , arise whenever the relaxed DNA contains a number of base pairs which is a nonintegral multiple of the average helix repeat number. In order to estimate δ , each topoisomer pair corresponding to increasing values of the integer $|n|$ was examined over the range $0 < |n| < 10$. If the topoisomer with linking difference closest to and greater than the nearest integer n had a higher mobility than the topoisomer with linking difference closest to and less than $-n$, then the value of δ was calculated by

$$\delta = -0.5 \left[1 - [\mu_n - \mu_{-n}] / [\mu_{-n-1} - \mu_{-n}] \right]$$

where μ_i refers to the mobility of the topoisomer closest to and greater than (for positive i) or less than (for negative i) the integer i . If the topoisomer with linking difference just less than $-n$ had the higher mobility, then δ was calculated by

$$\delta = 0.5 \left[1 - [\mu_{-n} - \mu_n] / [\mu_{n+1} - \mu_n] \right].$$

In the special case where $\mu_n = \mu_{-n}$, the value of δ was taken as either 0 or ± 0.5 , depending on whether the line of symmetry in a mobility vs. linking difference curve could be drawn through one topoisomer or between two topoisomers, respectively. The value of δ was then obtained by averaging over all sets of pairs.

pH Measurements

The pH of the electrophoresis buffer was measured at temperatures from

25° to 70° with a Beckman Research Model pH Meter equipped with a Fisher calomel Microprobe. The melting temperature of nicked DNA was calculated by the equation (19, 24)

$$T_m = 16.6 \log(C_s) + 41 X_{GC} + 81.5$$

where T_m is the melting temperature, C_s is the salt concentration, and X_{GC} is the (G + C) mole fraction, 0.482 for pSM1 DNA (21).

RESULTS

Two dimension electrophoresis was carried out on the distribution of pSM1 plasmids of different superhelix densities over a first dimension electrophoresis temperature range of 25° to 65°, in increments of 5°. The first dimension of electrophoresis is the critical one in fixing the effects of the solution environment, and temperature was controlled within $\pm 0.1^\circ$ with a thermostatted water circulator. The second dimension, performed in the presence of the intercalating agent chloroquine, is analytical only. Second dimension electrophoresis was carried out at room temperature (approximately 25°).

Fig. 1 presents the results of two dimension electrophoresis of pSM1 DNAs of different superhelix densities at first dimension electrophoresis temperatures of 35° to 60°. The chloroquine concentration used for experiments in which the first dimension electrophoresis temperature was 25° to 45° was 2 μ M. This decreases the twist of each DNA molecule by about 17 turns, based upon the experiment in which the first dimension electrophoresis temperature was 25°. The chloroquine concentration used for experiments in which the first dimension electrophoresis temperature was 50° to 65° was 1.5 μ M. This decreases the twist of each DNA molecule by about 14 turns, based upon a further experiment in which the first dimension electrophoresis temperature was 25° and the second dimension chloroquine concentration was 1.5 μ M. In addition, temperature changes bring about changes in twist (30). Taking the temperature dependence of the helix rotation angle to be -0.012 degrees/ $^\circ$ C (9, 27), the twist of pSM1 DNA (length = 5.68 kb) would be expected to increase by about 7 turns upon transfer from 60° (first dimension) to 25° (second dimension). Thus the expected total change in twist for pSM1 DNA, employing a chloroquine concentration of 1.5 μ M in the second dimension of electrophoresis, would be $-14 + 7 = -7$ turns. This shift was observed and is shown in Fig. 1, 60°.

More than 40 discrete topoisomers could be resolved in these gels, as shown in Figs. 1 and 2. The range covered was from a linking difference, ΔLk , of about +10 to -30. Again, taking the temperature dependence of the rotation

Figure 1. Two dimension gel electrophoresis of pSM1 topoisomers at different first dimension electrophoresis temperatures. A mixture of pSM1 topoisomers obtained by relaxing the native plasmid DNA with calf thymus type I topoiso-

merase at 2° for 30 min was loaded onto a tube gel for first dimension electrophoresis at the specified temperatures. Following electrophoresis, the tube was soaked in E buffer containing chloroquine at concentrations of 2.0 μM for gels used at 25° to 45° and 1.5 μM for gels used at 50° to 65°. The tube was cut to a length of approximately 12.5 cm and placed in a well at one end of a horizontal slab gel containing the same concentration of chloroquine for second dimension electrophoresis at room temperature. The E buffer was the same for both first and second dimension electrophoresis. The intense spot in the upper left hand corner of each gel corresponds to nicked circular plasmid DNA.

angle to be -0.012 degrees/°C, individual topoisomers could be tracked at different temperatures. The filled symbol at each temperature (Fig. 2) indicates the topoisomer which at 25° has a linking difference of zero. At temperatures 25° to 35°, mobility increases monotonically with increasing magnitudes of ΔLk of both positive and negative sign. However, at temperatures 37° (data not shown) and higher, mobility increases monotonically with increasing magnitudes of ΔLk only of positive sign. Increases in the magnitude of ΔLk of negative sign result in a cyclical variation of mobility with linking difference. This is shown most clearly in the 65° data, in which the

Figure 2. Mobilities of pSM1 topoisomers at different first dimension electrophoresis temperatures. The distance migrated by an individual topoisomer was calculated by measuring the distance migrated relative to nicked pSM1 DNA and adding the distance migrated by nicked circular pSM1 DNA in a control gel. The mobility was calculated as described in the text. The linking difference of each topoisomer was measured by the band counting method, with the fractional value estimated by a consideration of the symmetry of the distribution of topoisomer mobilities in the vicinity of the reference relaxed state (see Materials and Methods).

Figure 3. Mobilities of specific pSM1 topoisomers at different first dimension electrophoresis temperatures. The mobilities of topoisomers with linking differences closest to 0 (●), +8 (○), -8 (X), and -30 (■) were measured as described in the legend to Figure 2. The dashed line corresponds to the topoisomer with linking difference closest to -8. The electrophoresis conditions are described in the legend to Figure 1. Inset: the ratio of the mobilities of topoisomers having linking differences closest to -30 and 0, respectively, at different temperatures.

cyclical change in mobility with increasing negative linking difference begins at topoisomer -6.20, whereas with increasingly positive linking difference, mobility continues to rise up to the highest linking difference observed, $\Delta Lk = +12.80$ turns. Above 65°, most of the DNA sample was nicked during the first dimension of electrophoresis. Thus, data for temperatures above 65° were not obtainable.

An additional feature of the mobility curves is the presence of shoulders at linking differences of about +2 and -2. These shoulders become progressively more pronounced at increasing temperatures (Fig. 2). Unlike the maxima and minima which occur at greater negative values of ΔLk , the linking differences at which the shoulders are located appear to be independent of temperature.

Fig. 2 indicates that the mobility of that topoisomer with linking difference closest to zero increases with temperature. Fig. 3 presents the temperature dependence of mobility for this topoisomer as well as those topoisomers with linking difference closest to +8, -8, and -30. Like the topoisomer

Figure 4. Relative mobilities of pSM1 topoisomers at different first dimension electrophoresis temperatures. The relative mobility of a topoisomer is given by the ratio of its absolute mobility to the absolute mobility of the topoisomer which at that temperature has a linking difference closest to zero. The calculation of the absolute mobility is described in the text.

mer with linking difference closest to 0, the topoisomer with linking difference closest to +8 exhibits increasing mobility with increasing temperature. Those topoisomers with negative linking differences, however, pass through a maximum mobility. For the topoisomer with ΔLk closest to -8, the maximum mobility occurs at a temperature of about 60°; for the topoisomer with ΔLk closest to -30, it occurs at about 50°. The ratio of the mobilities of topoisomers with linking difference closest to -30 and 0 is presented in the inset to Fig. 3. The ratio remains constant until about 40°, and thereafter diminishes with increasing temperature. This diminution in the ratio is coincident with the appearance of the cyclical variation in mobility with linking difference (Fig. 2).

In Fig. 4, the temperature plots of Fig. 2 are normalized to the mobility of the topoisomer which at that temperature has a linking difference closest to zero. The curves for the relative mobilities of topoisomers of increasingly positive ΔLk are virtually superposable for all temperatures from 25° to 65°. The curves for the relative mobilities of topoisomers of increasingly negative ΔLk are markedly different for temperatures 45° to 65°. Each curve possesses several local maxima and minima.

The positions of the local maxima and minima shift towards less negative linking differences with increasing temperature (Fig. 4). The linking differences at which the maxima and minima occur are plotted against temperature in

Figure 5. Local maxima and minima of the curves of mobility versus linking difference (Fig. 2) plotted versus the temperature during the first dimension of electrophoresis. Max 1 refers to the linking difference at which the local maximum of least negative ΔLk occurs at the specified temperature. Min 1 refers to the linking difference at which the local minimum of least negative ΔLk occurs (excluding the local minimum at $\Delta Lk = 0$). Max 2, min 2, and so on refer to subsequent local maxima and minima.

Fig. 5. Maximum 1 refers to the local maximum with the least negative ΔLk , maximum 2 refers to the local maximum with the next least negative ΔLk , and so on. The lowest temperature at which local maxima and minima were observed was 37°. The distance between fixed maxima is relatively constant. A linking difference of 4-5 separates maxima 1 and 2, while a linking difference of 6-8 separates maxima 2 and 3. The mobility decreases especially sharply in passing from maximum 1 to minimum 1, which are separated in linking difference by about 2.

The cyclical variation in mobility with negative linking difference is reversible with temperature. A pSM1 DNA sample containing molecules of various linking differences was first heated to 60° for 30 min, then cooled. The sample was then loaded onto a tube gel for first dimension electrophoresis at 25° followed by second dimension electrophoresis in 1.5 μ M chloroquine. The result was identical to that obtained for the pSM1 sample loaded directly onto the tube gel at 25° without prior heating. This indicates that the effect is

reversible. Otherwise, one would have expected a set of topoisomers that migrated at mobilities intermediate between those of first dimension electrophoresis temperatures of 25° and 60°.

The cyclical variation in mobility with negative linking difference does not appear to be a slow kinetic process, in contrast to the rate of cruciform extrusion in superhelical DNAs (6, 11). A pSM1 sample was loaded onto a tube gel for first dimension electrophoresis at 50°. After 3 hr, electrophoresis was stopped for 9 hr. Electrophoresis was then continued for 15 hr. The result was identical to that obtained in the experiment in which first dimension electrophoresis at 50° was uninterrupted. In particular, the pattern of mobilities was not intermediate between results obtained for 50° and a higher temperature.

The cyclical variation in mobility with negative linking difference at 50° is qualitatively observed at agarose concentrations both lower (0.6%) and higher (0.9%) than those used in these experiments (0.7%). Other experiments with the second dimension of electrophoresis performed in the absence of chloroquine resulted in the same mobility distribution for those topoisomers that could be resolved. This result confirms that the chloroquine binding in the second dimension of electrophoresis does not influence the apparent distribution of mobilities in the first dimension of electrophoresis.

Preliminary experiments with pBR322 DNA at 50° and at 60° indicate that this plasmid exhibits electrophoresis behavior closely resembling that of pSM1. These results, which will be reported in detail elsewhere, reveal a similar cyclical variation in mobility with negative linking difference as well as a shift of the local maxima and minima towards less negative linking differences with an increase in temperature.

The pK_a of Tris decreases by about 1 unit from 25° to 65°. The pH of the E buffer was therefore measured and the actual ionic strength calculated at each temperature. This result was used (see Materials and Methods) to calculate the melting temperature (T_m) of nicked circular plasmid pSM1 DNA over this temperature range. The results show that the pH of E buffer decreases from 8.36 to 7.84 between 25° and 65°, and that the corresponding change in the melting temperature (T_m) of nicked circular pSM1 DNA is from 77.5° to 74.8°.

DISCUSSION

We show in the present paper that the electrophoretic mobility of superhelical DNA varies in a cyclic manner as the linking number is decreased from

that of relaxed DNA. The cyclic behavior is observed at temperatures 37° and greater, and increases in T and in $-\Delta Lk$ have comparable effects. We will discuss these phenomena in terms of possible mechanisms and in terms of the changes in free energy involved. The initial phase of a possibly similar cyclic behavior has also been observed during electrophoresis of a 1683 bp plasmid, pAO3, in a composite acrylamide-agarose gel (7). Although the temperature during this experiment was not specified, the superhelix density at which the first mobility reversal occurred was comparable to that seen by us with pSM1 DNA (5420 bp).

It has been known for some time that the mobility of closed DNA is quantized in terms of Lk (9, 14, 23). The mobility difference between adjacent topoisomer bands is a hydrodynamic property, and is therefore determined to first order by the associated difference in W_r . Changes in W_r between successive topoisomer bands might or might not be accompanied by changes in T_w . It has been estimated, based upon Monte-Carlo calculations of the variance of W_r for a nicked circular DNA, that a change in net winding is resolved about equally into changes in W_r and T_w for the special case in which $\langle W_r \rangle = 0$ and W_r and T_w are uncoupled (28). A more recent study (13), in which a lower limit for the torsional rigidity was obtained from the topoisomer distribution of small DNA circles, concluded that for DNAs of the size of pSM1 or pBR322, at least two-thirds of any winding change is partitioned into W_r . Both these estimates apply to the equilibrium distribution for which $\langle W_r \rangle = 0$, in the vicinity of which the contributions of changes in T_w are expected to be especially important (14).

Changes in T_w with increasing $|\Delta Lk|$ may be elastic or inelastic, and we apportion the changes in Lk accordingly: $\Delta Lk = W_r + \Delta T_{w_{el}} + \Delta T_{w_{in}}$. Since the relative contributions of W_r and $\Delta T_{w_{el}}$ to the electrophoretic mobility have not been ascertained, as noted above, we combine them as $W_r' = W_r + \Delta T_{w_{el}}$, obtaining $\Delta Lk = W_r' + \Delta T_{w_{in}}$. In the absence of inelastic changes in T_w , such as might result from local melting, the quantity W_r' is identical to the number of titratable superhelical turns (1). The interplay between changes in W_r' and in $T_{w_{in}}$ and the associated cyclical changes in mobility are examined over the region of the first maximum in Fig. 6. For clarity, the values of ΔLk and W_r' in the figure have been assigned integral values. The occurrence of the first maximum is envisioned as occurring in three stages, labelled I, II and III in the figure.

In Stage I (ΔLk ranging up to -10), only elastic changes in T_w occur so that W_r' decreases ($-W_r'$ increases) as ΔLk becomes more negative. Thus, as

Figure 6. Possible secondary structures associated with negative linking differences. The diagram in the lower portion represents an enlargement of the region of the first maximum in mobility, with integral values of ΔLk used for illustration. In the upper part of the figure two possible secondary structures have been placed above the graph at linking differences at which they would be expected to occur. The illustration of tertiary structures are schematic and intended only to indicate relative changes in writhe. (a) Cruciform formation. (b) Left-handed coil formation.

was observed for molecules of increasing positive linking difference, an increase in magnitude of ΔLk is transferred in a 1:1 ratio to an increase in magnitude of Wr' . The result is a monotonic increase in mobility with decrease in ΔLk . In Stage II (ΔLk from -10 to -11), a denatured loop forms. Its formation is driven by the free energy diverted by avoiding formation of an additional superhelical turn. The twist decreases inelastically in this helix-coil transition by a value of 1; Wr' remains constant (and so does mobility), so that a decrease in ΔLk is transferred in a 1:1 ratio to a decrease in Tw_{in} . In Stage III, Tw decreases and Wr increases as ΔLk decreases. It is necessary that Tw decrease in greater than a 1:1 ratio as ΔLk decreases in order that Wr' increase as ΔLk decreases.

The occurrence of stage III can be explained by at least two different hypotheses. As illustrated in Fig. 6, one possibility is that the falling portion of each cycle is due to cruciform formation (7, 17, 26). During Stage II (ΔLk from -10 to -11), a denatured loop of length 10 bp is formed at constant Wr' , and at $\Delta Lk = -12$ cruciform formation occurs. Instead of a 5 base pair stem, preserving the 1:1 decrease in Tw_{in} with decrease in ΔLk and maintaining $-Wr'$ at 10, a 10 base pair stem is formed. This cooperativity

would not extend to the full cruciform length because of other factors, such as mismatching at the base of the 10 base pair stem or loop-outs. Since the twist is decreased by 2 (10 base pairs on each side of the stem), Wr' increases by 1, from -10 to -9, and the mobility of the topoisomerase with $\Delta Lk = -12$ becomes less than that with $\Delta Lk = -11$. At $\Delta Lk = -13$, another 20 base pairs are unwound and 10 additional base pairs are added to the stem of the cruciform. The twist again decreases inelastically by 2 and Wr' increases by 1. The cruciform is now fully formed. At $\Delta Lk = -14$, Stage I again occurs.

An alternative explanation for Stage III is that the falling portion of each cycle reflects nonspecific left handed winding of the denatured coils about one another. This possibility, also illustrated in Fig. 6, is consistent with previous calculations (2) and experiments (8, 29) that local denaturation can be caused by supercoiling. During Stage II a denatured loop of length 10 base pairs is again formed at constant Wr' . At $\Delta Lk = -12$, 10 base pairs are added to the loop. However, because of the torsional stress on the single stranded DNA coils, left handed winding of the coils about each other occurs. In the illustration of Fig. 6, the left handed winding alone contributes -1 to the twist. Combined with the 10 base pairs added to the loop at $\Delta Lk = -12$, the total change in twist is -2. Consequently, Wr' must increase by 1, from -10 to -9. At $\Delta Lk = -13$, an additional 10 base pairs are added to the loop, allowing the left handed winding to change by -1 to -2. The twist again decreases by 2 and Wr' increases by 1. At $\Delta Lk = -14$, Stage I again commences.

Rigorous analysis of the energetics of these transitions would require the use of a statistical mechanical model (3, 4), as has been employed for the analysis of Z DNA transitions (22). This would require specification of separate energy terms for each part of the transition. For cruciform formation, this would include the hairpin loop, the paired bases in the stem, each possible defect, and the region at the base of the cruciform. For the left handed coil, this would include a detailed specification of the possible array of microstates. An alternative approach is to employ an equivalent two state model in which the transition is supposed to occur abruptly at the observed midpoint. A similar approach has been used to analyze the variation of mobility with ΔLk observed in the B to Z transition of a plasmid DNA containing various lengths of $d(TG)_n.d(CA)_n$ (12). The transition in this case was relatively broad, in contrast to that observed for a perfect palindrome in pBR322, which occurred over a very narrow range of ΔLk (6). In this analysis it is assumed that the initial transition is highly cooperative and that subsequent

elongation of the region is noncooperative (10, 12). This calculation culminates in an expression for the free energy of the transition, without regard to its detailed nature.

The cyclical variation of mobility with negative linking difference was observed at temperatures of 37° and greater (Fig. 2). The apparent free energy of the first transition (from maximum 1 to minimum 1) can be calculated by making the following assumptions: (1) The decrease in mobility with increasingly negative ΔLk measures the progress of this transition. (2) The transition can be analyzed in terms of an equivalent two state model. (3) The midpoint of the observed transition corresponds to the linking difference, ΔLk_m , at which the equivalent two state transition occurs. The apparent free energy of the transition, $\Delta G'$, is then the difference in free energy between the two states at this linking difference. This can be expressed as

$$(1) \quad \Delta G' = (K/N) [\Delta Lk_i^2 - \Delta Lk_f^2]$$

where K is a constant, N is the number of base pairs in the DNA molecule and ΔLk_i and ΔLk_f are the virtual linking numbers at ΔLk_m before and after the transition, respectively. The value of ΔLk_m was taken to be halfway between the observed first maximum and first minimum. We define the virtual linking number at each state to be that value of ΔLk which would have been obtained upon approaching the transition midpoint from either direction, had no prior transition occurred. The virtual linking number of molecules in the state before the transition, ΔLk_i , was taken to be equal to ΔLk_m . The virtual linking number of molecules in the state after the transition, ΔLk_f , was estimated by extrapolating the mobilities of the topoisomer at the first minimum and that immediately following it to ΔLk_m . This mobility was then interpolated between the mobilities of two topoisomers before the transition to obtain a linking difference value, which was taken as ΔLk_f . For example, at 60°, where maximum 1 occurred at $\Delta Lk = -10.29$ and minimum 1 occurred at $\Delta Lk = -12.29$, the midpoint of the transition was taken as $\Delta Lk_m = -11.29$. The values for ΔLk_i and ΔLk_f were -11.29 and -6.96 , respectively. Using a value of K of 1100 RT (9, 23), the apparent free energy of the first transition at 60° is +10.15 Kcal/mole DNA. The calculated results for temperatures from 37° to 65° are presented in Table 1. A plot of the apparent free energy of the first transition versus temperature is presented in Fig. 7. The slope of this plot may be used to calculate the apparent enthalpy, $\Delta H'$, and the entropy, $\Delta S'$, of the transition. Assuming that these quantities are temperature independent, we obtain the values $\Delta H' = 325$ Kcal/mole DNA and $\Delta S' = 940$ cal/mole DNA/deg.

Table 1. Estimates of the apparent free energy change, $\Delta G'$, and of the number of base pairs N_{bp} , involved in the first transition. Values of ΔLk_m and of ΔLk_f were obtained from Fig. 2, as described in the text, and ΔTw is their difference. $\Delta G'$ was calculated from Eq. (1). N_{bp} is the product of ΔTw and the average helical repeat (31), corrected for temperature (9) and taken to be 10.5 bp/turn.

$T(^{\circ}C)$	ΔLk_m	ΔLk_f	$-\Delta Tw$	$\Delta G'$, Kcal/mole	N_{bp}
37	26.1	19.7	6.4	35.1	67
40	24.5	19.2	5.3	27.7	55
45	21.5	16.3	5.3	24.3	55
50	18.4	13.6	4.8	19.3	52
55	15.4	9.9	5.4	17.4	58
60	11.3	7.0	4.3	10.2	46
65	7.7	2.9	4.8	6.6	50

The apparent free energy of the first transition extrapolates to zero at a temperature of 71.6° (Fig. 7). At this temperature, any $|\Delta Lk| > 0$ would be sufficient to initiate the first transition. This critical temperature is slightly less than the calculated melting temperature, $T_m = 74.4^{\circ}$, for this DNA under these conditions, which is consistent with the first transition being a premelting phenomenon. The values obtained for $\Delta H'$ and $\Delta S'$ correspond to 6.0 Kcal/mole base pair and 17.3 cal/mole base pair/deg, respectively, since the two state transition involved an average of 54.6 base pairs at the

Figure 7. Apparent free energy of the first transition as a function of temperature. The first transition occurs from maximum 1 to minimum 1 and was analyzed in terms of an equivalent two state model. The apparent free energy was calculated at the midpoint of the observed transition, employing Eq. (1), as described in the text.

midpoint. These values are comparable to those reported for the addition of a base pair to a double stranded (RNA) helix (5), which is consistent with our association of the transition with the formation of a denatured region of DNA.

The presence of shoulders at linking differences of about +2 and -2 (Fig. 2) cannot be readily explained by any of the above mechanisms. This phenomenon becomes more pronounced with increasing temperature, and it may represent either a transition from a toroidal to an interwound superhelical structure (27) or the onset of writhing (15). No indication of such shoulders was found in preliminary experiments with pBR322 DNA (in preparation), suggesting a possible effect of base composition or sequence.

The results obtained here indicate that two dimensional gel electrophoresis in which the first dimension of electrophoresis is carried out at elevated temperatures provides a sensitive assay for the detection of altered secondary structures in superhelical DNA. Native supercoiled DNA is first incubated with topoisomerase at low temperatures so as to produce a mixture of topoisomers. Two dimension electrophoresis is then conducted with such a sample at elevated temperature (50° to 60°). The resulting profile of mobility changes appears, at least tentatively, to be associated with the number and relative extents of duplex structural transitions. The relative free energies associated with these transitions will be indicated by the linking differences at which they appear, with the most stable altered local structure being formed at the least (negative) linking difference. Finally, the presence or absence of such duplex structural transitions under physiological temperatures and ionic conditions may be tested, in order that the possible biological role of such alterations be examined.

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