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Conformational fluctuations of DNA helix

(covalent closure by ligase/unwinding angle of ethidium/free energy of superhelix formation/gel electrophoresis)

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ABSTRACT Treatment of a homogeneous sample of circular DNA molecules that contain at least one single-strand scission per molecule with ligase yields a population of covalently closed circles heterogeneous in the topological winding number α. Under proper conditions, the product molecules with different values of α can all be resolved by gel electrophoresis. The distributions in α for several DNAs, covalently closed at different temperatures, have been determined and the results have been interpreted in terms of thermal fluctuations of the DNA helix.

There have been few experimental studies on conformational fluctuations of the DNA helix. The covalent closure of a circular double-stranded DNA molecule containing at least one single-strand scission (hereafter referred to as nicked circular DNA), offers a unique system for such a study. Because of thermal fluctuations within the helix, for a nicked circular DNA molecule the number of helical turns β varies about a certain mean value $\bar{\beta}$. Similarly, fluctuations in the spatial configurations of the DNA chain cause the number of tertiary turns τ to vary about a mean value $\bar{\tau}$, which is expected to be close to zero (for the definitions of β and τ, cf. ref. 1). It immediately follows that if a homogeneous population of nicked circular DNA molecules is sealed by ligase, the resulting covalently closed molecules are not homogeneous with respect to the topological winding number α, because the value of α for any product molecule is determined by the sum of β and τ at the time of closure (1).

Recently, Keller has presented evidence that covalently closed DNA circles differing by as little as one topological turn can be resolved by gel electrophoresis (2). Therefore, gel electrophoresis appears to be ideal for analyzing the distribution of topological turns among ligase-sealed circular DNA molecules. Indeed, Dugaiczyk *et al.* reported that ligase-closed circles of simian virus 40 (SV40) DNA gave multiple bands upon gel electrophoresis (3). In this communication, we present our analyses of the distribution in α for four DNAs covalently closed by ligase at several temperatures.

MATERIALS AND METHODS

DNAs. *Escherichia coli* 15 plasmid DNA, double-strand coliphage fd DNA, *pseudomonus* phage PM2 DNA were obtained in the covalently closed form by published procedures (4–6). SV40 DNA was a generous gift from Dr. M. Botchan. The DNAs were converted to the nicked circular forms by treatment with bovine pancreatic DNase I (7). Ligase treatment of the nicked circular forms was carried out in reaction mixtures containing 10 mM Tris·HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM Na_3EDTA, 5 μM NAD, 50 μg/ml of bovine serum albumin, and 10–40 μg/ml of a nicked circular DNA. After preincubation for 5 min at a given temperature, the reaction was started by the addition of *E. coli* ligase (purified to homogeneity by Dr. P. Modrich) to a final concentration of 0.05 μg/ml. The volume of the ligase stock added was 2% of the total volume and was delivered by a microsyringe with a fine needle (Hamilton) to avoid perturbing the temperature significantly. The reaction was terminated after 30 min by the addition of an equal volume of 20 mM Na_3EDTA. The DNA was phenol extracted and phenol was removed by dialysis against 10 mM Tris·HCl (pH 8.0), 10 mM Na_3EDTA.

Electrophoresis. Electrophoresis was performed on 0.3 cm thick slabs of agarose (Calbiochem or Bio-Rad) held in an apparatus constructed according to the design of Studier (8). The gel concentration was 0.7% (weight/volume) for PM2 DNA and 1.4% for the other DNAs. The electrophoresis buffer consisted of 40 mM Tris·HCl (pH 8.0), 5 mM sodium acetate, and 0.5 mM Na_3EDTA. A constant voltage gradient of 2–3.5 V/cm was applied for 8–18 hr. Gels were stained after electrophoresis by immersion in the electrophoresis buffer containing 0.5 μg of ethidium bromide per ml for 2 hr. The stained gel was placed on top of a short wavelength UV light source (Ultraviolet Products, Inc., model

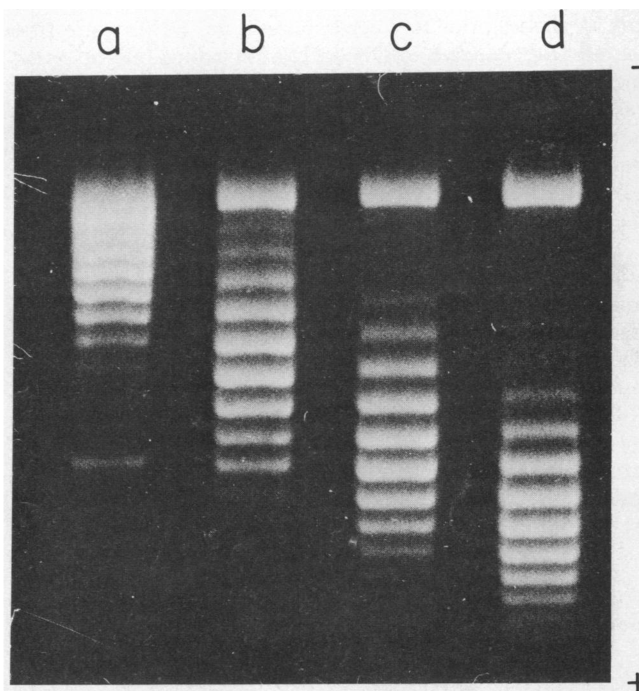

FIG. 1. Electrophoretic patterns of PM2 DNA samples covalently closed by ligase at different temperatures. Approximately 0.2 μg of a DNA sample was placed in each well of a 0.7% agarose slab gel and electrophoresed at room temperature for 17 hr at 2.5 V/cm. The temperatures of ligase reaction for the four samples a–d were 37, 29, 21, and 14°C, respectively.

Abbreviations: nicked circular DNA, circular double-stranded DNA molecule that contains at least one single-strand scission; SV40, simian virus 40.

C51) and photographed on Polaroid type 107 film through a filter which cut off light below 560 nm. The negatives were traced with a Joyce-Loebl microdensitometer.

The film response was calibrated by electrophoresis of different dilutions of a sample of λ DNA digested to completion with *Eco*RI restriction enzyme. From the known relative amounts of DNA in the bands, which are obtained from the known sizes of the restriction fragments (9), the area under each band of densitometer tracing was found to relate linearly to the amount of DNA present in the band with proper control of exposure time. We noted significant photochemical bleaching, however. Prolonged irradiation led to the lowering of fluorescence of all bands and the disappearance of bands of low intensities. Thus, the UV source was on only during photographing. For the group of covalently closed DNA bands in any sample, it was found that within an estimated experimental error of 10%, the area under each peak was directly proportional to the peak height. Therefore, the relative amounts of the bands were in most cases obtained from the peak heights. We have neglected any differences in ethidium binding by the group of covalently closed DNA bands in each sample due to their differences in the degrees of superhelicity, since during staining and photographing the DNA species were probably converted to the nicked form. The magnitude of such corrections is expected to be of the order of a few percent if the covalently closed DNAs remained intact.

RESULTS

Gel electrophoresis of DNA molecules covalently closed by ligase gives multiple bands

Fig. 1 depicts a fluorescence photograph of four PM2 DNA samples after zone electrophoresis in an agarose gel and subsequent staining with ethidium. As described in *Materials and Methods*, prior to electrophoresis the DNAs were first treated with pancreatic DNase I to introduce one or a few single-chain scissions into each molecule. The nicked DNA was then treated with *E. coli* DNA ligase at different temperatures followed by phenol extraction and dialysis, and then electrophoresed. The striking feature of the electrophoresis pattern is the multiplicity of sharp, well-resolved bands in each sample.

The slowest-moving band of each sample is the nicked circular DNA. Each of these samples also contained a small amount of contaminating linear PM2 DNA which is most readily seen as the fastest-moving band in the 37°C sample. The identity of this band was confirmed by comparing its mobility with linear PM2 DNA produced by treating native PM2 DNA with endonuclease *Hpa* II which makes one double-stranded cut per molecule (7). All other bands of each sample are covalently closed. This was shown by electrophoresis of the covalently closed species of each sample after fractionation from the nicked circular and linear species by CsCl-ethidium density gradient centrifugation (10).

The multiple bands are species of distinct electrophoretic mobilities.

To confirm that for each sample the multiple covalently closed DNA bands correspond to species of intrinsically distinct electrophoretic mobilities rather than resulting from electrophoretic artifacts, we performed a field reversal experiment. DNA samples covalently closed at 14°C were first electrophoresed until the multiple bands were well separated, corresponding to the pattern shown in Fig. 1d. The polarities of the electrodes were then switched, and electrophoresis was continued in the reverse direction for varying times. As expected for bands of intrinsically distinct electrophoretic motilities, upon reversal of the field the separated bands were found to merge again as they approached the top of the gel.

The multiple bands of ligase-joined closed circles are species of different topological winding numbers

Our interpretation of the group of covalently closed DNA bands in each sample is as follows. Because of thermal fluctuations of the DNA conformation, the covalent closure of nicked circular DNA molecules yields molecules of different topological winding numbers. The value of the topological winding number α of a molecule is determined by the sum of its number of the helical turns β and its number of tertiary turns τ *at the time of closure*. After covalent closure, both τ and β may fluctuate, but the sum of β and τ must be a constant. It is reasonable to assume that the instantaneous electrophoretic mobility of the covalently closed molecule is determined by its absolute number of tertiary turns at that instant. For molecules of a given α, if the fluctuations in τ are fast compared with the electrophoresis time scale one expects to see only one sharp band, with a mobility dependent on the time average of $|\tau|$ (11). Species with different values of α have different average values of τ under a given set of conditions, and therefore the electrophoretic mobilities of the species are different.

It has been shown previously (12) that the average helix rotation angle of the DNA duplex is dependent on temperature, in such a way that DNA molecules covalently closed at a lower temperature have a higher average topological winding number α than similar molecules closed at a higher temperature. As shown in Fig. 1, the present results for the four PM2 DNA samples closed at different temperatures indicate that the lower the temperature of the ligase reaction the greater the electrophoretic mobilities of the group of covalently closed DNA bands. Therefore, for the set of DNA samples shown in Fig. 1, the higher the average α value, the higher the average electrophoretic mobility. This in turn indicates that within each sample, the multiple bands arise from molecules with different α values. Furthermore, it is reasonable to expect that the electrophoretic mobility of a molecule increases with increasing absolute number of tertiary turns. Since for the four samples shown in Fig. 1 the average mobility increases with increasing α, it can be concluded that when electrophoresed at room temperature in the electrophoresis buffer described in *Materials and Methods*, the closed DNA circles prepared as described are all positively twisted. If the molecules were negatively twisted, the samples with higher α values would have fewer superhelical turns and would therefore migrate slower.

Adjacent covalently closed bands differ by one topological turn

An important conclusion which can be drawn from the electrophoresis pattern shown in Fig. 1 is that adjacent bands within a group of covalently closed DNA bands differ in α by one. Compare, for example, sample b, covalently closed by ligase at 29°C, and sample d, closed by ligase at 14°C. The group of bands of sample d corresponds to the group of bands of sample b shifted downfield by approximately five bands. The previously determined temperature coefficient of the average helix rotation angle (12) when corrected by taking the ethidium unwinding angle as 26° rather than 12°

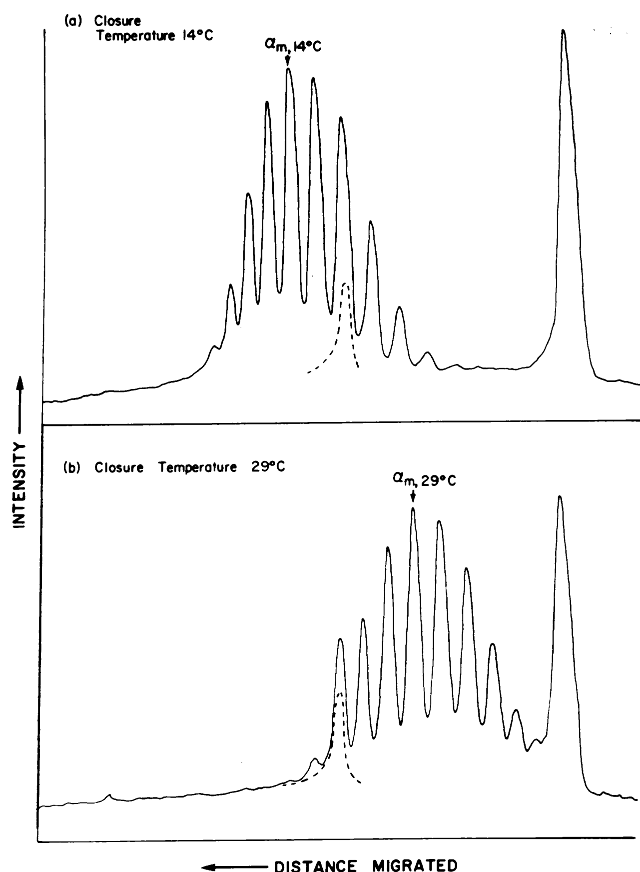

FIG. 2. Microdensitometer tracings of the electrophoretic patterns of two PM2 DNA samples covalently closed by ligase at (a) 14°C and (b) 29°C, respectively. The dotted line in each tracing indicates the position of linear PM2 DNA, which was present in small amounts in the PM2 DNA used.

(13, 14) is 0.010 to 0.011 degrees/base pair per °C. For a DNA the size of PM2 (6.5 megadaltons, ref. 15), the calculated change in α due to a difference in the temperature of ligase closure of 15° is 4.1 to 4.5 turns. Thus, the experimental result is that adjacent bands differ in α by 0.8 to 0.9 turns. However, the topological winding number of a covalently closed DNA molecule is a quantity which must be an integer. Therefore, the conclusion is that the adjacent bands differ in α by exactly one.

The distribution in α

Densitomer tracings of two frames of the negative of the photograph shown in Fig. 1 are shown in Fig. 2. For the

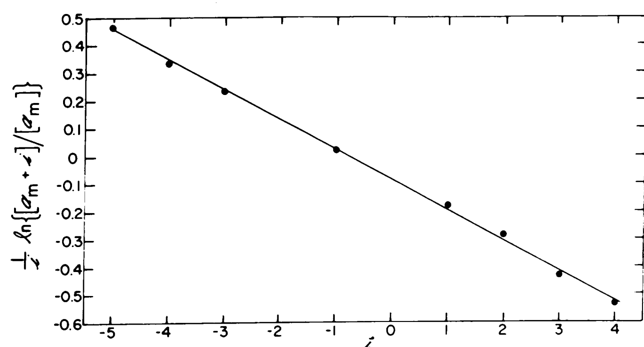

FIG. 3. A typical plot for the evaluation of constants K and ω_T. Data were obtained from the tracing shown in Fig. 2a.

group of covalently closed species, within experimental error, the relative amount of each species is proportional to the peak height (for detail, see *Materials and Methods*). For each sample we denote the winding number of the most intense band of the covalently closed species as $\alpha_{m,T}$, where the subscript T specifies the temperature at which covalent closure of the DNA sample was carried out. From the results of the previous sections, the winding numbers of the bands to the right of the most intense band are, in succession, $\alpha_{m,T} - 1$, $\alpha_{m,T} - 2, \ldots$, etc. Similarly, the winding number of the bands to the left of the most intense band are $\alpha_{m,T} + 1$, $\alpha_{m,T} + 2, \ldots$, etc. The dependence of the peak height (or the relative amount of a species) on the winding number is approximately a Gaussian distribution centering at a position $\alpha_{m,T} - \omega_T$. For the sample shown in Fig. 2a, the center is slightly to the right of $\alpha_{m,T}$.

We interpret the distribution as follows. In the ligase reaction medium at a temperature T, for the *most probable configuration* of a circular DNA molecule with one single-chain scission, the twist angle between the two base pairs on the two sides of the nick is in general not the same as the angle necessary for the joining of the end groups. Thus, the formation of the most probable closed circular species, within a winding number of $\alpha_{m,T}$, requires winding of this twist angle by $2\pi\omega_T$ radians or ω_T turns. We assume that this angular displacement is associated with a free energy difference proportional to the square of the displacement:

$$G(\alpha_{m,T}) = K\omega_T^2 \quad [1]$$

where K is a constant in a given medium at a given temperature. Similarly, for the formation of the species with $\alpha_{m,T} + i$ topological turns, the corresponding free energy difference is

$$G(\alpha_{m,T} + i) = K(i + \omega_T)^2. \quad [2]$$

The relative concentrations of the covalently closed species with $\alpha_{m,T}$ and $\alpha_{m,T} + i$ topological turns, $[\alpha_{m,T}]$ and $[\alpha_{m,T} + i]$, respectively, are related to the free energy differences by the classical thermodynamic result

$$-RT \ln \{[\alpha_{m,T} + i]/[\alpha_{m,T}]\}$$

$$= G(\alpha_{m,T} + i) - G(\alpha_{m,T}) \quad [3a]$$

$$= K(i + \omega_T)^2 - \omega_T^2. \quad [3b]$$

It follows immediately from Eq. **3b** that the concentration distribution is a Gaussian curve with a standard deviation of $\sqrt{RT/2K}$ and centering at $\alpha_{m,T} - \omega_T$.

Eq. **3b** can be rearranged to give

$$-\frac{RT}{i} \ln \frac{[\alpha_{m,T} + i]}{[\alpha_{m,T}]} = K(i + 2\omega_T). \quad [4]$$

For the PM2 DNA sample closed at 14°C, data obtained from Fig. 2a are plotted according to Eq. 4 in Fig. 3. A satisfactory straight line is obtained, which gives $K = 0.11$ and $\omega_T = 0.39$. Results obtained from similar plots for all samples are tabulated in Table 1.

Temperature dependence of the average helix rotation angle $\theta°$ of DNA

The temperature dependence of $\theta°$ can be directly calculated from the data in Table 1. Take the two PM2 DNA sam-

ples closed at 14°C and 29°C for example. A shift in temperature from 14 to 29°C decreased $\alpha_{m,T}$ by 5 turns, as one can see from the tracings in Fig. 2. The angular difference between the centers of the two Gaussian distributions is therefore 4.93 turns or 1760°. This has been interpreted previously as due to the temperature dependence of the average helix rotation angle $\theta°$ (12). Taking the number of base pairs of a PM2 DNA as $6.5 \times 10^6/660$ or 9850, we obtain $\Delta\theta°/\Delta T = -0.012$ degrees/°C; the negative sign indicates a decrease in $\theta°$ (unwinding) as T increases. The temperature coefficients of $\theta°$ obtained for all samples studied are tabulated in Table 2. The average value of $\Delta\theta°/\Delta T$ is -0.012 degrees per °C.

The temperature dependence of $\theta°$ has been previously determined by titrating DNA samples covalently closed at different temperatures with ethidium (12). If the unwinding angle ϕ_e of the DNA helix due to the binding of an ethidium is taken as 26° (13, 14), $-\Delta\theta°/\Delta T$ is calculated to be 0.0095 to 0.011 degrees/°C from published results (12). This is in good agreement with the value 0.012 degrees/°C from the present gel electrophoresis results.

The free energy of superhelix formation

When the absolute value i is large, the term $2\omega_T$ can be dropped from Eq. 4 and

$$-RT \ln \frac{[\alpha_{m,T} + i]}{[\alpha_{m,T}]} = K\, i^2. \qquad [4a]$$

The left-hand side of Eq. 4a is the free energy difference between a covalently closed DNA molecule with $\alpha_{m,T} + i$ topological turns and the same molecule with $\alpha_{m,T}$ topological turns. This free energy difference has been referred to in the literature as the free energy of superhelix formation ΔG_τ (16, 17). As discussed by a number of authors (16–18), the quantity

$$\Delta g_\tau \equiv \frac{\Delta G_\tau}{N} = NK(i/N)^2$$

where N is the number of base pairs per DNA molecule, is expected to be insensitive to the molecular weight of the DNA at a given i/N. In other words, NK is approximately a constant independent of molecular weight. This constant is tabulated in the last column of Table 1 in RT units.

The values of NK obtained from gel electrophoresis can be compared with the values of NK obtained from the difference in the binding of ethidium by superhelical and nicked DNAs. In concentrated CsCl solutions at neutral pH, the results of Bauer and Vinograd for SV40 DNA and the results of Hsieh and Wang for PM2 DNA, give $NK = 900\ RT$ and $550\ RT$, respectively, if ϕ_e is taken as 26° in these calculations (16, 17). The NK values obtained by gel electrophoresis are for a medium in which the ligase reactions were carried out (0.01 M Tris at pH 8, 0.002 M $MgCl_2$, 0.001 M Na_3EDTA). For PM2 DNA from the mature phage, in the ligase reaction medium NK has been measured to be 1200 RT by the ethidium binding method (Wang, unpublished results), in good agreement with the gel electrophoresis results.

DISCUSSION

We have shown that because of thermal fluctuations in DNA molecules, the conversion of nicked DNA circles to covalently closed circles by ligase results in a population heterogeneous in the topological winding number α. The experimentally measured distribution in α permits one to calculate the relative free energies of species of different α values.

It is not possible, however, to obtain the individual magnitudes of the fluctuations in β and τ of the nicked circular DNA molecules. Assuming that for a nicked circular DNA molecule fluctuations in β and τ are random events, then the experimentally measured variance in α can set upper limits for the variances in β and τ of the nicked molecule, since the sum of these is expected to be equal to the variance in α.

The high resolution and versatility of the gel electrophoresis technique make it ideal for studying the alteration of the DNA helix by various factors. In addition to the determination of the temperature coefficient of the average helix rotation angle of DNA, we have also applied this technique in measurements of the unwinding of double-stranded fd DNA by *E. coli* RNA polymerase (Jacobsen and Wang, unpublished results).

Finally, gel electrophoresis of ligase-joined circles might provide a method for physically distinguishing DNA molecules which differ by only a few base pairs in their lengths. Consider for example, a case in which two DNA molecules differing by a single base pair in length are covalently closed by ligase under identical conditions. The $\alpha_{m,T}$ values of the two are expected to be identical, since α must be an integer. The average numbers of helical turns of the two molecules differ by about 0.1 turn, however, since the numbers of base

Table 1. Parameters for the Gaussian distributions in the topological winding number α

DNA	N	Temperature of closure by ligase, °C	K	$-\omega_T$	NK
PM2	9850	14	0.106	−0.39	1040
		21	0.102	+0.35	1000
		29	0.097	−0.32	955
fd, double-stranded	5750	10	0.18	+0.25	1030
		20	0.16	+0.25	920
SV40	5300	0	0.23	+0.1	1220
		4	0.19	+0.3	1010
		15	0.23	−0.4	1220
		18	0.21	0	1110
		26	0.19	−0.3	1010
E. coli 15 plasmid	2200	0	0.65	+0.3	1430
		4	0.59	0	1300
		15	0.63	+0.3	1390
		18	0.71	+0.1	1560
		26	0.63	+0.5	1390

Table 2. Temperature coefficients of the average helix rotation angle obtained by analyzing the gel electrophoretic patterns of DNAs covalently closed by ligase

DNA	Temperature range °C	$-(\Delta\theta/\Delta T) \times 10^2$ (degrees/°C)
PM2	14–29	1.20
fd, double-stranded	10–20	1.23
SV40	0–26	1.22
E. coli 15 plasmid	0–26	1.13

pairs of the two molecules differ by 1. This in turn requires that the average values of τ of the two molecules differ by about 0.1. Since the mobilities of the molecules are dependent on the average values of τ, it should be possible to resolve the two molecules on a gel.

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