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Daunomycin unfolds compactly packed DNA

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Abstract

Daunomycin is an antitumor antibiotic known to inhibit DNA replication and transcription. Although the inhibition is assumed to be caused by a direct interaction of the drug with DNA, the exact effect of daunomycin on the higher order DNA structure remains uncertain. We studied the effect of daunomycin on DNA compacted states using fluorescence and electron microscopies. Structural changes in individual DNA molecules were examined under the following conditions. T4 phage DNA (166 kbp) was first compacted by spermidine followed by the addition of daunomycin to the compacted DNA. A direct observation of individual single duplex DNAs by fluorescence microscopy indicated that daunomycin induced unfolding of the compacted DNA. Electron microscopic observation of the morphological changes of the higher order DNA structure supported the results obtained by fluorescence microscopy. We discuss here the mechanisms of the unfolding of the compacted structure following intercalation of daunomycin into DNA particularly in terms of the free energy.

Keywords: Daunomycin; Spermidine; Higher order structure of DNA; Compacted DNA; Fluorescence microscopy; Electron microscopy

1. Introduction

Daunomycin is an anthracycline antibiotic used for cancer chemotherapy. The primary target of its antitumor activity is considered to be the nuclear DNA. Past studies on short DNAs or oligonucleotides have indicated that daunomycin inhibits both DNA replication and transcription through binding and intercalating into DNA base pairs [1–7]. However, little attention has been paid to the higher order structural change of long DNAs induced by daunomycin. This may be because of the lack of suitable methodology to study the higher order structure of long DNA chains.

DNA is often found in a compacted state in the living systems and the manner of packaging is closely related to the biological functions of DNA, such as replication and transcription [8]. Numerous in vitro studies have been undertaken in order to obtain deeper insight into the manner of in vivo DNA packing or folding. The in vitro compaction is induced by multivalent cations such as polyamines, hexammine cobalt (III) and peptides, as well as by inert polymers and alcohol [9–22]. However, most studies were performed without clear distinction between single molecular compaction and multi-molecular condensation. We have recently investigated the process of compaction in individual DNA molecules

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and demonstrated that the long duplex DNA undergoes discrete transition from an elongated coil state into a compacted globule state in the presence of condensing agents such as polyethylene glycol and cationic surfactant [19,22,23].

In the present study, we report that daunomycin induces unfolding of compacted single DNA molecule, T4 DNA (166 kbp), with the aid of the single molecular observation under fluorescence microscopy and electron microscopy. This finding is novel since it has been thought that intercalating drugs, such as daunomycin and actinomycin D, have almost no effect on the higher order structure of the compacted DNA molecules [24,25]. We used a naturally occurring polyamine, spermidine, as the condensing agent. It is known that spermidine stabilizes compacted DNA molecules and also increases the transcriptional activity of DNA [26–28]. Our results provide new insight into the pharmacological action of daunomycin on long DNA molecules.

2. Materials and methods

2.1. Materials

T4 phage DNA, 166 kbp with a contour length of 57 μm [29], was purchased from Nippon Gene (Toyama, Japan). A fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI), and an antioxidant, 2-mercaptoethanol (2-ME), were purchased from Wako Pure Chemical Industries (Osaka, Japan). Spermidine · 3 HCl was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Daunomycin · HCl was obtained from Sigma Chemicals Co., (St. Louis, MO).

2.2. Preparation of DNA solution

T4DNA was dissolved in 10 mM Tris-HCl buffer solution with 60 mM NaCl at pH 7.2. For fluorescence microscopic measurements, 0.3 μM DAPI and 4% (v/v) 2-ME were added to the DNA solution. It has been confirmed that the persistent and contour lengths of DNA remain essentially constant at low concentrations of DAPI used in the present experiment [29]. Compaction was induced by the addition of spermidine to the DNA solution. To avoid intermolecular DNA aggregation, measurements were conducted at a low DNA concentration, 0.3 μM or less in nucleotide units. Daunomycin was added to the compacted DNA solution under the condition that the spermidine concentration was maintained as the same before and after the addition of daunomycin.

2.3. Fluorescence microscopic measurements

Fluorescence DNA images were obtained using a microscope (Axiovert 135 TV, Carl Zeiss, Germany) equipped with a 100 × oil-immersion objective lens and a highly sensitive Hamamatsu SIT TV camera, that allowed recording of images on video tapes. The video image was analyzed with an image processor (Arugus 50, Hamamatsu Photonics, Hamamatsu, Japan). Observation was performed at 20°C.

2.4. Electron microscopic measurements

Samples used for electron microscopy were mounted on carbon-coated copper grids (# 200), negative-stained with 1% uranyl acetate, and observed with a transmission electron microscope (JEOL 1200EX, Tokyo) at 100 kV.

3. Results and discussion

To examine whether daunomycin is effective in unfolding the compacted DNA, we examined the conformational changes in individual DNA molecules in an aqueous solution using fluorescence microscopy. Fig. 1 shows fluorescent images of T4DNA molecules in an aqueous solution and the corresponding light intensity distribution in the photographs. Individual DNA molecules were observed in the buffer solution as random coil (Fig. 1(a)). In the presence of 300 μM spermidine, the DNA exhibited a compact structure, i.e., a globule state (Fig. 1(b)). With the addition of daunomycin onto the compacted DNAs, the process of unfolding was observed as shown sequentially in Fig. 1(c), 1(d) and 1(e). In this experiment, 25 μl of the 40 μM daunomycin solution was placed adjacent to the 25 μl solution of compacted DNA, and thus, time-depen-

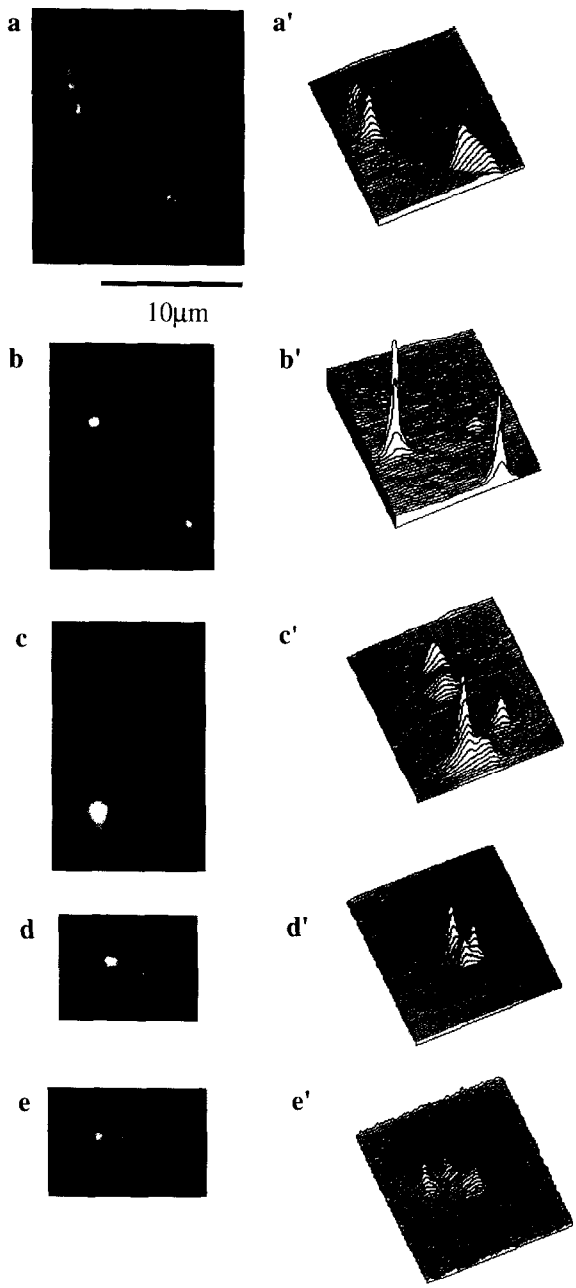


Fig. 1. Fluorescence microscopic images of individual T4DNA molecules moving freely in the solution and corresponding pictures of light intensity. (a and a') coiled DNA molecules in the buffer solution containing 10 mM Tris-HCl and 60 mM NaCl at pH 7.2; (b and b') 300 μM spermidine-compacted DNA molecules; (c-e and c'-e') elongated DNA molecules 40 min after the addition of daunomycin (final concentration, 20 μM) to the compacted DNA.

dent changes in the DNA structure were observed resulting from the gradual diffusion of daunomycin. In order to maintain a constant concentration of spermidine during the diffusion of daunomycin, equal concentrations, 300 μM, of spermidine were used in both solutions. It is clear from Fig. 1 that daunomycin induced significant changes in the higher order structure of the compacted DNA. We also noticed that the probability of the unfolded DNAs to aggregate with each other was higher than that observed in the compacted DNA. The fluorescent images of DNAs were blurred because of the resolution limit of the light wavelength and the high sensitivity of SIT camera. Since the blurring effect was estimated to be approximately 0.3 μm [9,23], the actual size of the compacted DNA was markedly different from the apparent size in the fluorescent image.

To evaluate the effect of daunomycin on the size of DNA in a quantitative manner, we measured the translational diffusion constant D for the individual DNA obstacles observed with the fluorescence microscopy. In order to attain an equilibrium, measurement of the diffusion constant was performed at least 2 h after preparation of the sample, including 300 μM spermidine and 24 μM daunomycin. The value D can be obtained from the mean square displacement (MSD) of the center of mass for DNA. Although we attempted to minimize the convective flow in the aqueous sample, a small but non-negligible convective flow was present during the measurement, possibly caused by the thermal effect of illumination. Since the rate and direction of the convective flow was almost constant during the period of observation, we could eliminate the effect of convective flow using the relationship in Eq. (1) [30].

$$< (\boldsymbol{R}(t) - \boldsymbol{R}(0))^2 > = 4Dt + At^2 \qquad (1)$$

where $\boldsymbol{R}(t) = (R_x, R_y)$, is the position of the center of mass for DNA, $\langle (\boldsymbol{R}(t) - \boldsymbol{R}(0))^2 \rangle$ is the mean square displacement, and A is a numerical constant related to the convective flow. Eq. (1) contains the implicit assumption that both rate and direction of the convective flow are constant, which was actually the case in our measurement at least for the period in the order of 10 s. The effective hydrodynamic radius ξ_H of a single DNA molecule was calculated from

the D value based on the Stokes-Einstein equation given in Eq. (2) [31,32].

$$\xi_H = \frac{k_B T}{6\pi\eta_s D} \quad (2)$$

where k_B is the Boltzmann constant and η_s is the viscosity of the solvent (1.002 mPa · s for pure water at $T = 293$ K). Fig. 2(a) illustrates the hydrodynamic radius ξ_H of a single DNA molecule. The mean ξ_H value of DNA treated with 300 μM spermidine and 24 μM daunomycin was 0.62 ± 0.17 μm, which was almost identical to that of untreated flexible coil-like DNA (0.62 ± 0.23 μm, Fig. 2(b)). In contrast, the mean ξ_H value for the spermidine-compacted DNA was only 0.063 ± 0.014 μm, suggesting that daunomycin induced elongation of the compacted DNA.

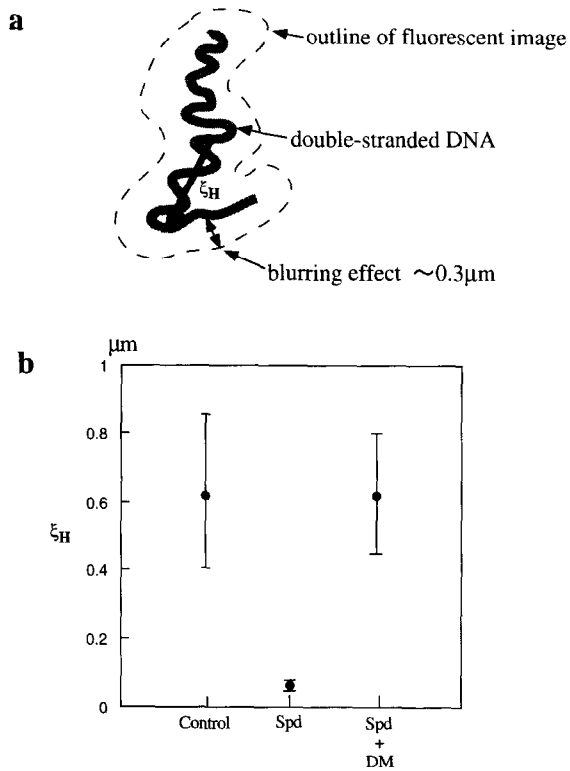


Fig. 2. (a) Schematic illustration of fluorescent image. (b) Hydrodynamic radii ξ_H of T4DNAs; Control, in the buffer solution; Spd, in the presence of 300 μM spermidine; Spd + DM, in the presence of 300 μM spermidine and 24 μM daunomycin. ξ_H was obtained from the diffusion constant for the Brownian motion of individual DNA molecules under fluorescence microscopy. Closed circles and vertical bars represent the ensemble average and the standard deviation for about 30 molecules, respectively.

In order to characterize daunomycin-induced morphological changes in the higher order structure of compacted DNA, we also examined the DNA structure using transmission electron microscopy. Fig. 3 shows a typical structure of the compacted DNA induced by spermidine, i.e., a toroidal structure. A large number of studies have demonstrated that DNA can form a toroidal structure in the presence of various condensing agents [12–16,33–39]. Most of these studies used small DNAs with sizes less than several tens kbp, such as lambda DNA or pBR322DNA, although few used giant DNA such as T4DNA (166 kbp). Two decades ago, Laemmli [16] reported the toroidal structure of compacted T4DNA with polylysine. In the present study, we observed a doughnut-shaped toroid T4DNA induced by spermidine. Our results further demonstrated that with further increase in the amount of spermidine, the toroidal structure tended to become tightly compacted (Fig. 3). The mean size of the toroid was calculated from 15 to 31 individual obstacles at a fixed spermidine concentration at least 3 h after the addition of spermidine. Table 1 shows that obstacles compacted with 600 μM spermidine had a mean edge to edge dimension S of 88 ± 16 nm, which was slightly larger than that compacted with 300 μM spermidine (74 ± 18 nm). In relation to this, Garcia-Ramìrez and Subirana [39] showed that increased ionic strength caused enlargement of particles compacted with histones H1, H5 and φ_0, and protamine. In the preceding section, we have described that the average radius of a compacted DNA with 300μM spermidine was about 63 nm in the aqueous solution (Fig. 2). In other words, the size in the aqueous solution is somewhat larger than the outer radius ($S/2 = 37$ nm) of the toroid observed with electron microscopy. These results suggest that, in aqueous solution, there may be remaining coiled parts around the toroid. Actually, careful inspection of the toroid on the electron micrograph supports this possibility.

We also measured the time-course of the daunomycin-induced unfolding using electron microscopy. Fig. 4 exemplifies the unfolding of compacted T4DNA. Exposure to daunomycin at a dose of 40 μM resulted in a partial DNA unfolding within 10 minutes (Fig. 4(a) and 4(b)). An extensively unfolded structure was observed 6 h after the addition of 40 μM daunomycin to 600 μM spermidine-compacted DNA

(Fig. 4(c)). Essentially the same unfolding process was observed for the DNA molecules compacted with 300 μM spermidine. To avoid the effect of dilution of spermidine on the unfolding of compacted DNA, the daunomycin solution was always prepared so as to maintain a constant concentration of spermidine during electron microscopic examination. Thus, the results obtained by transmission electron microscopy are similar to those measured by fluorescence microscopy.

It has been suggested that the binding of intercalating drugs, such as daunomycin, to extended coiled DNAs prevents subsequent packaging, while the compacted DNAs are no longer accessible to the drug, and, therefore, are stable against exposure to the drug (24). In contrast, our results clearly indicate

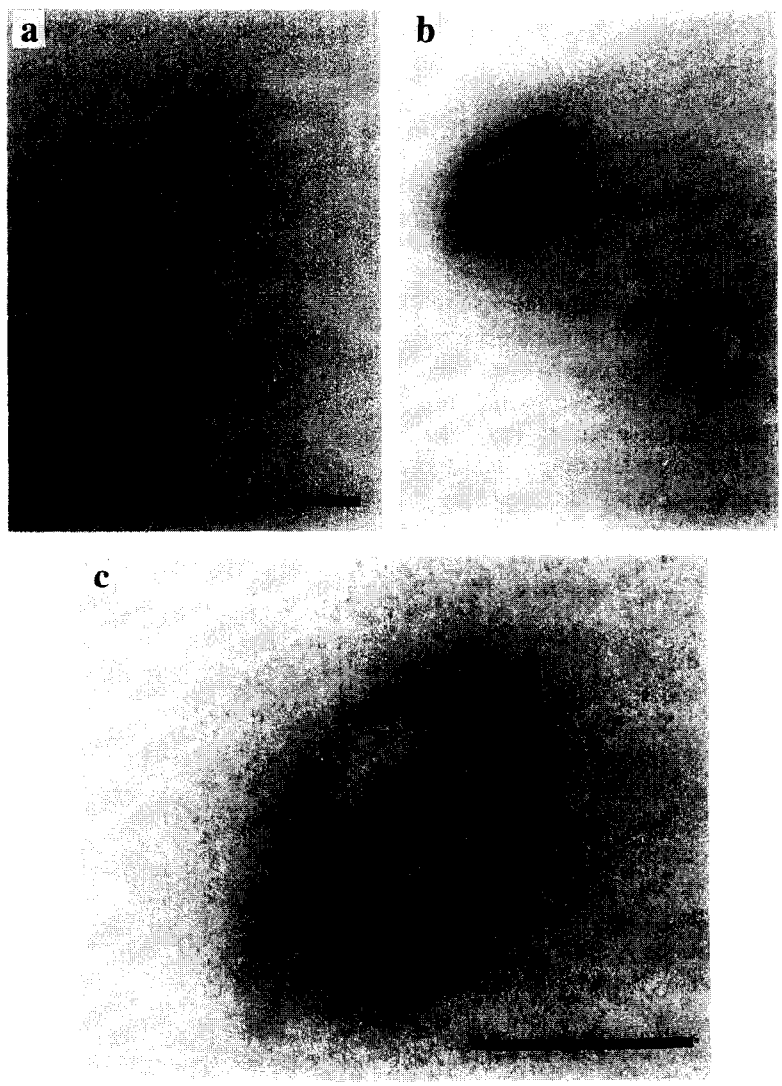
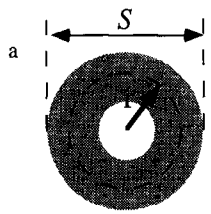


Fig. 3. Electron micrographs of spermidine-compacted T4DNA. All DNA preparations were contrasted with 1% uranyl acetate. DNA-spermidine complexes with 0.3 μM DNA in nucleotide unit and (a) 300 μM spermidine, and (b and c) 600 μM spermidine. (c) is the magnification of (b). Scale bar = 100 nm.

Table 1
Comparision of S (edge-to-edge dimension in nm) of the toroids

size S^a (nm)	Spermidine concentration		
	300 μM	400 μM	600 μM
range (n)[b]	50–100 (15)	56–100 (17)	50–126 (31)
mean ± S.D.	74 ± 18	82 ± 13	88 ± 16



[b] Number of counted particles

that daunomycin induces structural loosening of the compacted DNA, as evident from the examination of the morphology of individual DNAs. These results are important to our understanding of the therapeutic and cytotoxic effects of a variety of antitumor drugs.

How can daunomycin unfold the compacted DNA through intercalation? It is well established that intercalation increases the contour length of DNA. In addition, recent results from our laboratory indicate that intercalators markedly increase the persistence length λ [29], where λ is a measure of the average stiffness of polymer chain and is approximately 50 nm (i.e., approximately 150 base pairs) for typical double-stranded DNAs [40].

As the next step, we discuss the free energy difference, ΔF, of the toroidal compacted DNA compared with the coiled DNA. It is essential to consider the interaction between pairs of DNA segments [41,42] in the compacted state induced by spermidine. It is expected that spermidine, a trivalent polyamine, has an effect on bridging between double helix segments [43]. In addition to this effect, we have recently found that almost all of the negative charge in a DNA chain disappears at the collapse concentrations of spermidine (unpublished data). Thus, DNA segments becomes "attractive" each other resulting from the bridging effect together with the significant decrease in the repulsive Coulomb interaction.

Under conditions that the concentration of spermidine is high enough to induce DNA compaction, the free energy gain, ΔF_p, is given by the energy ϵ of the pair-attractive interaction between segments per unit length.

$$\Delta F_p = -n(2\pi r)\epsilon \quad (3)$$

where n is the number of pair-attractive interactions between DNA segments on the cross-section of toroidal DNA and is roughly proportional to the volume of the toroid. The average radius r between inner and outer radii of the toroidal ring (as is shown in the footnote of Table 1) is 30–40 nm, as measured by electron microscopy. One should also consider

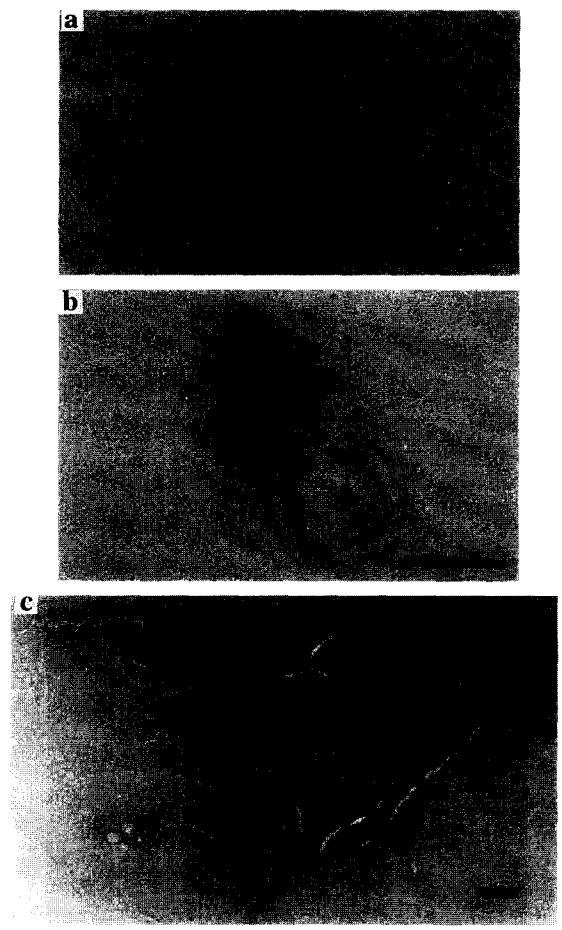


Fig. 4. Effect of daunomycin on spermidine-compacted T4DNA. Daunomycin was added to 600 μM spermidine-compacted DNA. The pictures were observed 10 mins (a and b), and 6 h (c) after the addition of 40 μM daunomycin. Scale bar = 100 nm.

the free energy loss, ΔF_b, caused by the bending energy necessary to form the toroid.

$$\Delta F_b = \frac{b}{2}\int_0^L \kappa^2 ds \quad (4)$$

where κ is the curvature of the toroid structure and b is the elastic constant per unit length of DNA. The elastic constant b is expected to be proportional to the persistence length λ [40,44].

$$b \cong k_B T\lambda. \quad (5)$$

As κ equals $1/r$ in the toroid structure,

$$\Delta F_b = \frac{k_B TL\lambda}{2r^2}. \quad (6)$$

Thus, the total free energy of the toroid with respect to the random coil structure, ΔF, is obtained by,

$$\Delta F \cong \Delta F_p + \Delta F_b = -n(2\pi r)\epsilon + \frac{k_B TL\lambda}{2r^2}. \quad (7)$$

If ΔF is negative, the toroid is stable. However, if ΔF is positive, the toroid should be unfolded. Based on the relationship described in the above equation, it is clear that any increase in λ contributes to the destabilization of the toroid. Thus, increased persistence length λ resulting from the intercalation of daunomycin has a destabilizing effect on the compacted DNA.

Here, let us briefly discuss on the structure unfolded by daunomycin. Fig. 4 indicates that a "bundle" of the unfolded DNA "fibers" has the thickness of the order of 10 nm, both for the partially and extensively unfolded structures. This implies that a bundle is composed with about 11–25 DNA fibers, considering that the effective diameter of a double-stranded DNA is 2.0–3.0 nm [34,36,38] and that the packing number of DNA segments may be proportional to the square of the relative ratio in the diameter ($10^2/2^2 \sim 10^2/3^2$). It is, thus, clear that destabilization with daunomycin does not mean the full-unfolding into an elongated coiled state. Instead, the DNA segments still tend to stick each other. Such a experimental trend can be explained that the pair-attractive interaction between the DNA segments is still effective after the unfolding of the toroidal structure (Fig. 5).

It should be mentioned that in order to understand the full mechanism of the unfolding of DNA induced by intercalators, one must also take into account other parameters, such as the electrostatistic effect, solvation, etc. However, in the present study, at least, we demonstrated clearly that the increased stiffness of the DNA chain by intercalation induces a marked change in the higher order structure.

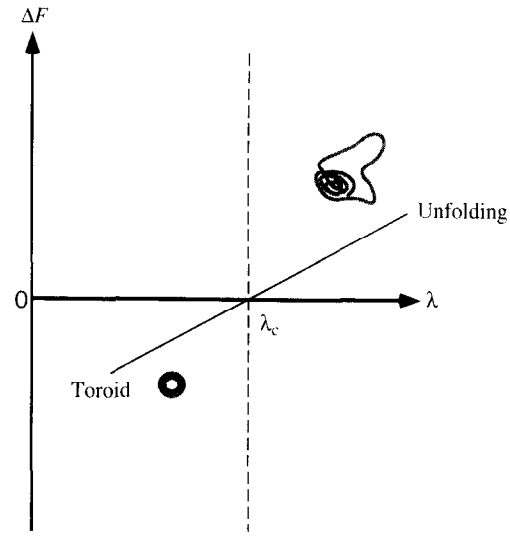


Fig. 5. Schematic representation of the free energy of toroidal DNA. The toroid becomes unstable when the persistence length (λ) is above a critical value, λ_c, as is indicated as the crossing point between the horizontal axis and the vertical dotted line. Here, $\Delta F = 0$ when $\lambda = \lambda_c$.

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