Folding transition of large DNA completely inhibits the action of a restriction endonuclease as revealed by single-chain observation

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Abstract The biochemical characteristics of lambda DNA chains in folded/unfolded states upon cleavage by the restriction enzyme *Apa*LI were investigated in the presence of spermine. These characteristics of DNA chains depending on their higher-order structure were studied at the single-molecule level using fluorescence microscopy. With a low concentration of spermine, lambda DNA takes a random coiled conformation and allows digestion by the enzyme, while under a high concentration of spermine, lambda DNA takes a compact folded structure and inhibits such attack. Together with comparative experiments on short oligomeric DNA, our results suggest that the transition in the higher-order structure causes on/off-type switching of sensitivity to the enzyme.

Key words: DNA; Spermine; Restriction enzyme; Fluorescence microscopy

1. Introduction

It is well known that genomic DNAs (mm–cm contour length) exist in a highly dense state in every living cellular environment (micrometer scale). To fold a long DNA chain into a compact structure, cationic substances such as histones, protamines, and polyamines are thought to play crucial roles. The 'condensation' of DNA induced by polyamines has been studied extensively [1] and the manner of such packing has been considered to play a role in the biological functions of DNA, such as replication and transcription [2]. It has been expected that condensation reduces accessibility to enzymes and blocks their function. In fact, an inhibition of endonuclease activity is observed in plasmid DNA with the addition of polyamines [3–5]. In contrast, some biochemical activities are known to be enhanced with polyamines, such as transcription, the specificity of enzymatic reactions, and the cyclization of DNA [5–9]. Until the last decade, the term 'condensation' had been used without clearly distinguishing between multimolecule condensation and single-molecule compaction [1]. Only recently has the folding transition of giant DNA been recognized as a quite different phenomenon from the condensation of multiple DNA molecules. Despite recent advances in the study of the folding transition of giant DNA, there have been almost no trials to clarify the structure–activity relationships at the level of single giant DNA molecules.

We previously studied the conformational changes of individual phage genome DNA molecules in the presence of polyamines using fluorescence microscopy [10–12]. We found that the conformation of a single large duplex DNA molecule switches from an elongated coil into a compact folded state in a discrete manner with the addition of polyamines. Theoretical considerations indicate that long semi-flexible polymer chains exhibit an all-or-none conformational transition [13]. Unfortunately, there have been few studies on the biochemical characteristics of DNA chains depending on the higher-order structure at the single-molecule level. Therefore, we investigated the correlation between the higher-order structure of a large DNA molecule at the single-molecule level and its sensitivity to a restriction endonuclease using fluorescence microscopy.

2. Materials and methods

2.1. Digestion of lambda DNA

To investigate the influence of spermine (4+) on the endonucleolytic digestion of large DNA molecules, we examined the products of digestion by agarose gel electrophoresis. Lambda phage DNA (48.5 kb, from Toyobo) and spermine (from Nacalai Tesque) were mixed in a buffer solution and incubated for 50 min at 37°C to reach an equilibrium state. The composition of the solution during incubation was lambda DNA, 5.3 ng/μl; Tris–HCl, 10 mM (pH 7.5); $MgCl_2$, 10 mM; dithiothreitol (DTT), 1 mM; and the desired amount of spermine. The restriction endonuclease *Apa*LI (New England Biolabs) was then added to the solution. Digestion was carried out for 40 min at 37°C. The composition of the solution during digestion was lambda DNA, 5 ng/μl; *Apa*LI, 15 units; Tris–HCl, 10 mM (pH 7.5); $MgCl_2$, 10 mM; and DTT, 1 mM. The concentration of spermine was varied up to 110 μM. The digestion reactions were stopped by adding sodium dodecylsulfate (SDS). The solutions were then diluted two-fold by adding distilled water to reduce the concentration of spermine for electrophoresis with 0.5 wt% agarose. After the electrophoresis, DNA bands were visualized by ethidium bromide and the gel image was acquired by a digital camera (EDAS 120, Kodak). The captured images were then analyzed by 1D image analysis software (Kodak).

2.2. Direct observation of lambda DNA molecules

To investigate the higher-order structure of individual lambda DNA molecules in the presence of various concentrations of spermine, fluorescence microscopy was used. After mixing DNA and spermine into a buffer, the solutions were incubated for 50 min at 37°C. Immediately before the observation, the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) and 2-mercaptoethanol (2-ME) were added. The final compositions of the samples were as follows: lambda DNA, 0.5 μM (in bp); DAPI, 0.5 μM; Tris–HCl, 10 mM (pH 7.5); $MgCl_2$, 10 mM; DTT, 1 mM; 2-ME, 5% (v/v); and spermine, 0, 50, 60, 70, 80, 90, 100, 110 μM. An inverted microscope (IX-70, Olympus)

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was used for direct observation with a high-magnification objective (100×) or a low-magnification objective (40×). The 100× objective was applied to observe the DNA molecules adsorbed on the glass surface. Using the 40× objective, individual DNA molecules were observed as bright spots that showed Brownian motion. After acquiring those DNA images of the bright spots from videotapes, the images were analyzed and the time developments of positions of the center of mass of individual DNA molecules were obtained using image analysis software (Cosmos, Library). The hydrodynamic radii, R_H, of individual DNA molecules were then calculated using the procedure reported previously [14]. Before each experiment, the lambda DNA sample was linearized by heating at 65°C and then rapidly cooled.

2.3. Digestion of a complementary oligonucleotide

To examine the influence of spermine on enzyme activity, we carried out digestion experiments with an 88-mer complementary oligonucleotide that forms a double-stranded structure in solution [15]. Fig. 1 shows a restriction map of the *Apa*LI cleavage sites in lambda DNA (top) and the sequence of the 88-mer complementary oligonucleotide (bottom). This complementary oligonucleotide has the same sequence as part of lambda DNA, including a cleavage site for *Apa*LI. The double-stranded complementary oligonucleotide and the desired amount of spermine were mixed in the buffer and incubated for 50 min at 37°C, and the restriction endonuclease *Apa*LI was then added to the solution. Digestion was carried out for 60 min at 37°C. The solution consisted of the complementary oligonucleotide, 3.3 ng/μl; *Apa*LI, 40 units; Tris–HCl, 10 mM (pH 7.5); $MgCl_2$, 10 mM; and DTT, 1 mM. The concentration of spermine varied: 0, 50, 100, and 200 μM. Digestion was stopped by adding SDS. The solutions were then diluted two-fold by adding distilled water and subjected to electrophoresis on 2 wt% agarose (Agarose for 50–800 bp fragment, from Nacalai Tesque) slab gel.

3. Results and discussion

3.1. Influence of spermine on the cleavage of large DNA molecules

Fig. 2A shows the effect of spermine on the cleavage of lambda DNA by *Apa*LI. The concentration of spermine varied: 0–110 μM. Below a spermine concentration of 70 μM, lambda DNA is digested as well as with 0 μM of spermine, and five DNA fragments appear. At 80 μM spermine (lane 5), a band of 48.5 kb, i.e. non-digested whole lambda DNA, appears. At 90 μM spermine (lane 6), digestion is suppressed and the band of non-digested lambda DNA becomes stronger. Above 100 μM spermine, digestion is completely inhibited and only a band of whole lambda DNA can be seen. Notice that at 80 and 90 μM spermine, faint smear bands appear at around 25–48.5 kb. These fragments can be interpreted as partially digested fragments. In addition, with 90 μM spermine, two faint bands (5.6 kb and 16.2 kb) are still seen. We will return to these findings later. To investigate these

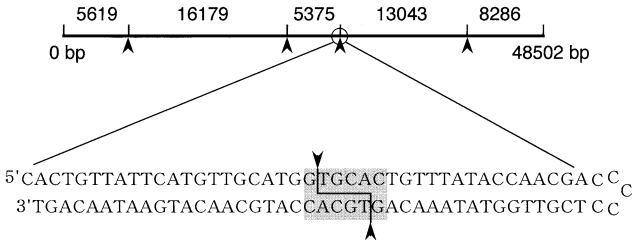

Fig. 1. Top: Restriction map of the *Apa*LI cleavage sites in lambda DNA. Arrows indicate cleavage points for *Apa*LI (GTGCAC). Bottom: Sequence of the 88-mer complementary oligonucleotide. The double-stranded part has the same sequence as the middle part of lambda DNA (indicated by a circle). The hairpin contains the sequence (hatched part) that is recognized and cleaved by *Apa*LI.

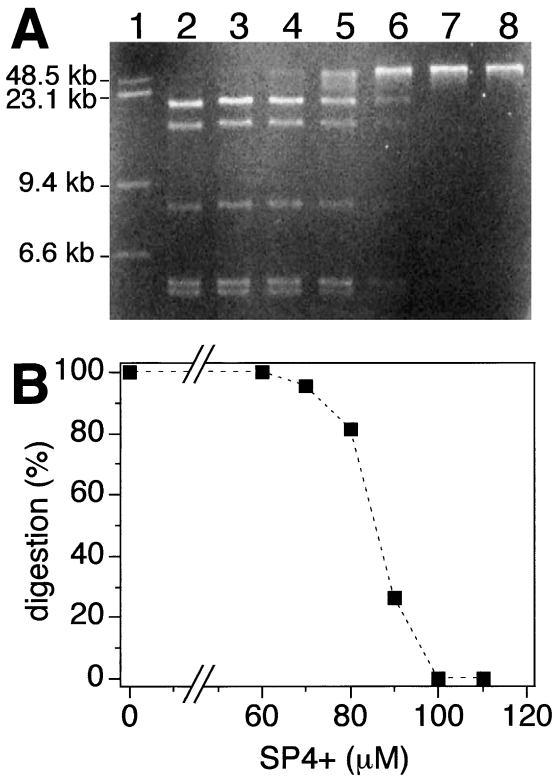

Fig. 2. A: Effect of spermine (SP4+) on *Apa*LI cleavage of lambda DNA. The spermine concentration was varied from 0–110 μM. Lane 1, marker, lambda/*Hin*dIII digest+lambda DNA; 2, 0 μM SP4+; 3, 60 μM SP4+; 4, 70 μM SP4+; 5, 80 μM SP4+; 6, 90 μM SP4+; 7, 100 μM SP4+; 8, 110 μM SP4+. B: The degree of the digestion of lambda DNA by *Apa*LI vs. spermine concentration.

results quantitatively, the degree of digestion was obtained from an image of the gel. First, the net intensity (the sum of the background-subtracted pixel values in the band) of each band was measured. Next, the ratio of the sum of the net intensities of the bands of digested fragments to the total net intensity of the lane (including the band of 48.5 kb) was calculated as the degree of digestion. Fig. 2B shows the degree of digestion for lambda DNA. The degree of digestion changes drastically between spermine concentrations of 70 and 100 μM.

3.2. R_H of lambda DNA molecules

Fig. 3A shows an example of fluorescence images of individual DNA molecules adsorbed on the surface of a cover glass obtained using a 100× objective lens. The higher-order structures of the DNA molecules are clearly different between the two conditions: random coiled conformation with 70 μM spermine and a folded structure with 110 μM spermine. However, in the bulk solution, it was impossible to evaluate the size of the lambda DNA molecules directly from the fluorescence image and to distinguish the higher-order structure (fold/unfold) using a 100× objective lens because of Brownian motion and the blurring effect of fluorescence. Therefore, low-magnification experiments with a 40× objective lens were carried out to obtain time developments of the center of mass of individual DNA molecules. Insets of Fig. 3B show trajectories of the center of mass of individual lambda DNA molecules over 5 s. Lambda DNA with 110 μM spermine diffuses faster than with 70 μM spermine. This means that the higher-

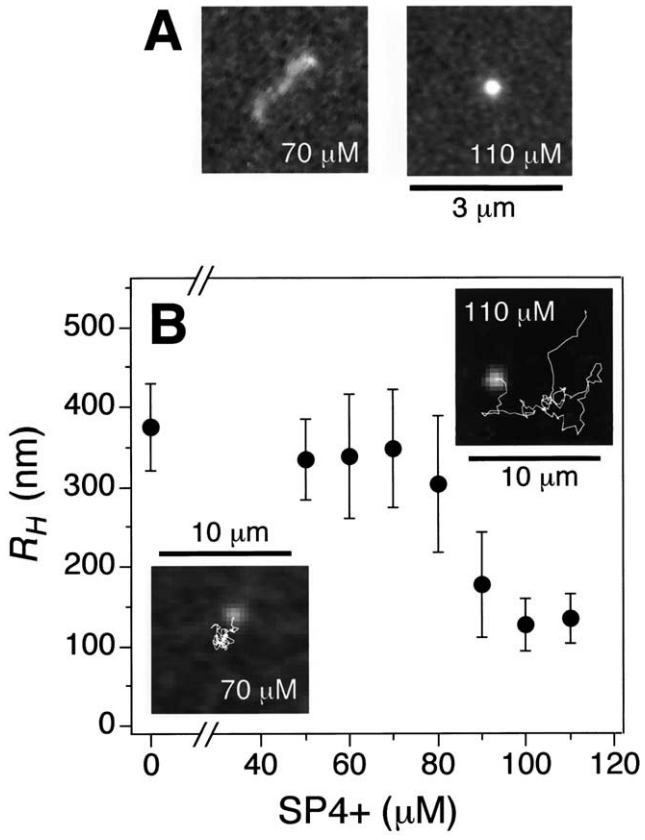

Fig. 3. A: Fluorescence images of individual DNA molecules on the surface of a cover glass obtained using a 100× objective lens. Scale bar = 3 μm. B: Ensemble averages of the hydrodynamic radii (R_H) of lambda DNA molecules at various spermine (SP4+) concentrations. At least 15 DNA molecules were analyzed for each spermine concentration. Error bars indicate the standard deviation in the distribution. Insets: Example trajectories of the center of mass of individual lambda DNA molecules over 5 s. Scale bar = 10 μm.

order structures of the DNA molecules are different between the two conditions. From these trajectories, the hydrodynamic radii were calculated under each condition. The distribution of the hydrodynamic radii, R_H, of lambda DNA molecules at various concentrations of spermine is shown in Fig. 3B. At lower concentrations of spermine (0–80 μM), R_H is almost constant at around 350 nm. This value is comparable to the reported size of the lambda DNA molecule in a random coiled conformation in buffer solution [14]. At 80 μM spermine, the average value of R_H seems to be slightly smaller and the standard deviation is large. Indeed, in this condition, brighter and quicker fluctuating fluorescence spots, indicating the appearance of folded DNA molecules, began to be observed. With a higher concentration of spermine (100, 110 μM), R_H is around 130 nm. This value is comparable to the reported size of folded lambda DNA in the presence of polyethylene glycol [16]. Figs. 2 and 3 show clearly that there is a strong correlation between the activity of the restriction endonuclease and the higher-order structure of individual DNA molecules. When DNA molecules have a random coiled conformation, the restriction endonuclease cleaves DNA. On the other hand, when DNA molecules have a compact folded form, digestion by the restriction endonuclease is inhibited. At 90 μM spermine, the average value of R_H is 176 nm. Under this condition, DNA molecules of intermediate size (150–200 nm) were frequently observed along with folded DNA (ca. 130 nm). This intermediate value for R_H may reflect a partially folded conformation (coexistence of folded and random coiled regions in a single chain). Recently, a partially folded conformation has been reported, i.e. individual DNA molecules have a stable partially folded conformation in polyamine solutions with a high salt concentration [12]. When the magnification of the objective lens was increased, partially folded DNA molecules were observed on the surface of the cover glass, although most of the DNA molecules were folded (not shown). Thus, DNA molecules in this conformation might be partially cleaved. The partial digestion seen in Fig. 2, lane 6, may reflect this phenomenon.

3.3. *Influence of spermine on the cleavage of short DNA molecules*

Next, we tried to confirm that the inhibition of digestion is due to the change in the higher-order structure of individual DNA molecules, rather than to a reduction in enzyme activity based on the high concentration of spermine. We synthesized an 88-mer complementary oligonucleotide that included a cleavage site for *Apa*LI (Fig. 1, bottom). This complementary oligonucleotide is much shorter than the persistent length of a double-stranded DNA molecule (typically 50 nm, ca. 150 bp [17]) when it forms a double-stranded structure and is expected to have a rod-like conformation in solution. Thus, this complementary oligonucleotide does not show a change in its higher-order structure. In addition, such short DNA molecules do not undergo multi-molecular condensation under the conditions described here [18]. Using this complementary oligonucleotide, we carried out digestion experiments with *Apa*LI under various spermine concentrations. Fig. 4A shows the effect of spermine on cleavage of the complementary oligonucleotide by the restriction enzyme *Apa*LI. The 88-mer oligonucleotide with a double-stranded structure migrates at about the same velocity as 50 bp double-stranded DNA (lane 1). In lanes 2–5 in Fig. 4A, the bands of the oligo fragments digested by *Apa*LI are slightly retarded compared to the 20 bp double-stranded DNA. Faint bands of undigested oligo can be seen because there were more cleavage sites for *Apa*LI than enzyme during the digestion. In Fig. 4B, the one-dimensional fluorescence intensity profiles along the lanes are similar to each other from 0 to 200 μM spermine, and indicate that the complementary oligonucleotide is digested regardless of the spermine concentration. Fig. 4C shows the degree of digestion, i.e. the ratio of the net intensity of the digested band to the sum of the total net intensity. Even at 200 μM spermine, the complementary oligonucleotide is digested as well as at a lower concentration of spermine. This is in strong contrast to the result obtained with lambda DNA (Fig. 2B). Thus, we can conclude that the activity of *Apa*LI is not reduced in the presence of a high concentration of spermine.

3.4. *Drastic change in segment density in a single large DNA molecule*

These results suggest that the biochemical character of large DNA molecules switches with a change in its higher-order structure induced by a counter polyamine. This change in the higher-order structure drastically changes the density of a large DNA molecule. The radius of gyration of a polymer in a random coil state is proportional to $N^{0.5}$, where N is the

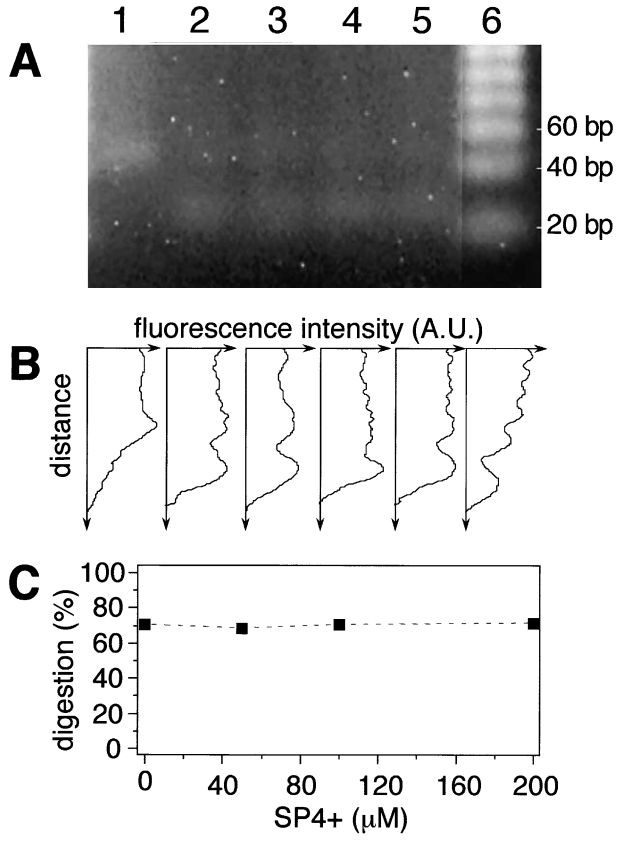

Fig. 4. A: Effect of spermine (SP4+) on *Apa*LI cleavage of the 88-mer complementary oligonucleotide. Lane 1, reference, without *Apa*LI; 2, 0 μM SP4+; 3, 50 μM SP4+; 4, 100 μM SP4+; 5, 200 μM SP4+; 6, marker, 20 bp ladder. B: One-dimensional profile of the fluorescence intensity along the lane corresponding to the lane immediately above. C: The degree of digestion of the complementary oligonucleotide by *Apa*LI vs. spermine concentration.

number of segments. Thus, the density of the segments decreases as $N^{-0.5}$ [19]. On the other hand, the density of segments in the folded state is independent of N, assuming that the folded state has the most highly packed structure. Thus, the difference in density between the folded and unfolded states increases when the polymer becomes longer. For instance, the density of segments in the folded state is 10^4 times greater than that in the random coil state for a 166 kb DNA molecule [16]. In our case, the distance between segments can be estimated from the hydrodynamic radius. In the folded state ($R_H \approx 130$ nm), the volume is about 120 times greater than that in the most highly packed state. Thus, the distance between DNA segments is around 20 nm. This distance is comparable to the size of the restriction endonuclease (a little less than 10 nm). Thus, this spatial restriction might explain the inhibition of the activity of *Apa*LI. On the other hand, when lambda DNA takes a random coiled conformation, the radius of gyration is estimated to be ~ 550 nm, based on its hydrodynamic radius, $R_H \approx 350$ nm [20]. Thus, the average distance between segments in the random coiled state is about 180 nm. This may be sufficient for both DNA and the enzyme to exhibit biochemical activity. Again, in this case, the ratio of the segment densities in the folded state to random coiled state is very large (ca. 74-fold). Therefore, this drastic change in segment density may explain the on/off-type switching of the biochemical characteristics of large DNA molecules.

Our results suggest that the sensitivity of a large DNA molecule to a restriction enzyme is regulated by its higher-order structure. Furthermore, the higher-order structure is controlled by the concentration of non-specific chemical agents such as spermine. Therefore, these results suggest that non-specific binding chemicals that determine the environment surrounding DNA may regulate the chemical properties of DNA by controlling its higher-order structure, which is more evident for long DNA molecules. This type of regulatory mechanism may complement lock-and-key-type regulation.

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