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CONVERSION OF CLOSED CIRCULAR DNA MOLECULES TO SINGLE-NICKED MOLECULES BY DIGESTION WITH DNAase I IN THE PRESENCE OF ETHIDIUM BROMIDE

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Summary

Closed circular DNA molecules were converted to single-nicked open molecules by digestion with DNAase I in the presence of ethidium bromide. Appropriate digestion conditions were obtained for the following DNA molecules: kinetoplast DNA minicircles from *Leishmania tarentolae*, SV40 DNA and PM-2 DNA. The nicking was shown to be non-strand specific in the case of ϕ X174 RF.

Introduction

The quantitative conversion of a population of closed circular DNA molecules into single-nicked open circular molecules is a valuable technique for research on circular DNA. Several methods are available for accomplishing this, but they are of limited general utility. Goebel and Helinski [1] described an Endonuclease I-transfer RNA complex which in 0.5 M NaCl would quantitatively introduce single nicks into closed circular Col E1 and ϕ X174 RF molecules. Beard et al. [2] showed that S1 endonuclease from *Aspergillus oryzae* would quantitatively introduce a single nick into closed SV40 DNA molecules. And Barzilai [3] showed that in the presence of ethidium bromide, a crude endonuclease activity from rat liver would quantitatively convert closed SV40 DNA molecules into apparently single-nicked open molecules. We have considered in detail the effect of ethidium bromide on the nicking activity of pancreatic DNAase I. We have investigated several parameters of this reaction, using as substrate the 0.29 μ m covalently-closed monomeric minicircles from the kinetoplast DNA of *Leishmania tarentolae* (Wesley and Simpson [4]), and have also obtained suitable conditions for complete conversion of PM-2 and SV40 closed DNA molecules into single-nicked open molecules.

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Abbreviations: kDNA, kinetoplast DNA; λ , average number of nicks per molecule. EGTA, ethyleneglycol-bis-(β -aminoethylether)-*N,N'*-tetraacetic acid.

Materials and Methods

Cell strain and reagents. The history and culture conditions of the strain of *L. tarentolae* used (Lt-C-1) have been described previously [4,5]. Pancreatic DNAase I (electrophoretically pure) was obtained from Sigma Chemical Co. [³H]thymidine (17Ci/mmol) was obtained from Schwartz/Mann Co. Bovine serum albumin (crystalline) was obtained from CalBiochem. Ethidium bromide was obtained from Sigma Chemical Co. All other reagents were of reagent grade.

Isolation of closed circular DNA molecules. ³H-labeled closed minicircles were isolated from *L. tarentolae* cells grown for 4 days in the presence of [³H]thymidine (10 μCi/ml) in the following culture medium:

Medium 199+:

Component:	Final dilution or concentration:
Morpholinopropane sulfonic acid buffer (pH 7.9)	0.06 M
Medium 199, 10 × concentrated — with glutamine and Hanks Salts — without Na bicarbonate (GIBCO)	1 → 10
Folic acid	1.7 μg/ml
Vitamin mixture, "Vn6A" (Trager, 1957)	1 → 10
Amino acid mixture, "LeIIIIE", without salts [8]	1 → 2
Hemin	10 μg/ml

The isolation of closed minicircles from sonicated closed kDNA networks by alkaline band sedimentation has been described [4,5]. Two preparations of minicircles were used for these experiments, with specific activities of 561 000 cpm/μg and 46 870 cpm/μg. Prior to enzyme digestion, these DNA preparations consisted of 92% and 68% closed molecules respectively. The DNAs were dialyzed versus 10 mM Tris · HCl (pH 7.4)/0.1 mM EDTA.

³H-labeled SV40 form I DNA (1300 cpm/μg) was a gift of Dr John Jordan, and φX174 RF I DNA, labeled with ³H in the viral strand and ³²P in the complementary strand was a gift of Dr Dan Ray. We obtained ³H-labeled PM-2 (5400 cpm/μg) DNA from Dr Wesley Brown. The SV40 DNA and the PM-2 DNA were in 10 mM Tris · HCl (pH 7.4)/0.1 mM EDTA/50 mM NaCl.

DNAase I digestion. DNAase I was dissolved (1 mg/ml) in either 0.125 M NaCl/20 mM MgCl₂/4 mM Tris · HCl (pH 8.0)/0.06% bovine serum albumin or (2 mg/ml) in 25 mM Tris · HCl (pH 7.4)/10 mM CaCl₂/50% glycerol and kept in small 100-μl aliquots at -20 °C. For the digestion reaction, the DNAase I stock was diluted with either 0.125 M NaCl/20 mM MgCl₂/4 mM Tris · HCl (pH 8.0)/0.06% bovine serum albumin, or 4 mM Tris · HCl (pH 8.0)/20 mM CaCl/0.06% bovine serum albumin to twice the desired final concentration. Unless specified, all the digestions described in Results were carried out using

the Mg^{2+} medium and the DNAase in the Mg^{2+} -containing buffer.

The reaction was initiated by addition of 50 μ l of the diluted DNAase solution to 50 μ l of the DNA ethidium bromide solution (0.3 μ g DNA in the case of the minicircles) at 30 °C. The reaction was terminated by cooling on ice and by simultaneous addition of 35 μ l of 0.2 M EDTA.

Alkaline band sedimentation. 5-ml of alkaline (pH 12.8) 5–20% linear sucrose gradients were prepared as previously described. The reaction mixtures were layered onto the gradients and the tubes centrifuged in an SW 65 rotor. The tubes were then fractionated from the top by means of an Isco Model D fractionator and drops were collected onto Whatman 3MM filter discs. Pelleted material was removed by suspension in a small volume of water which was then pipetted onto a 3MM filter disc. The discs were then processed through cold 5% trichloroacetic acid, 70% ethanol, 99% ethanol and ether, dried and counted in a toluene scintillation fluid (Omnifluor, New England Nuclear) in a Beckman Scintillation Counter. The graphs were computer-plotted. In all cases, the recoveries of acid-insoluble radioactivity from the gradients were greater than 95% of the radioactivity layered.

Results

Nicking of kDNA minicircles by DNAase I in absence of ethidium bromide.

Dose-response curves were obtained for the conversion of closed monomeric minicircles to open minicircles by digestion with DNAase I in the absence of dye for 15 min at 30 °C, as described in Methods. In the presence of 10 mM $MgCl_2$, the DNAase concentration which yielded an average of one nick per molecule (37% survival) was $5.8 \cdot 10^{-4}$ μ g/ml. The slope of the dose-response curve decreased 1.97-fold when 10 mM $CaCl_2$ was substituted for $MgCl_2$ and NaCl was omitted.

Alkaline sucrose band sedimentation profiles of minicircle preparations digested with DNAase I to average values of approximately two nicks and one nick per molecule, as calculated from the percent survival of closed molecules, are shown, respectively, in Fig. 1A and 1B. The two nearly equal peaks in Fig. 1B correspond to the more rapidly moving single strand circle and the slower unit length single strand linear fragment. The small amount of more rapidly sedimenting dimeric minicircles in this preparation can be neglected.

Theoretical distribution curves for random nicking of a population of closed circular molecules. The expected frequencies in alkaline gradients of single strand linears (unit circle contour length), single strand circles and closed circular duplexes were determined at different mean nicks per molecule, assuming random nicking. A plot of these values and the ratio of single strand linears to single strand circles is given in Fig. 2 as a function of the mean number of nicks per molecule (λ). The sum of the single strand linears and circles never exceeds 60%, and the ratio of linears to circles is always greater than one and increases steadily with increasing λ .

From the data previously given in Fig. 1B, at an average of approximately one nick per molecule (33% survival of closed circles), the single strand unit length linear and circular peaks represented roughly 30% and 20%, respectively, of the total DNA. These values are consistent with the values predicted in

Fig. 2 indicating that a random nicking situation existed in the absence of the dye.

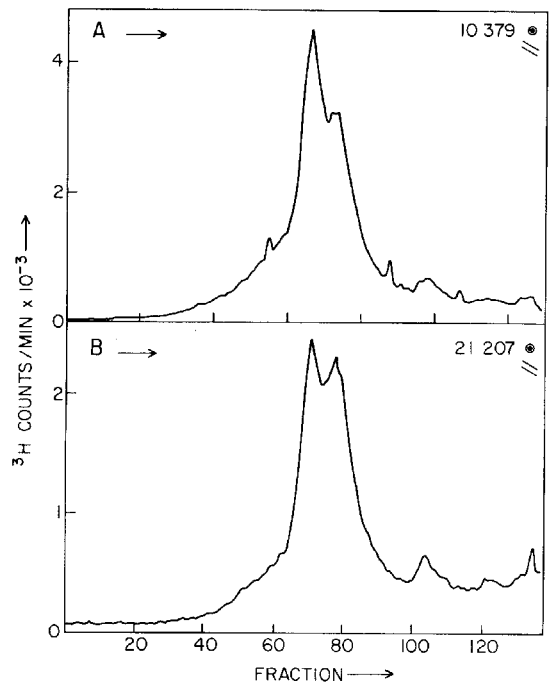


Fig. 1. Alkaline band sedimentations of ³H-labeled kDNA monomeric minicircles digested with low concentrations of DNAse I in the absence of ethidium bromide. The solutions were layered onto the gradients directly after termination of the reactions. The tubes were centrifuged in the SW 65 rotor for 12 h at 60 000 rev./min (5 °C). After collecting single-drop fractions from the top onto Whatman 3MM filter discs, any pelleted material was recovered by vigorous resuspension in a small volume of water and was pipetted onto a separate disc. A. DNAse concentration was 5 · 10⁻⁴ μg/ml. The pellet (●) contained 12.4% of the total radioactivity. B. DNAse concentration was 4.0 · 10⁻⁴ μg/ml. The pellet (○) contained 33% of the total radioactivity. The actual cpm in the pellets are indicated on the graphs.

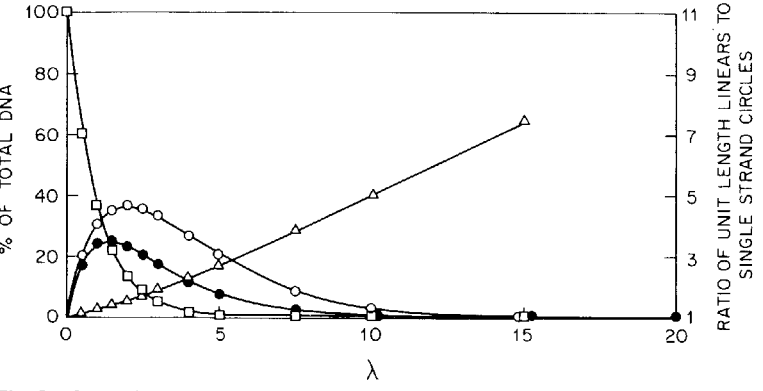


Fig. 2. Theoretical distribution curves for random nicking of a population of closed circular molecules. The derivation of the equations used to plot these graphs is presented in the Appendix. The concentrations of single strand linears (○—○), single strand circles (●—●), and circular duplexes (□—□), are shown in Fig. 3 as a function of λ, (λ=2nα, refer to appendix for definition of n and α), the average nick per duplex. The ratio of unit contour strand linears to single strand circles is also given (Δ—Δ).

Quantitative conversion of closed minicircles to single-nicked open minicircles as shown by alkaline band sedimentation. Dose-response curves were obtained for the conversion of closed monomeric minicircles to open minicircles by DNAase I in the presence of ethidium bromide. The slope of the dose-response curve at 89 μg ethidium bromide and an increase in the dye concentration to 444 $\mu\text{g}/\text{ml}$ further decreased the rate of reaction 2.8 fold.

Alkaline band sedimentation analysis (Fig. 3) of closed minicircles

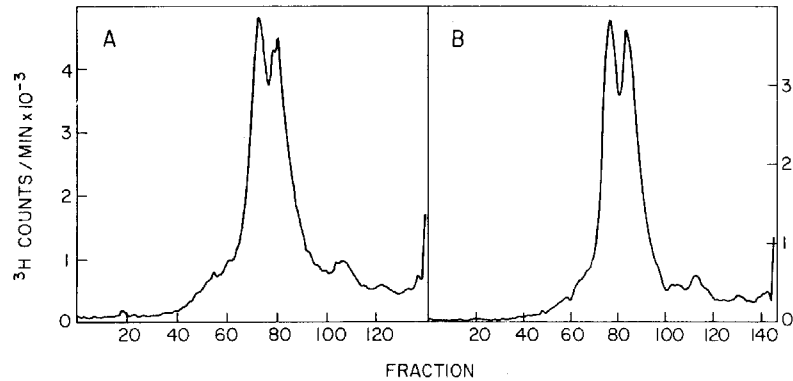


Fig. 3. Alkaline band sedimentation profiles of minicircles digested with 0.5 μg DNAase I/ml for 15 min at 30 $^{\circ}\text{C}$ in the presence of ethidium bromide. A. The standard Mg^{2+} -containing reaction medium was used. Dye concentration was 58 $\mu\text{g}/\text{ml}$. B. Ca^{2+} was substituted for Mg^{2+} and NaCl was deleted in the reaction medium. Dye concentration was 89 $\mu\text{g}/\text{ml}$. Tubes were centrifuged for 12 h at 60 000 rev./min in the SW 65 rotor at 5 $^{\circ}\text{C}$. Pellets are graphed as the final points in these and all following graphs.

digested with 0.5 μg DNAase I/ml for 15 min at 30 $^{\circ}\text{C}$ in the presence of 58–89 μg ethidium bromide revealed that the population of closed minicircles had been quantitatively converted to single-nicked open minicircles by this treatment. The digestion in Fig. 3A was performed in the presence of 10 mM MgCl_2 , and that in Fig. 3B was performed in the presence of 10 mM CaCl_2 . The profiles in the two instances are the same and have the following distinctive characteristics: (1) No closed minicircles remain undigested, (2) the open circular and single strand linear peaks are approximately equal, and (3) the peaks together represent 86% of the total DNA. These results are inconsistent with the idea that a single random nicking process is taking place in accordance with the equations diagrammed in Fig. 2, for in that instance, the combined percentage values of the open circle peak and the unit length single strand linear peak never exceed 60% of the total DNA. In addition, the theoretical maximum percentage values of the open circle peak and unit length single strand linear peak (25% and 37%) occur respectively at λ s of 1.5 and 2.0, and at no point would the frequencies of circles and unit length single strand linears be equal. To strengthen the argument, a comparison of the profiles in Figs 3A, 3B, and 1B, all representing a λ of 1, reveals the incomplete digestion of the closed DNA in the absence of ethidium bromide.

The failure to observe a Ca^{2+} -stimulated increase in the percentage of DNAase I-catalyzed double strand breaks (Fig. 5B) is probably due to the absence of Mg^{2+} in the incubation medium. Melgar and Goldthwait [6] showed that the presence of both Mg^{2+} and Ca^{2+} in the ratio of 10:1 was

optimal for the catalysis of double strand breaks by DNAase I. In the reaction shown in Fig. 3B, no Mg^{2+} was added and the predominant hydrolytic event was a single strand nick.

Effect of varying the DNAase concentration. The effect on the alkaline sedimentation profile of varying the DNAase concentration at a constant ethidium bromide concentration of $56 \mu\text{g/ml}$ is shown in Fig. 4A–F. The first

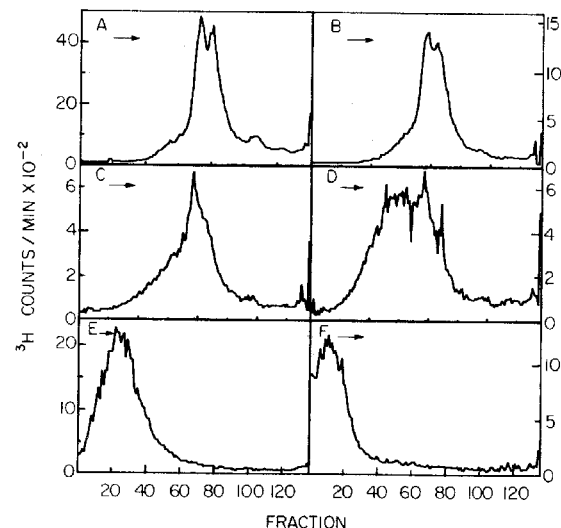


Fig. 4. Alkaline band sedimentations of minicircles digested for 15 min at 30°C with increasing concentrations of DNAase I at a constant ethidium bromide concentration. A. $0.5 \mu\text{g}$ DNAase I/ml. B. $1.0 \mu\text{g}$ DNAase I/ml. C. $5.0 \mu\text{g}$ DNAase I/ml. D. $7.5 \mu\text{g}$ DNAase I/ml. E. $50 \mu\text{g}$ DNAase I/ml. F. $500 \mu\text{g}$ DNAase I/ml. All reaction mixtures contained ethidium bromide at $58 \mu\text{g/ml}$. Tubes were centrifuged as in Fig. 3.

effect of increased enzyme concentration was a relative increase in the once-nicked linear peak and in the multiple-nicked trailing shoulder. Then a broad peak appeared with a lower average molecular weight. This peak became progressively lower in molecular weight as higher DNAase concentrations produced extensive fragmentation. This result argues that the protection against degradation at this dye concentration is not absolute, but is dependent on the enzyme concentration. However, this does not eliminate the possibility that the observed fragmentation occurs rapidly prior to onset of dye-induced protection.

Effect of varying the ethidium bromide concentration. The effect of varying the dye concentration on the extent of nicking was examined by alkaline band sedimentation as shown in Fig. 5. The preparations in 5A and 5B were digested with $0.5 \mu\text{g}$ DNAase/ml for 15 min at 30°C in the presence of 58 and $5.8 \mu\text{g}$ ethidium bromide/ml, respectively. Extensive fragmentation is seen only at the lower dye concentration.

The preparations in 5C and 5D were digested with $10 \mu\text{g}$ DNAase/ml in the presence of 445 and $89 \mu\text{g}$ ethidium bromide/ml, respectively. At this relatively high DNAase concentration, the presence of ethidium bromide affords only incomplete protection against fragmentation, but it is readily apparent that the extent of the protection is related to the concentration of dye in the reaction mixture.

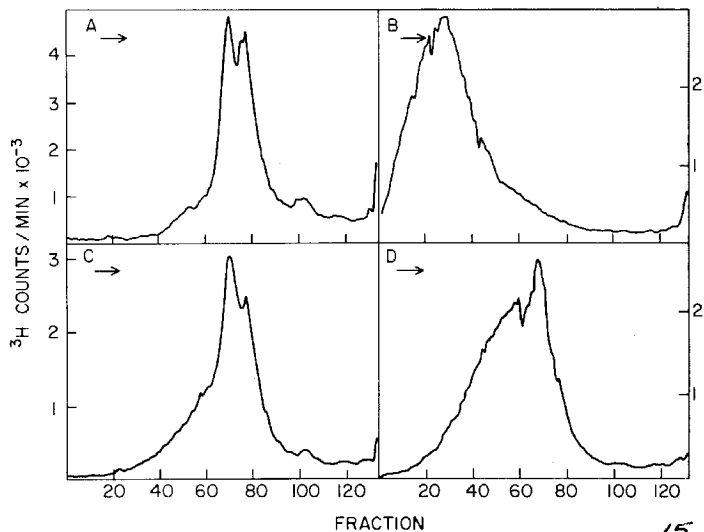


Fig. 5. Alkaline band sedimentation profiles of minicircles digested for ¹⁵ min at 30 °C with two concentrations of DNAase I in the presence of different concentrations of ethidium bromide. A. 0.5 μg DNAase I/ml; 58 μg ethidium bromide/ml. B. 0.5 μg DNAase I/ml; 5.8 μg ethidium bromide/ml. C. 10 μg DNAase I/ml; 44.4 μg ethidium bromide/ml. D. 10 μg DNAase I/ml; 89 μg ethidium bromide/ml. Tubes were centrifuged as in Figure 3.

Kinetics of nicking of minicircles in presence of ethidium bromide.

Alkaline sedimentation profiles were obtained of minicircle preparations digested with a relatively high DNase concentration of 10 μg/ml at a constant ethidium bromide concentration of 89 μg/ml for 5, 12.5, 15 and 30 min. The temporal sequence of profiles was similar to the sequence obtained with increasing concentrations of DNAase (Fig.4), in that the unit length linear peak increased first together with the trailing shoulder, and then at 30 min incubation time a broad secondary peak appeared. These results imply that the extensive fragmentation is not completed immediately but continues for at least 15–30 min.

To test this further, closed minicircles were first single-nicked and then were extensively fragmented by digestion with a high concentration of DNAase (50 μg/ml) in the presence of 89 μg ethidium bromide/ml for 5 min at 30 °C. In addition closed minicircles were digested under identical conditions and the two preparations were corun in alkaline sucrose. The resulting profiles were almost identical. If the extensive digestion of the closed molecule occurred during the intercalation of dye after introduction of the initial nick, then one would expect a more extensive fragmentation of the closed molecule than of the open molecule. The similarity of the two profiles argues against this possibility.

Furthermore, the extensive fragmentation of closed minicircles that occurred in short incubation times at a high DNAase concentration could be duplicated by increasing the time of incubation at a low DNAase concentration. Digestion of closed minicircles with 50 μg DNAase/ml for 5.5 min (89 μg ethidium bromide/ml) produced the same alkaline sucrose profile as digestion with 0.5 μg DNAase/ml for 10 h (89 μg ethidium bromide/ml).

Conditions to obtain single-nicked circles of SV40, PM-2 and ϕ X RF DNA.

Preliminary studies were performed with several larger circular molecules to obtain digestion conditions for complete conversion to single-nicked circles. In the cases of SV40 and PM-2 closed circular duplex DNA, complete conversion to single-nicked circular molecules was obtained by the conditions presented in Fig. 6A and 6B. The conditions employed for ϕ X RF digestion

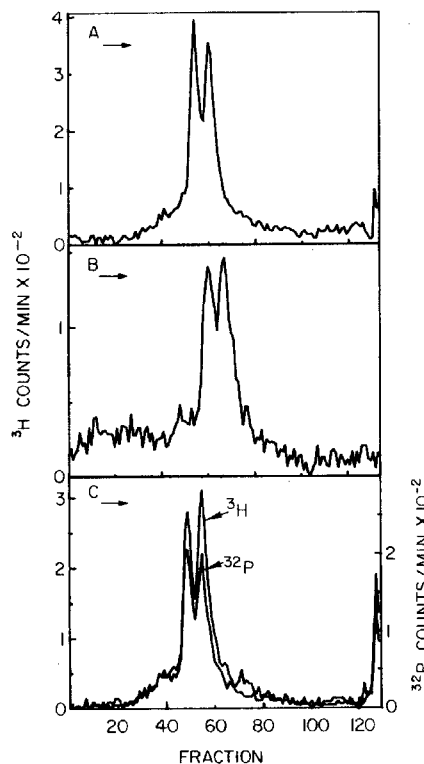


Fig. 6. Alkaline band sedimentation profiles of three larger circular DNA molecules digested with DNAase I in the presence of ethidium bromide. A. ^3H -labeled PM-2 DNA. Digested 0.9 μg with 0.1 μg DNAase I/ml and 296 μg ethidium bromide/ml in 150 μl volume for 15 min at 30 $^\circ\text{C}$. Centrifuged in the SW 65 rotor for 3.5 h at 60 000 rev./min (5 $^\circ\text{C}$). B. ^3H -labeled SV40 DNA. Digested 2.9 μg DNA with 0.1 μg DNAase I/ml and 296 μg ethidium bromide/ml in 150 μl volume for 15 min at 30 $^\circ\text{C}$. Centrifuged in SW 65 rotor for 5 h at 60 000 rev./min (5 $^\circ\text{C}$). C. ϕ X RF DNA labeled with ^3H in the viral strand and ^{32}P in the complementary strand. Digested with 0.1 μg DNAase I/ml and 444 μg ethidium bromide/ml for 15 min at 30 $^\circ\text{C}$. Centrifuged in SW 65 rotor for 4 h at 60 000 rev./min (5 $^\circ\text{C}$). The pellet (not graphed) contained 25% of the total radioactivity. In A and B the pellets were graphed. All digestions were performed in the standard Mg^{2+} -containing reaction medium.

yielded 75% conversion to open single-nicked molecules, and a slightly higher DNAase concentration is therefore indicated for complete conversion. The ϕ X RF profile demonstrated, however, that the nicking was not strand-specific. The molecules were labeled with ^3H in the viral strand and ^{32}P in the replicated strand, and as shown in Fig. 6C equal amounts of both isotopes were found under both the circle and linear peaks.

Discussion

We have confirmed and extended the findings of Barzilai [3] regarding the restriction behavior of ethidium bromide on the nicking of closed circular DNA molecules by endonuclease. In our studies, DNAase I was employed and the parameters of enzyme concentration, ethidium bromide concentration and incubation time were investigated. The main conclusion is that the nicking of closed circular molecules by DNAase I in the presence of ethidium bromide does not show all of the characteristics expected for a simple random nicking process as defined by the equations diagrammed in Fig. 2. Conditions were obtained for the digestion of closed minicircles in the presence of dye which yielded approximately 80–90% conversion to open molecules that possessed a single nick per molecule. The evidence for single-nicking was obtained by showing that these molecules yielded approximately equal numbers of single strand circles and unit length linears in alkaline band sedimentations. This deviation from the theoretical prediction for random nicking indicates that the dye does not merely lower the activity of the enzyme, but that there is a qualitative change in the nature of the process at some point. Our working hypothesis is that when the initial nick introduced into the supertwisted closed molecule rapidly relaxes the DNA molecule and allows the intercalation of more dye molecules, this increased intercalation further distorts the DNA double helix and thereby affects the rate of catalysis. Therefore, the rate of nicking of the relaxed molecules should be less than the rate of nicking of the closed molecule, thereby producing an increase in the relative proportion of single-nicked relaxed molecules. We did not measure the rate of nicking of relaxed molecules, but we did show that the protection provided relaxed molecules by high concentrations of dye was not absolute. It was also demonstrated that further nicking of the relaxed molecule did not occur immediately, as would be predicted by a model in which digestion occurred during the period of intercalation of dye into the relaxed molecule.

The initial nicking of the closed circular minicircle was $140 \times$ slower in the presence of $89 \mu\text{g}$ ethidium bromide/ml than in the absence of the dye. This could be due to a direct effect on the enzyme or to an effect on the substrate caused by the intercalation of dye by the closed circular molecule.

The optimal range of DNAase I concentration required to convert more than 90% of the closed minicircles to single-nicked open circles in 15 min at 30°C in the presence of ethidium bromide at $58\text{--}89 \mu\text{g/ml}$ was found to be approximately $0.2\text{--}1.0 \mu\text{g/ml}$. The optimal range of dye concentration at these enzyme levels was not investigated in detail, but it was observed that decreasing the dye concentration from 58 to $5.8 \mu\text{g/ml}$ at $0.5 \mu\text{g DNAase I/ml}$ led to extensive fragmentation, and also that increasing the dye concentration from 89 to $444 \mu\text{g/ml}$ decreased the rate of nicking 2.8-fold.

We have no evidence whether the limited nicking of closed minicircles in the presence of dye was site-specific. We have shown that the nicking of $\phi\text{X RF I}$ is not strand specific. It has been shown in the case of SV40 that there are several specific unpaired regions in the closed molecule that are susceptible to single strand-specific S-1 endonuclease [2]. It is possible that such regions, if characteristic of all closed circular molecules with superhelical

turns, are also the sites of the nicking of closed minicircles by DNAase I in the presence of ethidium bromide.

We did not systematically investigate the optimal single-nick conditions as a function of the molecular weight of the closed circular substrate molecule, but we did show that a decrease in the enzyme concentration was necessary to quantitatively convert several larger circular molecules to single-nicked open molecules, relative to that necessary in the case of 0.29 μm minicircles.

This method may prove to be of general utility for the quantitative introduction of single nicks into closed circular molecules.

Price [7] has recently demonstrated that both Ca^{2+} and Mg^{2+} are essential for the activity of DNAase I. In most of our reactions, exogenous Ca^{2+} probably came from trace contamination. This is a possible source of variability in applying this method to quantitatively introduce single strand nicks into closed circular molecules. Precise control of the level of Ca^{2+} in the reaction medium, perhaps by use of the selective chelator, EGTA, might eliminate some of the experimental variability. Another source of possible variation is the variable activity of commercial DNAase I preparations. Each preparation should be calibrated by running a dose-response nicking curve in the absence of dye.

Appendix

Derivation of theoretical distribution for random nicking of a population of closed circular molecules

Following is a derivation of the equations used to plot the graphs presented in Fig. 2 of the preceding paper.

At time zero, it is assumed that there are C_n single strand circles and that they are in pairs. The rate at which the circles are nicked is proportional to C_n , n (the number of bonds in each circle), and k , a rate constant, i.e.,

$$\frac{dC_n}{dt} = -knC_n \quad (1)$$

A similar equation for the rate of change of single strand linears (limited to unit contour length single strand linears, L_n) is also obtainable. This time, however, the rate is determined by the difference between the rate of formation of single strand linears from circles and the rate of their decomposition into smaller fragments. The formation of linear strands from circles is given by knC_n , and their decomposition is given by $-k(n-1)L_n$. The rate equation is then

$$\frac{dL_n}{dt} = knC_n - k(n-1)L_n \quad (2)$$

Eqns 1 and 2 are first order, linear differential equations. The initial conditions are that $L_n = 0$ and $C_n = C$.

It is convenient to express the solutions to Eqns 1 and 2 in terms of the fraction (α) of the number of bonds from the original nC bonds which have been broken. Then the solution of Eqns 1 and 2 is

$$C_n = C(1 - \alpha)^n, \text{ and } L_n = Cn((1 - \alpha)^{n-1} - (1 - \alpha)^n)$$

Originally, the circles are joined in pairs. At any stage of the reaction, some of the circles remain intact while others have been nicked. Upon alkaline denaturation, an intact pair remains as a covalently closed duplex. Any circle which is paired with a nicked one will separate from it and remain a single strand circle.

Based on the assumption of uniform random breakage of bonds at all stages of the reaction, we can calculate the expected concentration of circles which are in the unpaired state and the concentration in the paired state. In particular, after the reaction has progressed to the stage α , where α is the fraction of broken bonds as above, we get $C(1 - \alpha)^{2n}$ circles in pairs, and $C((1 - \alpha)^n - (1 - \alpha)^{2n})$ separate single strand circles (if α is not close to 1).

Acknowledgements

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