The Sequence-directed Bent Structure in Kinetoplast DNA Is Recognized by an Enzyme from *Crithidia fasciculata*

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*Crithidia fasciculata* nicking enzyme (Shlomai, J., and Linial, M. (1986) *J. Biol. Chem.* 261, 16219–16225) interrupts a single phosphodiester bond in duplex DNA circles from various sources, only in their supercoiled form, but not following their relaxation by DNA topoisomerases. However, this requirement for DNA substrate supercoiling was not observed using the natural kinetoplast DNA as a substrate. Relaxed kinetoplast DNA minicircles, either free or topologically linked, were efficiently nicked by the enzyme. Furthermore, bacterial plasmids, containing a unit length kinetoplast DNA minicircle insert, were used as substrates for nicking in their relaxed form. This capacity to activate a relaxed DNA topoisomerase as a substrate for nicking is an intrinsic property of the sequence-directed bend, naturally present in kinetoplast DNA. The 211-base pair fragment of the bent region from *C. fasciculata* kinetoplast DNA could support the nicking of a relaxed DNA substrate in a reaction dependent upon the DNA helix curvature.

Kinetoplast DNA (kDNA) is a unique extrachromosomal DNA network found in the single mitochondrion of parasitic hemoflagellate protozoa of the order Trypanosomatidae. It constitutes a multimeric DNA structure which consists in the species *Crithidia fasciculata* of about 5000 duplex DNA minicircles (2.5-kilobase pairs each) and about 50 duplex DNA maxicircles (37-kilobase pairs each), interlocked topologically to form a two-dimensional DNA network (reviewed in Refs. 1 and 2).

An unusual property of the monomeric kDNA minicircle is a region of bent DNA helix. The presence of this bend, first detected by the anomalous electrophoretic mobility of minicircle restriction fragments (3), was further supported by other physical studies (4–9). The bending of kDNA minicircle is an intrinsic property of the nucleotide sequence of the bent region, which consists of regularly spaced short oligomeric adenine residue runs (3, 6, 10, 11). This pattern has been found in association with other bent structures from various species of trypanosomatids (5). Its possible biological function in facilitating the packing of the kDNA network within the cell mitochondrion was suggested (3).

Several models have been proposed to describe the origin of the sequence-directed DNA bending that has also been observed in DNA from other sources (recently reviewed by Widom (12), Koo et al. (13), and Trifonov (14)). Their biological significance is still unknown, however, their location in the vicinity of sequences involved in the control of replication and transcription is intriguing.

The replication of kDNA minicircles has been described by Englund as a process in which each covalently sealed parental duplex DNA minicircle is released from the network and replicated as a free DNA monomer through a Cairns-type mechanism (1, 2, 15–17). The resulting progeny DNA minicircles, containing nicks and gaps, reattach to the network. The network increases in size until it doubles and then splits into two daughter networks. Englund's scheme (15) assumes the existence in the cell of an enzymatic system, catalyzing the topological interconversions of monomeric DNA circles and catenanes, which discriminates between newly replicated progeny and parental DNA minicircles. Since newly replicated DNA minicircles are nicked, while parental ones are covalently sealed (15–20), it has been previously suggested (1) that nicking might provide the signal for discrimination between replicated and prereplicated DNA minicircles. Such a discriminatory capacity is required in order to insure the release, and thereby the replication, of each of the minicircles only once in every generation.

We have studied the possibility that active nicking may play such a role in minicircle replication in *vitro*, using *C. fasciculata* DNA topoisomerase (21). We have recently described (22) a unique nicking enzyme purified to apparent homogeneity from *C. fasciculata* cell extracts. Nicking of kinetoplast DNA networks by the purified enzyme specifically inhibits their decatenation by the *Crithidia* DNA topoisomerase, but has no effect on the cationation of monomeric DNA minicircles into catenane networks. Based on its differential effect, observed in *vitro*, on the reversibility of the topological reaction, we have suggested (22) that active nicking by this enzyme may play a role in kDNA replication. Recent *in vivo* studies on the replication of kDNA minicircles in *C. fasciculata* (17) support the possible active nicking of kDNA.

Here we describe the topological considerations involved in the DNA substrate specificity of *C. fasciculata* nicking enzyme and discuss the role of the bend present in kDNA in the nicking reaction.

**MATERIALS AND METHODS**

**Nucleic Acids**—Kinetoplast DNA was prepared from *C. fasciculata* according to Englund et al. (23) and as described by Saucier et al. (24). kDNA networks were further purified using phenol extractions. Monomeric DNA minicircles were obtained by decatenating purified kDNA networks using *Crithidia* type II DNA topoisomerase (21). pBR322 and pUC18 were prepared as described by Maniatis et al.
pBR322, and M13mp8DN by the insertion of XhoI linearized fasciculata type
the concentration of chloroquine in the second dimension was 2 PM.
\( pH \) 8.3) for 16 h, at 200 turer. DNase I was purchased from Sigma, and calf thymus topoisom-
Biolabs, Bethesda Research Laboratories, or from Anglian Biotech-
Circle was prepared as previously described (26). The plasmid con-
prepared, as described by Kirkegaard and Wang (27), using
neutral 5% polyacrylamide gels (19:l Lo)

The final products of the relaxation of various DNA substrates by
relaxed DNA circle, in the presence of 2 yg/ml ethidium bromide,
Electrophoresis-Analysis of the reaction products in 0.8-1.5%
agarose gels under neutral or alkaline conditions and microdensito-
Gel Electrophoresis—Analysis of the reaction products in 0.8-1.5% agarose gels under neutral or alkaline conditions and microdensitometry were as previously described (22). DNA topoisomers separated by agarose gel electrophoresis were stained following their electrophoresis using 1 μg/ml ethidium bromide solution. When separation of nicked double-stranded DNA and covalently sealed (topologically relaxed) forms was required, electrophoresis analysis was carried out in the presence of 1 μg/ml ethidium bromide. Two-dimensional gel electrophoresis was carried out as described by Wang et al. (31), except that electrophoresis was performed in Tris acetate buffer and the concentration of chloroquine in the second dimension was 2 \( \mu M \).
Neutral 5% polyacrylamide gels (19:1 acrylamide:bisacrylamide) were electrophoresed in TBE solution (89 mM Tris borate, 2.5 mM EDTA, \( pH \) 8.3) for 16 h, at 200 V, at room temperature. Following their electrophoresis, gels were soaked in 20% methanol, 10% acetic acid solution for 20 min at room temperature, and then dried and autoradiographed.

**RESULTS**

*C. fasciculata Nicking Enzyme Introduces a Single Nick in Topologically Interlocked DNA Circles**—During replication,
kDNA minicircles are found in the trypanosomatid cell in two interconvertible topological forms of monomeric DNA circles and catenane networks. Nicking of kDNA minicircles in vitro by *C. fasciculata* nicking enzyme inhibits their decat-

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**FIG. 1.** Effect of *C. fasciculata* nicking enzyme on kDNA minicircles. 2 μg of each of kDNA networks (A) and decatenated free minicircles (C) were incubated with 2 units of *C. fasciculata* nicking enzyme under the standard assay conditions. DNA was extracted from the reaction mixture and 1 μg samples were digested with 12 units of S1 endonuclease in 40 μl of reaction mixture contain-

- 0.059 for plasmid form I DNA isolated from saurated cultures (30).

The linking number difference \( \Delta L \) between the supercoiled and relaxed DNAs \( (L-L_r) \) is determined by the quantitation of the number of supercoiled turns \( L_r \), calculated as the total number of base pairs in the molecule divided by the number of base pairs per helical turn (10.54, from Horowitz and Wang (29)). We have used the value of \( \Delta L \) to -0.059 for plasmid form I DNA isolated from saurated cultures (30).

Gel Electrophoresis—Analysis of the reaction products in 0.8-1.5% agarose gels under neutral or alkaline conditions and microdensitometry were as previously described (22). DNA topoisomers separated by agarose gel electrophoresis were stained following their electrophoresis using 1 μg/ml ethidium bromide solution. When separation of nicked double-stranded DNA and covalently sealed (topologically relaxed) forms was required, electrophoresis analysis was carried out in the presence of 1 μg/ml ethidium bromide. Two-dimensional gel electrophoresis was carried out as described by Wang et al. (31), except that electrophoresis was performed in Tris acetate buffer and the concentration of chloroquine in the second dimension was 2 \( \mu M \).
Neutral 5% polyacrylamide gels (19:1 acrylamide:bisacrylamide) were electrophoresed in TBE solution (89 mM Tris borate, 2.5 mM EDTA, \( pH \) 8.3) for 16 h, at 200 V, at room temperature. Following their electrophoresis, gels were soaked in 20% methanol, 10% acetic acid solution for 20 min at room temperature, and then dried and autoradiographed.

**RESULTS**

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nicked-interlocked circles (22) it is suggested that the single nick introduced during the nicking reaction (Fig. 1) has different effects upon the two topological forms of kDNA.

The Substrate for Nicking Is a Duplex DNA Circle—Interference with the reversibility of the topological reaction was unique to the action of C. fasciculata nicking enzyme and was not observed using randomly nicked kDNA networks (22). Such unique nicking may be directed by specific kDNA sequences which form a site for the enzyme at the level of either the primary or the secondary structure of the DNA molecule. In the latter case, the generation of a nicking site may also be dependent upon the tertiary structure of the DNA molecule.

To resolve these possibilities, we have assayed for the availability of a site for nicking in kDNA sequences of various conformations. The same analysis was applied to phage and plasmid DNA substrates. As shown in Fig. 2, C. fasciculata nicking enzyme catalyzes the hydrolysis of a phosphodiester bond in the circular duplex RF I-DNA of the M13 phage vector, but not in its single-stranded circular form. Neither was such a nicking displayed when an M13 recombinant phage DNA containing a unit length kDNA minicircle was used. Double-stranded DNA molecules provide such a site for nicking only in their circular form (Figs. 2 and 3). pBR322 DNA (not shown) or this vector plasmid containing a unit length DNA minicircle (the plasmid pSAL8) (Fig. 3A) are efficient substrates for nicking by C. fasciculata nicking enzyme. However, as judged by both the alkaline gel electrophoresis analysis (Fig. 3B) and S1 endonuclease digestion (Fig. 3C), neither the linearized plasmid DNA nor the linear kDNA insert could provide a site for nicking by the crithidial enzyme. Since only circular double-stranded DNA (Fig. 2) provides a substrate for the crithidial enzyme it was suggested that supercoiling of DNA may play a role in substrate recognition. This was further confirmed by the observation that pBR322 DNA which was nicked (using EcoRI endonuclease in the presence of ethidium bromide) and subsequently religated was not nicked by the crithidial nicking enzyme.

Action of C. fasciculata Nicking Enzyme Is Dependent upon the Superhelical Density of the DNA Substrate—Bacterial plasmids are efficient substrates for nicking by C. fasciculata nicking enzyme in their negative supercoiled form (Fig. 4A). Since negative supercoiling might promote the formation of secondary structures (30, 32–34), as well as the exposure of unwound regions in the DNA molecule, we have studied the properties of positively supercoiled DNA, in which these conformations are destabilized, as substrate in the nicking reaction. As demonstrated in Fig. 4B positively supercoiled toposomers of pUC18 plasmid DNA were as efficient substrates for nicking as were the negatively supercoiled ones. These observations suggest that the tertiary structure of the DNA substrate is, per se, an important factor in the nicking reaction.

Fig. 2. Action of C. fasciculata nicking enzyme on double-stranded and single-stranded DNA circles. 2 µg of each of M13mp18 RF I DNA (A), M13mp8 single-stranded DNA (O), and M13mp8DN single-stranded DNA (containing a unit length kDNA minicircle) (.), were incubated with 2 units of C. fasciculata nicking enzyme under the standard assay conditions. Samples, withdrawn at the time intervals indicated, were submitted to electrophoresis in 0.8% agarose gels and microdensitometry. Single-stranded DNAs were electrophoresed in 50 mM Tris borate, pH 8.3, 1 mM EDTA at room temperature and 5 V/cm; under these conditions the linear and circular forms of the single-stranded M13 phage vector DNAs are resolved. Percentage of nicking is from the total DNA used as a substrate. To account for the difference in ethidium bromide binding to nicked versus covalently closed DNA circles, we have used the value obtained for the fluorescence of the completely nicked substrate as a reference value.

Fig. 3. Double-stranded linear DNA as a substrate for nicking by C. fasciculata nicking enzyme. In A, 1 µg of form I pSAL8 DNA (lane 1) was incubated with 2 units of C. fasciculata nicking enzyme for 60 min at 30°C (lane 2). Analysis by 1% agarose gel electrophoresis in the presence of ethidium bromide was as described under "Materials and Methods." In B, 1 µg of linearized, radioactively labeled (see below) pBR322 DNA (lane 1) or kDNA minicircle (lane 3) were incubated with 2 units of C. fasciculata nicking enzyme as above (lanes 2 and 4, respectively). Reactions were stopped using alkaline stopping buffer and were subjected to alkaline gel electrophoresis analysis, followed by autoradiography of the dried gel (see "Materials and Methods"). Preparation of the linear DNA substrates was as follows: pBR322 was linearized by EcoRI endonuclease digestion. Linear DNA inserts containing a unit length (2500 base pairs) kDNA minicircles plus 450 base pairs (of flanking pBR322 sequences) were prepared by digestion of pSAL8 using NoI restriction endonuclease. The rescued DNA fragment was isolated by electrophoresis in low temperature melting agarose (Sigma) gel, phenol extraction from the gel, and ethanol precipitation. 3'-End labeling of the linearized DNA substrates was carried out using E. coli DNA polymerase I large (Klenow) fragment, and either by [α-32P]dATP or [α-32P]dCTP for pBR322 and kDNA, respectively. In C, 1 µg of the linearized labeled kDNA fragment (prepared as described above) was treated with S1 endonuclease (as described in the legend to Fig. 1), either prior (lane 2) or post (lane 3) its nicking by C. fasciculata nicking enzyme. Reaction products were analyzed by electrophoresis in neutral 1% agarose gel, followed by autoradiography of the dried gel. m1 and m2 are HindIII-digested λ DNA, labeled as above, used as size markers. (numbers represent the size in kilobase pairs) for the alkaline and the neutral gel electrophoreses, respectively. CC and NC are the supercoiled and nicked forms of pSAL8 DNA. L1 and L2 are the linear forms of pBR322 and the minicircle insert, rescued from pSAL8, under alkaline conditions. L3 is the double-stranded linear form of the kDNA minicircle insert.

Linial, M., and Shlomai, J., unpublished data.
**C. fasciculata Nicking Enzyme Recognizes a Bent DNA Helix**

Relaxed DNA topoisomers of bacterial plasmids, obtained through the actions of either calf thymus type I topoisomerase, ligase-joining of nuclease-nicked circles (not shown), or *C. fasciculata* type II topoisomerase (Fig. 4) could not support the nicking reaction. As demonstrated in Fig. 4C, the limit products of the *C. fasciculata* DNA topoisomerase-catalyzed relaxation of pUC18 DNA were unaffected upon their incubation with the nicking enzyme. The four different topoisomers resolved, having the linking difference values (\(\Delta L\)) of 0 to \(-3\) (determined by two-dimensional gel electrophoresis analysis) could not support the nicking reaction. These results indicated the dependence of the nicking reaction upon the degree of the topological winding of the DNA substrate.

To further define the topological requirements of the nicking reaction we have assayed for nicking activity using DNA topoisomers of various superhelical densities. When partially relaxed pUC18 DNA of 2686 base pairs was used, it was found that *C. fasciculata* nicking enzyme nicked only 12 out of the original 16 topoisomers calculated for pUC18 plasmid (as described under “Materials and Methods”). In the experiment presented in Fig. 4D we used partially relaxed pUC18 DNA as a substrate. Upon incubation with *C. fasciculata* nicking enzyme we observed the reduction of the ladder of bands down to the four slowest migrating DNA bands. This profile of the ladder remained unchanged following prolonged incubations with the nicking enzyme.

These results demonstrate that while the topoisomer, having the superhelical density (\(\sigma\)) of \(-0.012\) (calculated as described under “Materials and Methods”), was an inefficient substrate in the nicking reaction, the one having the value of \(\sigma = -0.016\) was nicked by the enzyme. Inasmuch as these two adjacent resolved topoisomers differ in \(\Delta L\) of only \(\pm 1\) (28, 35), it immediately follows that the part of this \(\Delta L\), which is expressed as a change in the writhe of this molecule, at that superhelical density, is required and sufficient to activate this topoisomer as a substrate for the nicking enzyme. This does not exclude the possibility that a change in the molecule writhe by only a fraction of a turn may have the same effect on the nicking reaction.

When such an analysis was applied to partially relaxed pBR322 DNA (of about 4.4 kilobase pairs) as a substrate, it was found that DNA topoisomers having a superhelical density (\(\sigma\)) of \(-0.017\) or higher (in absolute value) were nicked, while the ones with \(\sigma = -0.015\) or below (in absolute value) were inactive as substrates in this reaction. Similarly, as was found with partially relaxed M13mp8 RF DNA (of about 7.2 kilobase pairs) as a substrate for nicking, DNA topoisomers with \(\sigma > -0.015\) were inactive while those with \(\sigma < -0.016\) were nicked. It appears therefore, that in using DNA substrates of various length, the *Crithidia* nicking enzyme was acting upon DNA substrates of similar levels of superhelical density. These levels of the DNA substrate supercoiling required for nicking by the crithidial enzyme were much lower than those reported for other endonucleases (36).

**Kinotoplast DNA Minicircles Are Nicked by the Crithidial Enzyme in Their Relaxed Topological State—*C. fasciculata* nicking enzyme carries out the nicking of kDNA minicircles in their relaxed topological state. The analysis presented in Fig. 5 reveals that kDNA minicircles which were decatenated and relaxed using *C. fasciculata* DNA topoisomerase (Fig. 5) or relaxed post-decatenation with calf thymus DNA topoisomerase I (not shown), are efficient substrates for nicking by the purified enzyme. This phenomenon is further emphasized by the observation that the insertion of a unit length kDNA minicircle into pBR322 (to obtain the plasmid pSAL8), renders the bacterial plasmid an efficient substrate for nicking in its relaxed topological state (Fig. 6, A and B). The same vector that lacks the kDNA insert could be nicked by the crithidial enzyme only in its supercoiled form, but not post-relaxation by the DNA topoisomerase (Fig. 6, C and D).

pBR322 (of about 4.4 kilobase pairs) is considerably larger than the kDNA minicircles (of about 2.5 kilobase pairs). However, a possible effect of the size difference upon the

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**Fig. 4.** Dependence of *C. fasciculata* nicking reaction upon the DNA substrate supercoiling. Nicking reaction and electrophoresis analysis in 1.2% agarose gels were as described under “Materials and Methods.” In A, 0.5 \(\mu\)g of negatively supercoiled pUC18 DNA prior to (lane 1) and post (lane 2) incubation with 0.5 unit of *C. fasciculata* nicking enzyme for 70 min at 30 °C. In B, 0.5 \(\mu\)g of pUC18 positively supercoiled topoisomers (see “Materials and Methods”) (lane 1) and their products after a nicking reaction as above (lane 2). In C, 0.5 \(\mu\)g of the final products of *C. fasciculata* topoisomerase relaxation reaction (see “Materials and Methods”) using negatively supercoiled pUC18 DNA as a substrate before (lane 1) and after (lane 2) their nicking as above. In D, 0.5 \(\mu\)g of partially relaxed pUC18 (lane 1) was incubated in the nicking reaction for 60 min (lane 2). The DNA in lane 3 represents the negatively supercoiled form I pUC18 DNA substrate. NC and SC are the nicked and the supercoiled forms of pUC18, respectively.

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**Fig. 5.** Nicking of relaxed free kDNA minicircle by *C. fasciculata* nicking enzyme. Free kDNA minicircles where prepared by decatenation and relaxation of DNA networks using *C. fasciculata* DNA topoisomerase. Residual gapped and nicked circles in the preparation were covalently sealed using *E. coli* DNA polymerase I large (Klenow) fragment and T4 DNA ligase. 0.5 \(\mu\)g of phenol-extracted kDNA minicircles (A) were nicked using 0.5 unit of *C. fasciculata* nicking enzyme under the standard assay conditions and analyzed by electrophoresis in 1% agarose gel, containing ethidium bromide, and microdensitometry (B). The direction of electrophoresis is indicated by the polarity (from + to -). NET, NC, L, and CC are the network, nicked, linear, and covalently closed forms of kDNA minicircles, respectively.
trophoresis is indicated by the polarity (from bromide, was followed by microdensitometry. In PSAL8 negatively supercoiled DNA were relaxed using type I1 DNA topoisomerase, as described under "Materials and Methods." Phenol-extracted 1 pg samples of each of the relaxed pBR322 and pSAL8 circles were incubated with 1 unit of enzyme for the utilization of topologically relaxed kDNA minicircles, but not of their linear forms, in the

Topologically Relaxed DNA Circles Containing a Bent Structure Are Substrates for C. fasciculata Nicking Enzyme—As has been shown (Fig. 4), positively and negatively supercoiled topoisomerases are active substrates in the nicking reaction, while those relaxed through the action of DNA topoisomerases are inactive. Activation of relaxed topoisomerases in the presence of kDNA-derived sequences might, therefore, function through the re-induction of the required tertiary structure of the circular DNA substrate.

Since kDNA networks, which consist of DNA minicircles heterogeneous in their nucleotide sequences, were nicked in this reaction (Figs. 1 and 5), the presumption was that specific sequences, conserved in the heterogeneous minicircles, are involved in the activation of the relaxed substrate. It has been recently reported (8) that the sequence direct the bending of C. fasciculata kDNA is conserved throughout the heterogeneous minicircle population.

To further explore the possibility that the bent structure may play a role in the activation of the relaxed DNA substrates, we have studied the effect of a segment containing the bent region from C. fasciculata kDNA on the nicking reaction. A 211-base pair fragment, containing the bent region from C. fasciculata kDNA minicircle, cloned into the pSP65 plasmid (pPK201/CAT plasmid, described by Kitchin et al. (37)) was used in these experiments. Fig. 7 demonstrates that pPK201/CAT relaxed by the crithidia DNA topoisomerase, was an efficient substrate for nicking by C. fasciculata nicking enzyme. The quantitative analysis of the distinct DNA bands, constituting the limit products of pPK201/CAT relaxation reaction, revealed the utilization of all these different topoisomers, to the same extent, in the course of the subsequent nicking reaction (Fig. 7C). Furthermore, the rates of nicking, measured using relaxed pSAL8 versus pPK201/CAT DNA, relative to the rates measured using their respective super-

Fig. 6. Nicking of relaxed DNA topoisomers containing a unit length kDNA minicircle insert. 2 µg of each of pBR322 and pSAL8 negatively supercoiled DNA were relaxed using C. fasciculata type II DNA topoisomerase, as described under "Materials and Methods." Phenol-extracted 1 µg samples of each of the relaxed pBR322 and pSAL8 circles were incubated with 1 unit of C. fasciculata nicking enzyme for 75 min under the standard assay conditions. Electrophoresis of the reaction products in 1% agarose gels, containing ethidium bromide, was followed by microdensitometry. In A and C, topologically relaxed pSAL8 and pBR322 DNAs, and in B and D, their products of the nicking reaction, respectively. The direction of electrophoresis is indicated by the polarity (from − to +). NC and CC are the nicked and covalently closed forms of the DNA circles.

Fig. 7. Utilization of a plasmid containing a sequence directed bent structure by C. fasciculata nicking enzyme. 2 µg of negatively supercoiled pPK201/CAT DNA were relaxed using C. fasciculata type II DNA topoisomerase, as described under "Materials and Methods." The reaction was stopped using 20 mM EDTA and phenol extracted. The resulting relaxed pPK201/CAT DNA was incubated with C. fasciculata nicking enzyme under the standard assay conditions. Electrophoresis in 1% agarose gel was followed by ethidium bromide staining and microdensitometry, as described under "Materials and Methods." A-C represent the analysis of 0.5 µg of each of the original form I pPK201/CAT, its relaxed forms, and the supercoiled forms of pPK201/CAT DNA, respectively. The discrete peaks represent the final products of the relaxation reaction catalyzed by the topoisomerase, as resolved by this gel. NC and SC are the nicked and the supercoiled forms of pPK201/CAT DNA, respectively. The direction of electrophoresis is indicated by the polarity (from − to +).
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**FIG. 8. Utilization of a duplex DNA circle containing a kDNA minicircle derived fragment by C. fasciculata nicking enzyme.** 2 μg of each of negatively supercoiled (○) and relaxed (□) pORF1-17 DNA were nicked by C. fasciculata nicking enzyme under the standard assay conditions, as described under "Materials and Methods." Samples withdrawn at the time intervals indicated, were analyzed in 1% agarose gels and microdensitometry, as described under "Materials and Methods."

colored forms, were the same, indicating an equal effect of the unit length kDNA minicircle and its derived small bent fragment on the rate of the reaction. No such activation of a relaxed DNA topoisomerase could be observed using a plasmid containing a 610-base pair fragment derived from C. fasciculata kDNA minicircle (pORF1-17), which does not contain the bent sequence (Fig. 8). These results indicate that the capacity of the 2800 base pairs of the unit length kDNA minicircle to support a nicking reaction is fully retained by a fragment of only 211 base pairs containing the sequence-directed bent structure.

**Nicking of Topologically Relaxed DNA Topoisomers Is Dependent upon the Curvature of the DNA Helix**—To further explore the dependence of the nicking reaction upon the bending of the DNA helix we have studied the effect of the drug distamycin A1 on this reaction. This drug has been previously shown to bind to DNA and to abolish the anomalous electrophoretic behavior of DNA fragments containing a bent helix (6). Suppression of the bent structure, through the binding of distamycin A1, was recently also demonstrated using electron microscopy (9). Fig. 5 demonstrates the effect of increasing concentrations of distamycin A1 on the curvature of the 219-base pair bent fragment, and on the rate of nicking of a relaxed DNA circle containing this bent fragment, by C. fasciculata nicking enzyme. It shows that nicking of topologically relaxed pPK201/CAT circles is inhibited in the presence of the drug. This inhibition was of 50% in the presence of one distamycin A1 molecule added per 100 base pairs of DNA circles. Complete inhibition (>98%) of the nicking reaction was measured raising this ratio to one molecule of the drug per 25 base pairs. The possibility that inhibition of the nicking reaction is through the direct protection of the enzyme binding or cleavage sites by the bound drug (38, 39), cannot be excluded here. However, no such decrease in the rate of nicking could be measured using negatively supercoiled pPK201/CAT DNA as a substrate in this reaction (Fig. 9, inset). In fact, following a slight decrease (of about 15%) in the nicking activity in the presence of low concentrations of distamycin A1, we have reproducibly measured an increase of about 20% in the rate of the nicking reaction at high concentrations of the drug (at the ratio of one molecule of distamycin A1 per 5–10 base pairs). The decrease in the rate of nicking of relaxed DNA circles, in the presence of the drug, could be precisely correlated with the decrease in the extent of the bent-helix curvature, as was measured by the concomitant increase in its electrophoretic mobility (Fig. 9). These results indicate that the presence of a bent structure is essential for the nicking of relaxed DNA topoisomerases, and suggest that the rate of the reaction is dependent upon the extent of bending of the DNA helix.

**DISCUSSION**

C. fasciculata nicking enzyme introduces nicks into interlocked kDNA minicircles and thereby interferes with their decatenation by the trypanosomatid DNA topoisomerase (21, 22). This interference with the reversibility of the topological reaction was unique to this enzyme, implying the specificity of its action upon the DNA substrate. We have previously suggested a role for this enzyme in the process of kDNA minicircle replication, through the control of the release of kDNA minicircles from the network, prior to their replication, and their post-replication reattachment to it. Some of the topological considerations involved in the enzyme specificity and the role for the sequence-directed bent structure in this reaction are discussed in this paper.

From the nicking of natural kDNA networks, free kDNA minicircles (Fig. 1), and kDNA networks, relaxed by a type I DNA topoisomerase (21), it could have been erroneously concluded that the topological state of the DNA substrate is not a major factor to be considered in studying the substrate specificity of C. fasciculata nicking enzyme. However, analysis of the properties of various DNA substrates revealed the major role played by the tertiary structure of the DNA molecule in this reaction. The basic observation made in this study, that DNA molecules cannot serve as substrates in the nicking reaction in any other conformation but their covalently closed duplex form (Figs. 2 and 3), was instructive. It implied that a target site for nicking is not available in the DNA molecule as long as the termini of its strands are free to rotate about each other and, therefore, cannot adopt superhelical turns. Dependence of nicking upon the degree of supercoiling of the DNA substrate is clearly demonstrated in the case of bacterial plasmids and phage DNA circles used as substrates (Figs. 2 and 4). This dependence of the nicking reaction upon the substrate superhelicity was similar using DNA substrates of different sizes. This implies that at the size range used (2686 to 7229 base pairs for pUC18 and M13mp8 RF, respectively) no length dependence was involved.

An apparent exception to this general pattern is the nicking of kDNA and its derivatives in their topologically relaxed forms (Figs. 5–7). It implies that kDNA minicircles contain specific sequences which could render a relaxed DNA topoisomerase into an active substrate for nicking by the enzyme. It seems, therefore, that a DNA topoisomerase could be rendered an active substrate for nicking by either an increase in its superhelicity density (Fig. 4) or, alternatively, through the presence of kDNA sequences (Figs. 5–7).

The possibility that kDNA could form a direct site for nicking at the level of its primary structure, or in the form of a stable secondary structure is highly unlikely. This is in view of the observations that both positively and negatively supercoiled DNA could be used as substrates, whereas neither the single-stranded nor the linear duplex forms of kDNA minicircle were nicked in this reaction (Figs. 2 and 3). Furthermore, the strict dependence of the nicking of kDNA-containing circles upon the covalent closure of these substrates strongly suggest that their capacity to assume a change in the molecule writhe is essential for their nicking. Thus, it appears that in both the presence or the absence of kDNA minicircle se-
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Fig. 9. The effect of distamycin A1 on the rate of nicking by C. fasciculata nicking enzyme. 2 \( \mu \text{g} \) of each of relaxed (○) and supercoiled (●) (insert) pPK201/CAT DNAs were preincubated with distamycin A1 (Sigma), as described below, and then used as a substrate for nicking by C. fasciculata nicking enzyme, under the standard assay conditions. Electrophoresis of the reaction products in 1% agarose gels, was followed by microdensitometry of the ethidium bromide-stained bands to quantitate the rate of the reaction. 2 \( \mu \text{g} \) of pPK201/CAT DNA was digested by BamHI endonuclease to rescue a 219-base pair fragment containing the bent DNA helix from C. fasciculata kDNA (as described by Kitchin et al. (8)). The 219-base pair fragment, purified through a Bio-Gel A-0.5m column (1 \( \times \) 45 cm), was 3'-end labeled by [\( \alpha \text{-32P} \)]dCTP using E. coli DNA polymerase I large (Klenow) fragment, diluted with non-radioactive relaxed pPK201/CAT DNA, and incubated with distamycin A1, as described below. The drug-treated DNA was analyzed by electrophoresis in 5% polyacrylamide gel, followed by autoradiography, as described under "Materials and Methods." The apparent sizes (▲) deduced from the electrophoretic mobilities were measured using HindIII restriction endonuclease fragments of \( \lambda \) DNA, and HindPI restriction endonuclease fragments of pUC18 DNA which were 3'-end labeled as above, as size markers (at the range of 65-565 base pairs). In the absence of the drug, the apparent size measured for the 219-base pair fragment was of 370 base pairs. Treatment with the antibiotic drug distamycin A1 (diluted in 10 mM Tris-HCl, pH 7.4) was carried out by incubating the DNA substrates (2.5 \( \times \) 10^{-2} \( \mu \text{M} \)) in the presence of various concentrations of the drug (at the range of 4 \( \times \) 10^{-3} to 2 \( \times \) 10^{-2} M). Values for nicking activity presented are relative to the rate of the reaction in the absence of the drug.

As was found in the present study, a segment of 211 base pairs containing the bend from kDNA minicircles of C. fasciculata (8), has the capacity to promote in the DNA substrate the structural change required for its nicking by the crithidia1 enzyme (Fig. 7). Diekmann and Wang (40) have shown that the bent structure of kDNA has a zero writhe in its undistorted form, in either linear or nicked circular DNA. However, as these authors have pointed out, this sequence can be easily distorted, imposing a writhe on the DNA molecule. This change in the molecule writhe, induced by the bent structure, is expressed even at a relatively low superhelical density and in both negative and positive supercoiled topoisomers. Based on these data it is assumed that the bent structure used in our experiments promotes a change in the tertiary structure of the topoisomers used. In applying these data to the study...
presented here, one has to consider that the C. fasciculata bent fragment used here has a very extreme curvature. It comprises 18 runs of 4–6 adenine residues, 16 of which are on the same strand (8).

Finally, the general pattern of nick distribution in the bend-containing DNA circles suggests that the sequence directing the bending of the DNA helix is not, per se, a preferred site for cleavage by the crithidial enzyme. However, the dependence of the nicking reaction upon the presence of a bend in the relaxed DNA topoisomer (Figs. 5–7), and the correlation observed between the decrease in the helix curvature of the DNA substrate and the rate of its nicking (Fig. 9), are clearly demonstrated. Studies on the specific features of the preferred cleavage site for C. fasciculata nicking enzyme in various DNA substrates, including the bent DNA fragment from kDNA, reveal that no extensive sequence specificity is involved in defining the nicking site. However, recent observations reveal that the bent structure specifies the unique binding site for C. fasciculata nicking enzyme. Furthermore, such a site can be specified by sequence-directed bent structures from various prokaryotic and eukaryotic sources, as well as by the forcible bending of the helix resulting from its writhe in the supercoiled state.

We have previously proposed (22) a possible role for active nicking by C. fasciculata nicking enzyme in the replication of kinetoplast DNA minicircles through the effect on their reversible decatenation. The present study demonstrates the effect of the bent structure present in kDNA minicircles upon the nicking reaction. The physiological function of the bent structure in kDNA is not yet known. Marini et al. (3) have proposed that the bent structure might be functionally significant in facilitating the binding of specific proteins or in the packing of the kDNA network into the mitochondrion. Inasmuch as active nicking plays a role in kDNA minicircle replication, one might also speculate an involvement of the bent structure in this process.

Acknowledgment—We are grateful to Dr. P. T. Englund from the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, for the generous gift of the pPK201/CAT DNA and the information on the C. fasciculata kDNA bend region sequence.

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