Sequence-directed bent DNA helix is the specific binding site for Crithidia fasciculata nicking enzyme

(kinetoplast DNA/curved helix/minicircle replication)

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ABSTRACT The sequence-directed bent structure of kDNA minicircles specifies a unique binding site for Crithidia fasciculata nicking enzyme. Binding of the purified enzyme to the bent structure results in the formation of a tight enzyme-DNA complex that is highly specific to curved DNA. Recognition of the binding site is not determined by the nucleotide sequence at the site of binding per se but through the specific local variation in the DNA helix geometry. Both dynamic curved structures, which are generated by supercoiling, and static ones, which are sequence-directed, could support an efficient enzyme-DNA complex formation. Binding interactions are dependent upon the degree of the helix curvature and decrease with the straightening of the binding site. DNase I protection experiments identify distinct domains of enzyme binding within the bent structure and suggest the induction of structural changes within these regions as a result of protein–DNA interactions.

Sequence-directed bending of the DNA double helix is a conformational variation found in DNA molecules from both prokaryotic and eukaryotic organisms (1–10). Bent structures were found in association with sequences consisting of short oligomeric adenine residue runs, which are regularly spaced, in phase, with the helical repeat [recently reviewed by Widom (11), Koo et al. (12), and Trifonov (13)]. The biological significance of the local bending in the DNA helix is still unknown. However, the location of such conformational variations near sequences that are involved in the control of replication (2–4, 8–10), transcription (5, 6), and recombination (7) attracts interest to these structures as potentially important regulatory elements.

Bent DNA structures were first detected (1) in the minicircles of the kinetoplast DNA (kDNA) network (14) present in the mitochondrion of parasitic hemoflagellate protozoa of the order Trypanosomatidae (15). The sequence directing the bending of the DNA helix in Crithidia fasciculata kDNA minicircles has been analyzed and characterized (16), and its bent structure, consisting of 18 runs of four to six adenine residues (16), has been visualized by electron microscopy (17). Marini et al. (1) have proposed that the bent structure in kDNA minicircles might facilitate the binding of specific proteins or the packing of the kDNA network into the mitochondrion. A role for the bent sequence—as a signal recognized by an enzyme from C. fasciculata—is suggested in the present communication.

We have described (18) a unique nicking enzyme purified to homogeneity from C. fasciculata cell extracts. Based on the effect of the enzyme upon the reversible decatenation of kDNA networks, we have suggested a potential role for this enzyme in the replication of kDNA (18). Recently, we have described (19) the requirement of the reaction catalyzed by C.

fasciculata nicking enzyme for the presence of a bent sequence in the DNA substrate.

Here we describe the role of the sequence-directed bent structure in specifying a binding site for C. fasciculata nicking enzyme and discuss the possible role of the bend in the replication of kDNA minicircles.

MATERIALS AND METHODS

Nucleic Acids. kDNA was prepared from C. fasciculata as described by Sauzier et al. (20). pPK201/CAT (16) was a gift from Paul T. Englund (Department of Biological Chemistry, The Johns Hopkins University School of Medicine). The plasmid pSP65/8 was prepared from pPK201/CAT by excision of a 219-base-pair (bp) fragment containing the bent DNA structure from C. fasciculata kDNA minicircle, accomplished by digestion with BamHI endonuclease.

Filter Binding Assay. The 10-μl standard reaction mixture contained 25 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, 12% (vol/vol) glycerol, and 30 μg of bovine serum albumin per ml. Reactions were started by the addition of the enzyme [fraction V (18)]. The reaction mixture was incubated at 0°C for 15 min; diluted with 1.0 ml of washing solution containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, and 5% (vol/vol) dimethyl sulfoxide; and filtered through nitrocellulose filters (Schleicher & Schuell) that had been presoaked in the washing solution. Filters were washed with 1.0 ml of the above solution and dried, and the radioactivity was measured. Unless otherwise stated, 16 ng of C. fasciculata nicking enzyme [fraction V (18)] was added to the standard reaction mixtures containing pPK201/CAT DNA as substrate for binding, and 80 ng of the enzyme was added to reaction mixtures containing pSP65/8 DNA as substrate. The higher enzyme concentration used with the latter substrate was to obtain measurable levels of complex formation.

Mobility Shift Electrophoresis Analysis. Reaction mixture and assay conditions were as described above except that 1.25 μg of poly(C,I) (P-L Biochemicals) per ml was present in the reaction mixture. Reaction products were loaded onto a native 4% polyacrylamide gel in 24 mM Tris borate buffer (pH 8.3) containing 0.5 mM EDTA. Electrophoresis was for 2 hr at 8–10°C and 200 V.

DNase I Protection Experiments. Binding reactions were carried out in a 6-μl volume under the standard assay conditions and were supplemented with 1 μl of DNase I (Sigma) at 10 μg/ml. Reactions, carried out at 23°C for 90 sec, were stopped by the addition of loading buffer (80% vol/vol formamide/20 mM EDTA/0.1% bromophenol blue/0.1% xylene cyanol FF). Samples were heated for 2 min at 95°C and loaded onto a 6% polyacrylamide gel in 89 mM Tris borate buffer, pH 8.3/2 mM EDTA/7.8 M urea. Electrophoresis was at 1700 V for 90 min.

Abbreviations: dATP[α-32P], deoxyadenosine 5'-[α-32P]thio]triphosphate; kDNA, kinetoplast DNA.

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RESULTS

Binding \textit{C. fasciculata} Nicking Enzyme to Its DNA Substrate Is Dependent upon the Presence of a Bent-DNA Structure. Topologically relaxed DNA circles were inactive as substrates for \textit{C. fasciculata} nicking enzyme but could be rendered active substrates in two alternative ways: (i) through an increase in their superhelical density ($\sigma \leq -0.016$); or (ii) by the presence of the bent sequence from \textit{C. fasciculata} KDNA in a relaxed DNA topoisomer (19). Such dependence upon the presence either of a static-sequence-directed bend or, alternatively, of a topological structure in which dynamic bends in the DNA helix were induced suggested a major role for the bent-DNA helix in the nicking reaction.

To explore the possibility that bends in the DNA helix participate in specifying a binding site for \textit{C. fasciculata} nicking enzyme, we studied the effect of the presence of the bend in the DNA helix upon the binding of the enzyme to its DNA substrate.

Fig. 1 presents the results of an analysis in which we measured the binding of the enzyme (i) to a duplex DNA molecule containing the 211-bp bent-DNA sequence from \textit{C. fasciculata} kDNA [pPK201/CAT (16)] and (ii) to the same DNA molecule from which the bent fragment had been deleted (pSP65/8). Binding of the enzyme to the covalently closed DNA circles (Fig. 1A) showed that both DNA species bind the enzyme at about the same efficiency in their supercoiled form. However, the topological relaxation of these substrates resulted in a significant decrease in the capacity of the pSP65/8 DNA to bind the enzyme. This was further emphasized in the analysis of the enzyme binding to the corresponding linear DNA substrates (Fig. 1B), in which a difference of >2 orders of magnitude was observed in the capacity of these DNA substrates to bind the enzyme. Because binding of the enzyme was significantly reduced (>90%) as a result of the linearization of pSP65/8 DNA but not of pPK201/CAT, we attribute the lower level of binding measured with the relaxed pSP65/8 DNA—one-third that of linearized pPK201/CAT—to DNA topoisomers with superhelical density of $\sigma \leq -0.016$ constituting about 35% of the partially relaxed topoisomer population.

The pattern of binding of \textit{C. fasciculata} nicking enzyme found here corresponds well with the previously observed substrate specificity of the nicking reaction, suggesting that binding of \textit{C. fasciculata} nicking enzyme to the DNA template is dependent upon the presence of a bend in the DNA helix.

\textit{C. fasciculata} Nicking Enzyme Forms a Specific Complex with the Bend-Containing DNA Molecule. The interaction of \textit{C. fasciculata} nicking enzyme with a DNA molecule containing a bent structure results in the formation of a tight enzyme–DNA complex. In the experiment summarized in Fig. 2, radioactively labeled pPK201/CAT DNA competed in a protein binding assay with unlabeled DNA in which only the 211-bp bent fragment had been deleted. It was found that the deleted plasmid (pSP65/8) had lost its capacity to compete with the bend-containing DNA molecule in the binding of the enzyme. A large excess, >100-fold, of unlabeled pSP65/8 DNA had no measurable effect upon the binding of the enzyme to the labeled pPK201/CAT DNA, whereas in a reciprocal binding experiment, unlabeled pPK201/CAT DNA competed efficiently with radioactive pSP65/8 DNA (Fig. 2).

The higher affinity of \textit{C. fasciculata} nicking enzyme for the bend-containing DNA molecule is further emphasized by the effect of the ionic strength on the binding interactions during the formation of the enzyme–DNA complex and on the destabilization of a preformed complex. In the presence of 45 mM NaCl, a decrease of 50% was observed in the extent of complex formation with a DNA substrate lacking the bent structure, whereas an equivalent inhibition with the bend-containing DNA substrate required 4-fold higher concentrations of salt. A preformed complex of the nicking enzyme with DNA molecules lacking the bent sequence was more sensitive to salt than the one formed with the bend-containing DNA molecules. Whereas 93% of the labeled pSP65/8 DNA was released from the complex in the presence of 200 mM NaCl, no significant release (<3%) of the complexed pPK201/CAT DNA could be measured (data not shown).

Binding of \textit{C. fasciculata} nicking enzyme to the isolated 219-bp DNA fragment (containing the 211-bp bent sequence) was monitored by both the filter binding assay (Fig. 3) and the
Complexed DNA pPK201/CAT the enzyme formed between outside nicking enzyme protein higher (Cl) (Fig. 1 of (C2-C5) was of the reaction for (C1)). Inset). At required binding specificity of linear DNA molecules containing requirements of the reaction for (Fig. 1. The binding of the enzyme in the absence of the competitor DNA is taken as 100%. (100% values represent 2 fmol of bound pPK201/CAT DNA and 0.75 fmol of pSP65/8 DNA in reaction mixtures containing 16 ng and 80 ng of C. fasciculata nicking enzyme, respectively.)

Mobility-shift electrophoresis analysis (Fig. 3 Inset). The purified bent fragment retained the same capacity displayed by the pPK201/CAT DNA to efficiently bind the nicking enzyme. These data show that the bent sequence is both required (Fig. 1) and sufficient (Fig. 3) for DNA substrate recognition. This does not exclude the possibility that a smaller domain within the bent site could satisfy the reaction.

Studies on the substrate specificity of the nicking reaction (19) have revealed the requirement of the reaction for a bend in the DNA helix. These studies also have shown that the presence of the bent sequence per se could not satisfy the topological requirements of the nicking reaction, as bend-containing linear DNA molecules were not nicked by the enzyme. The data presented here indicate that specific binding of the enzyme to the bent site takes place in a curvilinear DNA molecule. Thus, it seems that the covalent closure of the DNA substrate, which is necessary for nicking, is required to activate the cleavage but not the binding component of this reaction.

Binding specificity was further demonstrated by using the mobility-shift analysis of protein–DNA complexes (Fig. 3 Inset). A series of distinct enzyme–DNA complexes was observed. At low protein concentration (lane 2), mainly one type of complex was formed (band C1). At protein concentration was increased (lane 3) larger complexes (C2–C5) were formed, which appeared to be less stable in the presence of competitor poly(C,I) than the initially formed one (C1) (Fig. 3 Inset, lanes 4–6). This appearance of several higher order complexes suggests the binding of more than one protein molecule to the bent site. Specificity of the complexes formed between the bent DNA sequence and C. fasciculata nicking enzyme was emphasized by the failure of sequences residing outside the bent domain to compete efficiently with the enzyme binding. Exchange of the bound bent fragment was much more efficient (by >1 order of magnitude) when the linear pPK201/CAT DNA was used as substrate than when pSP65/8 DNA was used. The efficient displacement of the complexed bent-DNA fragment by supercoiled topoisomerasers of both the bend-containing (pPK201/CAT) and the bend-lacking (pSP65/8) DNAs also supports specific binding of the enzyme to bent DNA structures (Fig. 3 Inset).

Binding of the Enzyme to the Bent Structure Is Dependent upon the Extent of the Helix Curvature. The nicking reaction catalyzed by C. fasciculata nicking enzyme is tightly dependent upon the degree of curvature of the DNA helix (19). To study the dependence of complex formation upon the extent of bending of the DNA helix we analyzed the effect of the drug distamycin A1 upon the binding of the enzyme to the 219-bp bent-DNA fragment from C. fasciculata kDNA. This drug has been shown to bind to DNA and to abolish the anomalous electrophoretic behavior of DNA fragments containing bent-DNA structures (16, 21). Fig. 4 shows the effect of the drug upon the binding of the enzyme to the 211-bp bent-DNA fragment. An inhibition of 50% was observed in the presence of one distamycin A1 molecule per 15–20 bp of the DNA fragment. Complete inhibition (>95%) of the enzyme binding was observed in the presence of one drug molecule per 4 bp. Under these conditions the bent DNA fragment was apparently straightened, as indicated by both its normal electrophoretic mobility in polyacrylamide gels (not shown) and as visualized by electron microscopy (17). Lack of inhibition of the enzyme binding to supercoiled DNA in the presence of distamycin A1 (Fig. 4 Inset) emphasizes the importance of specific local variation in the helix conforma-
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![Graph](image)

**Fig. 4.** Effect of distamycin A1 on the binding of *C. fasciculata* nicking enzyme to DNA. Eighty nanograms of the 32P-labeled 219-bp bent fragment (●) and 480 ng of 3H-labeled supercoiled pPK201/CAT (Inset) DNAs were used in a filter binding assay as described. Reaction mixtures (5 µl) containing distamycin A1 at the concentrations indicated were incubated for 20 min at 0°C prior to the addition of 80 ng of *C. fasciculata* nicking enzyme. The concentration range of the drug was 0.1–50 µM. Values presented are relative to binding in the absence of the drug. BP, base pair(s).

![Diagram](image)

**Fig. 5.** Protection of kDNA minicircle bent sequence from DNase I digestion by the binding of *C. fasciculata* nicking enzyme. A 263-bp fragment containing the bent sequence from *C. fasciculata* kDNA and a 275-bp fragment from pBR322 were obtained by digestion of pPK201/CAT with EcoRI and HindIII and of pBR322 with BamHI and SalI restriction endonucleases. The fragments were purified by electrophoresis in 1.6% agarose gel and by electroelution; 3'-end-labeling of the bent-DNA fragment at the HindIII site and of the pBR322 fragment at the SalI site was performed with *E. coli* DNA polymerase I large (Klenow) fragment and [α-32P]dCTP. The binding reaction mixtures contained 1 ng of the labeled bent DNA fragment (lanes 2–6) and pBR322 fragment (lanes 7 and 8). Binding conditions, the DNase I digestion reaction, and gel electrophoresis were as described. Reaction mixtures were without (lanes 2 and 7) or with the *C. fasciculata* nicking enzyme (lanes 3–6, 8) in the following amounts: 2 ng (lane 3), 8 ng (lane 4), and 32 ng (lanes 5, 6, and 8). In lane 6 DNase I was omitted. Sequencing was carried out by the Maxam–Gilbert procedure (23). In lane 1, the G ladder is presented as a reference. The arrowheads mark the location of the DNase I hypersensitive sites. The *Stu* I endonuclease site was used as a reference (16).

A structural change in the bent site as the result of its interactions with the bound enzyme. Binding of the enzyme resulted in protection from the nuclease action of a sequence flanking the 3' side of the bend (Fig. 5). We suggest that this protection of sequences flanking the bend is not the result of nonspecific binding of the enzyme but rather is the result of additional binding caused by the initial specific binding within the bent sequence. No such protective effect was observed.
with a nonbent pBR322 DNA in the presence of high levels of *C. fasciculata* nicking enzyme (Fig. 5, lane 8).

**DISCUSSION**

The location of bent-DNA structures in the vicinity of sequences that are involved in the control of DNA replication has been described in several systems (2–4, 8–10). This proximity has raised the possibility of a potential role for bent structures as signals recognized by specific proteins that are involved in the initiation of DNA replication. We have suggested (18) an essential role for *C. fasciculata* nicking enzyme in the process of kDNA minicircle replication.

The observations described in this paper show that the defined local variation in the conformation of the kDNA minicircle helix specifies a unique site for the binding of this enzyme. *C. fasciculata* nicking enzyme binding to the bend-containing DNA molecule (Fig. 1) results in the formation of a specific enzyme–DNA complex that could not be destabilized by an excess of >2 orders of magnitude of uncurved sequences (Fig. 2). Specificity of this complex was further emphasized by its stability under ionic-strength conditions at which the low-affinity complex, formed with uncurved DNA, completely dissociates. The substantial increase observed in the resistance of the preformed complex to high salt concentrations reflects an increase in binding interactions as a result of complex formation. Inasmuch as binding affinity is dependent upon the extent of the helix curvature (Fig. 4), these observations might reflect the further bending of the helix at the binding site because of protein–DNA interactions.

The observations presented suggest that it is the specific variation in the DNA helix geometry, rather than a specific nucleotide sequence at the curved site, that determines the specificity of the binding reaction. This is supported by the observation that foricible DNA bending, induced by the supercoiling of the circular duplex DNA molecule, supports efficient enzyme–DNA complex formation (Fig. 1). Relaxation results in a significant decrease in the capacity of the DNA molecule to bind the enzyme. Furthermore, there is an efficient displacement of the sequence-directed bent fragment from the complex by the supercoiled topoisomers but not by the linear form of the uncurved DNA (Fig. 2). In agreement with these data are our previous observations (19) on the dependence of the nicking reaction on the superhelical density of the DNA substrate.

The decrease in enzyme–DNA complex formation observed in the presence of distamycin A1 (Fig. 4) supports the notion that enzyme recognition is dependent upon the degree of the helix curvature. The gradual inhibition of enzyme binding correlates well with the straightening of bent helix at the enzyme binding site. These data are in agreement with our previous observation of the effect of distamycin A1 upon the nicking reaction.

The protection from nuclease digestion of the majority of the 211-bp bent sequence by the bound enzyme might suggest the concomitant presence of more than a single enzyme molecule in the enzyme–DNA complex. This is further supported by the filter binding assay data and by the generation of higher order enzyme–DNA complexes as observed in the mobility-shift gel analyses (Fig. 3 Inset).

Further studies on the protein structure, the stoichiometry of complex formation and the structural changes induced in the bent structure are required before a clear model, describing the structure of the enzyme–bent-DNA complex, can be drawn. However, our recent studies on the topological requirements of the nicking reaction (19) and the specificity of its cleavage site (24) have shown that the bent sequence per se does not constitute a preferred cleavage site for *C. fasciculata* nicking enzyme, but instead the cleavage site is determined by structural features of the DNA helix of a more general nature.

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