Site-specific interaction of DNA gyrase with DNA
(DNA sequence analysis/site-specific DNA breakage/oxolinic acid/DNase protection methods/Escherichia coli)

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Communicated by Gary Felsenfeld, March 31, 1981

ABSTRACT DNA gyrase, in the presence of the inhibitor oxolinic acid, can induce double-strand DNA breakage at specific sites. The sequences at several sites have been determined. In addition, the structure of complexes formed between DNA gyrase and restriction fragments containing an oxolinic acid-promoted cleavage site has been examined by DNase protection methods. DNA gyrase protects more than 120 base pairs of DNA against pancreatic DNase in a region surrounding the cleavage site. Protection is observed both in the presence and absence of oxolinic acid. Protected DNA flanking the cleavage site contains DNase I-sensitive sites spaced on the average 10 or 11 base pairs apart. This result supports the view that, in the DNA gyrase–DNA complex, the DNA is largely wrapped on the outside of the enzyme.

Recent work has shown that the mode of action of DNA gyrase involves the passage of a duplex DNA segment through a transient double-strand break in DNA. Evidence supporting this conclusion has come from two kinds of experiments. First, DNA gyrase can unknot knotted duplex DNA and can form and resolve DNA catenanes (1–3). Second, both the supercoiling and relaxing activities of DNA gyrase alter the linking number of the substrate DNA in steps of two (2, 4). Several mechanistic models incorporating transient double-strand DNA breakage have been proposed (2, 4, 5). In this paper, we describe experiments designed to examine the topography of complexes between DNA gyrase and DNA.

In the presence of oxolinic acid, DNA gyrase forms a complex with DNA that, on disruption with NaDodSO₄, generates double-strand breaks in the DNA (6, 7). Because of the likely relevance of this reaction to transient DNA breakage by DNA gyrase, we have mapped and determined the sequences of several oxolinic acid cleavage sites. Cleavage generates breaks on complementary strands staggered by 4 base pairs (bp) and results in covalent attachment of protein to each newly formed protruding 5'-phosphate end. These results complement previous studies on DNA gyrase cleavage sites (8–11).

By using nuclease protection methods (12), we have investigated the structure of complexes formed between DNA gyrase and DNA restriction fragments containing potential oxolinic acid-cleavage sites. Earlier studies on the binding of DNA gyrase to DNA established that the enzyme protects ~140 bp of DNA against micrococcal nuclease digestion (13, 14). Moreover, in the DNA gyrase–DNA complex, the DNA appeared to be wrapped on the outside of the enzyme (15). We find that, both in the presence and in the absence of oxolinic acid, DNA gyrase protects >120 bp of DNA against pancreatic DNase. A region of ~40 bp is most strongly protected. DNA flanking this region is less well protected and exhibits DNase I-sensitive sites spaced 10 or 11 bp apart in a pattern reminiscent of that obtained by DNase I digestion of nucleosomal DNA (16). These observations indicate that the DNA flanking the cleavage-site region is wrapped on the outside of DNA gyrase.

Somewhat similar results have recently been obtained from DNase protection experiments involving complexes formed with M. luteus DNA gyrase (11).

MATERIALS AND METHODS

Materials. [γ-³²P]ATP (3000 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was from New England Nuclear. ATP, [β, γ-imido] ATP, T₄ polynucleotide kinase, and terminal nucleotidyltransferase were from P-L Biochemicals. Calf intestine alkaline phosphatase was from Boehringer Mannheim. Escherichia coli DNA gyrase A and B proteins were purified to >99% homogeneity as described (2). Pancreatic DNase (DNase I) from Worthington was stored at 4°C in 2.5 mM HCl and diluted before use into 20 mM Tris·HCl, pH 7.5; 10 mM MgCl₂/0.5 mM CaCl₂/0.1 mM dithiothreitol. Restriction enzymes and E. coli exonuclease III (5.6 × 10⁴ units per ml) were from New England BioLabs. The supercoiled DNA of plasmids pBR322 and pVH51 was prepared by standard methods (2).

DNA Sequence Analysis. DNA restriction fragments were treated with alkaline phosphatase and then labeled at their 5' ends by using [γ-³²P]ATP and T₄ polynucleotide kinase. Restriction fragments specifically labeled at one 5' end were obtained by recutting with a second restriction enzyme. The desired fragments were isolated by acrylamide gel electrophoresis, and the sequence analyses were carried out by the method of Maxam and Gilbert (17).

The structures and sequences at sites of oxolinic acid-promoted DNA cleavage by DNA gyrase were determined as follows. Restriction fragments (100–250 bp long), known from rough mapping to contain potential DNA gyrase cleavage sites, were labeled with ³²P at both 5' ends and subjected to oxolinic acid-promoted gyrase cleavage followed by proteinase K treatment (for conditions, see ref. 6). The two resulting double-stranded fragments were separated and analyzed in each of two ways. (i) The lengths of the ³²P-labeled strands were determined by electrophoresis in a denaturing gel alongside the Maxam–Gilbert sequencing products obtained for each labeled strand of the original uncleaved restriction fragment. [Gyrase-cleaved fragments, having 3'-OH termini (see Results) run 0.5 nucleotide positions slower than the sequencing product of the same nucleotide length (18). This follows because the Maxam–Gilbert method generates DNA fragments that terminate in a 3' phosphate.] One must also note that the sequence analyses identify DNA fragments from which the nucleotide of interest has been removed. [The same considerations apply in locating the sequence position of 3'-OH termini generated by DNase I (12) or exonuclease III (see below).] (ii) The separated gyrase...

Abbreviation: bp, base pair(s).

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cleaved fragments were themselves subjected to the Maxam-Gilbert reactions and to denaturing gel electrophoresis-autoradiography to establish which base was at the newly formed 3'-OH end.

**DNase Protection.** The method of Galas and Schmitz was used (12). In this technique, a DNA restriction fragment labeled with \(^{32}P\) at one 5' end is lightly digested with pancreatic DNase in the presence or absence of the DNA binding protein under study. The resulting set of labeled DNA fragments is separated according to length. DNA sequences protected from nuclease attack are revealed as missing bands in the ladder of DNA fragments resolved by the gel.

For our experiments, the assay buffer contained (in 48 \(\mu\)l) 46 mM Tris-HCl, pH 7.5/6.9 mM MgCl\(_2/75\) mM KCI/0.38 mM CaCl\(_2/6\) mM diethiothreitol/0.19 mM Na\(_2\)EDTA/4.1% (wt/vol) glycerol, bovine serum albumin at 35 \(\mu\)g/ml, and = 0.05 \(\mu\)g of \(^{32}P\)DNA. Where included, an 8- to 10-fold molar excess of DNA gyrase over DNA was used (i.e., 500 units of gyrase B protein and an excess of gyrase A protein). Oxolinic acid when added was present at 73 \(\mu\)g/ml. Samples were incubated at 25°C for 75 min. Pancreatic DNase (3 \(\mu\)l at 5 \(\mu\)g/ml) was added, and the solutions were incubated at 25°C for 1 min. Reaction was terminated by the addition of 50 \(\mu\)l of stop solution [1.2 M ammonium acetate/0.1 M Na\(_2\)EDTA containing sonicated calf thymus DNA at 80 \(\mu\)g/ml]. The samples were heated to 75°C for 10 min, and then the DNA was ethanol precipitated and subjected to electrophoresis in a 10% polycrylamide/urea gel.

Oxolinic acid-induced DNA cleavage by DNA gyrase under the protection conditions was done by adding 5% NaDodSO\(_4\) (4 \(\mu\)l) and proteinase K (8 \(\mu\)l at 0.2 mg/ml) instead of DNase I. Samples were incubated at 37°C for 35 min, and then the DNA was isolated as described above.

Conditions for the protection of DNA against exonuclease III were the same as those used with DNase I (see above). Fifty-six units of exonuclease III was added to each sample, and incubation was at 25°C. Reaction was terminated at 1 min or 3 min by the addition of DNase I stop solution. DNA isolation and denaturing polyacrylamide gel electrophoresis were as described above.

**RESULTS**

**Site-Specific DNA Cleavage by DNA Gyrase.** As a first step in determining the structures and sequences of sites of oxolinic acid-mediated cleavage of DNA by DNA gyrase, we ascertained the nature of the DNA termini produced in the reaction. We found that labeling of ends was possible by using terminal nucleotidyltransferase but not by using polynucleotide kinase (19). This suggested the presence of free 3'-OH ends but blocked 5'-phosphate termini and parallels previous findings (9). Recently, it has been demonstrated that a protomer of the gyrase A subunit becomes covalently linked to each 5'-phosphate terminus generated by DNA gyrase cleavage (20, 21).

The Maxam-Gilbert sequence analysis method (17) was used to determine the structures and sequences of several preferred gyrase cleavage sites. We chose to examine sites in plasmid pBR322, whose entire sequence is known (22), and in the ColEl derivative pVH51, for which a large extent of the sequence has been determined (ref. 18; unpublished results). The sites studied include one in the tetracycline-resistance region of pBR322, one near the origin of replication of pVH51, and three in the Alu I D fragment of pVH51. Two of the three sites in the Alu I D fragment were spaced only three nucleotides from each other but were readily distinguished by their different cleavage efficiencies. The sequences around the cleavage sites are shown in Fig. 1. For all the sites, complementary DNA strands were broken with a 4-bp stagger, yielding protruding 5' ends.

**Fig. 1.** Sites of DNA cleavage induced by DNA gyrase in the presence of oxolinic acid. The sites are contained within the restriction fragments shown. The actual restriction fragments used in the sequence determinations and the site locations (i.e., the nucleotide position half-way between the two staggered breaks) were as follows. An \(Hpa\) II fragment of 206 bp is contained within the pVH51-Hae II C region. The cleavage site is centered at position (−77) relative to the origin of replication (18). The sequences of pVH51 and PNT1 are identical in this segment (unpublished results). Two fragments from the pVH51-Alu I D region were examined: a 105-bp \(Hpa\) II-Tac I fragment, designated \(Alu\) I D1 (1), which extends beyond the left end (in the restriction map of Tomizawa et al. (18) of \(Alu\) I D by 50 bp, and a 91-bp Tac I fragment (\(Alu\) I D2), located immediately to the right. The cleavage site in the first fragment is at +23 from the left end of the \(Alu\) I D fragment; the two sites in the second fragment are at +86 and +89, with stronger cleavage at +86. The sequence of this region has been determined (unpublished results). In pBR322, the 90-bp \(Hpa\) II fragment 15 contains a cleavage site at position 991 in the plasmid sequence (22).

From the sequence data (Fig. 1), it is seen that, for three sites, cleavage on one strand occurs within the dinucleotide GpG, but this feature is not found universally. Again, although several sites show adjacent clusters of guanines, other sites do not (see also, ref. 9). Inspection of several sites in pBR322 and pVH51 exhibiting less efficient cleavage than observed for sites shown in Fig. 1 (see, e.g., Fig. 3), also failed to reveal an obvious common sequence element. In summary, we find no readily apparent sequence rule determining sites of DNA cleavage by DNA gyrase.

**Binding of DNA Gyrase at DNA Cleavage Sites.** The binding specificity of DNA gyrase to restriction fragments containing potential cleavage sites was investigated by DNase I protection methods (12). Two different restriction fragments were used: a 203-bp \(Aca\) II/\(Alu\) I fragment from plasmid pBR322 DNA [spanning nucleotide positions 886–1089 (22)], containing the site in pBR322 centered 98 bp from the \(Alu\) I end (Fig. 1), and a 200-bp \(Hae\) II/\(Hae\) III fragment from pVH51, in which the cleavage-site sequence is centered 92 bp from the \(Hae\) III end. This \(Hae\) II/\(Hae\) III 8 fragment (18) contained the cleavage-site sequence located near the origin of replication of pVH51 (see legend to Fig. 1). The restriction fragments were labeled specifically at one or the other 5' end and each was subjected to protection analysis. In the presence of oxolinic acid, the complex of DNA gyrase competent to give DNA cleavage is formed slowly (6). To maximize the formation of this complex and to facilitate useful comparison of DNA protection afforded by DNA gyrase in the presence and absence of oxolinic acid, the enzyme and DNA were subjected to an initial incubation prior to the addition of the nucleases. The protection patterns are shown in Fig. 2. For both cleavage sites, DNA gyrase protected both DNA strands in an extensive region encompassing the cleavage site sequence. Protection of a region of DNA around each site occurred whether oxolinic acid was present in the incubation mixture (d lanes) or absent (e lanes). No protection was
observed when either the gyrase A or B subunit alone was used (results not shown).

Structure of Complexes Formed Between DNA and DNA Gyrase. The protection patterns shown in Fig. 2 contain structural information about the enzyme-DNA complex. Although the details of DNA protection were somewhat different for the two DNA binding sites studied (see below), in each case a stretch of at least 120 bp of DNA was protected. A region of \(-40 \) bp surrounding the cleavage-site sequence is most strongly protected. The DNA on either side of this region is less strongly protected, and there are bands corresponding to sites of enhanced sensitivity to DNase I. These features are made apparent in Fig. 3, which summarizes the data in Fig. 2, in particular, the locations of DNase I-sensitive sites. These are spaced 10 or 11 bp apart, and sites on complementary strands are on the average staggered by 2 bp.

The characteristics of DNA protection conferred by DNA gyrase are somewhat different for the two binding sites studied (Fig. 3). For the pBR322 site, protection is roughly symmetrical, extending at least 60 bp on either side of the cleavage site. For the pVH51 site, however, protection extends at least 90 bp on one side of the site while stopping 35 bp on the other side. Asymmetric binding of DNA gyrase at the pVH51 site is also observed in experiments involving E. coli exonuclease III. This enzyme processively degrades double-strand DNA in the 3' \( \rightarrow \) 5' direction starting at the 3' ends. The Hae II/Hae III \( \delta \) fragment of pVH51 labeled at one or the other 5'-phosphate end was treated with exonuclease III in the presence and absence of DNA gyrase. In the presence of bound DNA gyrase, digestion by the exonuclease was arrested at locations 15 and 18 bp on one side of the cleavage site and at 90 bp on the opposite side. The dissimilarity in protection by gyrase at the two sites is also highlighted by the patterns of DNase I-sensitive sites. It is evident that the number and location of DNase I-sensitive sites is rather different for the two DNA fragments.

Protection by DNA gyrase at a particular site was different in the presence and absence of oxolinic acid (Figs. 2 and 3). Incursion of the drug resulted in protection of a more extensive region of DNA (see, e.g., Fig. 3B). Moreover, DNA sequences immediately adjacent to the cleavage-site sequence became less accessible to the nuclease. The distribution of DNase I-sensitive sites is also different in the presence and absence of the drug (Fig. 3). The strong band at the cleavage site seen only when oxolinic acid was included (Fig. 2, d and e lanes) deserves comment. Cleavage at the same position was observed when the DNase I digestion was omitted (data not shown). Therefore, this band presumably arises by adventitious DNA cleavage induced by DNA gyrase during the incubation and isolation of the DNA.

Nucleotide Effects on DNA Cleavage and DNA Protection by DNA Gyrase. The effects of ATP and the nonhydrolyzable analogue [\( \beta, \gamma \)-imido]ATP on oxolinic acid cleavage were investigated for the site in the Aco II/Alu I fragment of pBR322 and that in the pVH51 \( \delta \) fragment. The inclusion of ATP had little effect on the efficiency or position of cleavage for the pBR322 site, although weaker double-strand cleavage was promoted at a secondary site (Fig. 4, lanes i and j). Results with [\( \beta, \gamma \)-imido]ATP were similar (data not shown). However, for the pVH51 \( \delta \) fragment, the addition of ATP or [\( \beta, \gamma \)-imido]ATP shifted the major cleavage site by 1 bp, altering the intensity of cleavage from \( \approx 35\% \) at site 1 to \( \approx 60\% \) at site 2 (Fig. 3B). Again, the addition of nucleotide induced weaker cleavage at secondary sites. The diverse effects of ATP on cleavage have previously been reported for several sites in ColE1, \( \phi X174 \), and simian virus 40 DNAs (10).

Nucleotide effects on the protection of DNA were studied for the gyrase binding site in the Aco II/Alu I fragment from pBR322 (Fig. 3A). DNA gyrase, in the presence or absence of oxolinic acid, gave essentially the same DNase I protection pattern irrespective of whether ATP or [\( \beta, \gamma \)-imido]ATP was included (Fig. 4).

DISCUSSION

An important question concerns the nature of the recognition element that directs DNA gyrase to bind to a particular DNA sequence—e.g., at DNA cleavage sites. From Fig. 1, it is apparent that there is no unique nucleotide sequence at or immediately adjacent to the cleavage site that is common to all sites studied here. Thus, DNA cleavage by DNA gyrase differs from that produced by type II DNA restriction enzymes (23), whose specificity is generally determined by sequences at or very near the restriction site. In previous work, it has been suggested that one of the two single-strand breaks generated by cleavage occurs predominantly in the sequence TpG (9). This generalization is not supported by our results for strong sites in the plas-
mids pBR322 and pVH51, where only one out of five examples shows this feature (Fig. 1). Scrutiny of DNA sequences extending on either side of the cleavage sites also failed to reveal any obvious sequence rule. Further analysis of DNA cleavage by DNA gyrase will be necessary to establish the determinant governing the binding of the enzyme to DNA.

The mechanism of DNA gyrase involves the passage of a duplex DNA segment through a transient double-strand break in DNA (2, 4). The enzyme is thought to be part of a "gate" through which the translocated DNA strand is passed. The intimate details of this process are at present poorly understood. Valuable mechanistic information can be provided by DNase I protection experiments as these reveal details of the time average topography of complexes formed between DNA gyrase and DNA. For the two binding sites examined here, DNA gyrase protects more than 120 bp of DNA against digestion by DNase I (Figs. 2 and 3). The following observations suggest that this protection arises from the binding of only one DNA gyrase molecule to each restriction fragment. First, it has been shown that a single DNA gyrase A2B2 tetramer protects 140 bp of DNA against digestion by micrococcal nuclease, a length similar to that protected against DNase I (14). Second, when either of the DNA–DNA gyrase complexes studied here was subjected to oxolinic acid-induced DNA cleavage, the DNA was cleaved asymmetrically rather than symmetrically. Non-specific binding of the DNA gyrase to DNA is not observed for the Hae II/Hae III δ fragment (Figs. 2 C and D and 3) in a 120 bp DNase I digestion of the Ato II/Alu I DNA restriction fragment of pBR322 containing the 203-bp Ato II/Alu I fragment shown in Fig. 3A. In the 312-bp fragment, the cleavage site is asymmetrically located 98 bp from the Alu I end. Nevertheless, the DNA protected by DNA gyrase was localized to the region immediately surrounding the cleavage site (data not shown). Thus, these findings suggest that the protection pattern observed are those of specific 1:1 complexes formed between DNA gyrase and the DNA binding site.

Within the DNA segment protected by DNA gyrase, a region of 40 bp is most strongly protected. This region contains the sequence at which oxolinic acid-mediated gyrase cleavage can be induced. If the view is adopted that sites of oxolinic acid-promoted gyrase cleavage are sites of transient double-strand DNA breakage during catalysis, then the most strongly protected region of DNA presumably forms part of the gate through which the other duplex DNA segment is passed. The relative inaccessibility of this portion of the DNA in the enzyme complex may arise because the DNA is recessed into a site on the enzyme at which transient DNA strand scission can take place. This binding locus quite likely involves the two gyrase A subunits; a protomer of the gyrase A subunit becomes covalently linked to each 5'-phosphate end of the double-strand break generated in the gyrase cleavage reaction (20, 21).

The protected DNA flanking the cleavage sequence appears to be wrapped on the outside of the enzyme in the DNA–DNA gyrase complex. This follows from the observation of pancreatic DNase digestion at sites separated by 10 or 11 bp. A similar pattern of DNase I-sensitive sites has been observed for nucleosomal DNA (16) and for DNA adsorbed to calcium phosphate precipitates (13). This feature is generally thought to be diagnostic of DNA adsorbed to a surface. The interpretation is consistent with the finding that DNA gyrase cleavage, in the absence of ATP, results in a positive superhelical wrapping of DNA on the enzyme (15). The coiling of DNA on the enzyme is thought to be the controlling factor that ensures that a negatively supercoiled product is formed in the supercoiling reaction (2, 5).
DNA strand passage catalyzed by DNA gyrase most likely involves conformational transitions within the enzyme-DNA complex. Detection of these changes is essential to understanding the mechanism of the enzyme. If a conformational transition alters the accessibility of the DNA to DNase I, then in principle it can be detected by a change in the DNase I protection pattern. It is interesting that the binding of oxolinic acid to DNA gyrase induces a marked change in both the degree and extent of protection conferred by the enzyme and results in a redistribution of DNase I-sensitive sites (Fig. 3 A and B). From the point of view of DNA supercoiling by DNA gyrase, the effects of ATP and the nonhydrolyzable analogue, [β,γ-imido]ATP, are especially relevant. The binding of either of these nucleotides to a DNA gyrase complex formed with the Aco I/Alu I DNA fragment of pBR322 does not result in any substantial change in the DNase footprinting pattern (Fig. 4). Protection of bulk PM2 DNA by M. luteus DNA gyrase was also unaltered by the inclusion of ATP (13). These results support the interpretation that the DNA is largely on the outside of the enzyme-DNA complex and not greatly affected by conformational changes induced by nucleotide binding.

The experiments presented here reveal several structural aspects of the DNA-DNA gyrase complex and how this is affected by the binding of various ligands. The results are compatible with recently proposed models for DNA gyrase (2, 4, 5). Further investigation of the interaction of DNA gyrase with DNA during catalysis will be necessary to delineate the topological pathway of the gyrase reaction and to determine whether the oxolinic acid cleavage sites are catalytically productive DNA binding sites for the enzyme.

Note Added in Proof. Another study describing protection of DNA by E. coli DNA gyrase has recently appeared (24).

We thank J. Tomizawa for help with early experiments and for valuable criticism of the manuscript. We are also grateful to J. C. Wang for communicating unpublished results. L. M. F. was supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Fund.