Escherichia coli DNA Topoisomerase III Is a Site-specific DNA Binding Protein That Binds Asymmetrically to Its Cleavage Site*

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The binding of DNA topoisomerase III (Topo III) to a single-stranded DNA substrate containing a strong cleavage site has been examined. The minimal substrate requirement for Topo III-catalyzed cleavage has been determined to consist of 7 bases: 6 bases 5’ to the cleavage site and only 1 base 3’ to the site. Nuclease P1 protection experiments indicate that the enzyme also binds to its substrate asymmetrically, protecting ~12 bases 5’ to the cleavage site and only 2 bases 3’ to the cleavage site. A catalytically inactive mutant of Topo III shows the same protection pattern as the active polypeptide, indicating that Topo III is a site-specific binding protein as well as a topoisomerase. Consistent with this view, an oligonucleotide containing a cleavage site is a more effective inhibitor and is bound more efficiently by Topo III than an oligonucleotide without a cleavage site.

Escherichia coli has been shown to possess four DNA topoisomerase activities. Two type I enzymes, DNA topoisomerase I (Topo I)‡ (1) and DNA topoisomerase III (2–4), have been purified and characterized. In addition, two type II enzymes, DNA topoisomerase II (DNA gyrase) (5) and DNA topoisomerase IV (Topo IV) (6), have also been purified and characterized. The role of these enzymes in DNA metabolism has been defined by studies, both genetic and in vitro. For example, DNA gyrase and Topo I have been shown to be involved in the maintenance of superhelical density of the E. coli chromosome (7). In addition, there is also evidence that DNA gyrase is involved in the terminal stages of DNA replication (8). Topo IV (9, 10) and Topo III (4, 11) have been shown to be potent decatenases in vitro, and it has been proposed that these enzymes are involved in the separation of nascent daughter chromosomes during the terminal stages of DNA replication. In addition, it has also been shown that DNA gyrase, Topo IV, and Topo III can support DNA polymerase chain elongation during the replication of plasmid DNA in vitro (12). The apparent redundant functions of these enzymes emphasizes the importance of these enzymes in DNA metabolism.

A model termed “sign inversion” (13) has been proposed that describes a unified mechanism for both type I and type II bacterial topoisomerases. In this model, topoisomerase binds to its substrate (either single-stranded DNA for a type I enzyme or double-stranded DNA for a type II enzyme), catalyzes a strand break (either single (type I) or double (type II)) and strand passage event, and then reseals the break. The “sign inversion” model was originally proposed to explain the mechanism of bacterial DNA gyrase (a type II DNA topoisomerase); however, it has been extended to explain the mechanism of E. coli DNA topoisomerase I, a type I enzyme. It has been proposed that in the case of Topo I, the strand passage event can be thought to involve the sign of the two strands of the helix (14), whereas, the strand passage event catalyzed by E. coli DNA gyrase, a type II enzyme, inverts the sign of a wrap of the entire helix (13, 15).

Although there is evidence supporting this model as a whole, there is very little information regarding the properties of the individual steps of topoisomerase catalysis; therefore, our laboratory has set out to perform a detailed characterization of the mechanism of DNA topoisomerase III. In this report, the binding of Topo III to its substrate is examined, and a model for Topo III-catalyzed cleavage of DNA is proposed.

MATERIALS AND METHODS

DNA and Nucleotides—X-174 RFI DNA was purchased from Life Technologies, Inc. DNA oligonucleotides were prepared by the University of Maryland Biopolymer Laboratory. Radiolabeled nucleoside triphosphate was purchased from Amersham Corp.

Enzymes and Reagents—Acrylamide and agarose were from Life Technologies, Inc. Bacteriophage T4 polynucleotide kinase was from New England Biolabs Inc. E. coli Topo III was purified as previously described (16). Nuclease P1 was purchased from Boehringer Mannheim.

Radiolabeling of Oligonucleotides—Oligonucleotides were 5’-end labeled using bacteriophage T4 polynucleotide kinase (Life Sciences) and [γ-32P]ATP as per the manufacturer’s recommendations. The labeled oligonucleotides were fractionated through a polyacylamide gel. The region containing the labeled oligonucleotide was excised, and the DNA was isolated by direct elution of the fragment into 10 mM Tris-HCl (pH 7.5 at 22°C), 1 mM EDTA. The radiolabeled oligonucleotides were diluted to a specific activity of 2000 cpm/μmol by the addition of excess unlabeled oligonucleotide.

Superhelical DNA Relaxation Assays—Superhelical DNA relaxation reaction mixtures (25 μl) contained 40 μM Heps-KOH buffer (pH 8.0 at 22°C), 1 mM magnesium acetate (pH 7.0), 0.1 mM bovine serum albumin, 40% (v/v) glycerol, and 200 ng of X-174 form I DNA. Reactions were incubated at 52°C for 10 min, and the reaction products were separated and visualized as previously described (4).

Oligonucleotide Gel Mobility Shift Assays—Reaction mixtures (10 μl) contained 40 μM Heps-KOH buffer (pH 8.0 at 22°C), 1 mM magnesium acetate (pH 7.0), 12% glycerol, and 5 μM of radiolabeled oligonucleotide. The reactions were incubated for 5 min at 37°C, and the products were separated through a 10% polyacrylamide gel (30:0.8) using 0.5 X TBE as the running buffer. The gels were photographed at 15 mA for 1.5 h, dried, and autoradiographed.

The 22-base oligonucleotide used in the assay was 5’-CAGAATCAGTGAGCCGCAACT-3’ where ↓ indicates the site of Topo III cleavage (16). The 22-base oligonucleotide, containing a Topo III cleavage site, was the following subsequence of the 45-base oligonucleotide: GAATTGAGCCGCAACT ↓ TCGGGAT. The 22-base oligonucleotide, without a Topo III cleavage site, was the following subsequence of the 45-base oligonucleotide: CAGAATCAGTGAGCCGCAACT-3’. Several exposures of the autoradiographs were...
quantified using a LKB-Pharmacia Ultrascan laser densitometer (in the case where a particular lane(s) were hard to visualize). In addition, bands from the gels, representing the indicated topoisomerase-oligonucleotide complexes, were also excised, and the amount of radiolabeled oligonucleotide was determined using a Bednacan LSM 5801 liquid scintillation counter.

Topoisomerase-induced DNA Cleavage Assay—Reaction mixtures (5 μl) containing 40 mM Hepes-KOH buffer (pH 8.0 at 22 °C), 0.1 μg/ml bovine serum albumin, 1 μM magnesium acetate (pH 7.0), and 30 fmol of radiolabeled oligonucleotide. Topo III was incubated for 3 min at 37 °C, and the reaction was stopped by the addition of SDS to 2%. The reactions were adjusted to 45% formamide, 10 mM EDTA, 0.025% bromphenol blue, 0.025% xylene cyanol and heat-denatured for 5 min at 90 °C. The reaction products were separated by electrophoresis through a polyacrylamide gel (19:1) containing 50% (w/v) urea. The gels were then dried and autodigraphed.

Nuclease P1 Protection Assay—Reaction mixtures (5 μl) containing 40 mM Hepes-KOH buffer (pH 8.0 at 22 °C), 0.1 μg/ml bovine serum albumin, 1 μM magnesium acetate (pH 7.0), and 200 fmol of radiolabeled oligonucleotide. Topo III was incubated for 3 min at 37 °C followed by addition of 3 × 10^{-2} units of P1 nuclease. The reactions were incubated an additional 10 min at 37 °C and terminated by the addition of EDTA to 10 mM. The reactions were adjusted to 45% formamide, 0.025% bromphenol blue, 0.025% xylene cyanol and heat denatured for 5 min at 90 °C. The reaction products were separated by electrophoresis through a polyacrylamide gel (19:1) containing 50% (w/v) urea. The gels were then dried and subjected to autoradiography.

**RESULTS**

Determination of the Minimum Substrate Requirement for Topo III-catalyzed Cleavage—The minimal sequence requirement of Topo III was determined by incubating the enzyme with oligonucleotides of various lengths and assessing the ability of the enzyme to cleave the substrate at the appropriate site. The oligonucleotides were designed to vary the length of DNA sequence both 5' and 3' of a strong Topo III cleavage site (16). Substrates as short as 7 bases were capable of being cleaved by Topo III; however, the length of sequence 5' to the cleavage site was critical in determining whether the enzyme could productively interact with the substrate.

Oligonucleotides, containing 5, 6, or 7 bases 5' of the cleavage site and 1 or 2 bases 3' to the cleavage site, were incubated with Topo III, and the cleavage products were resolved by electrophoresis through a polyacrylamide gel in the presence of urea (Fig. 1). When Topo III was incubated with a 9-base oligonucleotide containing 7 bases 5' of the major cleavage site, a major 7-base (corresponding to the previously identified cleavage site (16)) and a minor 6-base cleavage product were observed (lane 1). Incubation of Topo III with an 8-base oligonucleotide, containing 7 bases 5' and only one base 3' of the same major cleavage site, resulted in the production of identical cleavage products, although the 7-base cleavage product was diminished by 4.3-fold (lane 3). This data clearly establishes the minimal 3'-sequence requirement for Topo III to be only 1 base.

To establish the 5'-minimal sequence requirement for Topo III-catalyzed cleavage, the enzyme was incubated with an 8-base oligonucleotide containing 6 bases 5' of the major cleavage site. This reaction resulted in the production of the 6-base cleavage product; however, the expected 5-base minor cleavage product (observed in the reaction containing the oligonucleotide with 7 bases 5' of the cleavage site) was absent (compare lane 3 with lane 5). This result suggested that the minimal 5'-sequence requirement for cleavage was 6 bases. This conclusion was confirmed by incubating Topo III with an 7-base oligonucleotide containing the predicted minimal site of 6 bases 5' of the cleavage site (lane 7) and with a 7-base oligonucleotide containing only 5 bases 5' of the cleavage site (lane 9). A 6-base cleavage product was observed in the case of the former (although the cleavage was reduced 9.3-fold relative to the substrate containing 6 bases 5' and 2 bases 3' of the cleavage site (lane 5) and reduced 4.8-fold relative to the substrate containing 7 bases 5' and 1 base 3' of the cleavage site (lane 3)), but no 5-base cleavage product was observed for the latter oligonucleotide. A summary of the cleavage experiments performed with multiple oligonucleotides is presented in Fig. 2.

To further establish whether the minimal substrate was an actual characteristic of the enzyme rather than a peculiarity of the substrate, the minimal substrate requirement for Topo III-catalyzed cleavage was determined for two other independent cleavage sites. The minimal substrate requirement was identical using the different oligonucleotide substrates (data not shown).

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Containing a Cleavage Site

Topoisomerase III Is Preferentially Inhibited by an Oligonucleotide

The reaction products were resolved through a 25% polyacrylamide gel containing 50% (w/v) urea. The reactions contained no Topo III (lane 2) or 0.1 pmol (lane 1), 0.25 pmol (lane 3), 0.5 pmol (lane 4), 1 pmol (lane 5), or 2 pmol (lane 6) of Topo III. The topoisomerase III-induced cleavage product was obtained by incubation in the absence of P1 nuclease (lane 1). The solid bar indicates the position of the Topo III-induced footprint.

To demonstrate an attempt was made to footprint Topo III using the 45-base oligonucleotide shown in Fig. 2. A titration of Topo III revealed a distinct protection pattern that surrounded the cleavage site. Consistent with the minimal substrate experiments, the protected region was asymmetric relative to the cleavage site, encompassing 12 bases 5’ to the cleavage site to 2 bases 3’ to the cleavage site.

To demonstrate an attempt was made to footprint Topo III using the 45-base oligonucleotide shown in Fig. 2 as a substrate (Fig. 3). A titration of Topo III revealed a distinct protection pattern that surrounded the cleavage site. Consistent with the minimal substrate experiments, the protected region was asymmetric relative to the cleavage site, encompassing 12 bases 5’ to the cleavage site to 2 bases 3’ to the cleavage site.

Topoisomerase III Is A Site-specific Binding Protein—Although the P1 nuclease protection experiment revealed that Topo III generated a distinct footprint, it was unclear whether the protection pattern was generated by site-specific binding of the enzyme or dependent solely upon catalysis. To address this question, the nuclease P1 protection pattern of a catalytically inactive mutant of Topo III, Topo III-phe328 (which possesses a phenylalanine substitution for tyrosine 328, the putative amino acid involved in strand breakage [16, 17]), was compared to the protection pattern of the active enzyme (Fig. 4). Titration of the catalytically inactive mutant (lanes 8–11) revealed a similar footprint to the active polypeptide (lanes 2–5), indicating that Topo III is a cleavage site-specific binding protein. However, since the mutant polypeptide is inactive, the footprint of the mutant lacks the Topo III-induced cleavage product.

The Relaxation of Negatively Supercoiled DNA by Topoisomerase III Is Preferentially Inhibited by an Oligonucleotide Containing a Cleavage Site—The ability to detect a Topo III-dependent protection pattern surrounding a strong enzyme cleavage site suggested that interaction between the enzyme and its cleavage site served to stabilize the enzyme on its substrate. If this were the case, an oligonucleotide containing a strong Topo III cleavage site should be an effective inhibitor of catalysis. This question was addressed by comparing the ability of a 22-base oligonucleotide containing a strong Topo III cleavage site with a 22-base oligonucleotide without a cleavage site to serve as an inhibitor of Topo III-catalyzed relaxation of negatively supercoiled DNA (Fig. 5).

The 22-base oligonucleotides consisted of a subset of sequences derived from the 45-base oligonucleotide (shown in Fig. 2), which either contained (bases 9–30) or did not contain (bases 1–22) the cleavage site. The presence or absence of the cleavage site was confirmed by incubating the 22-base oligonucleotides with increasing levels of Topo III (Fig. 5, top panel). It is clear that only one of the oligonucleotides contains a strong cleavage site.

Each oligonucleotide was then assessed for its ability to serve
A 13-base oligonucleotide (a subsequence of the 45-base oligonucleotide) that did not show any evidence of interaction with Topo III (by footprint analysis) was also used as a substrate in a gel mobility shift assay. In accord with previous results, Topo III was capable of interacting with this substrate; however, the amount of stable topoisomerase-oligonucleotide complex was much lower than that formed with either the 22-base oligonucleotide or the 45-base oligonucleotide (data not shown). This length effect may be indicative of the mechanism by which Topo III locates its binding/cleavage site during catalysis.

**DISCUSSION**

The minimum substrate requirement for DNA topoisomerase III-catalyzed cleavage has been examined and found to consist of only 7 bases, asymmetrically encompassing a known cleavage site of the enzyme. Oligonucleotides containing as little as 1 base 3' of the cleavage site were cleaved by Topo III, establishing the minimal 3' requirement for the enzyme. An oligonucleotide containing 6 bases 5' of the cleavage site and 2 bases 3' of the cleavage site was also cleaved by Topo III; however, an oligonucleotide containing 5 bases 5' and 2 bases 3' of the site was not cleaved by the enzyme, establishing a 5'-sequence requirement of 6 bases. When a Topo III-induced cleavage assay was performed using the minimal substrate, cleavage was observed; however, it was reduced ~9-fold when compared to the efficiency of cleavage of an oligonucleotide containing one more base 3' of the minimal sequence and reduced ~5-fold when compared to an oligonucleotide containing one more base 5' of the minimal sequence.

The minimal sequence requirement for E. coli Topo I has previously been determined to be 7 or 8 bases (18, 19), but the substrates used in these studies were homopolymers and not an actual cleavage site of the enzyme. In any event, it appears likely that the minimal substrate requirement for the two enzymes is similar. It is unclear, however, if Topo I will show the same asymmetric sequence requirement for cleavage as Topo III.

Nuclease P1 footprinting experiments indicate that the enzyme also binds asymmetrically to its substrate relative to its cleavage site. Topo III was shown to protect a region of the substrate from 12 bases 5' to the cleavage site to 2 bases 3' of the site. An identical footprint was observed using a polypeptide incapable of cleaving the substrate, illustrating that catalysis is not a requirement for cleavage site recognition. Topo III, therefore, is a cleavage site-specific binding protein as well as topoisomerase.

The highly asymmetric sequence requirement of Topo III is consistent with the known catalytic mechanism of prokaryotic topoisomerases. These enzymes cleave DNA by making a nucleophilic attack 5' to the phosphate in the phosphodiester backbone. In this mechanism, the topoisomerase becomes covalently linked to the DNA 3' to the cleavage site via an enzyme-bridged phosphotyrosine linkage (reviewed in Ref. 7). Since the enzyme is covalently bound to the 3'-DNA fragment, it is not surprising that there may be only a minimal sequence requirement in this region. In fact, these experiments suggest that the enzyme may only require a single nucleotide to serve as a receptor for the nucleophilic attack. To prevent free rota-
The proposed mechanism of Topo III-mediated cleavage of DNA is presented. The reaction has been separated into three distinct steps (A-C). Briefly, Topo III (represented by the oval) binds randomly to single-stranded DNA. The enzyme then diffuses or tracks (arrows) along its substrate until it encounters a binding/cleavage site (diagonally filled rectangle). The enzyme forms a stable complex with its cleavage site through interactions within the active site of the enzyme and then catalyzes a transient break in the single-stranded DNA (the triangle represents the active site tyrosine residue). A detailed explanation of the model is given in the text.

The binding properties of Topo III are also reminiscent of a site-specific binding protein. First, an oligonucleotide containing a Topo III cleavage site is more efficiently bound by the enzyme than an oligonucleotide without a site. Second, an oligonucleotide containing a cleavage site is a better inhibitor of Topo III-catalyzed relaxation of negatively supercoiled DNA than an oligonucleotide lacking a cleavage site. Although gel mobility shift experiments indicate that substrates containing a cleavage site are bound more efficiently by the enzyme, Topo III is also capable of interacting with substrates that do not contain a cleavage site. These properties suggest a model for Topo III-catalyzed cleavage of DNA (Fig. 7).

In the first stage of interaction (which we term generalized binding), Topo III binds randomly along its substrate molecule (Fig. 7A). This is consistent with the finding that Topo III can bind to substrates with and without cleavage sites. This model would predict a length dependence of the binding reaction since, in a nonspecific binding interaction, a longer oligonucleotide provides a larger target site per mole of substrate than a smaller oligonucleotide. Once bound to the substrate, the enzyme diffuses, or tracks, along the DNA until it encounters a binding/cleavage site.

Locating a specific binding site via a nondirectional tracking process has been ascribed to Topo III because there is no evidence of a nucleoside triphosphate hydrolysis-driven directional search by these enzymes. In principle, a directional search is a more efficient mechanism to locate sequences that are relatively distant from one another, but the location of both Topo III and Topo I cleavage sites has been found to be, on average, only 10–20 nucleotides apart (4, 20). The relative abundance of these sites may not require a directional search.

Although the tracking-mediated search proposed in this model is consistent with our experimental results, kinetic experiments will be required to elucidate the actual mechanism by which Topo III locates its binding/cleavage site. Oligonucleotide mobility shift assays only give information on equilibrium binding; therefore, the length effect observed in our experiments, while consistent with the model, is only indicative of the more stable binding of Topo III to the longer oligonucleotide than to the shorter oligonucleotide.

In the second stage of the reaction (which we term sequence recognition and stabilization), the enzyme recognizes and forms a stable complex with its cleavage site (Fig. 7B). In this stage, interactions with amino acids near or within the active site of the enzyme stabilize the enzyme-DNA complex and properly position the substrate within the active site (as illustrated by the ability to generate a nuclease P1 footprint). Positioning of the substrate within the active site is independent of the generalized binding of the substrate since truncations within the carboxyl terminus that drastically reduce the enzyme’s binding affinity (>99%) do not alter cleavage site selection by the enzyme (16).

In the final stage of the reaction (which we term asymmetric cleavage), the enzyme cleaves the substrate (Fig. 7C). During this step of catalysis, the majority of noncovalent interactions occur with DNA 5’ to the cleavage site, with only minimal interactions occurring 3’ to the site.

The model above has been derived using data obtained from studies using small, single-stranded DNA oligonucleotides as model substrates. These observations can be extended to physiologically relevant reactions catalyzed by topoisomerase III upon double-stranded substrates. Topo III is a single-stranded DNA binding protein with a relatively low affinity for double-stranded DNA (16); therefore, Topo III-catalyzed relaxation of negatively supercoiled DNA would require the enzyme to recognize and bind to single-stranded regions present in a negatively supercoiled, predominantly double-stranded molecule. The presence of single-stranded regions within a covalently closed DNA substrate is influenced by the superhelical density of the molecule, the local base composition within the molecule, and the reaction conditions. The optimum reaction conditions for Topo III-catalyzed relaxation activity (i.e. low magnesium optimum and stimulation by high temperatures (4)) favor the formation of single-stranded DNA and are consistent with this interpretation.

In the case of the decatenation of multiply interlinked plasmid DNA molecules, the enzyme would bind to small single-stranded regions present in the replicated DNA. These regions would be a consequence of lagging strand DNA synthesis, particularly from the removal of the small RNA primers used to initiate DNA synthesis. This is also consistent with the finding that the decatenation of isolated plasmid DNA dimers by Topo III is dramatically stimulated by the presence of small gaps within the substrate (4).

In this report, we have addressed only a half-reaction in the total scheme of topoisomerase catalysis. There is very little known about the next step of catalysis, strand passage. We are in the process of trying to isolate mutants that affect the strand passage reaction in the hope of being able to further dissect the mechanism of topoisomerase III.

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