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Sequence specific interaction of *Mycobacterium smegmatis* topoisomerase I with duplex DNA

Tisha Bhaduri, Devanjan Sikder and Valakunja Nagaraja*

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

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ABSTRACT

We have identified strong topoisomerase sites (STS) for *Mycobacteruim smegmatis* topoisomerase I in double-stranded DNA context using electrophoretic mobility shift assay of enzyme–DNA covalent complexes. Mg^{2+}, an essential component for DNA relaxation activity of the enzyme, is not required for binding to DNA. The enzyme makes single-stranded nicks, with transient covalent interaction at the 5′-end of the broken DNA strand, a characteristic akin to prokaryotic topoisomerases. More importantly, the enzyme binds to duplex DNA having a preferred site with high affinity, a property similar to the eukaryotic type I topoisomerases. The preferred cleavage site is mapped on a 65 bp duplex DNA and found to be CG/TCTT. Thus, the enzyme resembles other prokaryotic type I topoisomerases in mechanistics of the reaction, but is similar to eukaryotic enzymes in DNA recognition properties.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes catalyzing the interconversion of topological forms of DNA (1). The enzymes transiently break the DNA backbone bonds and allow the passage of the intact DNA through these enzyme-operated DNA gates. As a consequence of these reactions, they play a major role in various biological processes involving DNA, such as replication (2), transcription (3), recombination (3) and chromosome dynamics (4). Chromosomal superhelical density in prokaryotes is homeostatically maintained as a result of cumulative action of topoisomerase I and DNA gyrase (5). Type I topoisomerases relax supercoiled DNA by concerted reactions involving single-strand cleavage and religation of the DNA backbone bonds after the passage of the other strand (1). During this process the enzyme becomes covalently linked to the transiently nicked DNA, and it is possible to identify the preferential cleavage site of topoisomerases by irreversible arrest of the covalent intermediate (6).

Generally, topoisomerases do not exhibit very high sequence specificity, unlike restriction enzymes, repressors and transcriptional activators. Yet, a few of them show some degree of site specificity while others show some sequence preference. The sequence specificities for gyrase (7) and eukaryotic type I (8,9) and type II (10) topoisomerases have been subjected to extensive studies. In the case of *Escherichia coli* DNA gyrase, a 140 base pair (bp) DNA segment is wrapped around the enzyme in the enzyme–DNA complex (11). A strong gyrase binding site (SGS) has been located within the *tet* gene at position 990 on pBR322 DNA (12). Bacteriophage Mu DNA has a SGS located towards the center of the genome which is indispensable for the optimal replicative transposition (13). *Tetrahymena* DNA topoisomerase I preferentially cleaves a hexadecameric sequence present in the DNaseI-hypersensitive regions in the non-transcribed spacer of the rDNA (14). In contrast, vaccinia virus DNA topoisomerase I recognizes short DNA sequences and exhibits a strong sequence specificity. The enzyme cleaves after the pentameric sequence motif (C/T)CCTT (15). A variety of topoisomerase inhibitors inhibit the enzyme activity by arresting enzyme–DNA covalent reaction intermediates. These topoisomerase poisons influence the sequence-specific cleavage by topoisomerases (16,17,18).

The type I topoisomerases from prokaryotes are known to prefer single-stranded regions in DNA (6). Initial studies with *E.coli* and *Micrococcus luteus* topoisomerase I showed that cleavage of single-stranded DNA substrates occurs preferentially at the sequence 5′-CXXX-3′. In the case of the *E.coli* enzyme, G residues are not found at position –1 in 80% of the cleavage sites studied (19). However, this sequence requirement is not sufficient to account for the observed non-random distribution of the cleavage sites. The cleavage sites were also mapped on single-stranded oligonucleotides (20) or double-stranded DNA with a nick (21) or a loop (22). Site-specific interaction of the *E.coli* topoisomerase III has been studied in some detail (23). The enzyme binds with high affinity to a single-stranded DNA substrate with a strong cleavage site (CT*T) and can cleave a substrate as small as a heptamer.

Here we report the identification of preferred cleavage sites for *Mycobacterium smegmatis* topoisomerase I. We have designed a new strategy for determining the preferred cleavage site for the enzyme on duplex DNA fragments. To our surprise, we find that the enzyme binds to double-stranded DNA with high affinity, a characteristic feature of eukaryotic type I topoisomerases.

MATERIALS AND METHODS

Enzymes and nucleic acids

Topoisomerase I was purified to apparent homogeneity from *M.smegmatis* SN2 cells as described (to be published elsewhere). In brief, the cell free extract was treated with 1% polyethyleneimine and the supernatant was purified through phosphocellulose, SP-Sepharose and Q-Sepharose columns. The enzyme

*To whom correspondence should be addressed. Tel: +91 80 3092598; Fax: +91 80 3341683; Email: vraj@cge.iisc.ernet.in

Table 1. Sequences of oligonucleotides used in this study

Sequence of oligonucleotides	Lengths	Purpose
5′-AACGAAGTGCTGAAC	15 mer	Covalent complex competition experiments
5′-ATGGATCCGATATCGGCCAACAGCTCG-3′	27 mer	Do
5′-GACTCTAGAGGATCCCCGGGTACCGAGCTC-3′	30 mer	Do
5′-TATTGGGCGCTCTTCCGCTTCCTCGCTCACTG-3′ 3′-ATAACCCGCGAGAAGGCGAAGGAGCGAGTGAC-5′	32 bp (Specific)	Non-covalent complex competition experiments
5′-GACTCTAGAGGATCCCCGGGTACCGAGCTC-3′ 3′-CTGAGATCTCCTAGGGGCCCATGGCTCGAG-5′	30 bp (Non-specific)	Do

has an apparent molecular mass of 110 kDa. One unit of enzyme catalyses 50% conversion of 500 ng of supercoiled pUC19 DNA into different relaxed topoisomers at 37°C in 30 min under standard assay conditions. The protein concentration was estimated by the method of Bradford (24), using crystalline bovine serum albumin as standard.

Plasmid DNA was purified by the standard procedures (25). Restriction enzymes were purchased from Boehringer Mannheim and New England Biolabs and used according to the manufacturer's specifications. Klenow fragment of *E.coli* DNA polymerase I, acrylamide and other electrophoresis reagents were obtained from Gibco-BRL. NAP10 gel filtration columns were purchased from Pharmacia. Radioactive nucleotides were obtained from Amersham International, UK.

Labeling of DNA fragments

DNA fragments generated after different restriction enzyme digestions were end-filled with either [α-^{32}P]dATP or [α-^{32}P]dCTP (5000 Ci/mmol) and other cold dNTPs, using Klenow fragment of *E.coli* DNA polymerase I. Radiolabeled fragments were purified using Sephadex G-25 spin columns. Radiolabeled nucleotide (10 µCi) was used per reaction and the specific activity of labeling was ~10^7 c.p.m./µg of DNA used.

For purification of the individual fragments, the labeled fragments were separated on native polyacrylamide gels. The gel pieces containing the required fragments were excised and DNA was eluted into 10 mM Tris–HCl pH 8.0 and 1 mM EDTA by constant shaking at 37°C. The eluant was passed through NAP10 gel filtration columns according to suppliers' specifications. NAP10 column fractions containing the radioactivity were precipitated with ethanol, using glycogen as carrier, and re-dissolved in sterile water.

Electrophoretic mobility shift assays (EMSAs)

Reaction mixtures (20 µl) contained 20 mM Tris–HCl pH 8.0, 1 mM EDTA, 20 mM NaCl, radiolabeled DNA fragments (25 fmol, 10 000 c.p.m) and topoisomerase I (0.1–1 pmol). The reactions were incubated at 37°C for 10 min and then arrested with 25 mM NaOH. Incubations were continued for another 5 min and then the reaction mixtures were neutralized using 25 mM HCl and 25 mM Tris–HCl pH 8.0. The complexes were treated with 50 µg/ml proteinase K in the presence of 1% SDS and 50 mM EDTA for 3 h at 37°C, wherever indicated. The products were separated through a 10% polyacrylamide gel (29:0.8) using 0.5× TBE (25) as the running buffer. The gels were electrophoresed at 100 V at room temperature, dried and then subjected to autoradiography.

The non-covalent binding of the enzyme to DNA was also assessed by EMSA to determine the K_d value. DNA (32mer duplex DNA with the cleavage site) was incubated with varying amounts of the enzyme in the reaction buffer for 10 min on ice and the samples were analyzed on 5% native polyacrylamide gels at 4°C. The amount of radioactivity in the free and bound fractions was determined by liquid scintillation counting. This 32 bp oligonucleotide and a 30 bp oligonucleotide were used as specific and non-specific competitors respectively, for the non-covalent complex formation. The sequences of the oligonucleotides used in the present study are presented in Table 1.

Determination of cleavage sites

The 65 bp *Hin*fI fragment of pUC19 (Fig. 5C) was differentially labeled on the top strand with [α-^{32}P]dCTP (depending on the identity of N in the *Hin*fI site, GANTC). Differential 5′-end labeling of the bottom strand was carried out as follows. The 65 bp fragment was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by digestion with *Hae*III which resulted in the removal of the label from the 5′-end of the top strand. Reaction mixtures (20 µl) containing 20 mM Tris–HCl, 20 mM NaCl, 5 mM $MgCl_2$ and radiolabeled DNA fragment were incubated with topoisomerase I at 4 or 37°C for 10 min. The covalent complex was arrested and the protein was removed from the complex with proteinase K treatment. Samples were extracted with phenol/chloroform and precipitated with ethanol in the presence of 5 µg yeast tRNA used as a carrier. The precipitated DNA samples were suspended in 10 µl of formamide loading dye and heat denatured for 5 min at 90°C. Sequencing of the DNA fragment by chemical method was according to Maxam and Gilbert (26). The reaction products were re-dissolved in 50% formamide, 10 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue, heat denatured for 5 min at 90°C and analyzed on 15% polyacrylamide–8 M urea gels. The gels were dried and subjected to autoradiography.

RESULTS

Identification of DNA fragments having preferred cleavage site(s)

A new method was used to identify strong topoisomerase sites (STS) on duplex DNA substrates. Figure 1A shows the scheme of the experiment. The rationale of the approach is based on the conjecture that the enzyme could interact with double-stranded DNA as it would normally encounter a duplex DNA *in vivo*. If the enzyme binds tightly at a site on the duplex DNA fragment, the complex could be arrested as an enzyme–DNA reaction intermediate. Such covalent complexes could be detected as slower moving bands on a native polyacrylamide gel. An example of such an experiment is shown in Figure 1B. DNA fragments generated by *Hin*fI digestion of pUC19 were radiolabeled and used as substrates for complex formation in the absence of Mg^{2+} (see later section). When the DNA–protein covalent complex was arrested with NaOH, a few of the fragments showed retarded electrophoretic mobility including the two smallest fragments (Fig. 1B, lane 3). Proteinase K digestion of the reaction products abolishes the retarded mobility of the fragments (Fig. 1B, lane 4). Even in the presence of larger DNA fragments (214 and 396 bp), covalent complexes are seen with the 75 and 65 bp fragments, indicating sequence preference of the enzyme. Specific interaction of the enzyme at preferred sites on duplex DNA was confirmed by carrying out the assay with fragments generated with other restriction enzymes (data not shown). The method seems to be a convenient approach to identify STS on double-stranded DNA fragments.

EMSA with individual fragments having STS

The purified 65 bp and the 75 bp *Hin*fI fragments were used for further analysis. The fragments were incubated with different concentrations of enzyme (Fig. 2). Increased amounts of complexes were formed with the increase in enzyme concentration (Fig. 2, lanes 4 and 5, and 10 and 11). When the NaOH treatment was omitted, no complex was observed (Fig. 2, lanes 3 and 9) as topoisomerase I cannot form a stable non-covalent complex under the conditions of the assay and electrophoresis. The higher amount of radioactivity in the complex of the 65 bp fragment indicates that it is a better substrate than the 75 bp fragment (Fig. 2, compare lanes 4 and 10). Proteinase K treatment abolishes the complexes (Fig. 2, lanes 6 and 12), demonstrating that the observed complexes were indeed protein mediated.

The ability of *M.smegmatis* topoisomerase I to form the covalent complex with the 65 bp fragment was compared with different DNA fragments. A 214 bp fragment (pUC19 digested with *Hin*fI), a 114 bp fragment (pUC19 digested with *Hin*fI and *Dra*I) and a 30 bp fragment (pUC19 digested with *Taq*I) did not form any detectable covalent complex with *M.smegmatis* topoisomerase I (not shown and Fig. 4, lane 9). Thus, it seems that not just any double-stranded DNA fragment is a substrate for the enzyme.

Mg^{2+} is not required for DNA binding and cleavage

The covalent trapping experiments described above were carried out in the absence of Mg^{2+}. This was necessary, as the presence of 5 mM Mg^{2+} in the reaction (concentration used for relaxation assay) resulted in lower yields of trapped complexes. The enzyme can efficiently bind and cleave double-stranded DNA in the

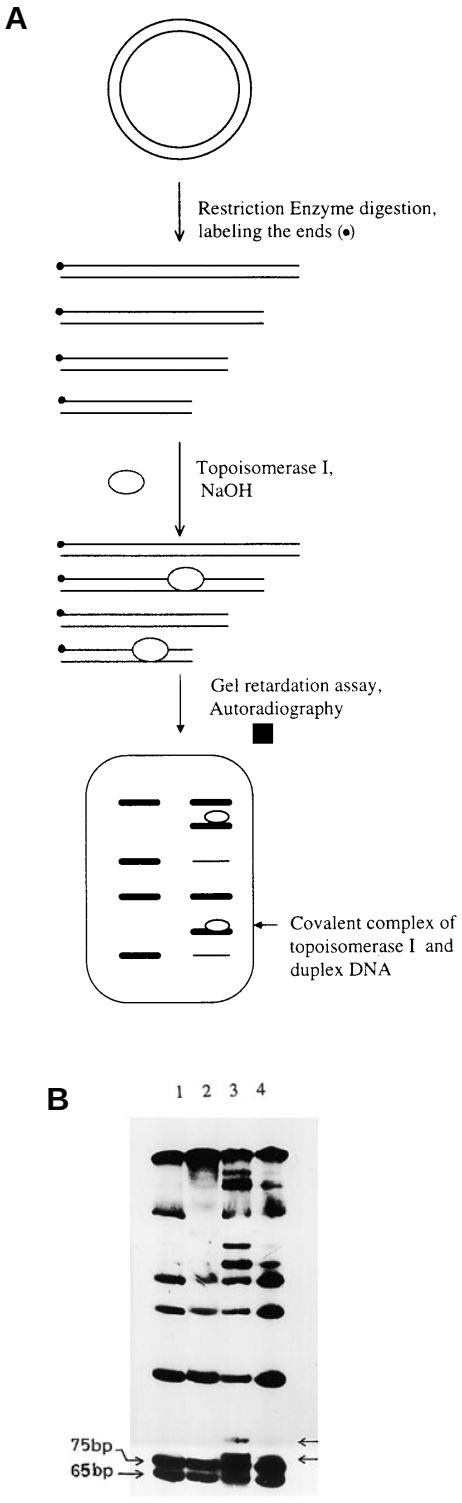

Figure 1. Method for identification of 'strong topoisomerase I site(s)'. (**A**) Scheme of the experiment. (**B**) 10% native PAGE to resolve covalent complexes. End-filled *Hin*fI digested pUC19 fragments (50 000 c.p.m., 5 ng) (lane 1) were incubated with 100 fmol of purified topoisomerase I (lane 2). Covalent complexes were not detected without alkali treatment. The largest fragment, however, shows retardation, possibly due to specific and non-specific interactions. Covalent complexes were trapped with NaOH (lane 3). Proteinase K treatment of the covalent complexes (lane 4) greatly abolishes the retardation of the complex. Arrows indicate the covalent complexes from the 65 and 75 bp fragments.

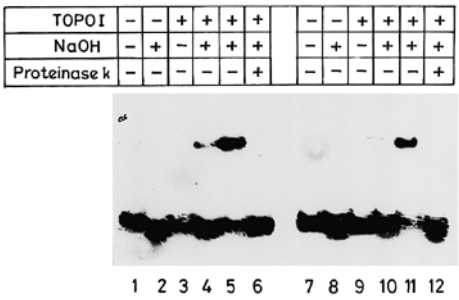

Figure 2. Electrophoretic mobility shift assay with the individual fragments. The end-labeled 65 bp (lanes 1–6) and 75 bp (lanes 7–12) DNA fragments were used as substrates (10 000 c.p.m., 25 fmol) in the absence of Mg^{2+}. Lanes 1, 2, 7 and 8, no topoisomerase I; lanes 4 and 10, 1 pmol, and lanes 3, 5, 6, 9, 11 and 12, 2 pmol of topoisomerase I. The samples were run in 10% native PAGE as described in Materials and Methods. Proteinase K treatment (lanes 6 and 12) results in abolishing the covalent complex.

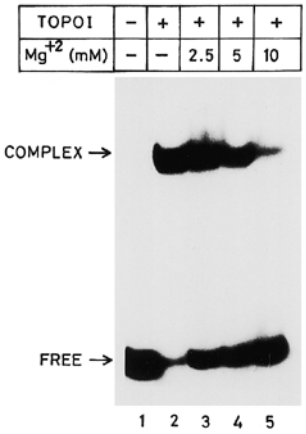

Figure 3. Effect of Mg^{2+} on DNA binding and cleavage. End-labeled 65 bp DNA fragment (lane 1) was incubated with 2 pmol of topoisomerase I and the complex was arrested with NaOH (lanes 2–5) in the presence of 2.5 mM (lane 3), 5 mM (lane 4) and 10 mM (lane 5) Mg^{2+}. Other details are as described in Materials and Methods.

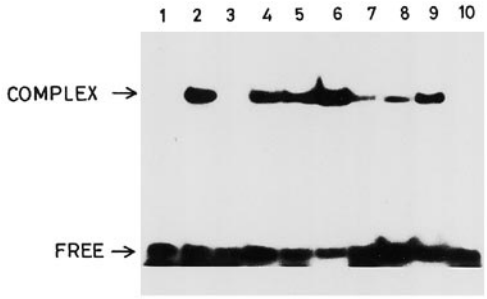

Figure 4. Competition assay with different single- and double-stranded DNA substrates. End-labeled 65 bp DNA fragment was used as the substrate. Lane 1, no topoisomerase; in all the other lanes 1 pmol of topoisomerase I was used and the complex was arrested with NaOH. Lane 2, no competitor; lane 3, single-stranded M13 DNA; lanes 4–6, 15 base, 26 base and 30 base oligonucleotides, respectively; lane 7, unlabeled 75 bp DNA fragment; lane 8, relaxed pUC19 DNA; lane 9, unlabeled 114 bp DNA fragment; lane 10, supercoiled pUC19 DNA. All the competitors were taken in 25-fold molar excess.

absence of Mg^{2+} (Fig. 3, lane 2). The amount of covalent complex decreased with the increase in Mg^{2+} ion concentration (Fig. 3, lanes 3–5). However, it should be noted here that there is an absolute requirement for Mg^{2+} ions for the DNA relaxation activity of the enzyme (27). These results indicate that Mg^{2+} is not required for DNA binding and cleavage, yet it is required at a subsequent step of catalysis. A noteworthy point is that eukaryotic type I topoisomerases do not require Mg^{2+} ions for their activity, but the metal ion can activate the religation (15) or cleavage step (28) of the enzyme. Mg^{2+} is also known to shift the enzyme from a processive to a distributive mode of catalysis (29). The decrease in the amount of covalent complex at 10 mM Mg^{2+} could be due to an ionic effect contributing to reduced interaction between protein and DNA. At this concentration of Mg^{2+}, DNA relaxation activity is also inhibited (not shown). The decrease in the covalent complex at higher concentrations of Mg^{2+} could also be due to an ionic effect resulting in reduced interaction. Examples of other DNA–protein interaction studies indicate that binding of protein to their specific sites may or may not be metal-ion-dependent. This aspect can best be illustrated by taking the examples of restriction endonucleases *Eco*RI and *Eco*RV. *Eco*RI can bind to DNA in the absence of Mg^{2+} but requires its presence for the cleavage at the specific site (30). In contrast, binding of Mg^{2+} to the restriction endonuclease *Eco*RV is a pre-requisite for the enzyme to bind to its cognate site. In the latter case, Mg^{2+} is also required for cleavage (31,32). In a recent study, *E.coli* topoisomerase I was shown to have bound Mg^{2+} ions in two different pockets of the enzyme. The occupancy at both the sites is required for the DNA relaxation activity of the enzyme (33). Additional experiments would delineate the precise role of Mg^{2+} in *M.smegmatis* topoisomerase I mediated reactions.

Competition assay with different DNA substrates

Prokaryotic type I topoisomerases are known to prefer single-stranded DNA or single-stranded regions in the double helical DNA as efficient substrates for the enzyme activity (34). Such substrates could efficiently compete with the 65 bp fragment for complex formation. When M13 single-stranded DNA (Fig. 4, lane 3) and supercoiled pUC19 DNA (Fig 4, lane 10) were used as competitors, no complex was observed with the 65 bp fragment. When single-stranded oligonucleotides of different lengths (15, 27 and 30mer) were used, the complex formation with the 65 bp fragment remained unaffected (Fig. 4, lanes 4–6). Thus, any single-stranded DNA is not a good substrate for the enzyme. *Escherichia coli* topoisomerase I is known to bind short single-stranded oligonucleotides. Even an 8mer oligonucleotide serves as a good substrate to the enzyme (20). Thus, the absence of competition with larger oligonucleotides (27 and 30mer) in our study is unlikely to be due to inadequate length. Amongst the other DNA substrates used as competitors, the 75 bp DNA fragment and the relaxed pUC19 DNA were found to compete partially with the 65 bp fragment (Fig. 4, lanes 7 and 8) under the present experimental conditions. A 114 bp fragment cannot inhibit the complex formation with the 65 bp fragment (Fig. 4, lane 9). Similarly, 30 and 214 bp DNA fragments were also unable to compete with the 65mer for covalent complex formation (not shown). These results confirm the point that the *M.smegmatis* topoisomerase I does not bind to any single- or

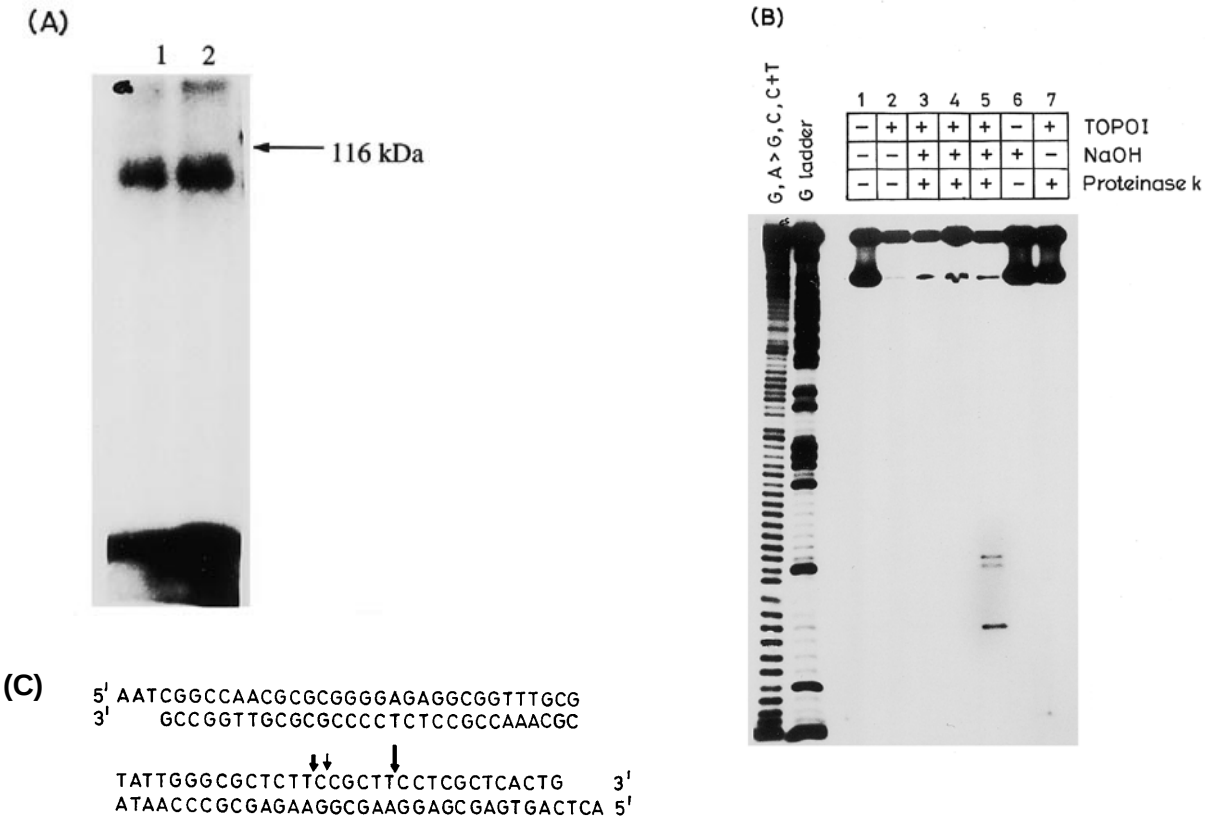

Figure 5. Localization and mapping of the cleavage site(s). (**A**) Autoradiogram of the SDS–PAGE. 1 and 2 pmol of topoisomerase I were used for complex formation (lanes 1 and 2). Position of a 116 kDa molecular weight marker is indicated by arrow. The radiolabeled protein band was not observed when the reaction was carried out under the following conditions: omission of alkali treatment, omission of enzyme or incubation of DNA with BSA and alkali. (**B**) Mapping of the cleavage site(s) on the 65 bp fragment. Differentially labeled 65 bp fragment (labeled at the 3′-end of the top strand) was used as the substrate. A mixture of chemical cleavage reactions (G, A>G, C+T) and G sequencing lanes are indicated. 65 bp DNA (1 pmol, 5×10^5 c.p.m.) was incubated with 2 pmol of topoisomerase I in indicated lanes, at 4°C (lane 4) or 37°C (lanes 3 and 5). Heat-denatured enzyme was used in lane 3. DNA was treated with only NaOH (lane 6), or the NaOH treatment was omitted (lane 7, also serves as a proteinase K control). DNA was incubated with protein without trapping of the complex (lane 2). (**C**) Sequence of 65 bp *Hin*fI fragment of pUC19 DNA. The arrows represent the position and extent of cleavage.

double-stranded DNA and exhibits sequence specificity for efficient binding.

Localization of the cleavage site(s) and characterization of covalent intermediate

The above experiments demonstrate that the 65 bp DNA fragment contains strong topoisomerase site(s). However, it does not indicate the location of the cleavage site(s) to a particular strand. The following set of experiments were carried out to determine the STS-containing strand. The 65 bp fragment labeled at either, or both, ends showed complex formation. Further *Hae*III digestion of the 65 bp fragment (labeled by end filling) results in removal of the label at the 3′-end of the bottom strand while the top strand retains the label. Detection of cleavage products in a denaturing polyacrylamide gel electrophoresis (PAGE) from such a fragment is an indication of the presence of STS in the top strand (data not shown). In a reverse experiment, cleavage products could not be detected when 5′-end labeled fragments digested with *Hae*III were used, indicating the absence of STS on the lower strand. The presence of STS on the top strand was confirmed by the differential labeling of the top strand of the 65 bp fragment with [α-^{32}P]dCTP as described in Materials and Methods. The uniquely labeled 65 bp fragment was incubated with *M.smegmatis* topoisomerase I, the DNA–protein complex was trapped using NaOH and the samples were analyzed on 1% SDS–7.5% PAGE (35). The autoradiograph of such a gel showed that the radioactivity has been transferred to the protein (Fig. 5A). These results demonstrate that (i) the top strand of the fragment has specific topoisomerase I cleavage site(s) and (ii) as label is present at the 3′-end of the DNA, protein–DNA covalent linkage must be via the 5′ phosphate group at the nick.

Mapping of the cleavage site(s)

The positions of cleavage site(s) were mapped by comparing the mobility of the cleaved fragment(s) with a sequencing ladder produced by chemical cleavage of the 65 bp fragment. The 65 bp fragment, labeled at the top strand, was incubated with the enzyme on ice or at 37°C respectively (Fig. 5B, lanes 4 and 5). Specific cleavage products were observed with the active enzyme incubated at 37°C (Fig. 5B, lane 5). Three sites of varied intensities were detected. The chemical cleavage yields fragments with 3′ phosphates while topoisomerase I cleavage products end with 3′-OH. This is taken into consideration while mapping the cleavage positions. Two major cleavage sites map after the

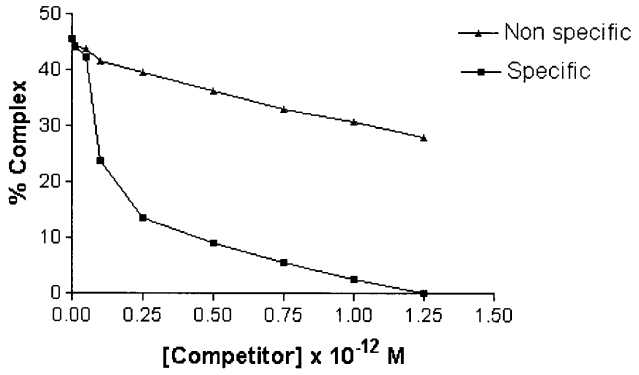

Figure 6. Competition assay with duplex oligonucleotides. Non-covalent complexes of topoisomerase I (10 fmol) and a labeled 32 bp specific oligonucleotide were resolved in a native PAGE (Materials and Methods) to obtain ~50% complex formation. The competition with specific (square) and non-specific (triangle) duplex oligonucleotides (Table 1) were carried out. The amount of complex was estimated by counting radioactivity in each lane using a liquid scintillation counter.

trinucleotide CTT (Fig. 5C). Experiments with the uniquely labeled bottom strand did not release any cleavage products indicating the absence of preferred sites.

High affinity binding of *M.smegmatis* topoisomerase I to duplex DNA

The competition experiments described earlier reflect high affinity of the enzyme for duplex DNA having STS. EMSA was employed to assess the binding of the protein to DNA and to determine the specificity of the interaction. Formation of non-covalent complexes (not shown) of the enzyme with a 32 bp duplex oligonucleotide having STS enabled us to make an estimate of the equilibrium dissociation constant for the *M.smegmatis* topoisomerase I. The duplex DNA was incubated with increasing amounts of the enzyme. The concentration of the enzyme required for 50% of the duplex oligonucleotide to form the complex was taken as the K_d value. The value of $1–5 \times 10^{-9}$ M is comparable to the affinity of many other site-specific DNA binding proteins, such as Arc and Trp repressors (36,37). The value is also comparable to the equilibrium dissociation constant of DNA gyrase of *E.coli* (38) and to that of *Tetrahymena* topoisomerase I (17), to their respective preferred sites. A competition assay was carried out with specific and non-specific duplex oligonucleotides (Table 1) to further assess the affinity of the enzyme. For this purpose, the topoisomerase and labeled 32mer duplex (specific) DNA were incubated to obtain 50% complex formation as in the case of K_d measurements. The ability of the specific and non-specific oligonucleotides to chase the non-covalent protein–DNA complex is shown in Figure 6. The absence of competition even at 1000-fold excess of the non-specific oligonucleotide also reflects the specific, high affinity interaction of the enzyme with DNA.

DISCUSSION

In this manuscript, we describe the site specific binding and cleavage reactions of topoisomerase I from *M.smegmatis*. We demonstrate that the enzyme binds to double-stranded DNA at regions having strong topoisomerase I sites with high affinity. The cleavage site within the binding region is determined, and is surprisingly similar to the cleavage sites of some of the well characterized eukaryotic topoisomerases. We also demonstrate that not just any double-stranded DNA is a good substrate. Further, in spite of binding tightly to the double-stranded DNA, the enzyme makes only single-stranded nicks with covalent attachment to the 5′-end at the nick.

The earlier method employed to identify the cleavage sites for different type I topoisomerases was to analyze the cleavage product obtained from an end-labeled single-stranded DNA fragment after trapping of the covalent complex. The cleavage sites of *E.coli* and *M.luteus* topoisomerase I were mapped on single-stranded DNA fragments by Tse *et al.* (19). Archaebacterial reverse gyrase (39) and *E.coli* topoisomerase III (23) cleavage sites have also been mapped on single-stranded DNA substrates. This approach while successful, produces a large number of cleavage products. This is expected as type I topoisomerases from prokaryotes are known to prefer single-stranded DNA for their binding and cleavage. Analyses of many cleavage sites of the enzymes do not reflect a high degree of sequence specificity. A notable exception is observed in the case of the *E.coli* topoisomerase III cleavage site, wherein a highly preferred site is mapped to a sequence CTT (23). The approach we employed to identify the preferred site is rather novel and developed on the following premise. In most circumstances, the enzyme would normally encounter DNA in double-stranded form, although single-stranded regions in superhelical DNA would also be available for its interaction. Thus, when the enzyme encounters a highly preferred site in double-stranded DNA, it should have the potential to bind tightly at the sequence to carry out the breakage and reunion reactions. The method employed here is easy to perform and only strong topoisomerase sites in double-stranded DNA context would be identified.

Analyses of the cleavage sites of different topoisomerases show considerable similarity in the cleavage pattern of the mycobacterial enzyme to that of topoisomerase III from *E.coli* (23) and topoisomerase I from *Tetrahymena* (14) and vaccinia virus (15). The cleavage is next to sequence CTT for topoisomerase I from *M.smegmatis*, which is very similar to that of vaccinia type I enzyme. A point to be noted here is that topoisomerase III from *E.coli* falls into the type 1A category making 5′-end covalent contacts, while vaccinia and *Tetrahymena* enzymes come under type 1B group, making 3′ covalent complexes during the intermediate stage of catalysis (40). The results presented in this paper, show that *M.smegmatis* topoisomerase I makes a 5′-end covalent contact similar to topoisomerase III of *E.coli* and hence belongs to the 1A group.

The site-specific, high-affinity binding of topoisomerase I from *M.smegmatis* to double-stranded DNA is rather unexpected and raises additional questions. Clearly, the ability to bind double-stranded DNA efficiently and then make specific single-stranded nicks is of physiological relevance. It is also conceivable that unlike other prokaryotic type I enzymes, the enzyme shows preference for double-stranded DNA like the eukaryotic counterparts, as organisms belonging to the actinomycetes are considered to be evolved prokaryotes (41,42).

We propose the following model to explain the action of topoisomerase I from *M.smegmatis*. Initially the enzyme would bind to any region of DNA in a random fashion. This binding could be considered as low-affinity binding. Once bound to DNA,

the enzyme could slide or track along the DNA as in the case of other site-specific DNA binding proteins such as repressors, RNA polymerase, restriction endonucleases, etc. This linear diffusion along the DNA is very likely to be random and could be in either direction. Encounter of STS by the enzyme while tracking would result in high-affinity binding. Thus, high-affinity binding at the preferred cleavage site would be the prerequisite for stable interaction of the protein with the DNA before cleavage of the duplex DNA to generate molecular gates.

In this report, we have addressed mainly the unusual aspects of the interaction of a new prokaryotic type I topoisomerase with DNA. A detailed analysis of the various steps of catalysis, strand passage, religation, etc. need to be carried out using variety of natural and synthetic DNA substrates to further delineate the molecular mechanisms of enzyme action. Of particular interest would be the study of inhibition of cleavage and/or relaxation activity by oligonucleotides having the specific cleavage sites. In the virtual absence of known inhibitors of prokaryotic type I topoisomerases, this approach would provide an opportunity for rational design of ligands targeted to this class of enzymes. This aspect gains further credence as mycobacterial infections are rampant world wide and there is an urgent need to develop new therapeutics due to the increasing emergence of multiple drug resistant clinical strains of *Mycobacterium tuberculosis* (43).

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