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DNA Topoisomerase I from *Mycobacterium smegmatis*

AN ENZYME WITH DISTINCT FEATURES*

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A type I topoisomerase has been purified to homogeneity from *Mycobacterium smegmatis*. It is the largest single subunit enzyme of this class having molecular mass of 110 kDa. The enzyme is Mg^{2+} dependent and can relax negatively supercoiled DNA, catenate, and knot single-stranded DNA, thus having typical properties of type I topoisomerases. Furthermore, the enzyme makes single-stranded nicks and the 5′-phosphoryl end of the nicked DNA gets covalently linked with a tyrosine residue of the enzyme. However, *M. smegmatis* enzyme shows some distinctive features from the prototype *Escherichia coli* topoisomerase I. The enzyme is relatively stable at higher temperatures and not inhibited by spermidine. It apparently does not contain any bound Zn^{2+} and on modification of cysteine residues retains the activity, suggesting the absence of the zinc-finger motif in DNA binding. Partially purified *Mycobacterium tuberculosis* topoisomerase I exhibits very similar properties with respect to size, stability, and reaction characteristics. Sequence comparison of topoisomerase I from *E. coli* and *M. tuberculosis* shows the absence of zinc-finger motifs in mycobacterial enzyme. Using a two-substrate assay system, we demonstrate that the enzyme acts processively at low ionic strength and switches over to distributive mode at high Mg^{2+} concentration. Significantly, the enzyme activity is stimulated by single strand DNA-binding protein. There is a potential to exploit the characteristics of the enzyme to develop it as a molecular target against mycobacterial infections.

DNA topoisomerases are ubiquitous enzymes that play a vital role in variety of cellular processes by maintaining the superhelical density of DNA (1). There are two distinct subfamilies of topoisomerases. Type I subclass consists of prokaryotic and eukaryotic topoisomerase I and topoisomerase III enzymes, which effect topological changes in DNA by transiently cleaving one DNA strand at a time to allow the passage of another strand (2). This results in the change in linking number of DNA by steps of one. Eukaryotic topoisomerase II, bacterial DNA gyrase, and topoisomerase IV belong to the type II subfamily. The enzymes of the latter group require ATP for catalysis and introduce transient double-stranded breaks, changing the linking number in steps of two (2). Topoisomerases have been found to participate in nearly all cellular transactions involving DNA and thus can be the ideal target enzymes for clinical medicine. The identification of DNA gyrase as a target for chemical inhibitors, antibiotics, toxins (3–5), and eukaryotic topoisomerases for a large number of anticancer agents (6) illustrates this point well.

The prokaryotic topoisomerase I has been extensively characterized from *Escherichia coli* (7, 8). Topoisomerase I from few other bacterial sources such as *Micrococcus luteus* (9), *Diplococcus pneumoniae* (10), and *Fervidobacterium islandicum* (11) have also been studied. Prokaryotic type I topoisomerases catalyze the relaxation of negatively supercoiled DNA in an energy independent manner. Other reactions catalyzed by this class of enzymes include knotting and catenation of single-stranded DNA or duplex DNA having a nick in one of the strands. It is suggested that in prokaryotes, topoisomerase I plays a crucial role to maintain the *in vivo* superhelical density of DNA in conjunction with the supercoiling activity of DNA gyrase (12). Topoisomerase I has been also implicated to have role in DNA replication, transcription, and recombination events (13). In certain pathogenic bacteria, regulation of virulence gene expression seem to be mediated by topoisomerases (14).

The genus *Mycobacterium* is of immense importance as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and other opportunistic pathogenic strains belong to this group. Two major diseases, tuberculosis and leprosy, continue to be serious health hazards in all the developing countries. Reappearance of tuberculosis in the Western world in the recent times and emergence of multiple drug resistant *M. tuberculosis* clinical strains have raised a global concern (15). There has been a dramatic increase in the occurrence of tuberculosis in human immunodeficiency virus-infected people, since the viral infection reduces the immunity in AIDS patients. These developments necessitate the identification of new molecular targets as a prelude to the drug discovery.

While therapeutic agents targeted toward prokaryotic topoisomerase I have not been realized yet, this class of enzymes remain as potential targets for a new class of inhibitors. Compared with many other bacterial species very little is known about the macromolecular events in mycobacteria. Thus, in addition to exploring it as a molecular target, detailed characterization of topoisomerase I from mycobacteria is also important to understand the basic biology of the organism. Comparatively slow growth rate of mycobacteria and their ability to lay dormant in host macrophages (15) is suggestive of some unusual regulatory features of DNA transaction events, which in turn may be related to the DNA modulating enzymes like topoisomerases.

In this article we report the detailed characterization of topoisomerase I from *M. smegmatis*. The enzyme in many respects, is similar to that of the prokaryotic prototype, characterized from *E. coli* topoisomerase I. Yet, it exhibits some

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distinct properties. Our analysis, quite unexpectedly, indicates that cysteines do not play a role in DNA binding.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—Polyethyleneimine, NEM,[1] MMTS, DTT, spermidine, yeast tRNA, proteinase K, *o*-phenanthroline, phosphoamino acid markers were obtained from Sigma. Buffers, solutions, and gel electrophoresis reagents were prepared using reagents from Life Technologies, Inc. SP-Sepharose and Q-Sepharose column materials were obtained from Pharmacia Biotech Inc. and phosphocellulose from Whatman. Micrococcal nuclease was purchased from Worthington. SSB protein was purchased from Bangalore Genie Ltd., India. Radioactive nucleoside triphosphates with specific activity 3000 Ci/mmol were obtained from Amersham Corp.

Plasmid pJW312-*Sal*I which was used for overexpressing *E. coli* topoisomerase I, was a generous gift from James C. Wang, Harvard University, Cambridge, MA. *E. coli* topoisomerase I was purified using the published protocol (16).

Purification of Topoisomerase I from M. smegmatis—Topoisomerase I activity was purified from *M. smegmatis.* Growth of *M. smegmatis* SN2 and preparation of the cells for cell-free extracts are as described before (17). The crude cell extract was centrifuged at 18,000 rpm and the supernatant was centrifuged at 40,000 rpm in a Beckman type 50 Ti rotor for 3 h, to obtain 100,000 × *g* supernatant (S100). This S100 supernatant was brought to 1% final concentration of polyethyleneimine, in the presence of 50 mM NaCl. The polyethyleneimine supernatant was subjected to 0–67% ammonium sulfate fractionation. The pellet was dissolved in buffer A (50 mM KPO_4, pH 7.4, 1 mM EDTA, 5% glycerol, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM KCl) and loaded onto a phosphocellulose column (150 ml). The bound proteins were eluted with a salt gradient of 200 mM to 1 M KCl. The active fractions (eluted at 450 to 650 mM KCl) were pooled, dialyzed (against buffer A), and subsequently loaded on to a SP-Sepharose column (30 ml). Elution of the column was with a salt gradient of 400 to 750 mM KCl in buffer A and active fractions corresponding to 450 to 500 mM KCl were pooled and dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% glycerol, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM KCl) and finally loaded onto a Q-Sepharose column (10 ml). The column was developed with linear gradient of buffer B having 50 to 600 mM KCl. The enzymatically active fractions eluted between 300 and 400 mM were pooled and dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20% glycerol, and 10 mM β-mercaptoethanol) and stored at −20 °C. Topoisomerase I from *M. tuberculosis* H_{37}Ra was partially purified (phosphocellulose chromatography) employing the procedure described above.

The topoisomerase relaxation assay was carried out as described before (17). One unit of enzyme catalyzes 50% conversion of 500 ng of supercoiled pUC19 DNA into different relaxed topoisomers at 37 °C in 30 min under standard assay conditions.

Catenation Assay—Catenation of M13 single-stranded DNA was carried out in a 20-μl reaction volume containing 20 mM Tris-HCl, pH 7.6, 20 mM KCl, 6 mM $MgCl_2$, 5 mM spermidine, 0.5 mM DTT, 50 μg/ml bovine serum albumin, 30% (v/v) glycerol, and 500 ng of DNA substrate. Incubation was at 37 and 52 °C for 1 h. Products were extracted with phenol-chloroform, and precipitated with alcohol before loading onto 0.8% agarose gel.

Processive and Distributive Mode of Relaxation Using Two Substrates—The relaxation reaction was carried out with one of the substrates with different concentrations of $MgCl_2$ for 10 min, with limiting enzyme concentration (0.2 units). Then the second substrate was added and the reactions were continued for another 10 min. Reactions were stopped with 1% SDS containing buffer and fractionated on a 1.2% agarose gel. Bands were visualized after ethidium bromide staining.

Transfer of Radioactivity from DNA to Enzyme—pUC19 DNA in 20 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA was nick-translated using [α-^{32}P]dATP and [α-^{32}P]dCTP (specific activity of labeling was 2×10^7 cpm/μg of DNA). The DNA was denatured by adding NaOH to a final concentration of 50 mM. After 10 min at 37 °C, the DNA was neutralized with 50 mM HCl and 0.1 volume of 1 M Tris-HCl, pH 8.0. A reaction mixture of 25 μl containing 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 50 μg/ml bovine serum albumin, 25 ng of denatured nick-translated DNA, and the topoisomerase I enzyme was incubated at 37 °C for 30 min. NaOH was added to a final concentration of 50 mM. Following incubation for an additional 10 min at 37 °C, the pH of the sample was readjusted to 8.0. $CaCl_2$ and micrococcal nuclease were added to a final concentration of 1 mM and 5 units/ml, respectively. The mixture was incubated overnight at 37 °C. The samples (protein-labeled nucleotide complex) were analyzed in a SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Covalent Attachment of the Enzyme at the 5′ End of the DNA Nicks—The experiment was carried out with minor modifications of the described procedure (18). 50-μl reaction mixtures containing 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 100 ng of either 3′-end filled or 5′-end labeled *Sau*3AI-digested pUC19 DNA (alkali denatured) was incubated at 37 °C for 1 h in the presence or absence of purified topoisomerase I. The reaction was stopped by adding 100 μl of a prewarmed solution containing 0.2 M NaOH, 2% SDS, 2 mM EDTA, 50 μg/ml tRNA. Samples were incubated for an additional 10 min at 37 °C, then neutralized by adding 50 μl of a solution containing, 0.4 M HCl, 0.4 M Tris-HCl, pH 8.0, and 0.25 M KCl and placed on ice for 10 min. Samples were then centrifuged at 12,000 rpm for 20 min at 4 °C. The resulting pellet was resuspended and washed with 200 μl of a solution containing 10 mM Tris-HCl, pH 8.0, 100 mM KCl, and 50 μg/ml tRNA at 65 °C. After 10 min samples were cooled on ice and recentrifuged. After two washes, pellets were resuspended in 200 μl of water by heating to 65 °C. The radioactivity in the suspension was estimated in a scintillation counter.

Identification of the Covalent Linkage—The protein-DNA complex, prepared as described in the earlier section, was precipitated with ice-cold acetone and free label was removed by repeated washing with 80% acetone. The precipitated protein-DNA complex was then proteolyzed with 50 μg/ml proteinase K at 37 °C for 1 h. The digested product was vacuum dried and resuspended in 100 μl of 5.6 M HCl. Hydrolysis was performed at 110 °C for 1 h. HCl was removed in vacuum and the dried hydrolysate was suspended in 15 μl of chromatographic buffer (375 ml of *n*-butanol, 250 ml of pyridine, 75 ml of acetic acid). Samples were spotted on a silica gel plate. Phosphoamino acid markers (phosphotyrosine, phosphoserine, and phosphothreonine) were also spotted on the same horizontal line. Thin layer chromatography, ninhydrin staining, autoradiography of the resolved radiolabeled amino acids, etc. were carried out according to the published procedures (19).

Modification of Cysteine Residues—Pure topoisomerase I (2–5 μM) was denatured by 6 M guanidium HCl treatment at 37 °C for 1 h. One aliquot of this denatured protein was sequentially dialyzed against the assay buffer (Tris-HCl, pH 8.0, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 μM $ZnCl_2$) containing 4, 2, of 1 M, and no guanidium HCl, respectively, to renature the protein. The remainder of the denatured protein was aliquoted and different aliquots were treated either with 1 mM NEM or 200 μM MMTS at 37 °C for 30 min. The NEM/MMTS-treated proteins were dialyzed in the same way against the assay buffer in the presence or absence of DTT. All the dialyzed fractions were assayed for DNA relaxation activity. Complete modification of cysteine residues was assessed by 5,5′-dithio(nitrobenzoic acid) estimation of free thiols (20, 21). Purified *E. coli* topoisomerase I (5 μM) was also subjected to similar treatments.

Estimation of Cysteine Residues—Determination of the free thiols was carried out by Ellman assay (20), essentially as described (21). 3 nmol of *M. smegmatis* topoisomerase I was diluted in 1 ml of 0.1 M phosphate buffer and treated with 100 μl of 10 mM 5,5′-dithio(nitrobenzoic acid). Molar concentration of cysteine was calculated from the $\Delta A_{412\ nm}$ as described (21). The value obtained was divided by the moles of the enzyme used to obtain the number of free thiols present in the protein.

Other Methods—Protein concentration was estimated at different stages of purification using the procedure of Bradford (22), using bovine serum albumin as standard. SDS-gel electrophoresis were carried out according to Laemmli (23). Standard DNA manipulations and autoradiography was carried out using established protocols (24). M13 single-stranded DNA was prepared using standard procedure and purified through a 5–30% sucrose density gradient (24). N-terminal sequencing of the protein was carried out in Central Protein Sequencing Facility of Indian Institute of Science using Shimadzu Gas Phase Sequenator PSQ-1.

RESULTS

Purification of the Enzyme—The purification scheme of a topoisomerase activity from *M. smegmatis* is presented in Fig. 1*a*. The protein fractions from different purification steps were analyzed in a SDS-polyacrylamide gel. The protein appears to

[1] The abbreviations used are: NEM, *N*-ethylmalamide; DTT, dithiothreitol; MMTS, methyl methanethiosulfonate; SSB, single-stranded DNA-binding protein.

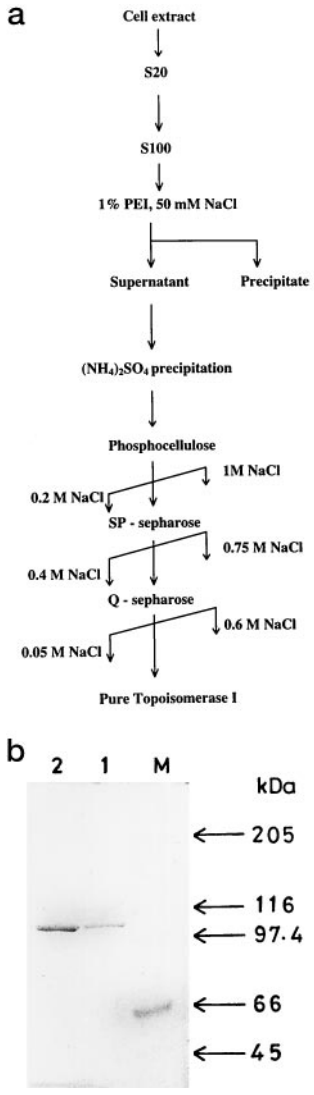

FIG. 1. **Purification of the *M. smegmatis* topoisomerase I.** *a*, purification scheme. *b*, silver-stained SDS-polyacrylamide gel electrophoresis (10%) of the purified topoisomerase. *Lanes 1* and *2*, 100 and 250 ng of purified topoisomerase I, respectively. Molecular weight markers are indicated by *arrows*.

be homogeneous as it migrates as a single band corresponding to the molecular mass of approximately 110 kDa in a silver-stained gel (Fig. 1*b*). Thus the enzyme appears to be larger than any of the prokaryotic type I topoisomerases characterized so far. The corresponding topoisomerase I from *E. coli* has a molecular mass of 98 kDa (25). The purified topoisomerase I from *M. smegmatis* was subjected to N-terminal sequence determination by the Edman degradation method. The sequence obtained, AGGSRGSGGA, is different from the N-terminal sequence of the *E. coli* enzyme and does not correspond to any other topoisomerases.

The DNA relaxation assay was used to monitor the enzyme activity at every stage of purification. The topoisomerase I activity was purified to 1263-fold and the specific activity of the enzyme was 2.17×10^5 units/mg of protein (Table I). The purified DNA relaxation activity had salient features of a prokaryotic type I topoisomerases. The enzyme has an efficient relaxation activity on negatively supercoiled DNA. Mg^{2+} is essential for the DNA relaxation activity, while nucleotide cofactor(s) and monovalent cations are not required. However, at higher salt concentrations (>250 mM) enzyme activity is abolished. DNA relaxation activity of *M. smegmatis* topoisomerase

TABLE I
Purification of M. smegmatis topoisomerase I
50 g of *M. smegmatis* SN2 cells were used.

Fractions	Protein	Activity units	Specific activity	Purification
	mg		*units/mg*	*-fold*
S20	584.65	ND[a]	ND	
S100	419.12	ND	ND	
$(NH_4)_2SO_4$	232.8	4.0×10^4	171.8	
Phosphocellulose	20.5	2.4×10^4	1.17×10^4	68.0
SP-Sepharose	0.125	1.1×10^4	8.0×10^4	465.6
Q-Sepharose	0.046	9982	2.17×10^5	1263.0

[a] ND, not determined.

I was not inhibited at 0.5 or 1 mM spermidine (Fig. 2*a, lanes 3* and *4*. This is in contrast to the properties of *E. coli* topoisomerase I which is inhibited at intracellular concentrations of the spermidine (26). Our results with *E. coli* topoisomerase I is in agreement with the earlier findings (26).

Thermal Stability of the M. smegmatis Topoisomerase I—To check the thermo-tolerance of the *M. smegmatis* topoisomerase I, standard assay mixtures were preincubated at different temperatures and the reactions were initiated by the addition of the enzyme. The results are presented in Fig. 2*b*. The mycobacterial enzyme retains some relaxation activity at 65 °C (*lane 11*), while the *E. coli* enzyme loses its activity above 42 °C (*lanes 4* and *5*), indicating that the *M. smegmatis* topoisomerase I is a more thermostable enzyme. Similar behavior is observed with partially purified *M. tuberculosis* topoisomerase I (not shown).

Positively Supercoiled DNA Is Not a Substrate—Topoisomerase I from *E. coli* catalyzes the relaxation of negatively supercoiled DNA but cannot use positively supercoiled substrates (7), whereas both kinds of DNA are relaxed with comparable efficiency by eukaryotic enzymes. The topoisomerase I from *M. smegmatis* was tested for its ability to remove positive supercoils, by comparing its relaxation activity with that of the *E. coli* and calf thymus enzymes. Positively supercoiled DNA was prepared by incubating relaxed DNA with 2 μg/ml ethidium bromide. This concentration is not inhibitory to the relaxation activity of the enzymes used. If the topoisomerase relaxes positive supercoils induced by ethidium bromide, the DNA would acquire negative supercoils after removal of the dye. On the other hand, if it failed to act on the positive supercoils, the product DNA would not show any topological changes following extraction of the dye molecule. This result shows that positive supercoiled DNA is not a substrate for the *M. smegmatis* topoisomerase I (Fig. 3*a, lanes 5* and *6*), unlike the calf thymus enzyme (*lanes 7* and *8*).

Catenation Reaction of Topoisomerase I—Prokaryotic topoisomerase I is known to catenate and knot single-stranded circular DNA (27, 28). Fig. 3*b*, shows the result of the catenation assay. M13 single-stranded DNA was used as the substrate. Catenated structures of higher molecular weight were observed at 52 °C (*lanes 5* and *6*). The catenanes were not detectable when the DNA was incubated with the buffer also at 52 °C (*lane 2*) or when the enzymatic reaction was carried out at 37 °C (*lanes 3* and *4*). Knotting assay was also performed with M13 single-stranded DNA as substrate, which led to the appearance of faster moving knotted molecules (data not shown).

Analysis of the Processive Behavior of the Enzyme Employing Two Substrates—The enzymes which operate on DNA exhibit two different modes of interaction, distributive and processive. Appearance of a large number of partially relaxed topoisomers before fully relaxed product in a typical topoisomerase I reaction, could be considered as distributive. In contrast, appear-

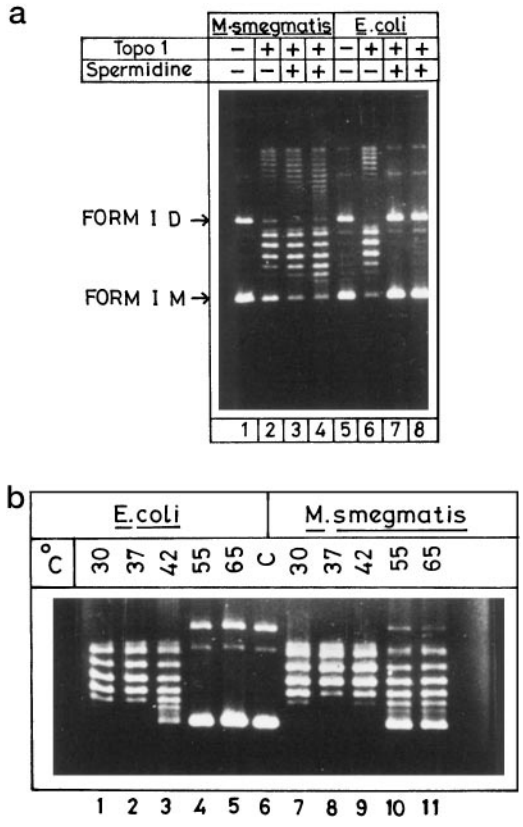

FIG. 2. **Properties of *M. smegmatis* topoisomerase I.** *a*, effect of spermidine. Topoisomerase I from *M. smegmatis* and *E. coli* (2 units each) were assayed for relaxation activity with 500 ng of supercoiled plasmid as substrate. *Lanes 1* and *5*, DNA alone; 0.5 mM (*lanes 3* and *7*) and 1 mM (*lanes 4* and *8*) spermidine incubated with enzyme. *M* and *D* represent the monomeric and dimeric forms of the supercoiled plasmid, respectively. *b*, thermal stability of the *M. smegmatis* topoisomerase I. Relaxation assay was carried out at the indicated temperatures for both *E. coli* (*lanes 1–5*) and *M. smegmatis* (*lanes 7–11*) topoisomerase I. *C* indicates the control reaction where the supercoiled DNA has been incubated at 65 °C for 30 min (*lane 6*).

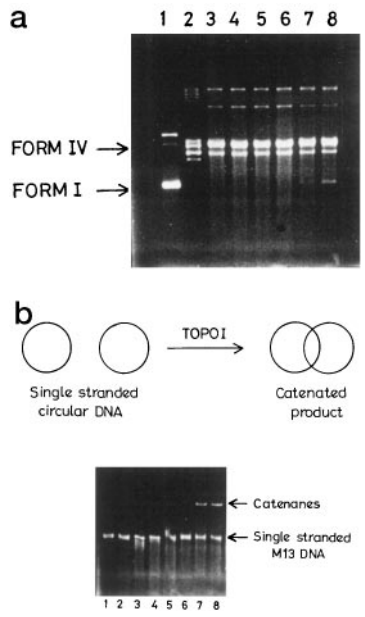

FIG. 3. *a,* relaxation of negatively and positively supercoiled DNA. 500 ng of negatively supercoiled DNA (Form I) (*lane 1*), was relaxed by 1 unit of *M. smegmatis* topoisomerase I in presence of 2 μg/ml ethidium bromide (*lane 2*). 1 μg of relaxed pUC19 DNA (Form IV) was used as substrate in *lanes 3–8*. All the assays mixtures contained 2 μg/ml ethidium bromide, with 1 and 2 units of *E. coli* (*lanes 3* and *4*), *M. smegmatis* (*lanes 5* and *6*), and calf thymus topoisomerase I (*lanes 7* and *8*), respectively. *b*, catenation assay with single-stranded M13 DNA. M13 single-stranded DNA (*lane 1*) was incubated with the assay buffer in absence of topoisomerase I at 52 °C (*lane 2*). The substrate was incubated with 2, 4, and 6 units of enzyme at 37 °C (*lanes 3, 4*, and *5*) and at 52 °C (*lanes 6, 7*, and *8*), respectively. The appearance of catenanes are indicated by *arrow* (*lanes 7* and *8*).

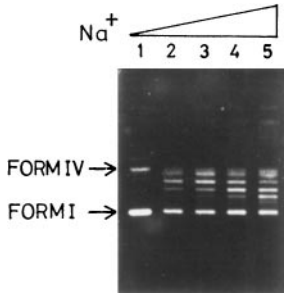

FIG. 4. **Effect of cations on processivity of the enzyme.** Relaxation assays were performed with 500 ng of pUC19 supercoiled DNA (*lane 1*) and 1 unit of enzyme (*lanes 2–5*) in the presence of 0 mM (*lane 2*), 10 mM (*lane 3*), 25 mM (*lane 4*), and 50 mM (*lane 5*) NaCl. All the reaction mixtures contained 2 mM $MgCl_2$.

ance of fully relaxed product during the course of the reaction while some supercoiled substrate still remains, indicates a processive mode (2, 29). The effect of NaCl on the mode of relaxation of *M. smegmatis* topoisomerase I is presented in Fig. 4. The enzyme shows a predominantly processive mode in the absence of monovalent salts and gradually shifts to a distributive mode with the increase in the salt concentration (Fig. 4, *lanes 2–5*). The results were similar when the influence of Mg^{2+} concentration on the relaxation activity of the enzyme is studied. With the increase in Mg^{2+} ion concentration there is an increase in the number of partially relaxed topoisomers, which reflects a distributive mode of action by the enzyme (not shown).

The effect of Mg^{2+} in changing topoisomerase I reaction from processive to distributive mode was demonstrated by employing two plasmid substrates. The assay is initiated with one of the supercoiled DNA substrates. The other substrate was added during the course of the reaction as shown in the scheme (Fig. 5*a*). In presence of higher concentration of Mg^{2+} (5–7.5 mM) the enzyme shifts more efficiently to the second substrate indicating a distributive mode of relaxation (Fig. 5, *b* and *c*). The enzyme does not discriminate either of the plasmids as both serve as efficient substrate when incubated together (not shown). Furthermore, the distributive behavior is observed irrespective of the order of addition of the plasmids (Fig. 5, *b* and *c*).

Analysis of the Reaction Intermediate—DNA topoisomerases have been shown to cleave the DNA phosphodiester bond and simultaneously get covalently linked to DNA at the cleavage site via a phosphotyrosine linkage (13). The formation of the covalent complex can be assessed by trapping the reaction intermediate followed by hydrolyzing the labeled DNA. This would result in transfer of the label in the nucleotide to protein (18). The active site residue involved in the protein-DNA linkage can then be identified, upon hydrolysis of the protein. Nick-translated, denatured pUC19 DNA was incubated with *M. smegmatis* topoisomerase I and the DNA-protein covalent intermediate was trapped by denaturing the protein with alkali. The DNA part of the complex was removed with micrococcal nuclease treatment, so that only the covalently linked nucleotide is attached to the protein (Fig. 6*a*). The partially purified *M. tuberculosis* topoisomerase I was subjected to such an analysis. The topoisomerase from both species have similar size and are larger than *E. coli* topoisomerase I. To identify the

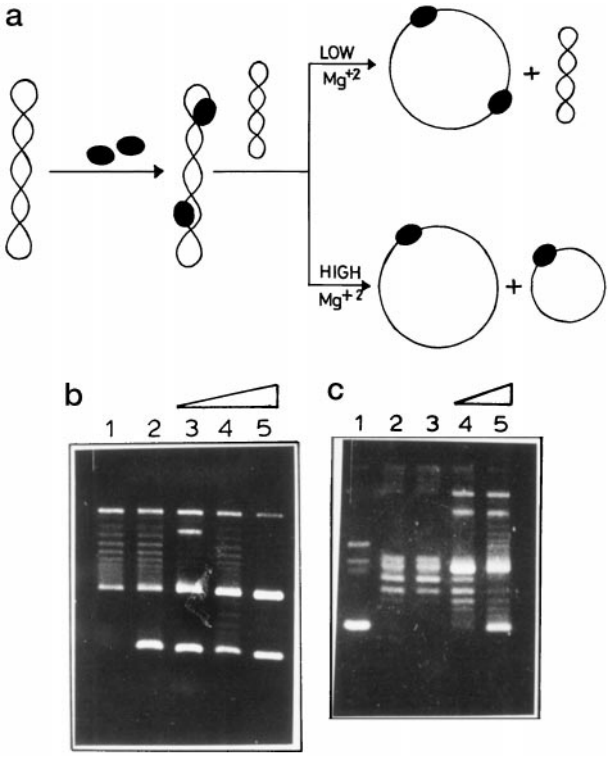

FIG. 5. **Enzyme switches over to distributive mode at high Mg^{2+} ion concentration.** *a*, schematic diagram of the experiment. *b*, relaxation reaction is carried out with plasmid pBR322 in presence of 2, 5, 7.5, and 10 mM $MgCl_2$ (*lanes 2, 3, 4*, and *5*) for 10 min with limiting enzyme concentration (0.2 unit), followed by the addition of plasmid pUC19. *Lane 1* shows relaxation of pBR322 DNA in the presence 2 mM $MgCl_2$ for 10 min. *c*, relaxation assay was performed with pUC19 DNA in absence of Mg^{2+} (*lane 1*), or in the presence of 5 and 7.5 mM Mg^{2+}, respectively (*lanes 2* and *3*). The second substrate, pBR322, was added to the reaction mixture after 10 min in the presence of 5 mM (*lane 4*) and 7.5 mM (*lane 5*) $MgCl_2$.

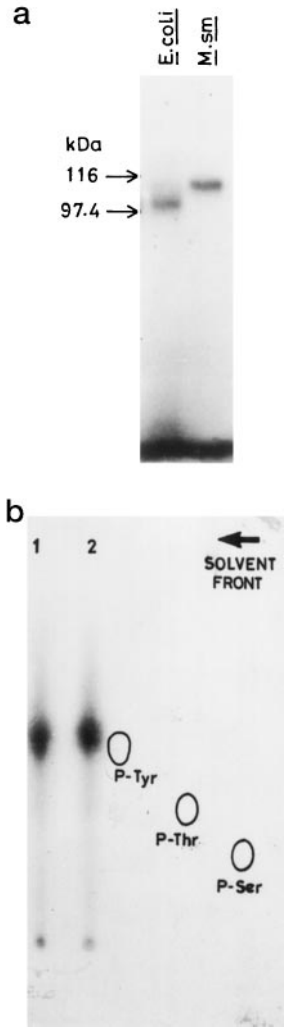

FIG. 6. **Identification of the active site residue.** *a*, DNA-topoisomerase I covalent complex was arrested for both *E. coli* and *M. smegmatis* enzymes. *Arrows* indicate the position of the molecular weight markers. *b*, the DNA-enzyme complexes for both *E. coli* (*lane 1*) and *M. smegmatis* (*lane 2*) were digested with proteinase K and then hydrolyzed. The resulting hydrolysates were analyzed by thin layer chromatography and subsequently stained and autoradiographed. The *circles* show the migration profile of the cold phosphoamino acid markers.

amino acid residue involved in the phosphoester bond formation with the DNA, the radiolabeled proteins (Fig. 6*a*) were processed further as described under "Experimental Procedures." The active site residue was identified by autoradiography of the thin layer chromatographic plate. Under our experimental conditions, the radiolabeled amino acids from *E. coli* and mycobacterial enzymes migrate at the same position (Fig. 6*b, lanes 1* and *2*). This result indicates that as in the case of other topoisomerases, *M. smegmatis* enzyme also has a tyrosine at the active site.

The covalent complex formation described above could be mediated either by the 3′- or 5′-end of nicked DNA. All the known prokaryotic type I enzymes make 5′ covalent contacts while eukaryotic type I enzymes make 3′ covalent linkage (19, 13). The experimental strategy to determine the linkage of *M. smegmatis* enzyme is depicted in Fig. 7*a*. Determination of the linkage could be established by employing DNA substrates labeled either at 5′-end or 3′-end. Most of the radiolabel DNA-protein complex was precipitated with an increase in enzyme concentration when the 3′-end labeled DNA substrates were used. Proteinase K treatment of the reaction mixtures reduces the precipitable counts, indicating that recoverable radioactivity is due to protein mediated complex formation. When 5′-end labeled DNA substrates were used, no appreciable complex formation was observed (Fig. 7*b*). These results demonstrate that covalent complex formation is mediated through the 5′-end of the nicked DNA.

Cysteines Are Not Involved in DNA Binding—E. coli topoisomerase I has zinc-finger structures at the C-terminal domain of the enzyme which participates in DNA binding and cleavable complex formation (30). This zinc coordination is via a cluster of 12 cysteine residues. When these cysteine residues are modified, the enzyme loses its relaxation activity (31). To determine whether cysteine residues have any role in the enzymatic activity of the *M. smegmatis* enzyme, two cysteine modifiers, NEM and MMTS, were used. NEM, a comparatively bulky reagent irreversibly alkylates thiols. MMTS, on the other hand brings about minimal steric change on modification and this alkylation can be reversed with DTT (32). *M. smegmatis* enzyme was not inhibited by either of the modifiers, while the *E. coli* enzyme was inhibited by MMTS but not NEM (Fig. 8*a*). The topoisomerase I from *M. smegmatis* could be resistant to inhibition due to the inaccessibility of the ligands to cysteines in the native form of the enzyme. To test this possibility, both *M. smegmatis* and *E. coli* enzymes were denatured with 6 M guanidium HCl and treated with either NEM or MMTS. 5,5′-Dithio(nitrobenzoic acid) estimation for free thiols was carried out to confirm complete modification. These modified proteins were then renatured slowly to their native forms in the presence or absence of DTT. DTT can relieve the MMTS modifica-

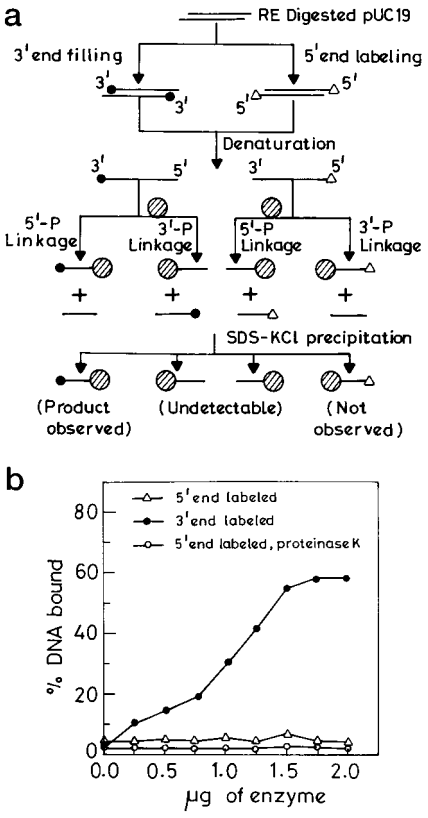

FIG. 7. **The enzyme makes covalent linkage with the 5′-end of the nick.** *a*, the experimental strategy. *b*, 5′- (Δ) and 3′- (●) end labeled fragments were complexed with protein and the complex was precipitated as described under "Experimental Procedures." Proteinase K treatment of the 3′-end labeled-DNA-topoisomerase I complex and subsequent KCl-SDS precipitation cannot recover any radioactivity (0).

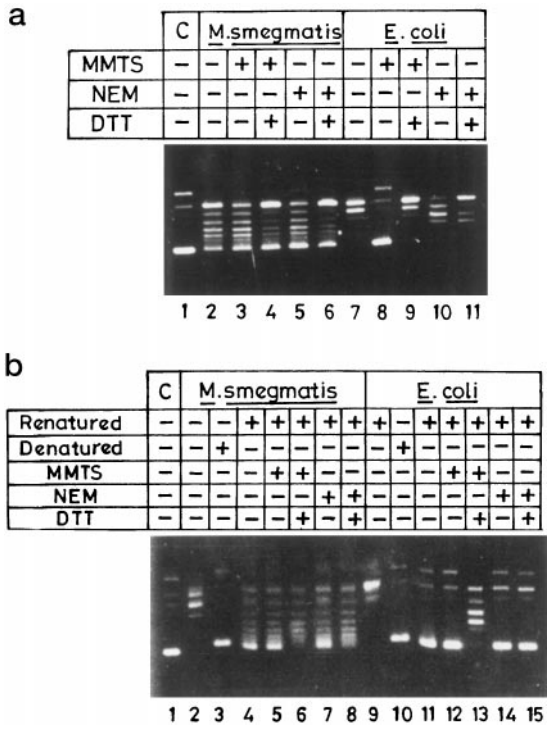

FIG. 8. **Effect of MMTS and NEM on topoisomerase activity.** *a*, MMTS and NEM inhibition of the *M. smegmatis* (*lanes 2–6*) and *E. coli* topoisomerase I (*lanes 7–11*) DNA relaxation activity. *Lanes 2* and *7*, no inhibitor; *lanes 3* and *8*, 100 μM MMTS; *lanes 4* and *9*, same as *lanes 3* and *8*, in the presence of 15 mM DTT; *lanes 5, 6* and *10, 11,* 1 mM NEM; in the absence (*lanes 5* and *10*) or presence (*lanes 6* and *11*) of 15 mM DTT. *C* indicates the control assay where no enzyme was added (*lane 1*). *b*, modification of cysteine residues on denatured protein. Both *M. smegmatis* and *E. coli* enzymes were denatured with 6 M guanidium HCl (*lanes 3* and *10*) and renatured as mentioned under "Experimental Procedures" (*lanes 4* and *9*). The denatured *M. smegmatis* and *E. coli* enzymes were treated with 100 μM MMTS (*lanes 5, 6, 12,* and *13*) and 1 mM NEM (*lanes 7, 8, 14,* and *15*). 15 mM DTT is present in *lanes 6, 8, 13,* and *15*. All the modified fractions were renatured by extensive dialysis. *E. coli* enzyme does not regain the activity when dialyzed in the absence of Zn^{2+} (*lane 11*). *Lane* 1 is the substrate pUC19 DNA and *lane 2*, activity of the native *M. smegmatis* enzyme.

tion but not the cysteine-NEM adduct (32). Enzyme from all the steps were assayed for the relaxation activity (Fig. 8*b*). The *M. smegmatis* topoisomerase I is not inhibited by either of the modifiers. On the other hand, *E. coli* topoisomerase I was inhibited by both the modifiers and DTT treatment could restore the activity only in the case of MMTS modification (*lane 13*). Ellman assay (20, 21) for free thiols was carried out to verify the complete modification of cysteines by MMTS and NEM in both the proteins. These results strongly suggest that cysteine residues do not contribute to *M. smegmatis* topoisomerase I activity. The primary amino acid sequence comparison of *E. coli* (25) and *M. tuberculosis* (33) enzymes reveal a difference in number and distribution of cysteine residues. While 12 cysteine residues (out of 14) form 3 clusters of cysteine (Fig. 9), none of those 12 residues are represented in mycobacterial enzyme. Only 5 cysteine residues found in the sequence are distributed at different regions of 900 amino acid protein. The Ellman assay (20) was used for the quantification of free thiols of topoisomerase I from *M. smegmatis* employing the standard assay conditions (21). Based on three independent measurements (Table II), we estimate that five cysteines are present per molecule of topoisomerase I of *M. smegmatis*. These results are in agreement with the number of cysteine residues found in the sequence of *M. tuberculosis* enzyme. Next, the histidine modifier diethyl pyrocarbonate was used to determine whether histidines could contribute for DNA binding. These set of experiments also show that histidines do not play a role in binding as DNA relaxation activity was not affected (data not shown). No detectable signal was observed when the binding of Zn^{2+} was directly assessed by zinc blotting using radioactive Zn^{2+} (36).

Stimulation of DNA Relaxation Activity by Single Strand-binding Protein—Type I topoisomerases from prokaryotes are known to prefer single-stranded regions in DNA as substrates. SSB binds and stabilizes the single-stranded region in a duplex DNA. Thus it is conceivable that SSB could influence topoisomerase activity by stabilizing single-stranded regions in double-stranded DNA. SSB was found to stimulate the relaxation activity of the enzyme (data not shown). SSB was either preincubated with the supercoiled DNA prior to the addition of topoisomerase I or co-incubated with enzyme and DNA. In both cases there was a stimulation in the relaxation activity of the enzyme. When DNA:SSB molar ratio was increased to 1:8 there was an inhibition of the enzyme activity (not shown).

DISCUSSION

In this article, we describe the detailed characterization of topoisomerase I from *M. smegmatis.* Other than *E. coli* topoisomerase I which has been studied extensively with regard to its structure, function, and mechanistics, detailed analysis from other prokaryotic systems is lacking (13). Thus the results presented here constitute an important study, also considering the medical importance of genus mycobacteria.

Although chromatographic behavior of the topoisomerase I from *M. smegmatis* is similar to the one from *E. coli*, the enzyme is considerably larger. The N-terminal sequence of the two enzymes are also different. Further comparison of the

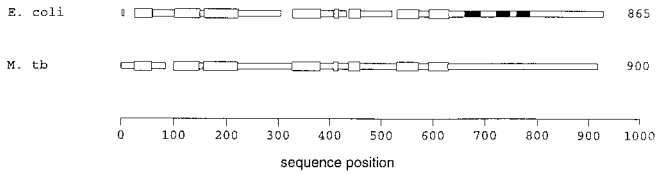

FIG. 9. **Alignment of amino acid sequences of *E. coli* and *M. tuberculosis* topoisomerase I.** Schematic representation of *E. coli* and *M. tuberculosis* topoisomerase I. *Open boxes* show the conserved regions in the two proteins. *Filled boxes* indicate the three cysteine clusters in *E.* coli topoisomerase I.

TABLE II
Estimation of Cysteine residues

Protein	Number of amino acids	Number of cysteines	Cysteines determined[a]	Reference
Hen egg lysozyme	129	8	7.7	34
C protein	140	3	2.8	35
Topoisomerase I *E. coli*	864	14	14.3	25
Topoisomerase I *M. smegmatis*			5.1	

[a] The values are based on three independent measurements.

primary sequence of the two proteins would require the molecular characterization of the gene for topoisomerase I from *M. smegmatis*. In our assessment of various biochemical properties and reactions of the enzyme, we have compared the results with the *E. coli* enzyme, arguably the prototype molecule. In many respects, the enzyme is similar to topoisomerase I from *E. coli*: 1) DNA relaxation, catenation, and knotting reactions; 2) absolute requirement for Mg^{2+} ion; 3) covalent linkage to the 5′-end of the nicked DNA; 4) tyrosine at the active site etc. The mycobacterial enzyme, however, displays certain different characteristics with respect to: 1) pH optima (17); 2) thermal stability; 3) effect of spermidine on the enzyme activity; and 4) role of cysteines in catalysis.

A rather surprising feature of the mycobacterial enzyme is its stability. The purified enzyme is active on long periods of storage and over broad pH range. Higher thermostability of the enzyme could as well be a reflection of the retention of activity over long duration on storage. Similar behavior is observed with partially purified topoisomerase I from *M. tuberculosis* H37Ra. We have considered the following points to explain some of these attributes of the enzyme. Many mycobacterial species live and survive in hostile environment within the macrophages of the host. Furthermore, free living mycobacteria exhibit relatively slow growth rates. The cell division time ranges from a few hours to 24 h in different species (37). Studies have shown that metabolic processes are slow in this group of organisms with lower rates of reactions (38). Thus, once synthesized, it is important that the key enzymes are relatively stable over a long period of time in this group of bacteria.

Another unexpected outcome of our analysis of the enzyme is that cysteines do not have a role in DNA relaxation activity. This is in contrast to the *E. coli* topoisomerase I, wherein 12 cysteine residues coordinate three Zn^{2+} atoms to form zinc-finger like structures involved in DNA binding. The derived amino acid sequence of topoisomerase I from *M. tuberculosis* (33) does not have a cysteine cluster found in *E. coli* (Fig. 9). Five cysteine residues are found in the entire sequence of *M. tuberculosis* enzyme. *M. smegmatis* topoisomerase I also contains only 5 cysteines based on our cysteine estimation results. We have considered the possibility that coordination could be through histidines in the mycobacterial enzyme. Our histidine modification experiments rule out a role for histidine as well. Moreover, Zn^{2+} blotting studies using radioactive Zn^{2+} do not show binding of zinc atoms to topoisomerase I from *M. smegmatis* or *M. tuberculosis.* Furthermore, *o*-phenanthroline, a known zinc chelator also does not inhibit the enzyme activity. Thus it seems likely that the binding to DNA could be mediated by some other structural motif in the protein. This particular difference in DNA binding characteristic, among other differences between the enzymes, form the basis to identify inhibitors of the enzyme activity.

Many DNA transaction enzymes function in two modes, processive or distributive. For example, DNA polymerase III (or DNA polymerase δ in eukaryotes) is a highly processive enzyme where as polymerase II (or DNA polymerase β in eukaryotes) functions distributively (39). Here we show that, depending on the cationic environment, mycobacterial topoisomerase I can switch from one mode to the other. We have assessed this characteristic by using two plasmids of different size as substrates. High concentration of Mg^{2+} could decrease the affinity of the enzyme to the substrate, thereby facilitating the "fall off" the enzyme from the initial plasmid. Currently no estimates of free Mg^{2+} in mycobacteria is available. Given the concentration of intracellular free Mg^{2+} in *E. coli* to be 1–2 mM (40) and if this applies to other bacteria as well, it is likely that the enzyme predominantly exhibits processive behavior *in vivo*. The ability of the enzyme to switch from one mode to the other in intracellular milieu may as well be an important characteristic.

One of the significant observations reported in this article is stimulation of enzyme activity by SSB. Although topoisomerase I is known to prefer single-stranded DNA, the natural substrate is the supercoiled form which is predominantly double-stranded. SSB, possibly by virtue of its ability to stabilize the single-stranded regions in DNA, stimulates the enzyme activity. Support for this functional cooperation between topoisomerase I and SSB also comes from studies where an SSB allele is shown to influence DNA supercoiling levels in the cells (41). One would expect increased local concentration of both topoisomerase I and SSB at regions of DNA actively involved in replication process. More recently SSB has been implicated in other important biological processes. *E. coli* SSB is shown to have a specific role in transcription activation of bacteriophage N4 promoters (42, 43). The protein could also have a role in influencing uracil DNA glycosylase-mediated excision reactions (44). The stimulation of topoisomerase I relaxation activity could be yet another function of SSB *in vivo*. It is likely that some of the other DNA transaction proteins could be involved in functional cooperation with topoisomerases.

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