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DNA topoisomerase I from *Mycobacterium smegmatis*

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DNA topoisomerase I has been purified from *Mycobacterium smegmatis* to near homogeneity using different column chromatographic techniques. The enzyme activity relaxes form I DNA into form IV DNA, requiring Mg^{2+}, but not ATP or any other cofactors for its activity. Several properties of the enzyme were found to be similar to that of the prototype enzyme, *Escherichia coli* topoisomerase I.

DNA topoisomerases are a class of enzymes that catalyse the interconversion of DNA topoisomers by concerted breaking and rejoining of phosphodiester bonds[1]. They influence a variety of vital cellular processes such as replication[2], gene expression[3], transposition[4], recombination[5], segregation and partitioning of daughter chromosomes[6] by modulating the three dimensional structure of DNA. These enzymes are essentially of two types: type I and type II. Type I topoisomerases catalyse reversible breakage and rejoining of one strand of DNA in the absence of any energy-donating cofactor(s), changing the linking number in multiples of one. Type II topoisomerases require ATP and catalyse the formation of transient double-stranded breaks, changing the linking number in steps of two. Type II topoisomerases are structurally and evolutionarily related as evident from the amino acid sequence comparison of the enzyme from both prokaryotic and eukaryotic systems[7]. In contrast, the bacterial and eukaryotic type I topoisomerases are clearly distinct with respect to their structure and function. *E. coli* topoisomerase I[8] is the prototype prokaryotic enzyme as it has been studied extensively. Topoisomerase I has also been purified from *Micrococcus luteus*[9] and *Diplococcus pneumoniae*[10], and they have properties similar to the *E. coli* enzyme.

Tuberculosis and leprosy caused by *Mycobacterium tuberculosis* and *Mycobacterium leprae* respectively have continued to be major health problems in developing countries[11]. Apart from these organisms, several opportunistic pathogens from *Mycobacteria* have been reported in immunocompromised hosts. With the resurgence of tuberculosis in developed countries, the problem has attained global dimension[11]. Further, alarming increase in resistance to frontline antitubercular drugs such as rifampicin, isoniazid, streptomycin, pyrazinamide etc. has been reported[12]. Thus, it has become important to develop new drugs to combat emerging multidrug resistant strains. Identification of suitable molecular targets to develop new antimicrobials is an important step in this direction. Presently, DNA topoisomerases are being considered as ideal candidates due to the wealth of information available on topoisomerase poisons[13].

We have initiated studies on DNA topoisomerases from *Mycobacteria*. Recently we have cloned DNA *gyr* A and *gyr* B genes encoding DNA gyrase A and B subunits of *M. tuberculosis*[14] and *M. smegmatis* (manuscript in preparation). Here we report our initial studies on DNA topoisomerase I from *M. smegmatis*.

Materials and Methods

Chemicals—Agarose, ethidium bromide, camptothecin and other chemicals were purchased from Sigma Chemical Company. DEAE sephacel and heparin sepharose were purchased from Pharmacia Ltd. Hydroxylapatite (Biogel HTP) was obtained from Bio-Rad Laboratories and Phosphocellulose (P11) from Whatman.

Cells—Mycobacterium smegmatis SN2, was grown in Youmans-Karlson medium as described earlier[15] for 12-14 hrs. Cells were harvested and washed with buffer A (50 m*M* Tris-HCl, *p*H 8.0, 30 m*M* NaCl) and stored at −70°C until use.

Purification Procedure

(*A*) *Preparation of crude extract*—The cells were sonicated and the crude extract was centrifuged at 18,000 r.p.m. for 1 hr. The S20 extract thus obtained was further processed by centrifugation at 40,000 r.p.m. in a Beckman type 50 Ti rotor. This S100

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supernatant was brought to 1% final concentration of polyethyleneimine (PEI). The PEI supernatant was subjected to a 0-67% ammonium sulphate fractionation. The pellet was dissolved in buffer B (50 m*M* KPO_4, *p*H 7.4, 1 m*M* EDTA, 5% glycerol, 10 m*M* beta-mercaptoethanol (β-ME), 0.1 m*M* phenyl methyl sulfonyl fluoride (PMSF) and 50 m*M* KCl)-(fraction I).

(*B*) *Phosphocellulose chromatography*—Fraction I (100 ml) was loaded onto a phosphocellulose column (60 ml). The column was eluted with a linear gradient of buffer B having 50 m*M* to 1 *M* KCl. DNA topoisomerase I was eluted at 450 m*M* to 650 m*M* KCl concentration. The most active fractions were pooled and dialysed against buffer B for 3 hrs (fraction II).

(*C*) *DEAE sephacel chromatography*—Fraction II (25 ml) was loaded onto a DEAE sephacel column (35 ml). The column was eluted with a linear gradient of buffer B having 50 m*M* to 800 m*M* KCl. The active fractions corresponding to 0.3-0.4 *M* KCl were pooled and dialysed against buffer C (50 m*M* KPO_4, *p*H 7.4, 1 m*M* EDTA, 5% glycerol 10 m*M* β-ME, 0.1 m*M* PMSF) for 3 hrs to obtain fraction III.

(*D*) *Hydroxylapatite column chromatography*—Fraction III (6 ml) was loaded onto a hydroxylapatite column (5 ml) previously equilibrated with buffer C. The column was eluted with a linear gradient of buffer C having 50 m*M* to 750 m*M* potassium phosphate, *p*H 7.4. The active fractions eluted between 0.3-0.4 *M* KPO_4 were pooled and dialysed against buffer B (fraction IV).

(*E*) *Heparin sepharose chromatography*—Fraction IV was purified from this column using a linear gradient of buffer B having 200 m*M* to 1 *M* KCl. The active fractions eluted between 650 to 750 m*M* KCl were pooled and dialysed against buffer D (20 m*M* KPO_4, *p*H 7.4, 1 m*M* EDTA, 50% glycerol, 10 m*M* β-ME, 0.1 m*M* PMSF) for 3 hrs. The enzyme was stored at −20°C (fraction V).

Topoisomerase I assay—The standard topoisomerase assay mixture contained in a final volume of 20 µl: 40 m*M* Tris-HCl, *p*H 8.0, 5 m*M* $MgCl_2$, 20 m*M* NaCl, 1 m*M* EDTA, 50 µg/ml BSA, 500 ng of pUC19 DNA and partially purified enzyme. Reactions were incubated at 37°C for 30 min, stopped by adding a 1 × stop buffer (0.4% SDS, 8% Ficoll, 0.6% bromophenol blue) and incubating at 65°C for 10 min. Samples were subjected to agarose gel electrophoresis, photographed and then scanned using a gel documentation system to quantitate the percentage conversion of supercoiled DNA (Form I). The percentage decrease in the band intensity of the supercoiled DNA reflects the extent of conversion of Form I DNA into different relaxed topoisomers. One unit 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.

Results and Discussion

Purification of topoisomerase I activity

The DNA topoisomerase I activity was assayed by separating the topoisomers of the plasmid DNA on agarose gels. DNA relaxation activity from both *M. smegmatis* and *M. tuberculosis* could be detected in crude extracts itself. Topoisomerase I activity was purified from *M. smegmatis* cells using successive steps of column chromatography. The ammonium sulphate fraction (fraction I) was passed through a phosphocellulose column. The eluted fractions from the column were assayed and the results are shown in Fig. 1A. The active fraction were then purified using DEAE sephacel, hydroxylapatite and heparin sepharose columns. The protein content and activity profile of eluant from the final column are represented

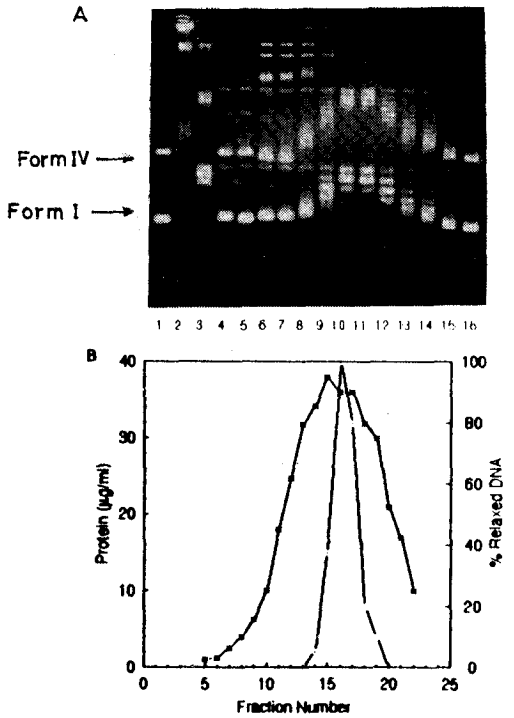

Fig. 1—Purification of topoisomerase I. (A): Agarose gel electrophoresis of phosphocellulose column fractions. [Lane 1, pUC19 without added protein; lane 2, S100 extract; lane 3, ammonium sulphate fraction; lanes 4-16, alternate fractions starting from fraction number 10. DNA bands on top half of the gel are due to the presence of pUC19 dimer and the resultant topoisomers. (B): Activity (---) and protein profile (-x-) from heparin sepharose chromatography]

in Fig. 1B. The active preparations were devoid of contaminating nuclease activity and hence found to be suitable for further characterziation of the enzyme.

Properties of *M. smegmatis* topoisomerase I

The eluant from heparin sepharose column (fraction V) showed a single major band on non denaturing as well as denaturing polyacrylamide gels (not shown). This preparation was used for studying the different properties of the enzyme. Under standard assay conditions employed, the enzyme activity was linear for 2 hrs (Fig. 2). The dilute preparations, however, do not show activity over a prolonged period of incubation. Moreover the enzyme activity was found to be proportional to the enzyme concentration used. The DNA relaxation activity of topoisomerase I was observed over a wide range of *p*H. However, higher activity was obtained between *p*H 7.0 and 8.5 (Fig. 3). The maximum activity on a wide *p*H range suggests that the enzyme is stable and the active site is not affected under varied ionic environment.

Requirement for Mg^{2+}

Several DNA binding proteins and enzymes involved in DNA metabolism are known to require Mg^{2+} for their activity. It has been shown that Mg^{2+} is essential for optimal relaxation activity of *E. coli* topoisomerase I[8]. On the other hand, eukaryotic topoisomerase I such as yeast TopA does not require Mg^{2+} for relaxation activity. We have examined Mg^{2+} dependence of *M. smegmatis* topoisomerase I. Results are shown in Fig. 4A. *M. smegmatis* topoisomerase I was found to be dependent on exogenously added Mg^{2+} ions. In the absence of Mg^{2+}

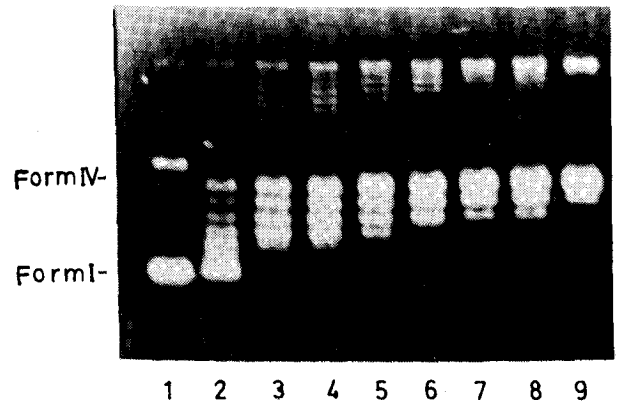

Fig. 2—Time course of topoisomerase I activity. [The standard topoisomerase I assay was performed as described in Materials and Methods. The assay mixtures were incubated for 0, 5, 10, 15, 30, 60, 90, 120 and 150 min in lanes 1-9 respectively]

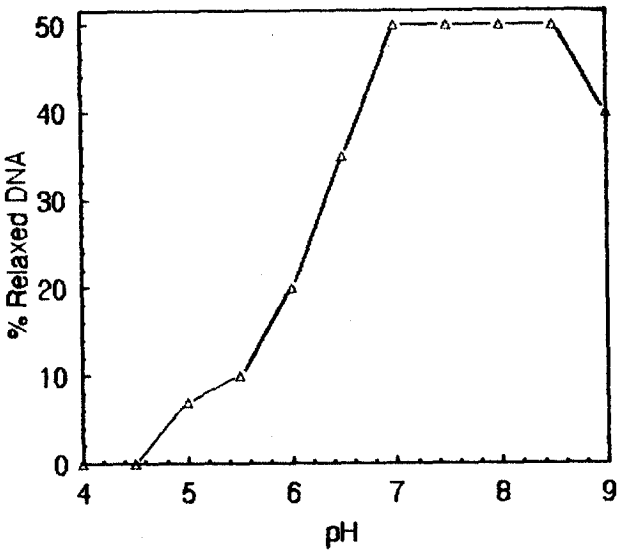

Fig. 3—Effect of *p*H on enzyme activity. [The enzyme was incubated in sodium acetate buffer (*p*H 4-5), MOPS buffer (*p*H 6-6.5), Tris-HCl buffer (*p*H 7-9) along with rest of the components and processed as described in Materials and Methods]

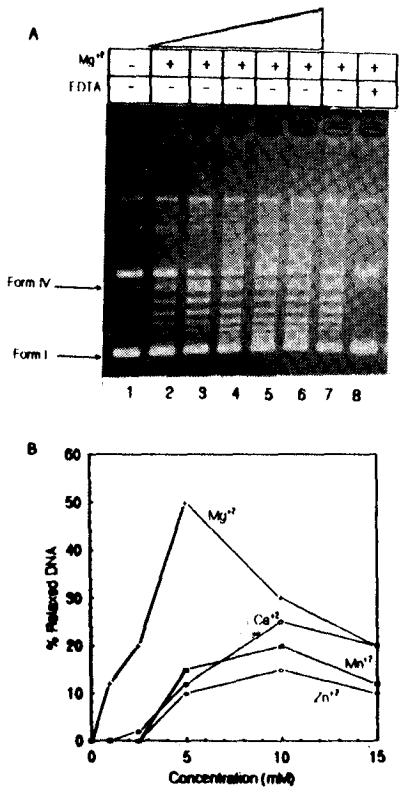

Fig. 4—Requirement for Mg^{2+} and other divalent cations. (A): Mg^{2+} dependance of topoisomerase I activity. [Lane 1, no Mg^{2+}; lanes 2-6, 2.5 m*M*, 5 m*M*, 7.5 m*M*, 10 m*M*, 15 m*M* respectively; lane 7, same as lane 3; lane 8, 10 m*M* EDTA in presence of 5 m*M* Mg^{2+}. (B): Enzyme was incubated in a standard assay mixture in presence of indicated concentration of divalent cations]

ions or in the presence of excess EDTA, no activity was detected. The optimal Mg^{2+} ion concentration for topoisomerase activity was about 5 m*M*. At higher concentration of $MgCl_2$, the enzyme activity was reduced (Fig. 4A,B). The influence of other divalent cations, such as Ca^{2+}, Mn^{2+} or Zn^{2+} were tested on the enzyme activity. These ions were not as effective as Mg^{2+} in supporting the reduction of negative supercoils by the enzyme (Fig. 4B). Next, the enzyme activity was assayed in total absence of Mg^{2+} or any other divalent cation, but in the presence of monovalent cation (Fig. 5A). At different concentrations of NaCl used, topoisomerase I activity was not observed. These results indicate that divalent cation requirement cannot be replaced by monovalent salts. This property of the enzyme is quite different from that of *E. coli* topoisomerase I. The divalent cation requirement can be substituted by higher concentrations of NaCl in the case of *E. coli* topoisomerase I[8].

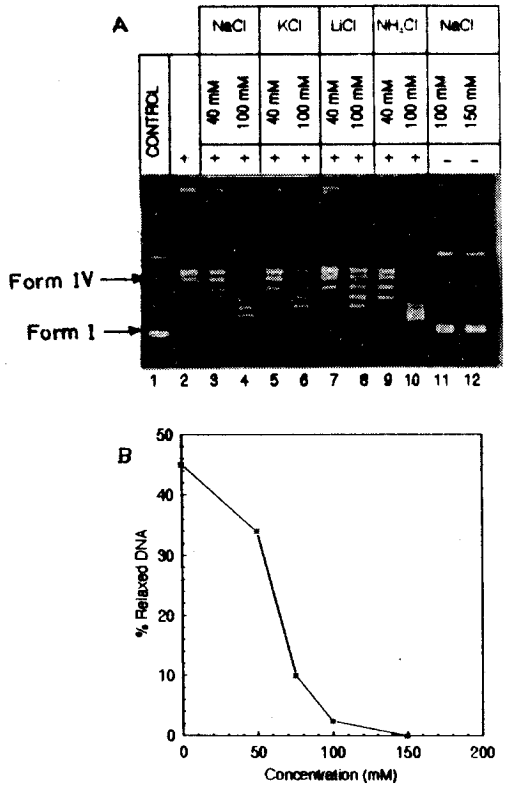

Fig. 5—Effect of monovalent cations and phosphate on enzyme activity. (A): Enzyme assay was carried out under standard conditions in presence of indicated amount of monovalent cations. [' + indicates the presence of 5 m*M* Mg^{2+} in the assay buffer. Lane 1, supercoiled control; lane 2, standard assay without monovalent cation; lanes 3-12, as labelled. (B): Indicated concentrations of phosphate in the form of potassium phosphate (*p*H 7.5) was used]

Effect of monovalent cations

Influence of different monovalent cations on topoisomerase I activity of *M. smegmatis* in presence of optimal Mg^{2+} concentration has been studied. Addition of monovalent cations (> 50 m*M*) resulted in marginal reduction in activity. Lower concentrations of NaCl has no negative influence on enzyme activity. Among the various monovalent cations tested (LiCl, NaCl, KCl and NH_4Cl) in the presence of 5 m*M* $MgCl_2$, NH_4^+ ions caused maximum inhibition (Fig. 5A). Relaxation activity of the enzyme was progressively lowered with increasing salt concentration. Enzyme activity was completely inhibited when NaCl concentration exceeded 0.3 *M*.

Cofactor requirements and effect of phospohate ion

All type I topoisomerases can relax supercoiled DNA in the absence of any cofactor, whereas type II topoisomerases require ATP[17]. *M. smegmatis* enzyme resembles a true type I enzyme in this respect. GTP, CTP and UTP also did not stimulate topoisomerase I activity.

Several enzymes are inhibited in presence of phosphate ion in the assay buffer[16]. Hence the effect of phosphate ion on the relaxation activity of the enzyme was studied. Marked inhibition of relaxation activity was observed with 100 m*M* KPO_4 concentration (Fig. 5B). Whereas when KCl is used in place of KPO_4 a comparable level of inhibition was seen only at a concentration above 300 m*M*. Thus this inhibition could be attributed to the effect of phosphate ion, as observed for many other DNA metabolising enzymes[16]. The significance of this observation is not clear at this stage as the enzyme does not require any nucleotide cofactors.

Prokaryotic type I topoisomerases differ from their eukaryotic counterparts in their inability to relax positively supercoiled DNA. The enzyme from *M. smegmatis* also failed to relax positively supercoiled DNA whereas under the same assay conditions, calf thymus DNA topoisomerase I could relax the substrate (not shown).

Effect of topoisomerase specific drugs

A large number of compounds have been identified as specific inhibitors of topoisomerases from different organisms. However, no compound has been shown to inhibit prokaryotic topoisomerase I at physiologically relevant concentrations. Table 1 shows the effect of different topoisomerase specific drugs on the topoisomerase I activity from *M. smegmatis* and *E. coli*. The compounds tested were camptothecin, oxolinic acid, norfloxacin and novobiocin. Camptothecin is a plant alkaloid which

Table 1—Effect of topoisomerase poisons on enzyme activity

Compound	Concentration	% Activity†	
		M. smegmatis	*E. coli*
Control	–	100	100
Camptothecin	50 μ*M*	100	100
	100 μ*M*	90	90
	200 μ*M*	70	75
	400 μ*M*	40	30
	500 μ*M*	10	<5
Oxolinic acid	15 μg/ml	100	100
	150 μg/ml	95	10
Norfloxacin	15 μg/ml	100	90
	150 μg/ml	60	20
Novobiocin	15 μg/ml	100	100
	150 μg/ml	100	100
	250 μg/ml	100	ND*

†The enzyme activity in control (no drug) is taken as 100% after scanning the gel (Materials and Methods).
*Not determined.

inhibits eukaryotic topoisomerase I within a concentration range of 10-50 μg/ml[18]. Oxolinic acid, norfloxacin and novobiocin are potent inhibitors of *E. coli* DNA gyrase[19]. None of these compounds inhibited the *M. smegmatis* enzyme activity significantly. Only at a very high concentration of camptothecin (500 μ*M*) and norfloxacin (150 μg/ml) was the relaxation activity partially inhibited, while oxolinic acid had no significant effect on enzyme activity. Under these conditions, the *E. coli* enzyme was inhibited to a similar extent by camptothecin but inhibition was more pronounced in presence of oxolinic acid. Further, norfloxacin which has slight inhibitory effect on topoisomerase I from *M. smegmatis* at higher concentration, also inhibited *E. coli* enzyme to a greater extent. Novobiocin had no inhibitory effect on enzymes from either sources. Thus, some of the quinolone compounds inhibit topoisomerase I activity although at a much higher concentration than that observed for DNA gyrase. Our data on inhibition of *E. coli* topoisomerase I by oxolinic acid are in agreement with earlier observations[19]. These results suggest that *in vivo*, topoisomerase I could be somewhat sensitive to quinolones and derivatives of camptothecin.

It should be mentioned here that DNA topoisomerases from both prokaryotic and eukaryotic sources are targets for several compounds which include antibiotics as well as anticancer drugs[13]. Most of these poisons are directed against either DNA gyrase in bacteria or topoisomerase II of eukaryotes. Camptothecin, a proven inhibitor of eukaryotic topoisomerase I[18], inhibits the mycobacterial enzyme at concentrations too high to be used as a drug. Nevertheless, the derivatives of the compound may have a strong inhibitory effect. Analysis of the structure and mechanism of action of topoisomerase I from mycobacteria should provide valuable information to design antimicrobial drugs.

This paper presents the first documented study on DNA topoisomerase I from this important group of bacteria. It should be noted here that as such not much information is available on topoisomerase I from prokaryotic systems except that of *E. coli*. Although similar to *E. coli* enzyme in many respects, the mycobacterial enzyme has its own characteristics with respect to *p*H, influence of cations and phosphate ion. Thus, our results would form the basis for detailed characterization of the structure and function of this vital protein from mycobacteria.

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