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. They influence a variety of vital cellular processes such as replication, gene expression, transposition, recombination, segregation and partitioning of daughter chromosomes 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. In contrast, the bacterial and eukaryotic type I topoisomerases are clearly distinct with respect to their structure and function. *E. coli* topoisomerase I is the prototype prokaryotic enzyme as it has been studied extensively. Topoisomerase I has also been purified from *Micrococcus luteus* and *Diplococcus pneumoniae*, 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. Apart from these organisms, several opportunistic pathogens from *Mycobacteria* have been reported in immuno-compromised hosts. With the resurgence of tuberculosis in developed countries, the problem has attained global dimension. Further, alarming increase in resistance to frontline antitubercular drugs such as rifampicin, isoniazid, streptomycin, pyrazinamide etc. has been reported. 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.

We have initiated studies on DNA topoisomerases from *Mycobacteria*. Recently we have cloned DNA gyrA and gyrB genes encoding DNA gyrase A and B subunits of *M. tuberculosis* 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-Karson medium as described earlier for 12-14 hrs. Cells were harvested and washed with buffer A (50 mM Tris-HCl, pH 8.0, 30 mM 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
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 mM KPO₄, pH 7.4, 1 mM EDTA, 5% glycerol, 10 mM beta-mercaptoethanol (β-ME), 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 50 mM 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 mM to 1 M KCl. DNA topoisomerase I was eluted at 450 mM to 650 mM 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 mM to 800 mM KCl. The active fractions corresponding to 0.3-0.4 M KCl were pooled and dialysed against buffer C (50 mM KPO₄, pH 7.4, 1 mM EDTA, 5% glycerol 10 mM β-ME, 0.1 mM 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 mM to 750 mM potassium phosphate, pH 7.4. The active fractions eluted between 0.3-0.4 M KPO₄ 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 mM to 1 M KCl. The active fractions eluted between 650 to 750 mM KCl were pooled and dialysed against buffer D (20 mM KPO₄, pH 7.4, 1 mM EDTA, 50% glycerol, 10 mM β-ME, 0.1 mM 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 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 20 mM NaCl, 1 mM 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 x 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
in Fig. 1B. The active preparations were devoid of contaminating nuclease activity and hence found to be suitable for further characterization 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 pH. However, higher activity was obtained between pH 7.0 and 8.5 (Fig. 3). The maximum activity on a wide pH range suggests that the enzyme is stable and the active site is not affected under varied ionic environment.

**Requirement for Mg²⁺**

Several DNA binding proteins and enzymes involved in DNA metabolism are known to require Mg²⁺ for their activity. It has been shown that Mg²⁺ is essential for optimal relaxation activity of *E. coli* topoisomerase I. On the other hand, eukaryotic topoisomerase I such as yeast TopA does not require Mg²⁺ for relaxation activity. We have examined Mg²⁺ dependence of *M. smegmatis* topoisomerase I. Results are shown in Fig. 4A. *M. smegmatis* topoisomerase I was found to be dependent on exogenously added Mg²⁺ ions. In the absence of Mg²⁺
ions or in the presence of excess EDTA, no activity was detected. The optimal Mg\(^{2+}\) ion concentration for topoisomerase activity was about 5 mM. 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.

### 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 mM) 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\(_4\)Cl) in the presence of 5 mM 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 phosphate ion

All type I topoisomerases can relax supercoiled DNA in the absence of any cofactor, whereas type II topoisomerases require ATP\(^1^7\). *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\(^1^6\). Hence the effect of phosphate ion on the relaxation activity of the enzyme was studied. Marked inhibition of relaxation activity was observed with 100 mM 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 mM. Thus this inhibition could be attributed to the effect of phosphate ion, as observed for many other DNA metabolising enzymes\(^1^6\). 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 I 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 µM         | 100        |
|             | 100 µM        | 90         |
|             | 200 µM        | 70         |
|             | 400 µM        | 40         |
|             | 500 µM        | 10 < 5     |
| Oxolinic acid| 15 µg/ml   | 100        |
|             | 150 µg/ml     | 95         |
| Norfloxacin | 15 µg/ml      | 100        |
|             | 150 µg/ml     | 60         |
| Novobiocin  | 15 µg/ml      | 100        |
|             | 150 µg/ml     | 100        |
|             | 250 µg/ml     | 100        |

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. Oxolinic acid, norfloxacin and novobiocin are potent inhibitors of E. coli DNA gyrase. None of these compounds inhibited the M. smegmatis enzyme activity significantly. Only at a very high concentration of camptothecin (500 µM) 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. 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. 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, 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 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 pH, 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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