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A complex of DNA gyrase and RNA polymerase fosters transcription in *Mycobacterium smegmatis*

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Abstract

We report here the existence of a complex between RNA polymerase (RNAP) and DNA gyrase in *Mycobacterium smegmatis*. The interaction between the two enzymes was detected during our attempts to purify DNA gyrase from *M. smegmatis*. RNAP subunits co-eluted along with DNA gyrase in two different affinity chromatography column procedures employed to purify the latter enzyme. A complex containing both the enzymes was isolated through gel filtration chromatography and sucrose density gradient centrifugation of the cell free extracts. The complex exhibited both DNA supercoiling and transcription activities. Reduction in the transcription activity of the complex in the presence of DNA gyrase inhibitor indicates a role for DNA gyrase in stimulating transcription.

Keywords: Affinity chromatography; DNA gyrase; Mass spectrometry; Mycobacteria; RNA polymerase

The type II DNA topoisomerase, DNA gyrase, is a distinct member in its family as it is the only enzyme identified so far, which can introduce negative supercoils into DNA in a reaction involving ATP hydrolysis [1]. The enzyme is ubiquitously found in all eubacteria and is obligatory for cell survival as it serves as a pivot for releasing the torsional strain during major DNA transactions such as replication and transcription. DNA gyrase also catalyzes other topological interconversions, namely catenation/decatenation, knotting/unknotting, and ATP-independent relaxation of negatively supercoiled DNA [2,3]. The enzyme is responsible for maintaining the homeostasis of DNA topology in the cell, in concert with the antagonistic and synergistic activities of other topoisomerases [4,5].

DNA gyrase from *Escherichia coli* has been extensively investigated with respect to structure, function, and reaction mechanism. The enzyme is a heterotetramer comprising of two GyrA and two GyrB subunits [3]. For molecular characterization of the enzyme from mycobacteria, we have cloned and overexpressed *gyrB* and *gyrA* genes from *Mycobacterium smegmatis* and reconstituted the enzyme activity [6]. Studies have revealed that the mycobacterial enzyme differs from the *E. coli* counterpart not only in its primary sequence [6,7] but also in several properties such as decatenation activity, susceptibility to various gyrase inhibitors, kinetics of DNA binding and ATP hydrolysis [8–10], and immunological cross-reactivity [11]. While purifying DNA gyrase from *M. smegmatis* using different approaches, few additional proteins were found to co-elute along with the purified fractions, irrespective of the purification protocol employed. Now, we demonstrate that RNA polymerase (RNAP) subunits are associated with DNA gyrase which led us to isolate the complex of the two enzymes from the cell free extracts wherein DNA gyrase stimulates transcription.

Materials and methods

DNA gyrase purification. DNA gyrase was purified from the cell free extracts of *M. smegmatis* by novobiocin-affinity chromatography,

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developed for the purification of *E. coli* DNA gyrase [8]. *M. smegmatis* SN2 cells were grown until mid-log phase and the cell pellet were resuspended in TGEM buffer (50 mM Tris–HCl, pH 7.5, 5% (v/v) glycerol, 1 mM EDTA, and 2 mM 2-mercaptoethanol). The cell suspension was then sonicated and S100 supernatant was obtained, which was fractionated using 70% ammonium sulfate. The pellet was dissolved in TGEM buffer and dialyzed against the same prior to loading onto the novobiocin–Sepharose column. The column was washed with TGEM buffer and gyrase subunits were eluted using 6 M urea.

A GyrB-specific monoclonal antibody, MsGyrB:A4, was covalently coupled to protein-A Sepharose beads and immuno-affinity purification of gyrase was performed as described previously [10]. The 30–55% ammonium sulfate fraction obtained from the S100 supernatant of *M. smegmatis* cell lysate was applied to anti-GyrB immuno-affinity column and washed extensively with the buffer containing 50 mM NaCl. To elute the GyrA subunit from the bound fraction, buffer containing 0.5 M NaCl was passed through the column. GyrB was dissociated from GyrB-antibody complex in the column by elution involving change in pH using glycine HCl (pH 2.8).

Mass spectrometry and N-terminal sequencing. For identification of the proteins co-eluted with DNA gyrase subunits during the novobiocin-affinity chromatography, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was employed. Protein bands were cut from the gel, washed thrice with 50% acetonitrile and 100 mM ammonium bicarbonate, dehydrated, and subjected to in-gel tryptic digestion at 37 °C for 12 h. Peptide masses of the digested products were obtained using Brucker Daltronics Ultraflex TOF/TOF mass spectrometer and searched against *M. smegmatis* protein sequence database in MASCOT server (www.matrixscience.com) to identify the proteins by peptide mass fingerprinting [12].

The proteins that co-purified with GyrA subunit from the immuno-affinity column were transferred onto polyvinylidene difluoride (PVDF) membrane, protein bands were excised, and detected by N-terminal sequencing.

Gel filtration analysis. Mycobacterium smegmatis SN2 cells were sonicated in TGEM buffer after three freeze-thawing cycles to prepare the cytosolic fraction (S100 supernatant) as described previously [9]. The S100 supernatant was subjected to 30–55% ammonium sulfate fractionation and the precipitate was resuspended in TEMK buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 100 mM potassium glutamate), and dialyzed against the same buffer. The dialysate was subjected to gel filtration on Sephacryl S-400 column (Amersham-Pharmacia, bed volume-124 ml), equilibrated with TEMK buffer, and the eluted fractions was analyzed by silver staining. Gel filtration fractions (1 ml each) covering the entire bed volume were collected individually after the initial void volume of 40 ml. The fractions were designated as per their elution volume and used for Western blotting (10 μl/fraction), supercoiling reactions (4 μl/fraction), and DE-81 filter-based transcription assays [13] (15 μl/fraction).

Sucrose gradient centrifugation. Ammonium sulfate fraction (30–55%) of the S100 supernatant from *M. smegmatis* SN2 was prepared in TEM buffer containing 50 mM potassium glutamate and layered on a 40 ml sucrose gradient (10–30% linear gradient with a cushion of 60% at the bottom). All sucrose solutions were also prepared in TEM buffer containing 50 mM potassium glutamate. After centrifugation at 100,000*g* (using an SW 28 rotor in a Beckman ultracentrifuge) at 4 °C for 16 h, 1 ml fractions were collected from the top of the gradient. To avoid any disturbance to the gradient while collecting fractions, fluorinert (Sigma) was passed through the inlet of the UA-6 Absorbance detector system (ISCO Inc., USA) and fractions were collected from the outlet at the flow rate of 200 μl/min. The distribution of various proteins in the fractions was then determined by Western blotting using anti-GyrA, anti-GyrB, anti-RpoC, and anti-RpoB antibodies.

Immunotechniques

Antibodies used. To detect the *M. smegmatis* gyrase subunits in immunoblotting experiments, polyclonal antibodies from the laboratory stock [11,14] recognizing both GyrA and GyrB subunits were used. Polyclonal antibodies specific for RpoB (β subunit) and RpoC (β′ subunit) of RNAP were prepared using techniques described earlier [11,14].

Immunoblotting. The proteins were resolved on SDS 8% polyacrylamide gels, transferred to nitrocellulose membrane at 200 mA for 3 h, and then probed with the anti-GyrA antibodies at 50 ng/ml concentration. The bound antibodies were detected by using donkey anti- (rabbit IgG) immunoglobulins, covalently linked to horseradish peroxidase and enhanced chemiluminescence reagents (Amersham-Pharmacia). The membrane was then stripped by incubating in buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, at 70 °C for 30 min. Subsequently, the blot was washed thrice with 1× PBST (Phosphate-buffered saline containing 0.1% Tween 20) at 15 min time intervals, following which the membrane was blocked using PBST containing 2% BSA. Re-probings were then performed with anti-GyrB, anti-RpoC, and anti-RpoB antibodies, similar to that as described above.

Supercoiling assays. Negatively supercoiled plasmid DNA was isolated from *E. coli* DH5α strain subsequent to fresh transformations, using standard methods. Relaxed circular pBR322 DNA was prepared by treating the negatively supercoiled DNA with *M. smegmatis* topoisomerase I as described [15]. Four hundred nanogram of the relaxed DNA was used as the substrate for supercoiling reactions, performed following the conditions described earlier [10]. Control supercoiling reactions were carried out using one unit of immuno-affinity purified DNA gyrase [10].

Transcription assays. In vitro [^{3}H]UMP-incorporation assays were performed following procedures described [13]. Briefly, 15 μl of the fractions was incubated for 30 min at 37 °C with 20 μg/ml of sheared salmon sperm DNA (unless otherwise indicated) in the presence of 40 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 200 μM each ATP, CTP, and GTP, 50 μM UTP, and 20 μCi/ml [^{3}H]UTP. The reactions were stopped by spotting onto DE-81 filters, presoaked with 5 mM EDTA. The dried filters were washed twice with 5% Na_2HPO_4, thrice with double-distilled water, and once with ethanol. The incorporated radioactivity in the dried filters was measured by scintillation spectrometry.

Results and discussion

DNA gyrase purification from M. smegmatis

A convenient one step method has been developed earlier to purify DNA gyrase from *E. coli* taking advantage of high affinity binding of novobiocin to the GyrB subunit of the enzyme [8]. We have adapted the procedure for purification of *M. smegmatis* DNA gyrase. Fig. 1A depicts the purification of DNA gyrase from *M. smegmatis* using novobiocin–Sepharose affinity chromatography. The method involves extensive washing with 100 mM KCl and 20 mM ATP before eluting the bound protein with 6 M urea. Few additional proteins eluted along with *M. smegmatis* GyrA and GyrB subunits, in the same fractions. Three of these co-eluting proteins were identified as the RpoC, RpoB, and RpoA (β′, β, and α subunits of RNAP, Fig. 1A) by mass spectrometric analysis (see Materials and methods), using MASCOT server (www.matrixscience.com). The probability based mowse score values obtained for RpoA and a mixture of RpoB and RpoC subunits were 67 and 113, respectively. These proteins bound to the column could be interacting with the gyrase or to the ligand covalently linked to the affinity matrix. Since β subunit also binds to ATP, the retention of RpoC and RpoB in the affinity column could be due to the binding of RpoB subunit to novobiocin, coupled to the column matrix. However, the retention of β subunit even after ATP wash of the column and its elution in the fractions

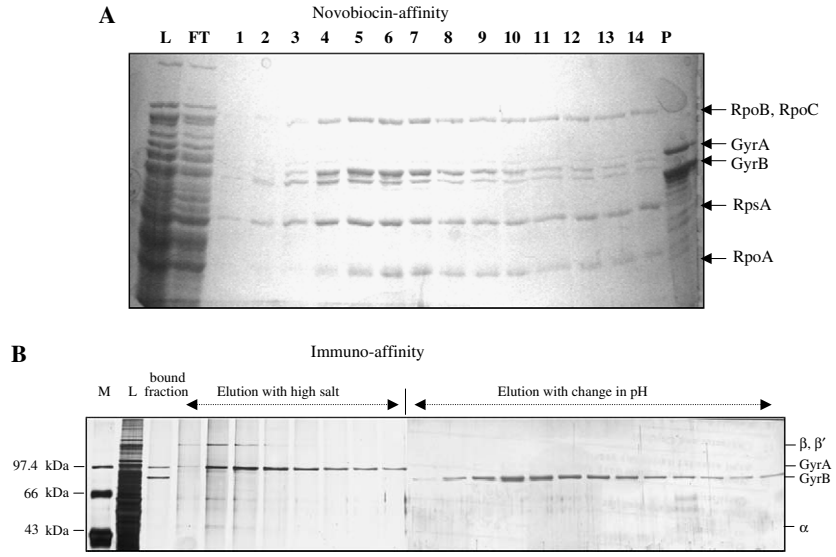

Fig. 1. DNA gyrase purification. (A) Novobiocin-affinity chromatography. Co-elution of β, β′, and α subunits of RNAP with gyrase subunits is shown on an SDS 8% polyacrylamide gel. Details of the procedure are given in Materials and methods. L denotes load to the column, FT indicates the flowthrough protein fraction from the column, P designates purified *M. smegmatis* GyrA, and GyrB used as markers. Fractions eluted using 6 M urea from the column are numbered from 1 to 14. (B) Immuno-affinity chromatography. SDS–PAGE (8%) profile of the fractions initially eluted with high salt (GyrA and RNAP subunits as indicated) and later with change in pH (GyrB) is shown. L represents load to the column and M indicates protein molecular weight markers.

containing gyrase subunits indicated the association of RNAP subunits with gyrase. To address this aspect further, immuno-affinity chromatography was employed as an alternate scheme of gyrase purification. In this latter scheme, MsGyrB: A4, an anti-GyrB monoclonal antibody, covalently coupled to Protein-A Sepharose column is used as the affinity matrix. Since the interaction between gyrase subunits is abolished at salt concentrations beyond 150 mM, GyrA subunit was eluted using high salt [10]. Subsequent to GyrA elution, GyrB was recovered from the antibody–antigen complex by a change in the pH. The data presented in Fig. 1B show the elution of proteins of sizes corresponding to those of β′, β, and α subunits of *M. smegmatis* RNAP in high salt along with GyrA. N-terminal sequencing of the co-purified proteins confirmed the authenticity of RNAP subunits. The co-elution of RNAP subunits along with GyrA in two different affinity chromatography procedures employed for gyrase purification overrules the possibility of strong interactions between novobiocin and β subunit of RNAP in the former purification.

Isolation of the complex containing both DNA gyrase and RNAP activities

The data presented above reveal that subunits of RNAP are associated with DNA gyrase in *M. smegmatis*, indicating the presence of a complex in the cell having both the enzyme activities. The results of sucrose density gradient centrifugation of the cell free extracts, carried out to investigate the existence of such a complex, are presented in Fig. 2. Immunoblotting of the fractions revealed the association of RNAP and gyrase subunits, in fractions 11–20 (Fig. 2).

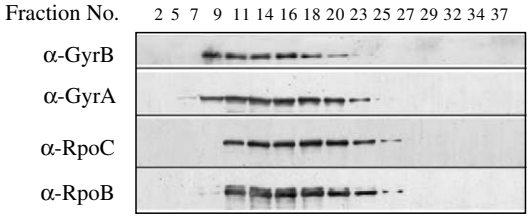

Fig. 2. Detection of RNAP–gyrase complex by sucrose density gradient centrifugation. Various sucrose gradient fractions of the cell lysate were checked for the distribution of RNAP and DNA gyrase subunits by Western blotting. Antibodies specific against GyrA, GyrB, RpoC, and RpoB were used. Five microliter of the indicated fractions was resolved on SDS 8% polyacrylamide gel prior to the transfer onto nitrocellulose membrane for Western analysis as described in Materials and methods.

To assess the interaction further, gel exclusion chromatography was also employed as described in Materials and methods. Western blot analysis showed that a complex comprising of subunits of both the enzymes eluted from the column in fractions ranging in molecular weight from 3500 to 1800 kDa (Fig. 3A). Since the fractions contain both enzymes subunits, we tested them for supercoiling and transcription activities. Supercoiling reactions were performed using relaxed DNA substrate and the activity profile is shown in Fig. 3B. These fractions also exhibited transcription activity when in vitro [^{3}H]UMP-incorporation experiments were carried out (Fig. 3C).

DNA gyrase stimulates transcription in the RNAP–gyrase complex

The transcription activity observed in the complex of DNA gyrase and RNAP could represent intrinsic catalytic

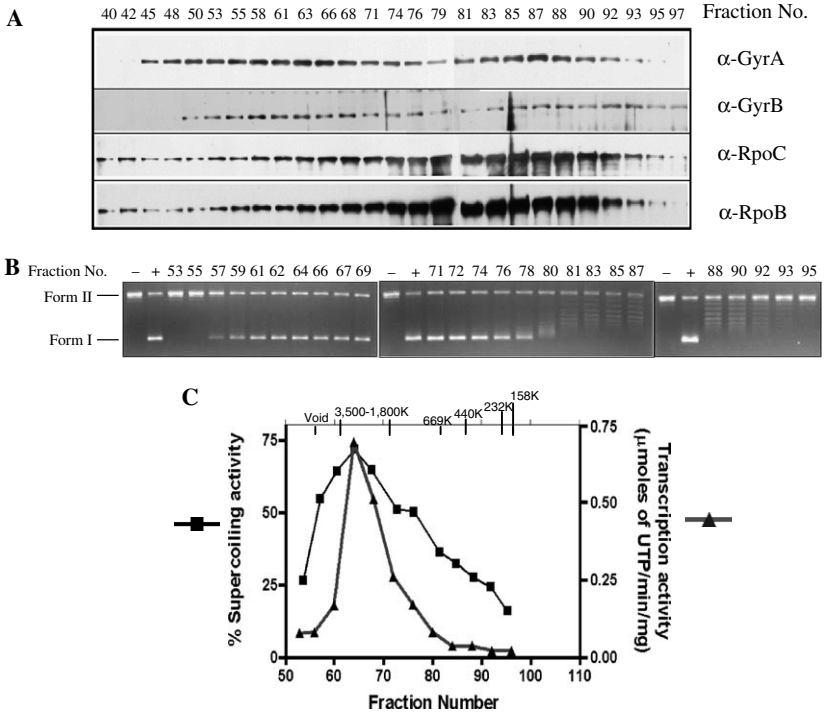

Fig. 3. Analysis of enzyme activities in the RNAP–gyrase complex. (A) Immunoblot analysis of gel filtration fractions using anti-GyrA, anti-GyrB, anti-RpoC, and anti-RpoB antibodies as described in Materials and methods. (B) Supercoiling assays with 4 μl of the gel filtration fractions. Lane −, no protein and lane +, supercoiling reaction control with purified gyrase, also used to estimate the % supercoiling activity of the fractions by densitometric analysis. Form I indicates the negatively supercoiled form of DNA and form II denotes the relaxed circular substrate for supercoiling reactions. (C) Activity profiles. DE-81 filter-based transcription assays were performed with various gel filtration fractions as detailed in Materials and methods. Transcription activity was calculated for each fraction (in μmol of UTP incorporated/min/mg of protein) and plotted as shown (triangles). Also, % supercoiling activity was calculated and plotted for each fraction (rectangles) based on the densitometric quantification of the activities shown in (B). Sizes of the complex and protein standards are depicted (top).

activity of RNAP without the influence from the other interacting component. Alternatively, the transcription activity seen may be an outcome of the association with gyrase. This was tested by carrying out in vitro RNA synthesis experiments in the presence of novobiocin, inhibitor of DNA gyrase. In the assays carried out using the conditions described in Fig. 3C, there was no significant effect on transcription in presence of the drug (data not shown). In these experiments, the template used was linear and hence the effect of gyrase may not be apparent. When topologically linked templates constructed from genomic sequences of mycobacteria containing promoter, pMN197B [6], were used for transcription in a similar [^{3}H]UMP-incorporation assay, considerable decrease in transcription was observed upon addition of novobiocin in the reactions (Table 1). It is known that negative supercoiling promotes transcription [16,17], and thus, negatively supercoiled templates serve as better substrates for transcription. The inhibition was less pronounced with negatively supercoiled templates and about 50% inhibition was observed with the relaxed circular templates (Fig. 4). Relaxed templates by themselves are not good substrates for transcription and would need to be unwound by gyrase for efficient transcription, unlike the negatively supercoiled templates. Consequently,

Table 1
Transcription by the complex on different topological templates

Template	Novobiocin (3 μg/ml)	Fraction No.[a]		
		64	66	68
−SC	−	0.793	0.715	0.676
	+	0.711	0.665	0.607
R	−	0.486	0.453	0.392
	+	0.249	0.219	0.203

−SC, negatively supercoiled DNA; R, relaxed DNA.

[a] The values are transcription activity represented as μmol of [^{3}H]UTP incorporated/min/mg.

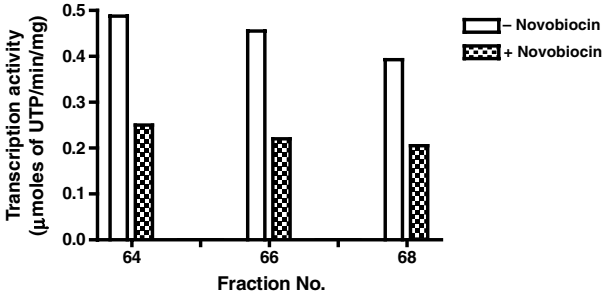

Fig. 4. Effect of novobiocin on transcription. Transcription assays were carried out using relaxed circular plasmid substrate (pMN197B) with the gel filtration fractions showing maximum supercoiling and transcription activities (see Fig. 3C), in the presence or absence of 3 μg/ml novobiocin. The specific activities obtained are shown in the bar diagram. Inhibition of the activity in the presence of novobiocin is shown as dotted bars.

in case of relaxed templates, when gyrase is inhibited by novobiocin a marked decrease in transcription is observed.

The observed inhibition of transcription by DNA gyrase specific inhibitor is not very surprising considering the vast difference in mycobacterial cellular organization as compared to that of *E. coli*. Many features of transcription in mycobacteria are yet to be understood and available data reveal numerous differences in mycobacterial and *E. coli* transcription machineries. For instance, promoter organization appears to be vastly different in these two distinct classes of bacteria and majority of mycobacterial promoters do not function in *E. coli* [18–21]. Further, analysis of *M. smegmatis* genome indicates that it contains ORFs, which encode for 17 putative sigma factors as compared to that of 7 σ factors found in *E. coli*. More recently, we have shown that the intrinsic terminators in mycobacteria are varied from those of *E. coli* [22]. A point of importance is that mycobacterial genomes are GC rich and as a result, transcription elongation rates could be much slower. An earlier study indeed has shown slower transcription elongation rates in mycobacteria [23]. Alternately, owing to the high G + C content of the genome, initiation of transcription at some of the mycobacterial promoters might be intrinsically unfavorable. In such a scenario, DNA gyrase–RNAP complex might facilitate transcription at a number of promoters, by enhancing promoter melting as a consequence of gyrase catalyzed negative supercoiling. Experiments with different promoter constructs and purified constituent enzymes would aid in further understanding the functional implications of the complex in mycobacterial transcription process.

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