A Mycobacterium smegmatis gyrase B specific monoclonal antibody reveals association of gyrase A and B subunits in the cell

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Abstract

DNA gyrase is a unique topoisomerase, which plays important roles in macromolecular events like DNA replication, transcription and genetic recombination. In this study a high affinity monoclonal antibody to the gyrase B (GyrB) subunit of Mycobacterium smegmatis was characterized, which did not cross-react with either the Escherichia coli GyrB subunit or with GyrB subunits from other mycobacterial species. The antibody recognized an epitope in the N-terminus, novobiocin-binding domain of GyrB. Immunoprecipitation of gyrase from M. smegmatis cell lysate revealed an association, mediated by ionic interactions, of gyrase A and GyrB subunits in the cell. This antibody is a valuable tool for structure-function analysis and immunocytological studies of mycobacterial DNA gyrase. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mycobacterium; DNA gyrase; Monoclonal antibody; Epitope mapping; Novobiocin; Surface plasmon resonance

1. Introduction

Conformational and topological changes in DNA that occur during many cellular processes such as replication, transcription and recombination are mediated by a class of enzymes known as DNA topoisomerases. Topoisomerases are essential and ubiquitous enzymes necessary for the viability of all organisms [1,2]. DNA gyrase is the only enzyme which can introduce negative supercoiling in DNA in an ATP dependent manner, and also perform other topological interconversions of DNA such as knotting/unknotting and catenation/decatenation [3]. DNA gyrase is well characterized from Escherichia coli and the purified enzyme is composed of gyrase A (GyrA) and gyrase B (GyrB) subunits that form an A2B2 complex in vitro. However, little information is available regarding the oligomeric status of the enzyme in vivo. DNA gyrase is essential for bacterial survival, and the enzyme is the target of a number of antibacterial agents including quinolones and coumarins [4].

The genus mycobacterium comprises several important species of public health concern. Apart from Mycobacterium tuberculosis and Mycobacterium leprae, the causative agents of tuberculosis and leprosy, respectively, many other mycobacteria such as M. avium, M. kansasii, M. chelonae and Mycobacterium fortuitum can cause opportunistic infections in immunocompromised hosts [5]. The resurgence of tuberculosis and the emergence of highly virulent multiple drug resistant M. tuberculosis clinical strains have raised a global alarm. Biochemical studies of mycobacterial DNA gyrase are still limited and poorly understood and could aid in the design and development of gyrase as a therapeutic target.

We have focussed our research efforts on structure-function analysis of mycobacterial DNA gyrase, to address the role of gyrase in important processes in mycobacteria. We have earlier cloned and over-expressed GyrA and GyrB subunits from Mycobacterium smegmatis [6]. In order to study the biochemical properties of the native enzyme we have generated a monoclonal antibody (mAb) to the GyrB subunit of M. smegmatis. This antibody reveals differences in structure of GyrB from different bacterial species, and also provides evidence for the first time that mycobacterial GyrB subunit exists in association with the GyrA in vivo.

Abbreviations: GyrB, DNA gyrase B subunit; mAb, monoclonal antibody
2. Materials and methods

2.1. Bacterial strains, plasmids and materials

*M. smegmatis* SN2 was a laboratory strain, *M. tuberculosis* H37Rv was obtained from National Tuberculosis Institute, Bangalore, India. *M. fortuitum* and *Mycobacterium gilvum* were provided by Dr. V.M. Katooch, Central JALMA Institute for Leprosy, Agra, India. All mycobacterial species were grown in modified Youmans and Karlson’s medium as described earlier [7].

2.2. Expression and purification of recombinant gyrase subunits

For the purification of *M. smegmatis* GyrB, *E. coli* strain BL21(DE3)pLysS harboring the recombinant plasmid pMK25 [6] was grown in Luria Bertani broth and the cells were induced with 500 µM isopropylthio-β-D-galactoside (IPTG) for 3 h at 18°C. Cells were lysed by sonication in buffer (20 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride, 1 mM benzamidine HCl and 1% Triton X-100). The cell lysate was centrifuged at 40,000 rpm and the supernatant treated with ammonium sulfate to obtain a 30–55% pellet fraction. The pellet was solubilized in TGEM buffer (50 mM Tris pH 7.6, 10% glycerol, 2 mM EDTA and 2 mM β-mercaptoethanol) and subjected to purification on MonoQ and Heparin Sepharose columns (Amersham-Pharmacia Biotech) using 0–1 M NaCl gradient elution. GyrB protein was eluted at 300 mM NaCl gradient. *M. tuberculosis* GyrB was over-expressed in BL21(DE3)pLysS cells harboring pMK15 and purified by electrodution from preparative sodium dodecyl sulfate (SDS)–polyacrylamide gels. Recombinant *E. coli* GyrA was purified as previously described by Maxwell and Hoyle [8]. The polyclonal antibodies were raised against purified *M. tuberculosis* recombinant GyrA and GyrB proteins in rabbits [9].

2.3. Generation of anti-GyrB mAb

Balb/c mice were immunized intradermally with 100 µg of *M. smegmatis* GyrB, and mAbs generated as previously described [10]. Enzyme-linked immunosorbent assay was performed using *M. smegmatis* GyrB (2 µg ml⁻¹) as described earlier [9]. An antibody-secreting clone, MsGyrB:A4, was subcloned to monoclonality by limiting dilution. Ascites fluid was prepared by intraperitoneal injection of 1×10⁶ hybridoma cells into Balb/c mice. Immunoglobulins were purified from ascitic fluid using Protein-A Sepharose affinity column chromatography and iso-typed as IgG1 with κ light chain.

Fab fragments of the IgG were prepared by papain digestion of the purified immunoglobulin and purified by Protein-A Sepharose affinity column. For Western blot analysis, proteins were resolved on an 8% SDS–PAGE [11]. Proteins were transferred to polyvinylidene difluoride membrane in transfer buffer (50 mM Tris–glycine buffer, pH 8.5, containing 20% methanol). The membrane was blocked with phosphate-buffered saline containing 2% bovine serum albumin and probed with polyclonal antiserum either to GyrA (1:20000) or GyrB (1:12000) or mAb MsGyrB:A4 (0.1 µg ml⁻¹). Bound IgG was detected using anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase and enhanced chemiluminescence reagents (Amersham-Pharmacia Biotech).

2.4. Immunoprecipitation of gyrase from mycobacterial cell-free extracts

Cell-free extracts were prepared from mycobacterial cultures grown to mid-exponential phase. Protein was estimated by the method of Bradford [12]. Protein (500 µg) was pre-incubated with 200 µg ml⁻¹ of anti-GyrB polyclonal antibodies or 50 µg ml⁻¹ of MsGyrB:A4 or normal mouse immunoglobulin in immunoprecipitation (IP) buffer (50 mM Tris pH 7.4, 1 mM EDTA, 5 mM β-mercaptoethanol, 50 mM NaCl, 1% Nonidet P-40 and 5% glycerol) for 6 h at 4°C. For immunoprecipitation under high salt conditions, MsGyrB:A4 (50 µg ml⁻¹) was pre-incubated with cell lysate (500 µg) in IP buffer containing 500 mM NaCl. The immunocomplex was recovered by adding 10 µl of Protein G agarose and incubation continued for 1 h. Immunocomplexes were washed and analyzed by SDS–PAGE followed by silver staining of the gel and Western blotting.

2.5. Novobiocin interaction with GyrB bound to MsGyrB:A4

Novobiocin was coupled to epoxy-activated Sepharose 6B as described earlier [13]. GyrB protein (1 µg) was incubated in the absence or presence of 100 µg of novobiocin at 37°C for 1 h in 50 µl of IP buffer followed by the addition of MsGyrB:A4 (3 µg). Incubation was continued for a further 1 h and the protein complex then recovered by addition of either 10 µl of Protein G agarose or novobiocin Sepharose (15 µl). The beads were washed with IP buffer extensively, followed by analysis by SDS–PAGE and Western blotting.

2.6. Generation of truncated GyrB protein

To generate the N-terminal *M. smegmatis* GyrB clone encoding 1–270 amino acids, oligonucleotide primers for PCR amplification (FP-CTGCGCATGGCTGCCCAG-AAG and RP-CTTGAGGAATTTCAGGACCCAGCC) were designed on *Nco*I site in the 5′ primer. The PCR product obtained with pMN6A [6] as template was digested with *Nco*I and *Eco*RI and cloned into pTrc99C predigested with *Nco*I and *Eco*RI to obtain pMsGyrB:AC.
plasmid, pMsGyrB:ΔC was transformed in *E. coli* DH10b

cells and induced with IPTG at an optical density of 0.6
(A600).

2.7. Surface plasmon resonance (SPR) spectroscopy

Binding kinetics were determined by SPR spectroscopy [14] using a BIAcore 2000 system, Uppsala, Sweden. The over-expressed and purified *M. smegmatis* GyrB was immobilized on the CM5 sensor chip at a concentration of 1200 resonance units as per manufacturer’s instructions. All measurements were carried out in a continuous flow of HBS buffer (10 mM HEPES, 150 mM NaCl, 5 mM EDTA and 0.05% Surfactant P-20, pH 7.4). The kinetic

data were analyzed using the BIA evaluation software (Version 3.0). The surface was regenerated by a pulse of 5 μl of 10 mM NaOH, for further experimentation as required.

3. Results

3.1. Epitope specificity in mycobacterial GyrB subunit

Western blot analysis using polyclonal antibodies raised against *M. tuberculosis* GyrB subunit was performed with purified proteins from *M. tuberculosis*, *M. smegmatis* and

Fig. 1. Species specific cross-reactivity of anti-GyrB antibodies. (A) Western blot analysis of GyrB proteins (1 μg per lane) purified from *M. smegmatis*, *M. tuberculosis* and *E. coli* was performed with a polyclonal antibody raised against *M. tuberculosis* GyrB. (B) Cell lysates were incubated with 200 μg ml⁻¹ IgG purified from the polyclonal antiserum or 50 μg ml⁻¹ MsGyrB:A4 in IP buffer. Immunocomplexes were recovered using 10 μl of Protein G agarose. Immunoprecipitates were analyzed by SDS-PAGE and Western blot was performed with polyclonal antibody or MsGyrB:A4.

Fig. 2. (A) A pair-wise amino acid sequence alignment of GyrB of *M. smegmatis* (MsGyrB, X84077) [6] and *M. tuberculosis* (MtGyrB, X78888) [23]. The alignment was obtained using GCG software. (B) Epitope mapping of MsGyrB:A4 mAb using GyrB truncated protein. (i) Schematic representation of *M. smegmatis* GyrB proteins. (ii) Immunoblot analysis of truncated *M. smegmatis* GyrB protein. Extracts prepared from *E. coli* cells harboring plasmid pMsGyrB:ΔC with or without induction with IPTG were analyzed on SDS-PAGE and Western blotting performed with MsGyrB:A4. Lane 1 contains 0.5 μg of purified *M. smegmatis* GyrB.
E. coli, and results indicated that all the three proteins were recognized (Fig. 1A). Interestingly, the mAb MsGyrB:A4 immunoprecipitated GyrB from *M. smegmatis* SN2 cell lysate but not from *M. tuberculosis* H37Rv cell lysate (Fig. 1B), whereas the GyrB specific polyclonal antibody immunoprecipitated GyrB from both the cell lysates. MsGyrB:A4 showed no reactivity with other fast growing mycobacterial species, i.e. *M. fortuitum* and *M. gilvum* (data not shown). The GyrB subunits of *M. smegmatis* and *M. tuberculosis* share an overall 88% identity at the amino acid level, but the observation that MsGyrB:A4 reacts with *M. smegmatis* GyrB alone indicates that this mAb binds to a unique region present in *M. smegmatis* protein.

Considerable differences in the amino acid sequences of *M. smegmatis* and *M. tuberculosis* are observed in the N-terminal region (Fig. 2A), where there is only 77% identity in the sequences of *M. smegmatis* and *M. tuberculosis* from residues 1 to 260, as opposed to 93% identity in the C-terminal region of the protein. It is therefore possible that MsGyrB:A4 interacts with the N-terminus of *M. smegmatis* GyrB. To confirm this, the N-terminal 1–259 amino acids of GyrB were over-expressed in *E. coli*, and Western blot analysis was performed with bacterial lysates prepared from cells expressing the truncated GyrB subunit. As shown in Fig. 2B, the epitope for MsGyrB:A4 lies in the N-terminus of *M. smegmatis* GyrB.

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The amino-terminal of *E. coli* GyrB 2–220 amino acids, a 24-kDa protein, binds coumarins, and this fragment has been crystallized with novobiocin [15]. Since both novobiocin and MsGyrB:A4 interact with the N-terminus of GyrB, we tested whether the interaction of novobiocin with GyrB modulated mAb-binding. *M. smegmatis* GyrB was pre-incubated with novobiocin, followed by the addition of MsGyrB:A4 mAb. The protein complex was then recovered with either Protein G agarose or novobiocin Sepharose (Fig. 3). Novobiocin bound GyrB could interact with MsGyrB:A4 (Fig. 3, lane 5). Pre-incubation of GyrB protein with novobiocin completely inhibited further interaction with novobiocin Sepharose (Fig. 3, lane 4), indicating that all the GyrB was bound to novobiocin under these experimental conditions. MsGyrB:A4 bound to GyrB complex could be precipitated with novobiocin Sepharose (Fig. 3, lane 3), indicated by the presence of the heavy and light chains of the antibody associated with the novobiocin Sepharose. MsGyrB:A4 antibody did not bind to novobiocin Sepharose in the absence of GyrB protein (data not shown). These results therefore indicate that binding sites for novobiocin and mAb do not overlap with each other, and binding of novobiocin to GyrB does not preclude binding of the antibody simultaneously.

![Diagram of interaction of MsGyrB:A4 and novobiocin with GyrB. Purified *M. smegmatis* GyrB was pre-incubated without (lanes 1–3) or with 100 μg of novobiocin (lanes 4 and 5) at 37°C for 1 h followed by addition of 3 μg of MsGyrB:A4 (lanes 2, 3 and 5). The complexes were isolated using novobiocin Sepharose (lanes 1, 3 and 4) or Protein G agarose (lanes 2 and 5). The precipitates were subjected to SDS-PAGE and Western blot analysis with MsGyrB:A4.](image)

![Analysis of interaction of MsGyrB:A4 and novobiocin with GyrB. Purified *M. smegmatis* GyrB was pre-incubated without (lanes 1–3) or with 100 μg of novobiocin (lanes 4 and 5) at 37°C for 1 h followed by addition of 3 μg of MsGyrB:A4 (lanes 2, 3 and 5). The complexes were isolated using novobiocin Sepharose (lanes 1, 3 and 4) or Protein G agarose (lanes 2 and 5). The precipitates were subjected to SDS-PAGE and Western blot analysis with MsGyrB:A4.](image)

![Electrophoresis and Western blot analysis of purified *M. smegmatis* GyrB.](image)

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![Electrophoresis and Western blot analysis of purified *M. smegmatis* GyrB.](image)

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3.2. Interaction of MsGyrB:A4 with DNA gyrase

The affinity of the mAb for M. smegmatis GyrB was determined by analysis of the binding kinetics by biosensor studies [14]. Analysis of the data obtained allowed the determination of kinetic constants of interaction and indicated that MsGyrB:A4 displayed a high affinity interaction ($K_d = 3.4 \times 10^{-9}$ M) with GyrB subunit. The E. coli gyrase is a heterotrimer comprising GyrA and GyrB polypeptides [2,3]. The DNA gyrase from Mycobacterium bovis has been shown to form GyrA-B complex when individual subunits are reconstituted onto DNA [16]. In order to determine if the mycobacterial enzyme also exists as a complex of GyrA and GyrB subunits in the cell, the cell lysate was treated with mAb and antibody bound enzyme collected with Protein G agarose. The immunoprecipitate was analyzed by SDS-PAGE followed by silver staining (Fig. 4A). MsGyrB:A4 specifically immunoprecipitated two polypeptides, which reacted with their specific antibodies on Western blot analysis (Fig. 4B,C). When immunoprecipitation was performed in the presence of 500 mM NaCl, no GyrA polypeptide was found in the immunoprecipitate (Fig. 4, lane 3), indicating GyrA–GyrB interaction is predominantly ionic in nature.

4. Discussion

To generate specific probes for the structure-function analysis of mycobacterial DNA gyrase, a mAb to M. smegmatis GyrB subunit was generated. The studies reported here are the first to describe the development and characterization of mAb to any mycobacterial DNA GyrB subunit. The type II topoisomerase family, which includes topoisomerase II of bacteriophage, eubacteria as well as eukaryotic enzymes, shares strong similarities in enzymatic properties and protein primary sequences [17]. We have however earlier noted a major difference in the amino acid sequence at the N-terminus of Gram-positive and Gram-negative bacterial GyrB, where a contiguous stretch of 165 amino acids is missing from Gram-positive bacteria [18]. While many epitopes in the protein are conserved, as shown by the cross-reactivity of polyclonal antibodies to M. tuberculosis GyrB with the E. coli enzyme (Fig. 1A), it is apparent that certain variations are located in the sequences at the N-terminus of the GyrB subunit, as detected by the extraordinary specificity of MsGyrB:A4 (Fig. 1B).

Earlier workers have proposed that GyrB could be a suitable phylogenetic marker for the identification and classification of bacteria [19]. These GyrB-based methods have already shown to be useful for the differentiation of closely related strains of Vibrio and Bacillus. Sequence analysis of the mycobacterial GyrB subunit has shown that more substitutions are found in the GyrB sequence than in 16S rDNA [20]. Since MsGyrB:A4 appears to distinguish between M. smegmatis and other strains of mycobacteria, this antibody could be used to characterize closely related strains of mycobacteria. It is therefore conceivable that mAbs specific to M. tuberculosis or M. leprae GyrB proteins could also be generated for rapid diagnosis of mycobacterial infections.

The 43-kDa amino-terminal domain of E. coli GyrB possesses a coumarin-sensitive ATPase activity, thereby localizing the coumarin drug-binding site to this part of the protein. The 43-kDa domain folds as two sub-domains, the amino-terminal 220 amino acids form the first domain which has been crystallized with novobiocin and the structure solved to high resolution [15] and amino acids 221–392 the second [21]. Since gyrase from mycobacteria was purified by novobiocin affinity chromatography [13], it can be concluded that the overall structure in the N-terminus of M. smegmatis GyrB is similar to that of E. coli enzyme. Even though MsGyrB:A4 interacts with the N-terminal domain of M. smegmatis protein, its interaction was not affected by novobiocin-binding to the GyrB subunit (Fig. 3), implying that the binding sites of novobiocin and the antibody are distinct.

The 47-kDa C-terminal domain of E. coli GyrB interacts with the DNA and GyrA subunit [3]. MsGyrB:A4 immunoprecipitates both GyrB and GyrA polypeptides (Fig. 4), indicating that the regions of interaction of GyrB with mAb and GyrA are distinct from each other. This would perhaps imply that the GyrA- and DNA-binding regions are located in the carboxy-terminal of GyrB, which is similar to the domain organization of E. coli GyrB.

Based on immunolocalization studies in E. coli [22], it was suggested that the GyrA and GyrB proteins are present within the cytoplasm as discrete subunits, which may or may not be DNA-associated. Till date, no information is available on the nature of the gyrase holoenzyme in mycobacterial cells. Our immunoprecipitation studies with the cell-free extracts reveal that at least in M. smegmatis, a significant amount of association of GyrA and GyrB subunits is observed, perhaps representing the fraction of active holoenzyme. This mAb would therefore be an ideal tool for immunolocalization studies of the holoenzyme or holoenzyme DNA complex in M. smegmatis. The interaction of GyrA and GyrB subunits is inhibited by the presence of sodium chloride, as shown by co-immunoprecipitation studies, indicating that predominantly ionic interactions are critical in mediating association of the two subunits.

Since MsGyrB:A4 specifically immunoprecipitated both subunits of gyrase, the mAb could be used as a ligand for the immunoaffinity purification of individual subunits of mycobacterial DNA gyrase. Our observation that GyrA–GyrB interaction can be disrupted with 500 mM NaCl should allow specific elution of the individual subunits,
thereby allowing a detailed biochemical characterization of this complex enzyme from mycobacteria.

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