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Purification, crystallization and preliminary X-ray crystallographic studies of the *Mycobacterium tuberculosis* DNA gyrase ATPase domain

Mycobacterium tuberculosis DNA gyrase, a nanomachine involved in the regulation of DNA topology, is the only type II topoisomerase present in this organism and hence is the sole target of fluoroquinolones in the treatment of tuberculosis. The ATPase domain provides the energy required for catalysis by ATP hydrolysis. Two constructs corresponding to this 43 kDa domain, Mtb-GyrB47^{C1} and Mtb-GyrB47^{C2}, have been overproduced, purified and crystallized. Diffraction data were collected from three crystal forms. The crystals belonged to space groups *P*1 and *P*2₁ and diffracted to resolutions of 2.9 and 3.3 Å, respectively.

1. Introduction

DNA gyrase and topoisomerase IV are type II topoisomerases that are found in almost all bacteria. These essential macromolecular assemblies are involved in the manipulation of DNA topology during replication, transcription and recombination. They maintain DNA topology by cleaving and religating DNA double-strand breaks (Champoux, 2001). DNA gyrase and topoisomerase IV are structurally and mechanistically related, but have acquired distinct biological functions during evolution (Champoux, 2001). DNA gyrase is unique in introducing negative supercoils and facilitates DNA unwinding at replication forks, and topoisomerase IV has a specialized function in mediating the decatenation of interlocked daughter chromosomes (Champoux, 2001; Levine *et al.*, 1998).

Bacterial type II topoisomerases are heterotetrameric enzymes assembled from two subunits (A and B). Each subunit consists of two domains: the breakage-reunion (BRD) and the C-terminal (CTD) domains for the A subunit, and the ATPase and the Toprim domains for the B subunit (Schoeffler & Berger, 2008). DNA breakage occurs at the catalytic reaction core formed by the BRD and Toprim domains. The energy required for catalysis is provided by ATP hydrolysis in the ATPase domain. Fluoroquinolone antibiotics block the catalytic core (Mayer & Aubry, 2012), whereas the ATPase domain is the target of other classes of drugs such as aminocoumarins (Lewis *et al.*, 1996).

In *Mycobacterium tuberculosis*, DNA gyrase is the single type II topoisomerase and therefore the sole type II topoisomerase that ensures the regulation of DNA superhelical density (Cole *et al.*, 1998); it is also the sole target of the fluoroquinolones, which are key antibiotics in the treatment of drug-resistant tuberculosis. As a result, the *M. tuberculosis* enzyme exhibits a different activity spectrum compared with other DNA gyrases, *e.g.* it supercoils DNA with efficiency comparable to that of other DNA gyrases, but shows enhanced relaxation, DNA-cleavage and decatenation activities (Aubry *et al.*, 2006). In order to establish the molecular basis of the specificities of *M. tuberculosis* DNA gyrase, we solved the structures of the GyrB Toprim domain, the GyrA BRD domain and the GyrA CTD domain (Piton *et al.*, 2009, 2010; Darmon *et al.*, 2012).

To provide further structural data with regard to the specificities of *M. tuberculosis* DNA gyrase and to facilitate the discovery of new antituberculous agents, we have investigated the role of the ATPase domain, which possesses a specific insertion of 32 amino acids that is present only in mycobacteria and other Corynebacteriae. The *M. tuberculosis* GyrB ATPase domain was expressed, purified and

Table 1

Primers used for cloning.

Construct	Primers (5'–3')
pET-TBgyrBFL3His	a AGATCTGTGGCTGCCAGAAAAAGAAGGCCAAG
pET-TBgyrBFL3His	b GAAGGCCCAAG CTCGAGTTAGACATCCAGGAACCG-AACATC
Mtb-GyrB47 ^{C1}	c AGATCTGTGGCTGCCAGAAAAAGAAGGCCAAG
Mtb-GyrB47 ^{C1}	d CTCGAGTTACACCAACTCTCGTGCCTTACGTGC
Mtb-GyrB47 ^{C2}	e GACGACGACAAGATGGCTGCCAGAAAAAGAAGGCC
Mtb-GyrB47 ^{C2}	f CCGGTTACACCAACTCTCGTGCCTTACG

crystallized independently in two laboratories. Here, we report the preliminary crystallographic analyses obtained from three crystal forms.

2. Materials and methods

2.1. Cloning, protein production and purification

Two constructs (Mtb-GyrB47^{C1} and Mtb-GyrB47^{C2}) of the gene encoding the *M. tuberculosis* DNA gyrase ATPase domain (residues 1–427 of subunit B) were independently cloned and expressed in two laboratories (GlaxoSmithKline, UK, and the Pasteur Institute, Paris). At GlaxoSmithKline, full-length *M. tuberculosis* gyrase B (accession No. CAA55486.1) was cloned by PCR from genomic DNA strain H37Rv provided by GlaxoSmithKline (Tres Cantos, Spain) using primers *a* and *b* (Table 1); it was subcloned into pET-20b (Novagen) cleaved with *Bam*HI and *Xho*I, and contained an N-terminal six-histidine tag and a PreScission protease cleavage site to make pET-TBgyrBFL3His. The construct encoding the ATPase domain of gyrase B (Mtb-GyrB47^{C1}) was obtained by PCR of the full-length clone using primers *c* and *d* (Table 1). PCR products were subcloned into pET-20b (Novagen) cleaved with *Bam*HI and *Xho*I and contained an N-terminal six-histidine tag and a PreScission protease cleavage site.

Mtb-GyrB47^{C2} was obtained by PCR from full-length DNA gyrase subunit B (Aubry *et al.*, 2006) using primers *e* and *f* (Table 1) which each include a ligation-independent cloning sequence (bold). The amplified product was ligated into the vector pRSF-2 Ek-LIC (Novagen, USA) after being treated with LIC-qualified T4 DNA polymerase according to the manufacturer's instructions. This construct contains an additional hexahistidine tag at the N-terminus followed by an enterokinase cleavage site. The two recombinant plasmids, pET-20b/Mtb-GyrB47^{C1} and pRSF-2 Ek-LIC/Mtb-GyrB47^{C2}, were transformed into *Escherichia coli* strain BL21(DE3)pLysS (Novagen).

Cells freshly transformed with pET-20b/Mtb-GyrB47^{C1} were grown overnight at 303 K in LB medium containing 100 µg ml⁻¹ ampicillin, 34 µg ml⁻¹ chloramphenicol and 1% glucose. The culture was diluted 1:10 into 5 l similar broth (without glucose), equilibrated for 30 min at 303 K and induced overnight at 303 K with a final isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration of 0.5 mM. Following harvesting, the cell pellet was resuspended in 50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM β-mercaptoethanol, 1 mg ml⁻¹ lysozyme, 1 µg ml⁻¹ each of pepstatin A, bestatin, leupeptin and aprotinin, and then sonicated on ice and centrifuged at 30 000g. The soluble fraction was purified on a 50 ml Ni-NTA column (Qiagen) with a 0–500 mM imidazole step gradient in lysis buffer without lysozyme. The protein was buffer-exchanged into 50 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM dithiothreitol, and 2 U PreScission protease per 100 µg protein were added overnight. The protease was removed with glutathione Sepharose (the protease is GST-tagged), and any uncleaved protein was removed by adsorption

to Ni-NTA at 300 mM NaCl. The conductivity was reduced to <10 mS by dilution and the protein was purified over a 20 ml SourceQ ion-exchange column (GE Healthcare). Peak fractions were pooled, concentrated and applied onto a 500 ml Superdex 200 column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA. The protein was concentrated to 6 mg ml⁻¹; it was >95% pure as judged by Coomassie staining and its identity was confirmed by mass spectrometry and N-terminal sequencing. The final protein contains five extra residues at the N-terminus (GPLGS prior to Val1) with a total molecular weight of 46.7 kDa.

Cells freshly transformed with pRSF-2 Ek-LIC/Mtb-GyrB47^{C2} were grown at 310 K in LB medium containing 50 µg ml⁻¹ kanamycin and 40 µg ml⁻¹ chloramphenicol. At an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 1 mM and the cells were grown at 310 K for 4 h. Following harvesting, bacteria were resuspended in 50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole and lysed by sonication on ice. After centrifugation at 18 000g for 1 h at 277 K, the

Figure 1
Crystals of the Mtb-GyrB47^{C2} construct. (a) Initial screening resulting in urchin-shaped crystals. (b) Crystals obtained using the Additive Screen.

Table 2

Data-collection statistics.

Values in parentheses are for the outermost shell.

	Mtb-GyrB47 ^{C1}	Mtb-GyrB47 ^{C1}	Mtb-GyrB47 ^{C2}
Beamline	ID23-1, ESRF	ID23-1, ESRF	PX-1, SOLEIL
Wavelength (Å)	0.97955	0.97855	0.91942
Resolution range (Å)	40–2.95 (3.0–2.95)	25–2.9 (2.95–2.9)	50–3.3 (3.5–3.3)
Space group	<i>P1</i>	<i>P1</i>	<i>P2</i> ₁
Unit-cell parameters (Å, °)	<i>a</i> = 71.9, <i>b</i> = 97.8, <i>c</i> = 138.1, <i>α</i> = 108.2, <i>β</i> = 105.2, <i>γ</i> = 91.0	<i>a</i> = 101.4, <i>b</i> = 138.2, <i>c</i> = 147.7, <i>α</i> = 105.3, <i>β</i> = 92.3, <i>γ</i> = 107.2	<i>a</i> = 78.6, <i>b</i> = 171.9, <i>c</i> = 109.2, <i>β</i> = 110.2
No. of unique reflections	64548 (3266)	159199 (7913)	41603 (6419)
Multiplicity	1.7 (1.6)	1.9 (1.9)	3.8 (3.7)
Completeness (%)	84.5 (86.2)	98.3 (97.7)	98.7 (95.0)
Molecules per asymmetric unit	8	16	6
<i>V</i> _M (Å ³ Da ^{−1}) (Matthews, 1968)	2.2	2.5	2.3
Solvent content (%)	44.4	51.4	46.8
<i>I</i> / <i>σ</i> (<i>I</i>)	10.8 (1.9)	14.1 (1.9)	16.3 (2.4)
<i>R</i> _{merge} [†] (%)	7.9 (27.2)	5.3 (42.2)	5.9 (56.9)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl) \rangle$ is its mean value.

supernatant was loaded onto a Ni²⁺-chelating HisTrap HP column (GE Healthcare) and the 6His-Mtb-GyrB47^{C2} protein was eluted with 50 mM Tris pH 8.0, 500 mM NaCl, 500 mM imidazole. After dialysis overnight at 277 K against 50 mM Tris pH 8.0 and 50 mM NaCl, the 6His-Mtb-GyrB47^{C2} protein was concentrated at 277 K using Amicon Ultra 10K (Millipore). To remove the His tag, the 6His-Mtb-GyrB47^{C2} protein was cleaved overnight at 277 K by enterokinase at a molar ratio of 1:100. Any uncleaved protein was removed by adsorption to a Ni²⁺-chelating HisTrap HP column pre-equilibrated in 50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole. The flowthrough containing Mtb-GyrB47^{C2} was dialysed overnight at 277 K against 50 mM Tris pH 8.0, 50 mM NaCl and was concentrated at 277 K using Amicon Ultra 10K (Millipore). The concentrated Mtb-GyrB47^{C2} protein was purified further by size-exclusion chromatography on a Superdex 75 10/30 column (GE Healthcare) pre-equilibrated with 50 mM Tris pH 8.0, NaCl 50 mM. Alternatively, the 6His-Mtb-GyrB47^{C2} was purified according to the same protocol but without the His-tag cleavage step. The purified proteins were concentrated to 15 mg ml^{−1} in size-exclusion buffer using Amicon Ultra 10K (Millipore). The purity of the proteins was examined by 12% SDS-PAGE and determined to be >95% pure. The final 6His-Mtb-GyrB47^{C2} protein contains 14 extra residues at the N-terminus whereas the final Mtb-GyrB47^{C2} starts at codon 1 (valine) and contains 427 amino acids with a molecular weight of 46.3 kDa.

2.2. Crystallization and data collection

Crystallization screens on the purified Mtb-GyrB47^{C1} construct at 12 mg ml^{−1} were run on protein either lacking ligand or in the presence of 5 mM MgCl₂ plus either 1 mM AMPPNP, 1 mM novobiocin (a coumarin) or 1 mM GR122222X (a cyclothialidine). Crystals were only obtained in the presence of AMPPNP and GR122222X. Crystals grown in the presence of GR122222X in 0.1 M bis-tris pH 5.5–6.5, 20% PEG 3350 were not well ordered and failed to diffract beyond 4 Å resolution. Crystals with AMPPNP were obtained in a variety of conditions, but initially all crystals tested occurred in a *P1* cell which had 16 subunits in the asymmetric unit. Analysis of the data sets with *phenix.xtriage* (Adams *et al.*, 2010) gave no signs of twinning or a lower symmetry cell. The best data set obtained for this *P1*(16) crystal form, collected on beamline ID14-4 at the ESRF, is shown in Table 2 (crystal grown from 0.1 M Tris pH 8.5, 0.2 M NaCl, 25% PEG 3350). After rescreening crystallization conditions, investigating many different crystallization parameters and testing many crystals, a second crystal form was found. This was

again in space group *P1*, with eight subunits in the asymmetric unit. The best data set we obtained from this *P1*(8) crystal form was an anisotropic data set to 2.95 Å resolution (Table 2); the crystal was grown in 0.1 M Tris pH 8.5, 0.2 M MgCl₂, 20% PEG 8000. Data on the AMPPNP crystals were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski *et al.*, 2003).

Initial crystallization screening of Mtb-GyrB47^{C2} (10 mg ml^{−1}) was performed on a Mosquito robot (TTP LabTech) using several commercial screening kits (600 conditions). Preliminary results were obtained only in the presence of AMPPCP (5 mM) with 15% PEG 1500 as precipitant (Fig. 1). This condition was further screened and resulted in urchin-shaped crystals that did not diffract beyond 7 Å. Using the Additive Screen (Hampton Research) and extensive optimization of the crystallization conditions, the best diffracting crystals were grown by the hanging-drop vapour-diffusion method against 24% PEG 1500, 0.1 M MES pH 6.5, 5 mM MgCl₂, 1.2% myo-inositol (Fig. 1). After successive soaking in cryoprotectant solutions

Figure 2
Diffraction pattern obtained on beamline PROXIMA-1 (SOLEIL, Saint-Aubin) from optimized crystals of the Mtb-GyrB47^{C2} construct.

consisting of mother liquor with increasing glycerol contents (30% final), crystals were flash-cooled in liquid nitrogen. X-ray diffraction data were collected on beamline PROXIMA-1 (PX-1) at the SOLEIL synchrotron (Fig. 2). The crystals belonged to space group $P2_1$ with unit-cell parameters $a = 78.6$, $b = 171.9$, $c = 109.2$ Å, $\beta = 110.2^\circ$, and contained six monomers in the asymmetric unit. Data were processed with *XDS* (Kabsch, 2010) and scaled with *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). Processing statistics are summarized in Table 2.

Molecular replacement was performed with *Phaser* (Storoni *et al.*, 2004). For the Mtb-GyrB47^{C1} structure, the 43 kDa *E. coli* DNA gyrase ATPase domain (40% amino-acid identity) was used as the search model (PDB entry 1ei1; Brino *et al.*, 2000). The first round of refinement was performed with *REFMAC* (Murshudov *et al.*, 2011) using strict non-crystallographic symmetry for the GHKL and transducer subdomains. For the Mtb-GyrB47^{C2} structure, using the 43 kDa *E. coli* ATPase domain as search model did not give any results. Phases could be obtained using the GHKL and transducer subdomains of a monomer of the preliminary refined Mtb-GyrB47^{C1} as the search model.

3. Results and discussion

Two constructs for the ATPase domain of *M. tuberculosis* H37Rv GyrB, Mtb-GyrB47^{C1} and Mtb-GyrB47^{C2}, were independently cloned and expressed in two laboratories (GlaxoSmithKline, UK, and the Pasteur Institute, Paris). The same ends of the domain (residues 1–427) were identified in both laboratories and the proteins are identical apart from having different N-terminal tags. Several start sites have been reported for *M. tuberculosis* GyrB (reviewed in Maruri *et al.*, 2012) resulting in four different numbering schemes used throughout the literature. Analyses at GlaxoSmithKline suggested that the start site reported for *M. smegmatis* GyrB (Revel-Viravau *et al.*, 1996), which has 80% amino-acid identity with *M. tuberculosis* GyrB, is most likely correct. This start site is 40 residues downstream of the longest reported start site and starts at a GTG codon (valine), which is not uncommon in mycobacteria (Jacobs *et al.*, 2002). This was also identified as the start site at the Pasteur Institute and we use the new consensus numbering system for *M. tuberculosis* GyrB (Maruri *et al.*, 2012).

High-throughput crystallization screenings were first performed on both constructs. Mtb-GyrB47^{C1} and Mtb-GyrB47^{C2} crystallized only in the presence of an ATP analogue (AMPPNP and AMPPCP, respectively). Two crystal forms were obtained for Mtb-GyrB47^{C1} in space group $P1$ with 16 and eight molecules in the asymmetric unit. The structure could be determined by molecular replacement with the structure of the *E. coli* GyrB ATPase domain using the $P1(8)$ diffraction data. Surprisingly, in the case of Mtb-GyrB47^{C2}, molecular replacement using the *E. coli* GyrB ATPase domain as the starting

model gave no solution. In contrast, phases could be determined using the two ATPase subdomains, the GHKL and the transducer, as templates, revealing that the structures of Mtb-GyrB47^{C1} and Mtb-GyrB47^{C2} are in a different conformational state. Structural details will be described in a separate paper.

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