Novel T9 loop conformation of filamenting temperature-sensitive mutant Z from *Mycobacterium tuberculosis*

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As of 2017, tuberculosis had infected 1.7 billion people (23% of the population of the world) and caused ten million deaths. *Mycobacterium tuberculosis* (Mtb) is quickly evolving, and new strains are classified as multidrug resistant. Thus, the identification of novel druggable targets is essential to combat the proliferation of these drug-resistant strains. Filamenting temperature-sensitive mutant Z (FtsZ) is a key protein involved in cytokinesis, an important process for Mtb proliferation and viability. FtsZ is required for bacterial cell division because it polymerizes into a structure called the Z-ring, which recruits accessory division proteins to the septum. Here, the crystal structure of the MtbFtsZ protein has been determined to 3.46 Å resolution and is described as a dimer of trimers, with an inter-subunit interface between protomers AB and DE. In this work, a novel conformation of MtbFtsZ is revealed involving the T9 loop and the nucleotide-binding pocket of protomers BC and EF.

1. Introduction

Tuberculosis (TB) is a deadly disease and is the leading cause of death from infectious diseases worldwide (World Health Organization, 2018). *Mycobacterium tuberculosis* (Mtb) is the bacterium responsible for this potentially lethal illness (Kumar *et al.*, 2007). People with human immunodeficiency virus (HIV) are especially vulnerable, since Mtb is active when the immune system is weakened. Infection by Mtb makes this the number one opportunistic infectious disease that causes death among the population with HIV (Lawn & Zumla, 2011). People with HIV are not the only population at high risk. Diabetes, alcohol abuse and drug abuse are factors that increase the chance of infected people developing TB (Lawn & Zumla, 2011; Restrepo, 2007). Mtb is quickly evolving and new strains classified as multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis have emerged that are outpacing current treatments (Gomez & McKinney, 2004; Raviglione & Smith, 2007). It is imperative that new treatments are developed because as of 2017 tuberculosis has been responsible for ten million new cases and 1.3 million deaths annually (Segar & Reuters, 2018).

An important protein involved in Mtb cell division is filamenting temperature-sensitive mutant Z (FtsZ; Romberg & Levin, 2003). This protein undergoes a process called dynamic polymerization (Fig. 1), whereby GTP-bound MtbFtsZ polymerizes into protofilaments that form a structure called the Z-ring, which is necessary for cell division to occur. In this process, the cytoplasm of a cell that is ready to divide elicits nucleotide exchange with rapid equilibrium in favor of guanosine 5'-triphosphate (GTP)-bound MtbFtsZ until it

reaches a critical concentration. At this point, polymerization begins, forming protofilaments. As polymerization occurs, GTP hydrolysis is also taking place in a process called 'steady-state turnover'. GTP-bound MtbFtsZ favors polymerization in a straight conformation, while guanosine diphosphate (GDP)-bound MtbFtsZ prefers a curved conformation. Once the cell has divided, regulation of GTP stops and GDP-bound polymers begin to disassemble, reverting to GDP-bound monomers.

The mechanism by which protofilaments are formed is accomplished by the insertion of the T7 loop of an FtsZ

protomer into the nucleotide-binding site of another FtsZ protomer in a longitudinal head-to-tail fashion (Fig. 2; Oliva *et al.*, 2004). The T7 loop contains Asn205, Asp207 and Asp210, which are necessary for GTP hydrolysis (Oliva *et al.*, 2007; Scheffers *et al.*, 2002; Wang *et al.*, 1997). GTP hydrolysis is not required for FtsZ to assemble into protofilaments, but is responsible for the conformational changes that protofilaments undergo during steady-state turnover (Huecas & Andreu, 2004; Lu *et al.*, 2000).

MtbFtsZ is a GTPase-activated protein that contains two globular domains separated by helix H8 (Fig. 2) and has the

Figure 1

Schematic of protofilament polymerization. (a) Nucleotide exchange takes place in favor of GTP. (b) The concentration of GTP-bound FtsZ reaches a critical point and polymerization occurs. (c) GTP hydrolysis occurs, which forces the protofilaments to adopt a curved conformation. (d) Cell division is complete and nucleotide exchange in favor of GDP-bound FtsZ occurs, which induces depolymerization of the protofilaments.

Figure 2

Polymerization of FtsZ in a longitudinal head-to-tail fashion. (a) The T7 loop (red) inserts into the nucleotide-binding site. The N-terminal domain is shown in green and the C-terminal domain in blue. GTP γ S is represented in orange. (b) Crystal structure of MtbFtsZ (PDB entry 2q1y, chain A). The two globular domains are separated by helix H8 (shown in yellow). Switch I (sH2) and switch II (T3 loop) are shown in magenta in their ON position.

tubulin signature motif 103-GGGTGSG-109 (Supplementary Fig. S3), with one substitution where S (serine) is replaced by T (threonine). MtbFtsZ also has the ability to bind other nucleotides such as citrate (Oliva *et al.*, 2007). Switch I and switch II (shown in magenta in Fig. 2) have been identified in MtbFtsZ to be helix sH2 (where 's' denotes switch) and the T3 loop, respectively (Leung *et al.*, 2004).

Helix sH2 in MtbFtsZ is spatially analogous to the G-protein switch I (Leung *et al.*, 2004). It contains the highly conserved Asn41, Thr42 and Asp43 residues (Supplementary Fig. S5) which are required for GTPase activity. Mutagenesis studies of the corresponding sH2 residues, Asn43 and Asp45, in *Escherichia coli* FtsZ showed a significant reduction in GTPase activity (Lu *et al.*, 2001). These three residues form an intricate hydrogen-bond network that is determined by the ligation state of FtsZ (Fig. 3; Leung *et al.*, 2004). When GTP is bound, the side-chain O atom of Asn41 is directed to the β - and γ -phosphate O atoms by a water molecule. The Thr42 O atom from the side chain coordinates to the Thr106 side-chain O atom, which interacts with the γ -phosphate O atom through a hydrogen bond. The side chain of Asp43 is directed away and interacts with the Ala46 N atom. When the switch is in the OFF position, the side chains of these residues flip their orientation, breaking the bridge. Once this happens, the entire sH2 region takes the form of a β -sheet or a loop and in rare cases remains a helix.

Switch II, which is also known as the T3 loop, needs to be in the OFF position for switch I to change conformation from a helix to a β -sheet or loop (Leung *et al.*, 2004). The T3 loop becomes disordered in the presence of GDP or citrate or when no nucleotide is present. Residues from the T3 loop, Leu66, Gly69, Ala68 and Ala70, become rigid when the nucleotide is GTP(γ)S (Fig. 3). The T3 loop collapses inwards when it interacts with these residues. Ala68 and Ala70 coordinate directly to the γ -phosphate, and Leu66 and Gly69 are stabilized by a water molecule. After GTP hydrolysis, this system is broken and the T3 loop becomes disordered (its OFF

position), which allows sH2 to adopt its β -sheet or loop form (Leung *et al.*, 2004).

MtbFtsZ was selected as a target for drug-discovery efforts because of its role in cell division (Slayden *et al.*, 2006). Studies identified that one of the key steps in Mtb proliferation is cell division, which is triggered by FtsZ (Huang *et al.*, 2006). Once MtbFtsZ protofilaments form the cytoskeletal framework, called the Z-ring, FtsA is recruited and serves as the linker between MtbFtsZ and the bacterial cell wall, which can now be constricted (Ma *et al.*, 1996; Pichoff & Lutkenhaus, 2005). In a study published by Respicio and coworkers, residue Asp210 in MtbFtsZ was mutated to a glycine, which significantly reduced polymerization (100-fold) and lowered GTP hydrolysis (fivefold) when compared with the wild type. This mutation resulted in reduced viability of the Mtb merodiploid strain (Respicio *et al.*, 2008).

Ojima and coworkers have synthesized small molecules that exhibit anti-TB activity which target MtbFtsZ (Kumar *et al.*, 2010). These novel compounds are trisubstituted benzimidazoles that reduce the polymerization of MtbFtsZ (Awasthi *et al.*, 2013). One of the most promising drugs developed is SB-P17G-A20 (Supplementary Fig. S1), which has been shown to target MtbFtsZ protofilaments with a minimum inhibitory concentration (MIC) of $0.16 \mu\text{g ml}^{-1}$ (Knudson *et al.*, 2014).

Here, we present a crystal structure of MtbFtsZ which contains six protomers in the asymmetric unit that form a dimer of trimers. These trimers revealed the hinge mechanism proposed by Li *et al.* (2013) and a novel conformation involving the insertion of the T9 loop into the nucleotide-binding pocket.

2. Materials and methods

2.1. Macromolecule production

Protein purification was carried out as reported by White *et al.* (2000). *E. coli* expression plasmids constructs that carry the FtsZ gene from Mtb were used to grow bacteria expressing MtbFtsZ. These colonies were grown in selective Luria-Bertani medium. Protein expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside and the cells were pelleted and flash-frozen for storage. The cells were later thawed and suspended in 50 mM Tris pH 7.5, 500 mM NaCl, 100 mM KCl, 0.01% NP-40 and lysed in a cell disrupter. Centrifugation was performed and the clear lysate was added to Ni^{2+} -charged His-Bind resin. The protein was washed in 50 mM Tris pH 7.5, 300 mM NaCl, 100 mM KCl, 500 mM imidazole. The protein was concentrated to 3 mg ml^{-1} and checked using the Bradford assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the protein purity (Fig. 4a). The N-terminal His₆ affinity tag was removed by treatment with thrombin (0.25 units of biotinylated thrombin per milligram of tagged FtsZ protein). To remove biotinylated thrombin and uncut FtsZ protein, free unbound affinity tags were passed successively through streptavidin agarose and fresh Ni^{2+} -charged His-Bind resin. Further purification was performed

Figure 3
Hydrogen-bond network between the γ S phosphate (orange), switch I residues (light blue) and switch II residues (gray) in PDB entry 1rlu (Leung *et al.*, 2004). The N-terminal domain is shown in green and GTP is shown in orange. Red spheres represent water molecules and yellow dashed lines represent hydrogen bonds.

Table 1
Macromolecule-production information.

Source organism	<i>M. tuberculosis</i> CDC1551
DNA source	<i>M. tuberculosis</i> CDC1551
Forward primer (BamHI site)	CGTTTGGATCCACTTTGATGACCCCCCGCAC
Reverse primer (XhoI site)	TTTGGCCTCGAGATGTTAGCGGCGCATGAA
Cloning vector	pET-28b
Expression vector	pET-28b
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MTTPHNYLAVIKVVGIGGGVNAVNRMIEQGLKGVFI AINTDAQALLMSDADVKLDVGRDSTRGLGAGADPEVGRKAAEDAKDEIEELLRGADMVFTAGEGGGTGTGGAPVVASIARKLGALTGVVTRPFSFEGKRRSNQAENGIAALRESCDTLIVIPNDRLLQMGDAVSLMDAFRSADEVLLNGVQGITDLITTPGLINVDFAVKGIMSGAGTALMGIGSARGEGRSLKAAEIAINSPLLEASMEGAQGVLSIAGGSDLGLFEINEASLVQDA AHPDANI IFGTVIDDSLGEVVRVTIAAGFDVSGPGRKPFVMGETGGAHRIESAKAGKLTSTLFEFVDAVSVP LHTNGATLSIGGDDDDVDVPPFMRR

by size-exclusion chromatography using an ÄKTA-driven Superdex S200 60/16 column in 50 mM Tris pH 7.8, 200 mM NaCl, 100 mM KCl storage buffer (Fig. 4*b*). The protein was concentrated to 3 mg ml⁻¹ and flash-frozen. Table 1 shows the macromolecule-production information.

2.2. Crystallization

MtbFtsZ crystals (Supplementary Fig. S2) were produced by the hanging-drop vapor-diffusion method at 293 K. The initial crystallization condition used was that reported by Leung *et al.* (2000). The condition in which these crystals grew is shown in Table 2. The drop was composed of 1 µl reservoir solution and 1 µl protein solution containing 0.5 mM SB-P17G-A20. The crystals were soaked in reservoir solution that

Table 2
Crystallization.

Method	Hanging-drop vapor diffusion
Plate type	15-well
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	3.0
Buffer composition of protein solution	50 mM Tris pH 7.8, 200 mM NaCl, 100 mM KCl
Composition of reservoir solution	0.1 M sodium citrate pH 5.6, 0.3 M ammonium acetate, 15% PEG 4000
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (ml)	0.5

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.	
Diffraction source	Beamline X25, NSLS
Wavelength (Å)	1.1000
Temperature (K)	100
Detector	PILATUS 6M
Rotation range per image (°)	0.2
Total rotation range (°)	90
Exposure time per image (s)	1
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.1, 180.9, 220.2
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.205
Resolution range (Å)	55.05–3.46 (3.55–3.46)
Total No. of reflections	123964 (9116)
No. of unique reflections	37287 (2754)
Completeness (%)	96.4 (97.3)
Multiplicity	3.3 (3.3)
$\langle I/\sigma(I) \rangle$	9.3 (1.8)
<i>R</i> _{int}	0.053 (0.366)
Overall <i>B</i> factor from Wilson plot (Å ²)	73.698

containing 30% glycerol before being cryocooled in liquid nitrogen.

2.3. Data collection and processing

Crystals were screened on beamline X6A and data were collected on beamline X25 of the National Synchrotron Light

Figure 4
(*a*) SDS-PAGE gel showing that most of the protein (>95%) has a molecular mass of 40 kDa. Molecular-mass markers are labeled in kDa. (*b*) FPLC profile of MtbFtsZ without the His tag. Peak 4 was the peak used in this study and represents a dimeric form of 80 kDa. The rest of the peaks represent higher order structural forms (the protomer mass of MtbFtsZ is ~40 kDa) that are greater than 80 kDa. Peak 4 was used because the other peaks represent a mixture of protein molecules of different masses bound together.

Table 4
Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	55.05–3.46 (3.55–3.46)
Completeness (%)	95.5
No. of reflections, working set	35388 (2597)
No. of reflections, test set	1881 (143)
Final R_{cryst}	0.243 (0.348)
Final R_{free}	0.309 (0.370)
No. of non-H atoms	
Protein	11880
Ligands	66
Water	0
R.m.s. deviations	
Bonds (Å)	0.011
Angles (°)	1.470
Average B factors (Å ²)	
Protein	119.80
Ligands	96.59
Ramachandran plot	
Most favored (%)	83.79
Allowed (%)	11.60
Disallowed (%)	4.60

Source (NSLS) at Brookhaven National Laboratory. Frames were processed with *xia2* using the *-3dii* option (Winter *et al.*, 2013). The crystals belonged to space group $P2_12_12_1$, with six molecules in the asymmetric unit, corresponding to a Matthews coefficient of $3.13 \text{ Å}^3 \text{ Da}^{-1}$ (a solvent content of 60.68%; Matthews, 1968, 1976; Kantardjieff & Rupp, 2003). The data-collection statistics are shown in Table 3.

2.4. Structure solution and refinement

The structure was solved by molecular replacement with *PHENIX* (Adams *et al.*, 2002, 2010; Terwilliger *et al.*, 2012) using MtbFtsZ (PDB entry 2q1y; 100% sequence identity; Respicio *et al.*, 2008) as a model. Initial model building was also performed with *PHENIX* (Terwilliger *et al.*, 2008). Iterative cycles of building and refinement were conducted, and final refinement to 3.46 Å resolution was performed using *REFMAC* 5.8.0 (Murshudov *et al.*, 2011). The structure was submitted to the Protein Data Bank and assigned PDB code 5v68 (Berman *et al.*, 2000, 2003; Bernstein *et al.*, 1977). Figures were prepared using *PyMOL* (Schrödinger). Table 4 shows the structure-solution and refinement statistics.

3. Results

M. tuberculosis filamenting temperature-sensitive mutant Z (MtbFtsZ) was purified from an *E. coli*-based expression system (Table 1). The purified protein was crystallized at 293 K (Table 2). The structure was solved to 3.46 Å resolution by molecular replacement using PDB entry 2q1y (Respicio *et al.*, 2008) as a search model (Table 4). The final model was refined to an R factor of 24.3% and an R_{free} value of 30.9% using data between 55.05 and 3.46 Å resolution (Table 4). 95.39% of residues were in the favored and allowed regions of the Ramachandran plot (Table 4).

The crystal structure is comprised of six protomers in the asymmetric unit that form a dimer of trimers: chains A, B, C

and chains D, E, F (Fig. 5*a*). When superimposed, trimers ABC and DEF have an r.m.s.d. of 1.13 Å (Fig. 5*b*). This shows that the trimers are very similar. The structure has two GDP moieties in the nucleotide-binding pockets of protomers B and E, along with two phosphates in the nucleotide-binding sites of protomers C and F. The relative orientation of the receptive subunits (protomers C and F) and the other two subunits has not been reported before. Even propagating crystallographic

Figure 5
(*a*) The asymmetric unit contains six protomers that form a dimer of trimers. Protomers A and C are shown in blue, protomer B is shown in gray, protomers D and F are shown in red, protomer E is shown in cyan, GDP is represented by orange spheres and phosphate is represented by green spheres. (*b*) Trimer ABC superimposed onto trimer DEF. The same color scheme is used as in (*a*).

Figure 6

(a) PDB entry 5v68 superimposed onto PDB entry 4kwe. The nucleotide in both is GDP. (b) PDB entry 5v68 with bound GDP superimposed onto PDB entry 2q1y with bound GTP. (c) PDB entry 4kwe with bound GDP superimposed onto PDB entry 2q1y with bound GTP. The color scheme is as follows: PDB entry 5v68, blue; PDB entry 4kwe, yellow; PDB entry 2q1y, green; nucleotides, orange spheres; Glu231, green spheres; phosphate, red spheres.

symmetries in all possible permutations does not reproduce the observed orientations of subunits *C* and *F* (Fig. 6).

Comparison of other MtbFtsZ structures with our trimer *ABC* reveals that protomers *A* and *B* superimposed well but the conformation of protomer *C* is vastly different (Fig. 6). In Fig. 6(a), our trimer *ABC* is compared with a curved MtbFtsZ protofilament (PDB entry 4kwe; Li *et al.*, 2013). Based on an r.m.s.d. of 1.2 Å between protomers *AB* of PDB entry 5v68 and protomers *CB* of PDB entry 4kwe, our protomers *A* and *B* are similar to protomers *C* and *B* of PDB entry 4kwe. Glu231 (shown as green spheres) of protomer *C* from PDB entry 5v68 and Glu231 of protomer *A* from PDB entry 4kwe are far apart, with a distance of 58.3 Å. Fig. 6(b) shows a superimposition of our structure (PDB entry 5v68) with that of MtbFtsZ with bound GTP (PDB entry 2q1y). To generate this trimer, A1A2A3, the crystal symmetry molecules neighboring chain *A2* that exhibit the inter-subunit interface were used. Only chains *A1* and *A2* were used in the superimposition. Again, protomers *A* and *B* from our study are similar to the top two protomers *A1* and *A2* from PDB entry 2q1y, with an r.m.s.d. of 2.1 Å. The distance of their respective Glu231 residues is approximately 63.8 Å. Superimposition of PDB entries 4kwe and 2q1y in Fig. 6(c) reveals an r.m.s.d. of 2.8 Å between the trimers and a 12.5 Å distance between the respective Glu231 residues of protomers *A* and *A3*, showing that these structures are relatively similar. The distances between Glu231 of PDB entry 5v68 from protomer *C* are far greater than 12.5 Å and the interaction with the middle protomers of PDB entries 4kwe and 2q1y with protomer *C* of 5v68 involve the T9 loop residue Glu231, which is not the case in the other structures.

The top two protomers of all three structures (*AB* for PDB entry 5v68, A1A2 for PDB entry 2q1y and *CB* for PDB entry 4kwe) all exhibit a similar inter-subunit interface (Fig. 6). This interaction between protomers *A* and *B* of PDB entry 5v68

involves the T6 and T7 loops and helices H11, η_1 and H7 (Fig. 7a). Protomer *A* of our structure ‘sits’ on helices η_1 and H7 and loop T6 (all shown in blue) from protomer *B*. This brings residues Asn205, Asp207 and Asp210 of the T7 loop (shown in red) of protomer *A* of PDB entry 5v68 within 16 Å of GDP from protomer *B*, forming the GTPase active site. The T3 loop is disordered or in its OFF position (surrounded by a magenta cloud). Helix sH2 is also in the OFF position because there is no hydrogen-bond network (shown in magenta).

Protomer *C* of our structure (PDB entry 5v68) has a novel conformational change involving protomer *B*. In this conformation, the T9 loop of protomer *B* is inserted into the nucleotide-binding pocket of protomer *C* (Fig. 7b). Loop T11 (cyan) maintains the T3 loop in the OFF position; thus, the hydrogen-bond network to sH2 is not present. An enlarged view of the loop T9 interaction where the residues involved are fitted into a $2F_o - F_c$ electron-density map contoured at 1σ is shown in Fig. 7(b). The residue from the T9 loop involved in this conformation is Glu231 from protomer *B*, where the side-chain O atom forms a hydrogen bond to the side-chain N atom of Arg140 from protomer *C*. The Glu274 side-chain O atoms from protomer *B* coordinate to the amine N atoms of Arg140 of protomer *C*. The nucleotide-binding pocket is large enough to accommodate a phosphate bound by protomer *C* residues Gly17 and Gly18 from helix H1 and Gly105 and Thr106 from helix H5. All of these residues are conserved throughout other FtsZ systems, which could be an indication that these residues play a biological role in FtsZ (Supplementary Fig. S3).

4. Discussion

MtbFtsZ protofilaments form the Z-ring, which can provide FtsA in order to attach itself to the bacterial cell wall, forming the septum (Ma *et al.*, 1996; Pichoff & Lutkenhaus, 2005). This

septum begins the circumferential invagination of the bacterium, which mechanically moves by switching from straight to curved protofilaments, which is powered by GTP hydrolysis (Lutkenhaus, 1993; Lu *et al.*, 2000). The interface between protomers *C* and *B* (PDB entry 4kwe) that was observed by Li *et al.* (2013) (Fig. 6a) demonstrates the ‘hinge’

where protomer *C* from PDB entry 4kwe can pivot and form either straight or curved protofilaments depending on the nucleotide (Li *et al.*, 2013). This inter-subunit interface was observed by us in protomers *AB* and *DE* (Figs. 5 and 7a) as well as in *Staphylococcus aureus* FtsZ (SaFtsZ; PDB entry 4dxd; Tan *et al.*, 2012). Mutagenesis studies were performed

Figure 7

(a) Crystal structure of protomers *A* and *B* of PDB entry 5v68 (gray). The box represents an enlargement of the inter-subunit interface with bound GDP (orange). Helix H11 is shown in green. Helices $\eta 1$ and H7 and loop T6 are shown in blue. Switch I (the T3 loop) is disordered (surrounded by a magenta cloud). Switch II (sH2) is shown in magenta. (b) Crystal structure of protomers *B* and *C* of PDB entry 5v68. Protomer *B* is shown in white and the T9 loop in black; protomer *C* follows the same color scheme as in Fig. 2, where the N-terminal domain is shown in green, the C-terminal domain in blue, helix H8 in yellow, the T7 loop in red, the T11 loop in cyan and the switches in magenta. The blue cloud indicates the region that is shown in the enlargement, which shows the T9 loop conformation of protomer *C*. Glu231 from protomer *B* is shown in black, while Glu274 from protomer *B* is shown in white. The residues in green are from protomer *C*. The $2F_o - F_c$ map is contoured at 1σ . Hydrogen bonds are represented by yellow dashed lines.

for MtbFtsZ and SaFtsZ on several residues involved in this inter-subunit interaction by Li *et al.* (2013) and demonstrated reduced GTPase activity, thus showing that this inter-subunit interaction plays a biological role.

In the GTP-hydrolysis mechanism proposed by Li *et al.* (2013), when the protomer switches are OFF, as seen in Fig. 7(a), GTP is able to enter the nucleotide-binding pocket. Once GTP has bound to the active site, the switches are turned ON, as shown in Fig. 3. Protomers with GTP can now assemble in a head-to-tail fashion, as seen in Fig. 2, forming straight protofilaments. This brings the T7 loop residues Asn205, Asp207 and Asp210 to complete the GTPase site needed to cleave the γ -phosphate. After GTP hydrolysis, coordination with sH2 no longer exists and the T3 loop is turned OFF or becomes disordered; when the T3 loop is disordered, the protofilaments are also curved (Fig. 7a). This ON/OFF cycle continues and powers the constriction of the Z-ring until cytokinesis is complete (Li *et al.*, 2013).

Here, we propose a molecular mechanism for the observed insertion of Glu231 from the T9 loop into the nucleotide-binding pocket in MtbFtsZ function. The T9 loop conformation of protomer C with protomer B occludes the nucleotide-binding pocket of protomer C (Fig. 7b). This interaction stabilizes the T3 loop from protomer C in the OFF position via the T11 loop from protomer B. Helix sH2 is maintained OFF and the T7 loop no longer forms the GTPase active site. If GTP has no access to the nucleotide-binding pocket, the switches are maintained OFF and the GTPase active site is no longer present, causing GTPase activity to cease. If GTP hydrolysis is halted, the opening and closing of the hinge mechanism ceases (Li *et al.*, 2013). With the protofilaments no longer able to switch from a curved to a straight conformation, the mechanical constriction force required for cytokinesis is removed and ultimately prevents the organism from dividing (Lu *et al.*, 2000; Lutkenhaus, 1993; Li *et al.*, 2013).

The trisubstituted benzimidazole SB-P17G-A20 that was used in the crystallization condition targets protofilament formation by reducing MtbFtsZ polymerization (Knudson *et al.*, 2014). No electron density for SB-P17G-A20 was observed in our structure (PDB entry 5v68). This could be owing to the relatively high salt concentration in our crystallization condition (Table 2). In general, studies of ligand–macromolecule interactions have shown that when salt concentrations are increased the binding constant decreases (Riggs *et al.*, 1970; Latt & Sober, 1967; Poliakov *et al.*, 1972). Currently, there is no evidence that the trisubstituted benzimidazole SB-P17G-A20 plays a role in the conformational change of protomer C in our structure. This study may serve as a basis for future studies with trisubstituted benzimidazoles and MtbFtsZ to determine whether there is a correlation by discovering other crystallization conditions that favor binding or by soaking protein crystals with SB-P17G-A20.

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