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Crystal structure of VapC21 from *Mycobacterium tuberculosis* at 1.31 Å resolution

Paulo Jardim, Isadora Cristina da Silva Santos, João Alexandre Ribeiro Gonçalves Barbosa, Sônia Maria de Freitas, Napoleão Fonseca Valadares*

Laboratory of Molecular Biophysics, Department of Cell Biology, University of Brasília, Brasília, Brazil

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ABSTRACT

Persisters are individual bacterial cells that exhibit a phenotype characterized by slow growth, low metabolic rate and multidrug tolerance. The processes that drive cells into a persistence state constitute an active but incipient research field, and structural data regarding its components are scarce. The molecular targets of many therapeutic drugs are involved in cell wall synthesis and cell division, and these cellular processes are down-regulated in persister cells, consequently these cells are more likely to survive antibiotic treatment. Toxin-antitoxin systems were shown to have a leading role in the formation of persisters, and several pathogenic bacteria display a wide array of these systems. The *Mycobacterium tuberculosis* H37Rv genome presents 88 toxin-antitoxin loci, of which 47 code for members of the VapBC protein family. To date, only four crystal structures of *Mycobacterium tuberculosis* VapBC complexes are available, and all of them present the toxin bound to and inhibited by the antitoxin. We present the 1.31 Å resolution structure of VapC21, the first structure of a *Mycobacterium tuberculosis* VapC toxin in the absence of its cognate inhibitory antitoxin. Our data show that VapC21 is a dimer in solution, with conserved active site architecture and an extensive antitoxin binding groove. Additionally, the strategy used to mutate a putative catalytic residue allowing the expression and purification of soluble VapC21 will pave the way for the resolution of more toxin structures in the absence of antitoxins. Taken together, our findings represent an important step in unraveling the molecular mechanisms related to persistence, which will contribute for the design of faster and more efficient therapeutic approaches for the treatment of tuberculosis, particularly for infections with multidrug-resistant strains.

1. Introduction

Mycobacterium tuberculosis (Mtb) is a widespread human pathogen that currently infects billions of individuals. Most of them present a latent form of infection that does not display any clinical symptom, but might develop into active tuberculosis later in life [1,2]. It is estimated that around half a million cases of tuberculosis resistant to isoniazid and rifampicin, defined as multidrug-resistant tuberculosis, occurred in 2014, further complicating this global public health issue [3].

In addition to drug-resistance and an efficient mechanism of infection, persistence is a less known factor that contributes to the high prevalence of Mtb in the human population [4]. Unlike drug-resistance, bacterial persistence do not arise from mutations, plasmid gene incorporation, or any other genomic alteration, but from a random change of few individual antibiotic sensitive bacterial cells into a state marked by very low metabolic rate and transient multidrug tolerance [5]. Since the effects of most antibiotics rely on the inactivation of specific active protein targets, the drug tolerance of persisters arises as a consequence of their low activity non-growing or dormant state.

Toxin-antitoxin (TA) systems are part of the main molecular machineries that drive bacterial cells into a persistent state [6]. The *Mycobacterium tuberculosis* H37Rv genome presents 88 toxin-antitoxin loci [7], of which 47 correspond to the *vap*BC family (*v*irulence *a*ssociated *p*rotein). These are type II TA systems that participate in the general stress response, including hypoxia and nutritional stress [1,8]. Type II TA systems function as operons expressing a toxin (VapC) presenting a PIN domain associated to assorted ribonuclease activities [8–11] and an antitoxin (VapB) that binds and inhibit the toxin [6,7]. Recent studies show that VapC

* Corresponding author.
E-mail address: napo@unb.br (N.F. Valadares).

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toxins display various RNase activities, including cleavage of mRNA, cleavage of the Sarcin–Ricin loop of 23S rRNA and selective cleavage of tRNAs [8–12], and that these proteins are inhibited by the binding of their cognate VapB antitoxins. In these complex self-regulated systems the antitoxin is capable of binding specific DNA sequences to regulate the transcription of the operon, and the binding affinity to DNA is increased when the antitoxin is bound to a toxin. The antitoxins exhibit higher susceptibility to proteases, and their degradation renders the toxins free to exert their cytotoxic effects [5,13].

The relevance of toxin-antitoxins systems extends beyond their role in transient multidrug tolerance. In a wide variety of bacteria these systems were shown to participate in the general stress response, biofilm formation, cell motility and modulation of pathogenicity [6,8,14,15]. Additionally, these systems may influence bacterial fitness, competitiveness and multidrug resistance in biofilms. Therefore a better understanding of their molecular mechanisms is central to the study of persistence in pathogenic bacteria, and of general importance in the study of prokaryotes [13,16].

Currently, only 4 of the 47 members of the VapBC family in Mtb H37Rv present crystal structures deposited in the Protein Data Bank (PDB), VapC3, VapC5, VapC15 and VapC30 [17–20], and in all these cases the structures reported are toxin-antitoxin complexes. In the present study, we report the expression, purification and crystallization of VapC21 from Mtb, followed by its crystal structure at 1.31 Å resolution, which is the first structure of a Mtb toxin in the absence of its cognate antitoxin. We anticipate that this atomic resolution structural data will contribute to the understanding of the molecular mechanisms that drive cells into a persistent state.

2. Material and methods

2.1. Reactants

pET-24a+ expression vectors harboring the sequence of vapC21 cloned using the sites *Nde*I and *Xho*I were purchased from GenScript.

Protino Ni-NTA metal affinity column were purchased from Macherey-Nagel and a Superdex 75 10/30 column was purchased from GE. Crystallization screening kits were purchased from Hampton Research. Imidazole, kanamycin, chloramphenicol and all other reactants were purchased from Sigma.

2.2. Protein expression and purification

Competent *E. coli* BL21 (DE3) pLysE cells were transformed using pET24a+ coding for vapC21. Single colonies were grown overnight at 37 °C and 220 RPM on LB media containing 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. 1 mL of this cell culture was transferred to a 1 L conical flask containing 500 mL of LB supplemented with 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. After the OD_{600} reached 0.8, IPTG was added to a final concentration of 0.3 mM and the cells were grown at 16 °C and 220 RPM for 12 h. The cells were harvested by centrifugation for 30 min at 12900g, and the pellets containing the cells were re-suspended in standard buffer (75 mM Tris, pH 8, 300 mM NaCl, 3 mM 2-mercaptoethanol) and lysed by sonication in an ice bath. Cell debris were removed by centrifugation at 7200g for 40 min at 4 °C. The supernatant was filtered through a 0.45 μM disposable filter, supplemented with imidazole to a final concentration of 15 mM and loaded in a 1 mL Protino Ni-NTA metal affinity column pre-equilibrated with standard buffer. The column was washed with 30 mL of standard buffer supplemented with 15 mM imidazole, and a flow rate of 1 mL min^{-1} was used through all the process. A second wash with standard buffer supplemented with 90 mM imidazole was performed for 20 min, and protein was eluted in standard buffer containing 400 mM imidazole and kept on ice. The protein was loaded in a Superdex 75 10/30 column (GE) previously equilibrated with standard buffer and connected to an AKTA Purifier (GE) using a flow rate of 1 mL min^{-1}. Fractions corresponding to the protein were collected, pooled and concentrated in an Amicon Ultra-15 centrifugal filter concentrator with cutoff of 3.000 Da (Millipore). Samples were collected throughout the process to monitor the presence of the VapC21 by 15% SDS-PAGE.

2.3. Crystallization assays

The pure VapC21 samples were concentrated to 5 mg mL^{-1} and used in automated crystallization screens using a Mosquito HTS robot and the Crystal Screen and Crystal Screen 2 reagent kits from Hampton Research. 96-well plates were prepared, and hanging drops containing 200–300 nL of a solution containing a mixture of protein and well solutions were incubated at 20 °C. Promising conditions were further developed in manual assays with freshly prepared protein and mother liquor solutions. Crystals were observed after 4 days in a condition containing 0.1 M Hepes pH 7.5, 8% v/v ethylene glycol, 10% w/v polyethylene glycol 8000.

2.4. Data collection, processing, structure solution and refinement

X-ray diffraction data were collected at the MX2 beamline of the Laboratório Nacional de Luz Síncrotron (Campinas, Brazil) at 100 K and wavelength 1.240 Å on a Pilatus 2 M detector from Dectris. Immediately before diffraction data collection, crystals were cryoprotected in mother liquor supplemented with ethylene glycol to a final concentration of 18%. Crystals were cryo cooled directly in the cryo nozzle in use at the MX2 beamline. The diffraction data were indexed, integrated and scaled using the program XDS [21]. The crystal belong to the space group C121 with cell dimensions $a = 61.34$, $b = 44.35$, and $c = 56.53$ Å, angles $\alpha = 90.00°$, $\beta = 101.72$, $\gamma = 90.00°$, and diffracted to a resolution of 1.31 Å.

The initial molecular replacement attempts using as template the structures of VapC3, VapC5, VapC15, VapC30 (PDB codes 3H87, 3DBO, 4CGH and 4XGR) [17–20] were unsuccessful. Innumerous attempts using these templates and the program CHAINSAW [22] to mutate their residues to alanine or glycine, as well as manually removing the loops and other parts of the templates were not successful.

To circumvent these difficulties, a model of VapC21 was generated using only chain A of PDB 3H87 as template and the program MODELLER 9.15 [23] with a combination of parameters ensuring that the φ and ψ angles of the model were very similar to those of the template. All residues were deleted with the exception of those in helices 5, 6 and 7, and most residues were mutated to alanine. This template was submitted to a series of molecular replacement procedures in PHASER [24], followed by visual inspections in the program COOT [25] with the goal of observing plausible electron density for Trp74 and/or Trp78. A promising solution was identified and confirmed by a steady reduction in the R_{work} and R_{free} values after each round of chain extension and density fitting in COOT followed by refinement in PHENIX [26]. The structure refinement was performed using PHENIX and COOT for model building and density fitting, while monitoring the values of R_{work} and R_{free} and evaluating the stereochemistry of the resulting models using Molprobity [27]. The coordinates and structure factors are deposited in the PDB under the code 5SV2.

3. Results and discussion

The *M. tuberculosis* genome presents 88 loci corresponding to

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