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Crystallization and preliminary X-ray crystallographic studies of *Mycobacterium tuberculosis* chorismate mutase

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Chorismate mutase catalyzes the first committed step in the biosynthesis of the aromatic amino acids phenylalanine and tyrosine in bacteria, fungi and higher plants. The recent re-annotation of the *Mycobacterium tuberculosis* genome has revealed the presence of a duplicate set of genes coding for chorismate mutase. The mycobacterial gene Rv1885c bears <20% sequence homology to other bacterial chorismate mutases, thus serving as a potential target for the development of inhibitors specific to the pathogen. The *M. tuberculosis* chorismate mutase was crystallized in space group *C*2 and the crystals diffracted to a resolution of 2.2 Å. Matthews coefficient and self-rotation function calculations revealed the presence of two monomers in the asymmetric unit.

1. Introduction

Mycobacterium tuberculosis possesses intricate mechanisms for survival inside the hostile environment of the host and an ability to acquire essential nutrients from this environment (Wayne, 1994; Betts *et al.*, 2002). It has previously been shown that amino-acid auxotrophs of *M. tuberculosis* do not survive or multiply within macrophages, suggesting that these nutrients are not available within the compartment of the macrophage in which the bacteria reside (Bange *et al.*, 1996; Gordhan *et al.*, 2002). Moreover, genes coding for proteins involved in the biosynthetic pathway of aromatic amino acids have been reported to be essential for *M. tuberculosis* (Parish & Stoker, 2002).

Chorismate mutase (EC 5.4.99.5) is a branchpoint enzyme of the shikimate pathway and frequently exists as a fusion protein. The most common associations of chorismate mutase are seen with other enzymes of the aromatic amino-acid biosynthesis pathway such as prephenate dehydrogenase, prephenate dehydratase, deoxy-*arabino*-heptulosonate phosphate (DAHP) synthase *etc*. Chorismate mutase channels its substrate, chorismate, towards the biosynthesis of phenylalanine and tyrosine in lieu of tryptophan. It thus plays an important role in regulating the balance of aromatic amino acids in the cell. Since the shikimate pathway and hence chorismate mutase exist exclusively in bacteria, fungi and higher plants, the enzyme is likely to be a useful target for drug development (Kishore & Shah, 1988).

Chorismate mutases have previously been classified into three categories: the AroH class, the prokaryotic AroQ class and the eukaryotic AroQ class. Interestingly, the tertiary structures of AroQ and AroH class enzymes are completely unrelated. While the AroQ proteins belong to the all-α fold (Lee *et al.*, 1995), the AroH proteins possess a trimeric pseudo-α/β-barrel structure (Chook *et al.*, 1993). The presence of completely different tertiary folds in different organisms suggests a separate evolutionary origin for chorismate mutases with convergent enzymatic activity. Moreover, a few of the prokaryotic AroQ enzymes are known to be periplasmic and many others are known to possess large regulatory domains.

The annotated genome sequence of *M. tuberculosis* shows the presence of two open reading frames (Rv1885c and Rv0948c) with weak sequence similarity to the known chorismate mutases (Cole *et al.*, 1998; Camus *et al.*, 2002). A recent study suggests that the hypothetical protein coded by Rv1885c represents the chorismate

mutase enzyme of this organism (Sasso *et al.*, 2005; Prakash *et al.*, 2005). The study shows that *M. tuberculosis* chorismate mutase does not possess any associated prephenate dehydratase or dehydrogenase activity, indicating its monofunctional nature. Size-exclusion chromatography reveals the existence of the *M. tuberculosis* chorismate mutase as a dimer. Extensive biochemical and biophysical characterization of the recombinant protein suggests its resemblance to the AroQ class of chorismate mutases, prototypical examples of which include the *Escherichia coli* and yeast chorismate mutases. Considering the fact that the enzyme does not have a human homologue, understanding the mechanism of its action would open up avenues for the development of novel therapeutic interventions against tuberculosis.

2. Materials and methods

2.1. Expression and purification

E. coli BL21 (DE3) cells harbouring the expression vector pET23a with the gene Rv1885c, cloned in *Nde*I and *Xho*I sites with a C-terminal 6×His tag, grown in 1 l LB medium supplemented with 100 µg ml^{-1} ampicillin and 10% glycerol. The culture was induced at an OD_{600} of 0.4 with 0.1 m*M* IPTG at 300 K and 150 rev min^{-1} to allow protein expression. The cells were harvested by centrifugation and resuspended in lysis buffer (10 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl and 10% glycerol) supplemented with 0.1 m*M* PMSF. After sonication, the supernatant was applied onto a Talon cobalt affinity resin column (Clontech, USA) pre-equilibrated with lysis buffer, followed by washing with five bed volumes of lysis buffer supplemented with 30 m*M* imidazole. The recombinant protein was eluted with lysis buffer supplemented with 250 m*M* imidazole.

2.2. Crystallization

The purified protein was dialysed against 10 m*M* Tris–HCl pH 7.5 and then concentrated to 5 mg ml^{-1} using a Centricon concentrator (Amicon; 3 kDa molecular-weight cutoff). Previous biochemical studies had indicated that L-tryptophan is an allosteric inhibitor of *M. tuberculosis* chorismate mutase and moreover provided protection against trypsin cleavage (Prakash *et al.*, 2005). Thus, the protein was incubated with 1 m*M* L-tryptophan before setting up crystallization trials. The recombinant protein with the 6×His tag was used for crystallization trials in a variety of random conditions using the Magic 96 matrix (Sarfaty & Hol, personal communication). The hanging-drop vapour-diffusion technique was used for random screening of crystallization conditions. 2 µl protein was mixed with 3 µl well solution and allowed to equilibrate against 500 µl well solution. Tiny crystals were obtained at 298 K in 0.2 *M* Li_2SO_4 and 22% PEG 8000 in 0.1 *M* sodium acetate buffer pH 4.6 in 24 h. Crystals grew to average dimensions of 10 × 10 × 10 µm over a period of 4–5 d.

2.3. Data collection

Crystals of chorismate mutase were soaked in artificial mother liquor (0.2 *M* Li_2SO_4 and 28% PEG 8000 in 0.1 *M* sodium acetate buffer pH 4.6) supplemented with 26% glycerol as the cryoprotectant. Intensity data were collected from two crystals at 100 K at the BL41XU beamline at the SPring-8 synchrotron facility using an Area Detector Systems Corporation (ADSC) Q-315 detector. The crystal-to-detector distance was maintained at 300 mm. Individual 1° oscillation frames were collected covering a range of 128 and 70° for the two crystals. The exposure time for each frame was 30 s. Data were processed, scaled and merged with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Self-rotation function calculations were performed using the *POLARRFN* program as available in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell (2.14–2.07 Å)

Wavelength (Å)	1.0
Space group	*C*2
Unit-cell parameters (Å, °)	a = 124.4, b = 83.8, c = 62.7, β = 93.4°
Resolution limits (Å)	70–2.07
Unique reflections	36542 (2491)
R_{merge}† (%)	11.3 (29.3)
Average $I/\sigma(I)$	10.07 (2.24)
Completeness (%)	93.4 (64.1)
Redundancy	3.6 (1.7)
Matthews coefficient (Å^3 Da^{-1})	3.6

† $R_{merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum |I_{hkl}|$, where I_{hkl} are the intensities of symmetry-redundant reflections and $\langle I_{hkl}\rangle$ is the average intensity over all observations.

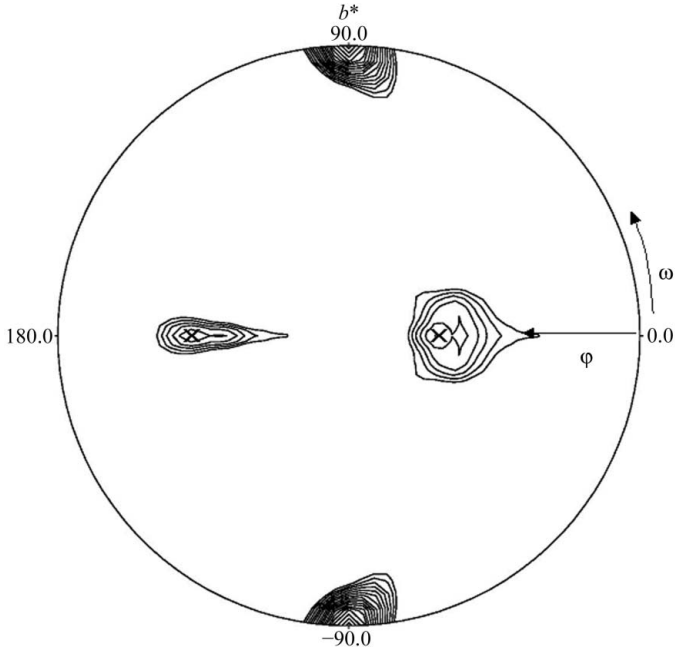

Figure 1
Self-rotation function plot at κ = 180°. The rotation function was calculated with a radius of integration of 18 Å in the resolution range 20–3 Å. The two strongest peaks at φ = 90° indicate the crystallographic twofold symmetry (b*), while the other two peaks marked with '×' correspond to the non-crystallographic twofold axes.

3. Results and discussion

Sequencing of the *M. tuberculosis* chorismate mutase gene confirmed its sequence and that the encoded protein possessed two extra amino acids, Leu and Glu, between its C-terminus and the 6×His tag. The purified protein was found to be homogenous as analyzed by SDS–PAGE, with a molecular weight corresponding to the expected 22 kDa. Crystals of chorismate mutase grew to maximum dimensions of 10 × 10 × 10 µm. Attempts at microseeding after pre-equilibration of the protein droplet did not increase the size to a great extent. Crystals were found to belong to space group *C*2. The merged and scaled data were found to be useful to a resolution of 2.07 Å, with a completeness of 93.4%. Data-collection and resolution statistics are

shown in Table 1. A Matthews coefficient of 3.6 Å^3 Da^{-1} suggested the presence of two molecules in the asymmetric unit, corresponding to a solvent content of 66% (Matthews, 1968).

Previous work had established the dimeric state of this protein (Prakash *et al.*, 2005). Because of the presence of two molecules in an asymmetric unit, calculations of the self-rotation function were used to establish the non-crystallographic symmetry. The self-rotation function showed distinct peaks for $\kappa = 180^\circ$, thus confirming the orientation of the molecular dyad axis (Fig. 1).

In conclusion, therefore, we have crystallized the chorismate mutase from *M. tuberculosis*. The dimeric nature of the molecule has been confirmed using the self-rotation function. However, the low sequence homology with other known chorismate mutases and the failure to obtain a good molecular-replacement solution indicate a need for structure solution using experimental phasing techniques such as multiwavelength anomalous dispersion (MAD) or multiple isomorphous replacement (MIR). Owing to the absence of chorismate mutases in mammals and its essentiality in *M. tuberculosis*, the protein is a promising drug target. The crystal structure of this protein will therefore be a useful starting point for structure-based inhibitor-design studies.

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