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Crystallization and preliminary X-ray diffraction analysis of prephenate dehydratase from *Mycobacterium tuberculosis* H37Rv

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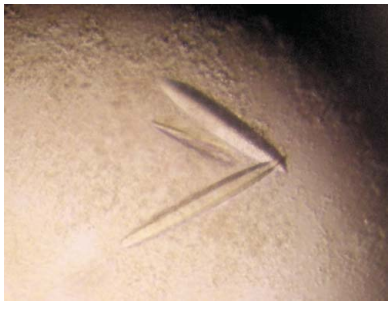

Tuberculosis remains the leading cause of mortality arising from a bacterial pathogen (*Mycobacterium tuberculosis*). There is an urgent need for the development of new antimycobacterial agents. The aromatic amino-acid pathway is essential for the survival of this pathogen and represents a target for structure-based drug design. Accordingly, the *M. tuberculosis* prephenate dehydratase has been cloned, expressed, purified and crystallized by the hanging-drop vapour-diffusion method using PEG 400 as a precipitant. The crystal belongs to the orthorhombic space group $I222$ or $I2_12_12_1$, with unit-cell parameters $a = 98.26$, $b = 133.22$, $c = 225.01$ Å, and contains four molecules in the asymmetric unit. A complete data set was collected to 3.2 Å resolution using a synchrotron-radiation source.

1. Introduction

According to the World Health Organization, one-third of the world population is asymptomatically infected with *Mycobacterium tuberculosis* and approximately eight million people will develop active tuberculosis (TB) and three million people will die every year (World Health Organization, 2005). The increasing prevalence of TB, the emergence of multi-drug-resistant strains (MDR-TB) as a consequence of inappropriate treatment regimens or patient non-compliance in completing the prescribed courses of therapy and the devastating effect of co-infection with HIV have highlighted the urgent need for the development of new antimycobacterial agents. The shikimate pathway is present in algae, higher plants, fungi, bacteria and parasites from the phylum Apicomplexa, but is absent in mammals (Pittard, 1996; Roberts *et al.*, 1998). The essentiality of this pathway in *M. tuberculosis* has been demonstrated by disruption of the *aroK* gene (Parish & Stoker, 2002), which codes for the shikimate kinase that catalyzes the fifth step in chorismate biosynthesis. In mycobacteria, chorismate is the precursor of aromatic amino acids, naphthoquinones, menaquinones and mycobactins (Ratledge, 1982). Amino-acid auxotrophs of mycobacteria have displayed restricted growth in macrophages (Bange *et al.*, 1996; Gordhan *et al.*, 2002), thereby suggesting that amino-acid availability is limited in the vacuoles of the macrophages within which the bacillus resides (Russell, 2005). Accordingly, the aromatic amino-acid biosynthesis pathway is an attractive target for the development of antimycobacterial agents. Homologues of enzymes of the phenylalanine-biosynthesis pathway have been identified in the genome sequence of *M. tuberculosis* H37Rv strain (Cole *et al.*, 1998), including the *pheA*-encoding prephenate dehydratase gene (Rv3838c). Prephenate dehydratase catalyzes the decarboxylation and dehydration of prephenate to form phenylpyruvate, which in turn is converted to phenylalanine by the tyrosine aminotransferase enzyme. In *Amycolatopsis methanolica*, the prephenate dehydratase was characterized as a homotetrameric protein (150 kDa) with a subunit molecular weight of 34 kDa (Euverink *et al.*, 1995). In *Escherichia coli*, the *pheA*-encoded bifunctional P-protein possesses chorismate mutase and prephenate dehydratase activities. More recently, the *M. tuberculosis pheA* gene has been shown to encode an allosterically regulated monofunctional prephenate dehydratase that only catalyzes the dehydration and concomitant decarboxylation to form phenylpyruvate (Prakash *et al.*, 2005). Here, we report an alternative cloning

strategy and the expression, purification to homogeneity and preliminary crystallographic analysis of recombinant prephenate dehydratase from *M. tuberculosis*. The results presented here will pave the way for the three-dimensional structure determination of wild-type *M. tuberculosis* prephenate dehydratase, which in turn should provide a framework on which to base the rational design of chemotherapeutic agents to treat TB. In addition, this crystal structure will provide insight into the allosteric activation of prephenate dehydratase by phenylalanine, tyrosine and tryptophan.

2. Materials and methods

2.1. Cloning, protein expression and purification

The *pheA* gene (Rv3838c) encoding prephenate dehydratase from *M. tuberculosis* was amplified by the polymerase chain reaction (PCR) from genomic DNA. The forward (5′-TG**CATATG**GTGC-GTATCGCTTACCTCGGTCC-3′) and reverse (5′-AC**AAGCTT**T-CATGCTTGCGCCCCCTGGTCG-3′) synthetic oligonucleotide primers were based on the amino-terminal coding and carboxy-terminal non-coding strands of the *pheA* gene (Cole *et al.*, 1998) containing 5′ *Nde*I and 3′ *Hin*dIII restriction sites (shown in bold), respectively. The PCR product was cloned into pET-23a(+) expression vector (Novagen) and the recombinant plasmid was sequenced to confirm the identity of the cloned DNA fragment and to ensure that no mutations had been introduced by the PCR amplification step. To overexpress *M. tuberculosis* prephenate dehydratase protein, *E. coli* BL21(DE3) electrocompetent cells were transformed with the recombinant plasmid. Transformed cells were grown in Luria–Bertani (LB) medium containing 50 µg ml^{-1} carbenicillin at 310 K to an OD_{600} value of 0.5 and were then grown for an additional 3, 6, 9, 12 or 24 h in the presence or absence of 1 m*M* isopropyl β-D-thiogalactoside (IPTG). Cells were harvested by centrifugation (17 300*g* for 15 min at 277 K), resuspended in 50 m*M* Tris–HCl pH 7.8 and lysed by sonication. All subsequent steps were performed on ice or at 277 K. The cell lysate was centrifuged at 48 000*g* for 1 h at 277 K. The supernatant was incubated with 1%(*w*/*v*) streptomycin sulfate for 30 min and centrifuged (48 000*g* for 30 min at 277 K). A saturated solution of ammonium sulfate was added to the supernatant to a final concentration of 30% and the mixture was centrifuged at 48 000*g* for 30 min at 277 K. The resultant pellet was resuspended in 50 m*M* Tris–HCl pH 7.8 and clarified by centrifugation. The supernatant was loaded onto a Q-Sepharose Fast Flow (2.6 × 8.2 cm) anion-exchange column (GE Healthcare) and fractionated using a 0.0–0.5 *M* NaCl linear gradient. The fractions were pooled and ammonium sulfate was added to a final concentration of 0.6 *M*; the mixture was then loaded onto a HiLoad 16/10 Phenyl Sepharose HP hydrophobic interaction column (GE Healthcare). The active fractions were loaded onto a Mono Q HR 16/10 anion-exchange column (GE Healthcare) and eluted using a 0.0–0.5 *M* NaCl linear gradient.

2.2. Enzyme assay

Prephenate dehydratase activity assays were carried out as described elsewhere (Gething *et al.*, 1976). In summary, 400 µl reaction mixture containing 0.5 m*M* barium prephenate, 1.0 m*M* EDTA, 0.01% bovine serum albumin and 50 m*M* DTT in 20 m*M* Tris–HCl pH 8.2 was pre-incubated for 5 min at 310 K. Recombinant prephenate dehydratase was added to this mixture and incubated for 5 min at 310 K. The reaction was stopped by the addition of 800 µl 1.5 *M* NaOH and phenylpyruvate formation was measured at 320 nm. Appropriate controls were performed in the absence of recombinant enzyme solution. The molar extinction coefficient value of 17 500 M^{-1} cm^{-1} (Cotton & Gibson, 1965) was used to calculate the phenylpyruvate concentration.

2.3. Determination of protein concentration

Protein concentrations were determined by the method of Bradford *et al.* (1976) using the Bio-Rad Laboratories protein-assay kit and bovine serum albumin as standard.

2.4. Crystallization

Crystallization trials were initially performed by the hanging-drop vapour-diffusion method at 292 K. Hampton Crystal Screen and Crystal Screen 2 kits (Hampton Research) were used to determine the initial crystallization conditions. Hanging drops were prepared by mixing 1 µl of a solution containing 10 mg ml^{-1} recombinant protein in 50 m*M* Tris–HCl pH 7.8 and 1 µl reservoir solution. Crystals were obtained with a reservoir solution containing 0.1 *M* HEPES pH 7.5, 28%(*v*/*v*) PEG 400, 0.2 *M* calcium chloride.

2.5. Data collection

The data set for recombinant *M. tuberculosis* prephenate dehydratase was collected at a wavelength of 1.438 Å using a synchrotron-radiation source (Station MX1, LNLS, Campinas) and a MAR CCD detector. The crystal was flash-frozen at 100 K in liquid nitrogen. The oscillation range used was 0.8°, the crystal-to-detector distance was 150 mm and the exposure time was 90 s. The crystal diffracted to 3.2 Å resolution. All data were processed and scaled using the programs *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The probable 963 bp *pheA* gene was successfully PCR amplified from *M. tuberculosis* H37Rv genomic DNA. Under the experimental conditions tested, the presence of 10% dimethyl sulfoxide (DMSO) proved to be essential for PCR amplification. DMSO is a co-solvent that improves the denaturation of GC-rich DNA, consistent with the 65.6% GC content of *M. tuberculosis* genomic DNA (Cole *et al.*, 1998). The PCR fragment was cloned into pET-23a(+) expression vector between *Nde*I and *Hin*dIII restriction sites. Nucleotide-sequence analysis confirmed the identity of the cloned gene and demonstrated that no mutations had been introduced by the PCR amplification step. The cloning strategy previously published for *M. tuberculosis* recombinant prephenate dehydratase resulted in a mutant protein containing 5′-Leu-Glu-$(His)_6$-3′ extra C-terminal amino-acid residues (Prakash *et al.*, 2005). The cloning strategy reported here results in *M. tuberculosis* recombinant prephenate dehydratase with no extra amino-acid residues (wild type). Recombinant plasmid was introduced into into *E. coli* BL21 (DE3) host cells and the best experimental protocol for prephenate dehydratase protein expression was determined to be 9 h of cell growth in the absence of IPTG induction after a value of 0.5 for OD_{600} had been reached. Analysis by SDS–PAGE showed a significant amount of a 33 kDa protein, in agreement with the predicted molecular weight for this protein. In pET expression vectors, the target genes are cloned under control of the bacteriophage T7 late promoter and the expression hosts contain a chromosome copy of T7 RNA polymerase gene under *lacUV5* control, the expression of which should be induced by IPTG (isopropyl β-D-thiogalactoside) to ensure tight control of recombinant gene basal expression (Studier & Moffatt, 1986; Dubendorff & Studier, 1991). In agreement with the results

Table 1
Summary of data-collection statistics for *M. tuberculosis* prephenate dehydratase.

Values in parentheses refer to the highest resolution shell.

X-ray wavelength (Å)	1.438
Temperature (K)	100
Resolution range (Å)	65.94–3.20 (3.37–3.20)
Total/unique reflections	71611/23215
Space group	$I222$ or $I2_12_12_1$
Matthews coefficient (Å^3 Da^{-1})	2.7
Unit-cell parameters	
a (Å)	98.26
b (Å)	133.22
c (Å)	225.01
$\alpha = \beta = \gamma$ (°)	90
Mosaicity (°)	0.43
Data completeness (%)	94.4 (97.2)
Average $I/\sigma(I)$	5.7 (1.5)
Multiplicity	3.1 (3.0)
$R_{\rm merge}$† (%)	0.120 (0.434)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

presented here, leaky expression has been shown to occur in the pET system (Kelley *et al.*, 1995; Basso *et al.*, 2001; Oliveira *et al.*, 2001; Magalhães *et al.*, 2002; Silva *et al.*, 2003). It has been proposed that leaky protein expression is a property of the *lac*-controlled system as cells approach stationary phase in complex medium and that cyclic AMP, acetate and low pH are required to achieve high-level expression in the absence of IPTG induction, which may be part of a general cellular response to nutrient limitation (Grossman *et al.*, 1998).

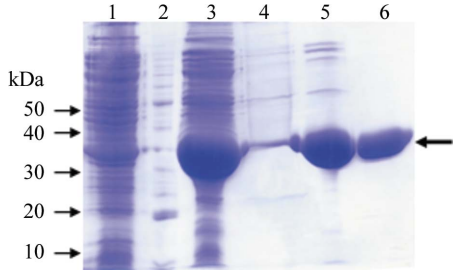

Figure 1
SDS–PAGE analysis of recombinant *M. tuberculosis* prephenate dehydratase protein purification. Lane 1, crude extract; lane 2, BenchMark Ladder protein standards (Invitrogen; 10–220 kDa); lane 3, ammonium sulfate precipation; lane 4, Q-Sepharose Fast Flow anion exchange; lane 5, phenyl Sepharose HP hydrophobic interaction; lane 6, Mono Q HR anion exchange.

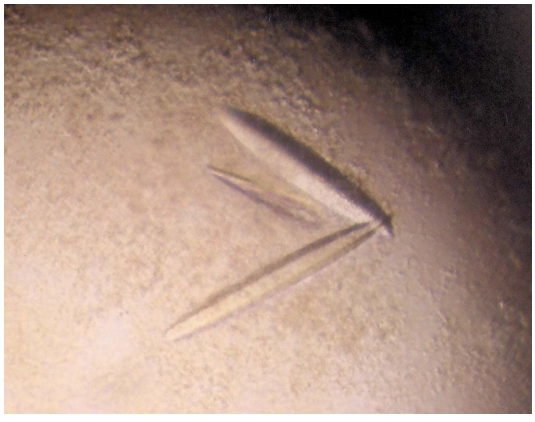

Figure 2
Crystal of recombinant *M. tuberculosis* prephenate dehydratase with approximate dimensions of 0.5 × 0.01 × 0.02 mm.

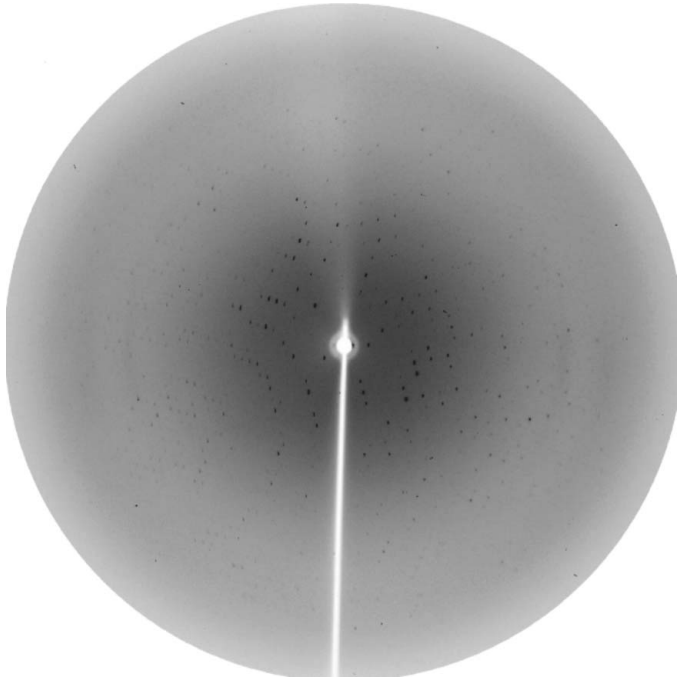

Figure 3
X-ray diffraction pattern of recombinant *M. tuberculosis* prephenate dehydratase.

The protocol described in §2 resulted in a 2367-fold protein purification, yielding approximately 70 mg of homogeneous recombinant *M. tuberculosis* prephenate dehydratase (Fig. 1) from 3 l growth medium (approximately 15 g cell paste) with a specific activity value of 1.42 U mg^{-1}. This value for specific activity is lower than that previously reported (Prakash *et al.*, 2005) probably because the prephenate substrate used in the assay described here is not saturating. Small crystals of prephenate dehydratase appeared after 7 d (Fig. 2). The crystals diffracted to 3.2 Å resolution and belong to the orthorhombic space group $I222$ or $I2_12_12_1$ (Fig. 3). Assuming the asymmetric unit content to be four monomers of molecular weight 33 600 Da, the $V_{\rm M}$ value is 2.7 Å^3 Da^{-1} (Matthews, 1968). Assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume, the calculated solvent content in the crystal is 54.7%. Unit-cell parameters and other statistics are given in Table 1. Attempts were made to solve the three-dimensional structure by molecular replacement using the program *AMoRe* (Navaza, 1994) with phenylalanine hydroxylase as the search model (Kobe *et al.*, 1999; PDB code 2phm), since it showed the highest degree of similarity (60%) using the *MULTALIGN* program. However, no meaningful result was obtained, which could be a consequence of the low identity (20%) between the search model and the structure under study. Heavy-atom screening is currently under way in order to try to solve the structure by multiple isomorphous replacement. The work described here will pave the way for *M. tuberculosis* prephenate dehydrogenase structure determination, which in turn should provide a three-dimensional framework on which to base the design of antimycobacterial agents.

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