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Crystallization and preliminary X-ray diffraction analysis of biotin acetyl-CoA carboxylase ligase (BirA) from *Mycobacterium tuberculosis*

Vibha Gupta,[a] Rakesh K. Gupta,[a,b] Garima Khare,[a] Avadhesha Surolia,[c] Dinakar M. Salunke[c] and Anil K. Tyagi[a]*

[a]Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India, [b]Ram Lal Anand College, University of Delhi, Benito Juarez Road, New Delhi 110 021, India, and [c]National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India

Correspondence e-mail: aniltyagi@south.du.ac.in

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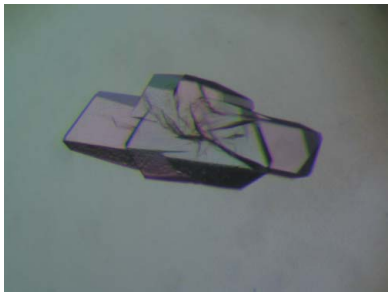
The gene encoding biotin acetyl-CoA carboxylase ligase (BirA) from *Mycobacterium tuberculosis* was cloned and expressed in *Escherichia coli* with a C-terminal Strep-tag. PEG 4000 as well as PEG 8000 were used as precipitants at pH 7.5 to crystallize the protein using the vapour-diffusion technique. X-ray characterization of crystals at room temperature indicated that the crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 79.7$, $b = 62.8$, $c = 105.8$ Å. Assuming the presence of two BirA molecules in the asymmetric unit, the solvent content of the crystals was 44% (V_M = 2.2 Å^3 Da^{-1}). When transferred to a cryoprotectant, crystals grown in the same drop exhibited a difference in one unit-cell parameter, with $a = 60.1$, $b = 64.0$, $c = 103.6$ Å, but belonged to the same $P2_12_12_1$ space group. These crystals, with two molecules of BirA present per asymmetric unit, appeared to have a very low solvent content of 28% (V_M = 1.7 Å^3 Da^{-1}).

1. Introduction

Mycobacterium tuberculosis remains one of the most damaging human pathogens, infecting approximately one-third of the world's population. It is responsible for the loss of ~2–3 million lives due to tuberculosis every year (http://www.who.int). The reasons for its being one of the most effective infectious agents include (i) its extreme infectivity, which occurs *via* aerosol by inhalation of just a few bacilli, and (ii) its ability to persist dormantly under adverse conditions and be reactivated subsequently upon weakening of the host's immune system. Moreover, the emergence of multidrug-resistant strains of *M. tuberculosis*, the advent of AIDS and the absence of an effective TB vaccine have made the control of tuberculosis difficult (O'Brien & Nunn, 2001). The currently used antitubercular drugs inhibit only a small number of known *M. tuberculosis* targets (Zhang *et al.*, 2005). This has necessitated a search for the identification and characterization of new pathways essential for mycobacterial growth that can be used as targets for the design of novel and effective antitubercular drugs (http://www.doe-mbi.ucla.edu/TB/).

M. tuberculosis has a unique cell envelope with a high lipid content which plays a crucial role in the pathogenesis of this organism. Mycobacterial acyl-CoA carboxylases, which catalyze the first committed step of lipid biosynthesis, require a biotin moiety for their enzymatic activity (Norman *et al.*, 1994). Biotin-dependent acetyl-CoA carboxylases/transcarboxylases are a class of enzymes that, in addition to fatty-acid biosynthesis, are important for gluconeogenesis as well as propionate catabolism (Knowles, 1989). Attachment of the biotin moiety to these enzymes is catalyzed by another enzyme, biotin acetyl-CoA carboxylase ligase (BirA), in a two-step process: (i) synthesis of biotinyl-5′-AMP from the substrates biotin and ATP and (ii) transfer of the activated biotin to a specific lysine residue on the



biotin carboxyl carrier protein (BCCP) domain of acetyl-CoA carboxylase (Beckett & Matthews, 1997; Chapman-Smith & Cronan, 1999). By virtue of being a key enzyme in the biotinylation of these carboxylases, which catalyze several reactions in essential metabolic processes, BirA represents a promising target for the development of antitubercular drugs against *M. tuberculosis*. Moreover, owing to the marginal sequence homology of mycobacterial BirA to other homologues, as indicated by sequence comparison with its counterparts from *Escherichia coli* (∼32% identity) and *Pyrococcus horikoshii* (∼37% identity), it would be interesting to elucidate the structure of mycobacterial BirA alone and in complex with its proteinaceous substrate (BCCP) in order to explore the unique features that it might offer towards the design of structure-based inhibitor molecules. In this communication, we report the crystallization and preliminary X-ray diffraction analysis of recombinant BirA from *M. tuberculosis*.

2. Experimental methods

2.1. Cloning

The gene encoding BirA (Rv3279c) was amplified by PCR using genomic DNA of *M. tuberculosis* H37Rv as template. The primers for amplification were designed based on the sequences available from EMBL/GenBank. The sequences of the forward and reverse primers were 5′-GGATCCGCTAGCGAATTCACCGACCGCGATCGGCTC-3′ and 5′-GAATTCAAGCTTATTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTACGCAAATGCACCACGTCGCCCGC-3′, respectively. The reverse primer was designed in such a manner that it adds a Strep-tag at the C-terminus of the expressed recombinant protein in order to facilitate its purification. The PCR product was digested with the restriction enzymes *Nhe*I and *Hin*dIII and ligated with the expression vector pASK-IBA43plus (IBA) previously digested with the same restriction enzymes. *E. coli* XL1-Blue cells were transformed with the ligation mixture, recombinants were selected and the nucleotide sequence of the cloned gene was verified by DNA sequencing.

2.2. Protein expression and purification

E. coli BL21 (DE3) cells were transformed with the recombinant plasmid and the transformants were directly used to inoculate LB medium (1 l) containing 50 µg ml^{-1} ampicillin. The culture was grown at 310 K (200 rev min^{-1}) to an $A_{600\,\mathrm{nm}}$ of 0.6. Protein expression was induced by the addition of 1 m*M* IPTG and the culture was grown for a further 3 h at the same temperature. Cells were harvested by centrifugation at 4500*g* for 5 min at 277 K. The harvested cells were resuspended in 50 ml buffer *A* (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* PMSF and 2 m*M* β-mercaptoethanol) and then lysed using a French press. The resulting lysate was centrifuged at ∼27 000*g* for 30 min at 277 K. The supernatant was loaded onto 10 ml Strep-Tactin resin (IBA) pre-equilibrated with buffer *A*. The protein was eluted with 2.5 m*M* desthiobiotin in 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* PMSF and 1 m*M* DTT. The purity of the protein fractions was analyzed by electrophoresis on a 12.5% SDS–polyacrylamide gel. The protein was concentrated (to ∼5 mg ml^{-1}) using an Amicon concentrator and YM30 membrane and aliquots of the protein were stored at 203 K.

2.3. Crystallization

Various commercial screens (Hampton Research, USA; Jena Biosciences, Germany; Qiagen, USA) were set up at 293 K with concentrated protein to screen the initial crystallization conditions for recombinant BirA fused at the C-terminus with a Strep-tag. The hanging-drop vapour-diffusion technique in 24-well EasyXtal Tool plates (Qiagen, USA) was employed for all setups. Clusters of crystals with a common nucleation source appeared overnight in several PEG 4000 and PEG 8000 precipitant-based conditions, which were further optimized (Fig. 1). The best crystals grew using 12–16%(*w*/*v*) of both PEG 4000 and PEG 8000 in 0.1 *M* HEPES pH 7.5 reservoir solution, with the drop containing 3 µl protein solution (5 mg ml^{-1}) in 20 m*M* Tris–HCl pH 8.0, 2.5 m*M* desthiobiotin, 50 m*M* NaCl, 1 m*M* PMSF and 1 m*M* DTT and an equal volume of reservoir solution. Preliminary X-ray characterization of these crystals indicated differences in unit-cell parameters when the crystals were transferred directly from the mother liquor for characterization at room temperature (crystal form *A*) or to a cryoprotectant for characterization at cryogenic temperature (crystal form *B*).

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Crystal form *A*	Crystal form *B*
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 79.7, b = 62.8, c = 105.8	a = 60.1, b = 64.0, c = 103.6
Temperature (K)	295	120
Wavelength (Å)	0.8088	1.5418
Crystal-to-detector distance (mm)	170	100
Resolution limits (Å)	15–2.8 (2.9–2.8)	20–2.69 (2.82–2.69)
Exposure time per image (s)	4	300
No. of observed reflections	89516	44885
No. of unique reflections	15831	13250
Average redundancy	5.7 (5.8)	3.19 (3.25)
Completeness (%)	99.4 (99.6)	98.7 (96.8)
Mean $I/\sigma(I)$	17.2 (2.2)	4.3 (2.1)
R_{merge}† (%)	9.8 (46.2)	8.7 (34.9)
No. of molecules in ASU	2	2
Matthews coefficient (Å^3 Da^{-1})	2.2	1.7
Solvent content (%)	44	28

† $R_{\mathrm{merge}} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices *hkl* and $\langle I(hkl)\rangle$ is the mean intensity of redundant measurements of that reflection.

2.4. Data collection and processing

An initial data set was collected to 2.8 Å resolution from crystal form *A* (four crystals) using a synchrotron-radiation source [beamline X13, European Molecular Biology Laboratory (EMBL), Hamburg, Germany] at room temperature. Owing to the sensitivity of the

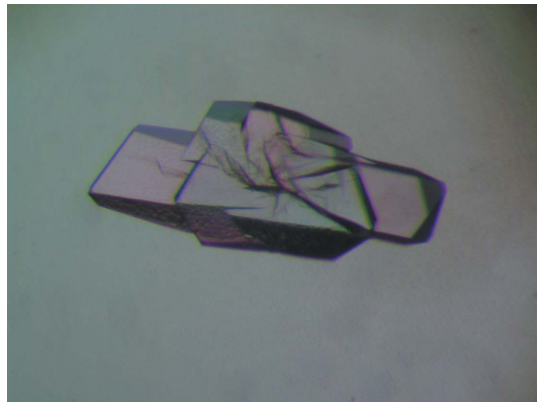


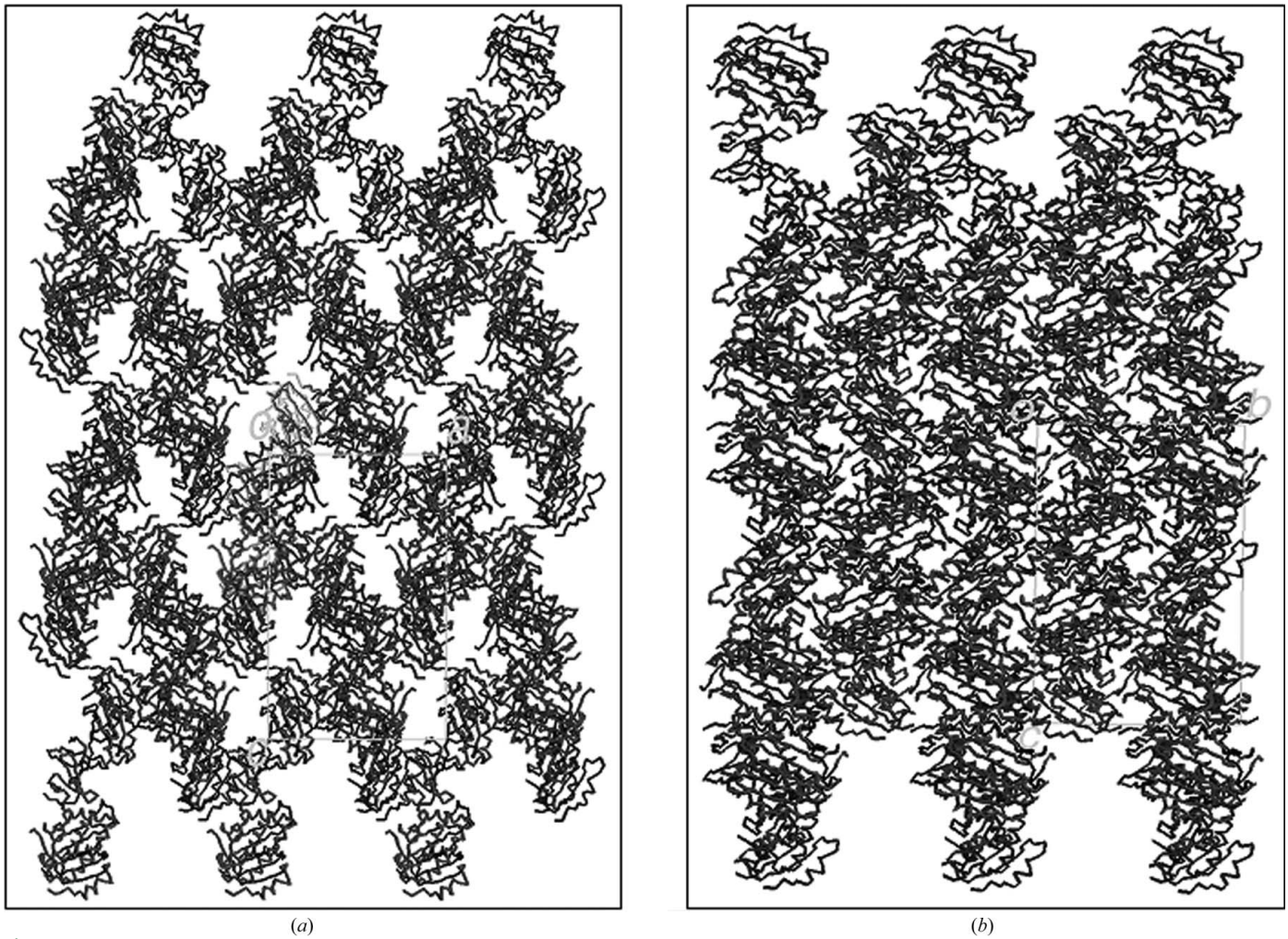
Figure 1
Crystals of *M. tuberculosis* BirA grown by the hanging-drop method.

crystals to radiation, cryocooling was necessary during data collection to avoid crystal decay at room temperature. To collect data from cryocooled crystals, many cryoprotectants such as glycerol, dextrose and oils were tried, but they lowered the resolution and led to a diffuse pattern. A 1:1 mixture of paraffin and Paratone-N oil was then used as a cryoprotectant for the collection of a low-temperature data set. These crystals are referred to as form *B* and diffracted to 2.69 Å resolution at the home source. A total of 93 images were recorded on a MAR345 dtb detector (MAR Research) using a rotating-anode generator (Rigaku) with a crystal-to-detector distance of 100 mm, an oscillation range of 1° and an exposure time of 5 min per image. The data collected from crystal form *A* at the synchrotron were processed using the *HKL*-2000 package (Otwinowski & Minor, 1997), whereas the data collected from crystal form *B* at the home source were processed with the *AUTOMAR* program suite (Bartels & Klein, 2003). The data-collection and processing statistics for both data sets are summarized in Table 1.

3. Results and discussion

BirA from *M. tuberculosis* H37Rv was expressed in BL21 (DE3) cells, resulting in most of the recombinant protein being expressed in the soluble fraction. The protein was purified by single-step Strep-Tactin affinity chromatography with a purity of 99% as estimated by electrophoresis using a 12.5% SDS–polyacrylamide gel. The final yield of purified recombinant protein was ∼15 mg per litre of culture.

Initial crystallization screening resulted in several leads. The best crystals grew to dimensions of about 0.4 × 0.1 × 0.05 mm in 1–2 weeks. A 2.8 Å resolution data set was collected at room temperature for crystal form *A* on beamline X13 at EMBL, Hamburg, Germany and was processed using the *HKL*-2000 package. Assuming the presence of two molecules in the asymmetric unit of the $P2_12_12_1$ cell, the crystals had a Matthews coefficient of 2.2 Å^3 Da^{-1} and a solvent content of 44% (Matthews, 1968). Better resolution data to 2.69 Å were collected at the home source from a flash-frozen crystal (form *B*). On indexing and scaling with *AUTOMAR*, this crystal form also belonged to space group $P2_12_12_1$; Matthews coefficient calculations suggested that each asymmetric unit contained two monomers with an unusually low solvent content of 28%. The data-collection and processing statistics for both data sets are summarized in Table 1. It is most likely that the unit cell of the crystal at room temperature (form *A*) is transformed into form *B* on subjecting it to cryoprotectant and low-temperature conditions, as is evident by the significantly reduced solvent content of crystal form *B* (see also Fig. 2). The shrinkage of the unit cell by about 25% in crystal form *B* probably results from



(*a*) (*b*)

Figure 2
Crystal-packing diagrams of (*a*) the *ac* plane looking down the *b* axis in crystal form *A* solved at 2.8 Å resolution and (*b*) the *bc* plane looking down the *a* axis in crystal form *B* solved at 2.69 Å resolution. Protein molecules are drawn as C^{α} traces and the grey box represents the unit cell. Both figures were produced using *Coot* (Emsley & Cowtan, 2004).

transfer of the crystal to cryoprotectant and flash-freezing. However, the small possibility that the two crystal forms coexist in the same crystallization drop cannot be ruled out.

To begin with, we used homologous protein templates from *E. coli* (32% sequence identity; PDB code 1bia) and *P. horikoshi* (37% sequence identity; PDB code 1wnl) to determine the structure of *M. tuberculosis* BirA by molecular replacement using *Phaser* (McCoy *et al.*, 2007). Although solutions could be obtained from both the templates, the maps were noisy, probably owing to the low homology. Little progress could be made in rebuilding the structure, with many regions remaining undefined, and further progress awaited unbiased phase information. In the meantime, the coordinates for the crystal structure of BirA from *M. tuberculosis* at 1.8 Å resolution were deposited in the Protein Data Bank (PDB code 2cgh). The crystallization conditions for this BirA were 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* trimethylamine *N*-oxide and 20%(*w*/*v*) MPEG 2000 (as obtained from the PDB) and the crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 62.1$, $b = 81.0$, $c = 101.9$ Å, which are almost identical to those of our crystal form *A*. A run of the *Phenix AutoMR* wizard (Adams *et al.*, 2002) in default mode with 2cgh (dimer) as a template resulted in a clear single solution with an LLG gain of 1992, a rotation-function *Z* (RFZ) score of 23.4 and a translation-function *Z* (TFZ) score of 32.0 (EULER 32.9 0.1 237.1 FRAC −0.76 0.74 −0.23). A molecular-replacement solution could also be obtained with the same program suite for crystal form *B* with a dimer as a search template. The LLG gain, RFZ and TFZ in this case were 323, 10.1 and 20.6, respectively (EULER 95.5 169.8 92.4 FRAC 0.57 1.29 0.38). The crystal-packing arrangements for both crystal forms are shown in Fig. 2. Molecules in both crystal forms are evenly distributed throughout the whole unit cell and no clashes are observed between molecules.

We are presently attempting to crystallize BirA in complex with its proteinaceous substrate in order to understand how activated biotin is transferred to a specific lysine residue of the BCCP subunit of acetyl-CoA carboxylase.

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