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Cloning, expression, purification, crystallization and preliminary X-ray studies of argininosuccinate lyase (Rv1659) from *Mycobacterium tuberculosis*

The last enzyme in the arginine-biosynthesis pathway, argininosuccinate lyase, from *Mycobacterium tuberculosis* has been cloned, expressed, purified and crystallized, and preliminary X-ray studies have been carried out on the crystals. The His-tagged tetrameric enzyme with a subunit molecular weight of 50.9 kDa crystallized with two tetramers in the asymmetric unit of the orthorhombic unit cell, space group $P2_12_12_1$. Molecular-replacement calculations and self-rotation calculations confirmed the space group and the tetrameric nature of the molecule.

1. Introduction

We have been pursuing a long-range programme of structural studies of proteins from *Mycobacterium tuberculosis* and related organisms with a view to contributing to the understanding of the basic biology of the pathogen and to build a platform for structure-based inhibitor design (Vijayan, 2005; Arora *et al.*, 2011; Abhinav *et al.*, 2013). Much of our work has been concerned with DNA recombination, protein synthesis and stringent response (Vijayan, 2005; Krishna *et al.*, 2007; Selvaraj *et al.*, 2007; Kaushal *et al.*, 2008; Roy *et al.*, 2008; Prabu *et al.*, 2009; Chandran *et al.*, 2010). Biosynthetic pathways offer another attractive area for structural investigation. In this context, we have already carried out detailed studies on pantothenate kinase, the first enzyme in the CoA-biosynthetic pathway, from *M. tuberculosis* (Das *et al.*, 2006; Chetnani *et al.*, 2009, 2010, 2011). We have now initiated work on selected proteins in the pathway involved in the biosynthesis of arginine.

The biosynthesis of arginine, which is essential for the growth, survival and sustenance of many eubacteria, involves eight steps, in the first of which L-glutamic acid is converted to N-acetyl-L-glutamic acid with the concurrent conversion of acetyl-CoA to CoA. In the final step, L-argininosuccinate is converted in an elimination reaction into arginine and fumaric acid by the enzyme argininosuccinate lyase, which is the subject of the present study. Argininosuccinate lyase is a 470-amino-acid enzyme with a molecular mass of 49.7 kDa that is essential in *M. tuberculosis* (Griffin *et al.*, 2011). Crystal structures of the enzyme from *Escherichia coli* (Bhaumik *et al.*, 2004), *Thermus thermophilus* (PDB entry 2e9f; M. Goto, unpublished work), *Anas platyrhynchos* (Sampaleanu *et al.*, 2001), *A. anser* (Lee *et al.*, 2005), *Meleagris gallopavo* (Sampaleanu *et al.*, 2001) and *Homo sapiens* (Turner *et al.*, 1997) have been determined. Here, we report the preliminary biochemical and crystallographic studies of the enzyme from *M. tuberculosis*.

2. Materials and methods

2.1. Cloning, expression and purification

The argininosuccinate lyase or ArgH (Rv1659) open reading frame was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA (Cole *et al.*, 1998). The sequence of the forward primer used was 5'-CTCGCCACCATATGATGAGCACCAACGAGGGGTC-3' (NdeI restriction site in bold) and that of the reverse primer was 5'-GAGGCCCTCGAGGCGCACCAGCTGGC-3' (XhoI restriction site in bold). PCR involved 30 cycles comprising of denaturation at 368 K (1 min), annealing at 337 K (1 min) and extension at 345 K

(1 min). The *NdeI*–*XhoI* flanking fragment consisting of a 1413 bp *argH* open reading frame was then cloned into pET-22b(+) vector. Recombinant cloning was performed using *NdeI* and *XhoI* endonucleases and the integrity of the DNA sequence was established by sequencing. The recombinant construct thus obtained, pET22b-Rv1659 (*NdeI*–*XhoI*), with an extra methionine at the N-terminus and an extended sequence LEHHHHHH at the C-terminus of Rv1659, was employed for protein overexpression.

ArgH protein tagged with a C-terminal hexahistidine stretch was then expressed in *E. coli* strain BL21 (DE3) under the influence of a T7 promoter. Cells transformed with the recombinant plasmid were grown in Luria–Bertani medium supplemented with 100 µg ml^{−1} ampicillin at 310 K. When the A_{600} reached 0.6, the culture was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and was incubated for a further 3 h. The cells were harvested by centrifugation at 6000 rev min^{−1} for 10 min at room temperature. The pellet was washed with phosphate-buffered saline and was then resuspended in ice-cold lysis buffer (30 mM Tris–HCl pH 8, 500 mM NaCl, 2 mM PMSF, 2 mM β-mercaptoethanol). The solution was then sonicated at 25% amplitude for 5 min with an on–off cycle of 2 and 5 s, respectively. The lysate was centrifuged at 14 000 rev min^{−1} for 45 min at 277 K. The recombinant ArgH protein with the hexa-His tag in the supernatant was purified using an Ni–NTA metal affinity column (Novagen). The supernatant was left to bind to the column for 45 min, followed by subsequent washing with lysis buffer containing 20, 40 and 60 mM imidazole. The protein was then eluted with an elution buffer consisting of lysis buffer containing 100, 200 and 300 mM imidazole, respectively, as a competitor. The protein was further dialyzed against 25 mM potassium phosphate buffer and stored in the same buffer at 277 K. The final yield of the protein was 15 mg per litre of culture.

The purity of the protein, which had a molecular mass of 50.9 kDa including the hexahistidine tag, was determined by SDS–PAGE analysis (Fig. 1). Native PAGE and gel-filtration experiments were carried out to confirm the quaternary association.

2.2. Crystallization

The microbatch-under-oil method was employed for all crystallization experiments, using Greiner 72-well plates with equal

Figure 1
Purification profile of argininosuccinate lyase from *M. tuberculosis*. Lane 1, molecular-mass marker (labelled in kDa); lane 2, supernatant loaded onto the column; lane 3, pellet after cell lysis; lane 4, flowthrough; lane 5, wash with 60 mM imidazole. Lanes 6–8 correspond to fractions eluted with 100, 200 and 300 mM imidazole, respectively.

Table 1

Data-collection and processing statistics.

Values in parentheses are for the outermost resolution shell.

No. of crystals	1
Wavelength (Å)	0.9537
Detector	MAR CCD
Crystal-to-detector distance (mm)	200.3
Rotation range per image (°)	0.50
Total rotation range (°)	241
Exposure time per image (s)	8
Resolution range (Å)	40.48–2.40 (7.59–2.40)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 127.55$, $b = 183.82$, $c = 190.60$
Mosaicity (°)	0.30
Total No. of measured intensities	890784 (129432)
Unique reflections	174848 (25300)
Multiplicity	5.1 (5.1)
Mean $\langle I/\sigma(I) \rangle$	7.3 (2.1)
Completeness (%)	100.0 (100.0)
$R_{\text{merge}}^{\dagger}$ (%)	16.8 (70.9)
$R_{\text{meas}}^{\ddagger}$ (%)	18.6 (78.7)
Overall B factor from Wilson plot (Å ²)	33.3

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\ddagger R_{\text{meas}} = \frac{\sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th observation of reflection hkl , $\langle I(hkl) \rangle$ is the weighted average intensity for all i observations of the reflection with indices hkl and $N(hkl)$ is the multiplicity.

proportions of silicon and paraffin oil. Various conditions were screened using commercially available kits from Hampton Research, Molecular Dimensions and Jena Biosciences. All drops consisted of 2 µl protein solution (9.5 mg ml^{−1}) in 25 mM phosphate buffer pH 7.4 and 2 µl precipitant solution. Somewhat irregularly shaped diffraction-quality crystals (0.05 × 0.05 × 0.15 mm) were obtained from Index (Hampton Research) condition No. 42 in about 45 d at 295 K (Fig. 2). The precipitant solution consisted of 100 mM bis-tris pH 5.5, 25%(w/v) polyethylene glycol 3350.

2.3. X-ray data collection and processing

Intensity data were initially collected to 2.9 Å resolution at 100 K using a MAR 345 image plate mounted on a Bruker MICROSTAR ULTRA II Cu $K\alpha$ rotating-anode X-ray generator. Subsequently, a 2.4 Å resolution data set could be collected using synchrotron radiation on the BM14 beamline at the ESRF using a MAR CCD detector, again at 100 K. No separate cryoprotectant was required during data collection. The diffraction data thus obtained were

Figure 2
Crystal of argininosuccinate lyase from *M. tuberculosis*.

processed using *MOSFLM* (Leslie, 2006) and scaled using *SCALA* (Evans, 1993) from the *CCP4* suite (Winn *et al.*, 2011). Crystal data and data-collection statistics are given in Table 1.

2.4. Molecular replacement and rotation function

Molecular-replacement calculations were carried out using *Phaser* (McCoy *et al.*, 2007), while *MOLREP* (Vagin & Teplyakov, 2010) was used for evaluating the self-rotation function.

3. Results and discussion

Argininosuccinate lyase from *M. tuberculosis* with a hexahistidine tag has been purified to homogeneity (Fig. 1). The tetrameric molecule has a subunit molecular mass of 50.9 kDa. The protein has been crystallized (Fig. 2) and preliminary X-ray analysis of the crystals has been carried out. A value of $2.65 \text{ \AA}^3 \text{ Da}^{-1}$ for the Matthews coefficient (Matthews, 1968) suggested the presence of two tetrameric molecules in the asymmetric unit of the orthorhombic cell. Systematic absences clearly indicated the space group to be $P2_12_12_1$.

The crystal structures of the homologous enzymes from *E. coli* (PDB entry 1tj7; Bhaumik *et al.*, 2004), *T. thermophilus* (PDB entry 2e9f; M. Goto, unpublished work), *A. platyrhynchos* (PDB entries 1hy0 and 1hy1; Sampaleanu *et al.*, 2001), *M. gallopavo* (PDB entry 1i0a; Sampaleanu *et al.*, 2001), *A. anser* (PDB entry 1xwo; Lee *et al.*, 2005) and *H. sapiens* (PDB entry 1aos; Turner *et al.*, 1997) are available. They exhibit sequence identities ranging from 39.4 to 42.9% with the *M. tuberculosis* enzyme.

Molecular-replacement calculations with the tetrameric enzyme from *T. thermophilus* as the search model led to an acceptable structure solution with a log-likelihood gain of 2771 and a Z-score of 53, thus confirming the space group. The results of the calculations were consistent with the self-rotation function, indicating the tetrameric nature of the molecule with 222 symmetry.

X-ray intensity data were collected at the Facility for Protein X-ray Crystal Structure Determination and Protein Design at the Indian Institute of Science, supported by the Department of Science and Technology, and on the BM14 beamline at the ESRF, Grenoble, supported by EMBL and the Department of Biotechnology and manned by Dr Hassan Belrhali and Dr Babu A. Manjasetty. Financial support to AS from the Indian Council of Medical Research (ICMR No. 5/8/5/6/2012/ECD-1) is acknowledged. AM is a Junior Research Fellow of the Council of Scientific and Industrial Research (CSIR).

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References

- Abhinav, K. V., Sharma, A. & Vijayan, M. (2013). *Proteins*, **81**, 644–657.
- Arora, A., Chandra, N. R., Das, A., Gopal, B., Mande, S. C., Prakash, B., Ramachandran, R., Sankaranarayanan, R., Sekar, K., Suguna, K., Tyagi, A. K. & Vijayan, M. (2011). *Tuberculosis*, **91**, 456–468.
- Bhaumik, P., Koski, M. K., Bergmann, U. & Wierenga, R. K. (2004). *Acta Cryst.* **D60**, 1964–1970.
- Chandran, A. V., Prabu, J. R., Manjunath, G. P., Patil, K. N., Muniyappa, K. & Vijayan, M. (2010). *Acta Cryst.* **F66**, 1093–1095.
- Chetnani, B., Das, S., Kumar, P., Surolia, A. & Vijayan, M. (2009). *Acta Cryst.* **D65**, 312–325.
- Chetnani, B., Kumar, P., Abhinav, K. V., Chhibber, M., Surolia, A. & Vijayan, M. (2011). *Acta Cryst.* **D67**, 774–783.
- Chetnani, B., Kumar, P., Surolia, A. & Vijayan, M. (2010). *J. Mol. Biol.* **400**, 171–185.
- Cole, S. T. *et al.* (1998). *Nature (London)*, **393**, 537–544.
- Das, S., Kumar, P., Bhor, V., Surolia, A. & Vijayan, M. (2006). *Acta Cryst.* **D62**, 628–638.
- Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.
- Griffin, J. E., Gawronski, J. D., Dejesus, M. A., Ioerger, T. R., Akerley, B. J. & Sasseti, C. M. (2011). *PLoS Pathog.* **7**, e1002251.
- Kaushal, P. S., Talawar, R. K., Krishna, P. D. V., Varshney, U. & Vijayan, M. (2008). *Acta Cryst.* **D64**, 551–560.
- Krishna, R., Prabu, J. R., Manjunath, G. P., Datta, S., Chandra, N. R., Muniyappa, K. & Vijayan, M. (2007). *J. Mol. Biol.* **367**, 1130–1144.
- Lee, H.-J., Lai, Y.-H., Wu, S.-Y. & Chen, Y.-H. (2005). *Biochem. J.* **392**, 545–554.
- Leslie, A. G. W. (2006). *Acta Cryst.* **D62**, 48–57.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Prabu, J. R., Thamotharan, S., Khanduja, J. S., Chandra, N. R., Muniyappa, K. & Vijayan, M. (2009). *Biochim. Biophys. Acta*, **1794**, 1001–1009.
- Roy, S., Saraswathi, R., Chatterji, D. & Vijayan, M. (2008). *J. Mol. Biol.* **375**, 948–959.
- Sampaleanu, L. M., Vallée, F., Slingsby, C. & Howell, P. L. (2001). *Biochemistry*, **40**, 2732–2742.
- Selvaraj, M., Roy, S., Singh, N. S., Sangeetha, R., Varshney, U. & Vijayan, M. (2007). *J. Mol. Biol.* **372**, 186–193.
- Turner, M. A., Simpson, A., McInnes, R. R. & Howell, P. L. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 9063–9068.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Vijayan, M. (2005). *Tuberculosis*, **85**, 357–366.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.