**Sanket Kaushik, Avinash Singh,
Mau Sinha, Punit Kaur, Sujata
Sharma and Tej P. Singh***

Department of Biophysics, All India Institute of
Medical Sciences, New Delhi 110 029, India

Correspondence e-mail:
tpsingh.aiims@gmail.com

Received 16 December 2012
Accepted 24 April 2013

Cloning, expression, crystallization and preliminary structural studies of dihydrodipicolinate reductase from *Acinetobacter baumannii*

Acinetobacter baumannii is a virulent pathogenic bacterium that is resistant to most currently available antibiotics. Therefore, the design of drugs for the treatment of infections caused by *A. baumannii* is urgently required. Dihydrodipicolinate reductase (DHDPR) is an important enzyme which is involved in the biosynthetic pathway that leads to the production of L-lysine in bacteria. In order to design potent inhibitors against this enzyme, its detailed three-dimensional structure is required. DHDPR from *A. baumannii* (*AbDHDPR*) has been cloned, expressed, purified and crystallized. Here, the preliminary X-ray crystallographic data of *AbDHDPR* are reported. The crystals were grown using the hanging-drop vapour-diffusion method with PEG 3350 as the precipitating agent. The crystals belonged to the orthorhombic space group *P*222, with unit-cell parameters $a = 80.0$, $b = 100.8$, $c = 147.6$ Å, and contained four molecules in the asymmetric unit. The complete structure determination of *AbDHDPR* is in progress.

1. Introduction

Acinetobacter baumannii is an aerobic Gram-negative non-motile bacterium generally found in hospitals (Oncolül *et al.*, 2002; Zeana *et al.*, 2003; Towner, 2009). Being a hospital pathogen, *A. baumannii* mainly infects patients in intensive care units and emergency wards of hospitals (Somerville & Noble, 1970). The *Acinetobacter* species has become resistant to many classes of antibiotics (Takahashi *et al.*, 2000; Turton *et al.*, 2006; Dijkshoorn *et al.*, 2007). Hence, it has become imperative to identify and characterize new drug targets within the bacterium and to consequently determine their three-dimensional structures for facilitating the structure-based design and development of new drugs against them.

Dihydrodipicolinate reductase (DHDPR; EC 1.3.1.26) is one of the key enzymes in the lysine-biosynthesis pathway (Kobayashi, 2003) as it catalyses the production of L-lysine which is essential for protein synthesis in bacteria. Therefore, DHDPR from *A. baumannii* (*AbDHDPR*) forms an important drug target.

To date, the structure of *AbDHDPR* is not available, although crystal structures of DHDPR from several other species of bacteria are available. These include *Escherichia coli* (*EcDHDPR*; PDB entry 1dih; Scapin *et al.*, 1995), *Mycobacterium tuberculosis* (*MtDHDPR*; PDB entry 1yl5; Cirilli *et al.*, 2003), *Thermotoga maritima* (*TmDHDPR*; PDB entry 1vm6; Pearce *et al.*, 2008), *Bartonella henselae* (*BhDHDPR*; PDB entry 3ijp; Seattle Structural Genomics Center for Infectious Disease, unpublished work), *Burkholderia thailandensis* (*BtDHDPR*; PDB entry 4f3y; Seattle Structural Genomics Center for Infectious Disease, unpublished work) and *Staphylococcus aureus* (*SaDHDPR*; PDB entry 3qy9; Girish *et al.*, 2011). *AbDHDPR* shows amino-acid sequence identities of 60, 30, 34, 40, 53 and 31% to *EcDHDPR*, *MtDHDPR*, *TmDHDPR*, *BhDHDPR*, *BtDHDPR* and *SaDHDPR*, respectively. Because of the significant variations in the sequences of various DHDPRs from different species, and owing to conformational flexibilities at the substrate-binding sites (Reddy *et al.*, 1996) as well as the occurrences of substrate-binding specificities for the cofactors, NADH and NADPH (Girish *et al.*, 2011), structure determinations of DHDPRs

from all species are essential if specific inhibitors are to be designed against them. Here, we report the results of cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of *AbdDHDPR*.

2. Materials and methods

2.1. Gene cloning

Genomic DNA from *A. baumannii* was isolated and the *dapB* gene was amplified using the forward and reverse primers DHDPRf, CATATGTCAGCAGCTCCACGC, and DHDPRr, CTCGAGTTACACCTGTACGTCGTTC, respectively. The forward primer contained an *NdeI* restriction-enzyme site and the reverse primer contained a *XhoI* restriction-enzyme site. The resultant PCR product was ligated in the pGEM-T Easy (Promega, Wisconsin, USA) vector. The resulting construct was digested with *NdeI* and *XhoI* restriction enzymes and was further subcloned in the pET28a (Novagen, Darmstadt, Germany) vector for expression. The construct obtained with pET28a was transformed in *E. coli* BL21(DE3) cells for expression of DHDPR.

2.2. Expression and purification

A single colony obtained after transformation in *E. coli* BL21(DE3) cells was inoculated in LB medium supplemented with 50 mg ml⁻¹ kanamycin and grown at 310 K. This overnight culture was used as a seed culture. The seed culture was re-inoculated in 1 l fresh LB medium; the required amount was inoculated with 1% of this culture and grown at 310 K with shaking (200 rev min⁻¹) until the OD at 600 nm reached approximately 1.0. The culture was cooled to 300 K, induced with 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and grown for a further 16 h at 289 K with slower shaking (180 rev min⁻¹). Cells were harvested by centrifugation at 8000g for 5 min at 277 K. Approximately 5 g of the wet pellet was suspended in 15–20 ml 50 mM Tris-HCl buffer containing 150 mM NaCl pH 8.0 (lysis buffer) and stored at 193 K until further use. Frozen cells were thawed on ice and protease inhibitor was added. The cells were disrupted using a Constant cell-disruption system at 152 MPa

Table 1

Crystallographic data.

Values in parentheses are for the outermost shell.

Space group	P222
Unit-cell parameters (Å)	$a = 80.0$, $b = 100.8$, $c = 147.6$
Resolution range (Å)	38.7–2.5
Total no. of measured reflections	3382
No. of unique reflections	2560
V_M (Å ³ Da ⁻¹)	2.6
Solvent content (%)	53
Data completeness (%)	65 (50)
$R_{\text{merge}}^{\dagger}$ (%)	12.9 (48)

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

(Labmate, Chennai, India). The ruptured cells were centrifuged at 13 000g for 30 min at 277 K. The cleared lysate was applied onto a Ni-NTA Superflow column (Qiagen, Maryland, USA) pre-equilibrated in lysis buffer and purified using stepwise washing with 30 mM followed by 300 mM imidazole in lysis buffer. The protein contents of each fraction were examined using 10% SDS-PAGE. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue R250 (Sigma, Missouri, USA). The fractions corresponding to *AbdDHDPR* as indicated by their molecular mass were pooled. The imidazole was removed by dialysis in lysis buffer and the sample was concentrated using ultrafiltration with molecular mass cut-off of 3 kDa centricon (Millipore, Massachusetts, USA). The concentrated protein was further purified using fast protein liquid chromatography (Bio-Rad, California, USA) using a Superdex G75 column in buffer consisting of 20 mM Tris-HCl, 50 mM NaCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β -mercaptoethanol (ME) pH 7.5. The purity of the protein was established by SDS-PAGE (Fig. 1).

2.3. Crystallization

The purified protein was crystallized by the hanging-drop vapour-diffusion method at 298 K using 24-well Linbro crystallization plates. An initial crystallization screening was carried out using Crystal Screen and Crystal Screen 2 (Hampton Research). Crystals were obtained by equilibrating a 10 μ l protein drop consisting of a mixture of 7 μ l 20 mg ml⁻¹ protein solution and 3 μ l reservoir solution against a reservoir solution consisting of 25% PEG 3350 in 0.3 M Tris-HCl buffer pH 8.0. Crystals grew in 2 weeks to approximate dimensions of 0.4 $\times$ 0.4 $\times$ 0.2 mm (Fig. 2).

Figure 1

SDS-PAGE showing the purity of the protein. Lane 1 contains a single band for *AbdDHDPR* and lane 2 shows bands for molecular-weight markers (labelled in kDa).

Figure 2

Crystals of dihydrodipicolinate reductase from *A. baumannii* grown using 25% PEG 3350 in 0.3 M Tris-HCl buffer pH 8.0.

Figure 3

Amino-acid sequence comparison of *AbDHDPR* with DHDPRs from various bacterial species for which crystal structures have been determined: *EcDHDPR*, *BtDHDPR*, *BhDHDPR*, *SaDHDPR*, *TmDHDPR* and *MtDHDPR*. The sequence identities of *AbDHDPR* to *EcDHDPR*, *BtDHDPR*, *BhDHDPR*, *TmDHDPR*, *SaDHDPR* and *MtDHDPR* are 60, 53, 40, 34, 31 and 30%, respectively. The residues that roughly form the NADPH binding site are shaded in pink. The conserved residues in the DHDPR proteins from the seven bacterial species are shaded in cyan.

2.4. X-ray data collection and processing

Crystals were examined in the X-ray beam using a MAR Research 345 mm diameter imaging-plate scanner (MAR Research, Norderstedt, Germany) mounted on a rotating-anode X-ray generator (Rigaku, Tokyo, Japan) operating at 50 kV and 100 mA. The crystals diffracted to 2.5 Å resolution. However, these crystals were not very stable in the X-ray beam. Despite the use of various crystallization conditions and additives, crystals of *AbDHDPR* could only survive for a short time, thereby allowing only a partial data collection. Efforts are being made to improve the quality of the crystals. The partially collected data were processed using the *HKL-2000* suite of programs (Otwinowski & Minor, 1997). The crystals belonged to the orthorhombic space group *P222*, with unit-cell parameters $a = 80.0$, $b = 100.8$, $c = 147.6$ Å. The crystals showed a high mosaicity of 0.80°. Assuming the presence of four molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) was calculated to be

$2.6 \text{ Å}^3 \text{ Da}^{-1}$, which corresponded to a solvent content of approximately 53%. The preliminary crystallographic data are given in Table 1.

3. Results and discussion

The amino-acid sequence of *AbDHDPR* shows moderate to low sequence identity to its counterparts from other species. The sequence identities were found to vary from 60 to 30%, with the highest being with *EcDHDPR* and the lowest with *MtDHDPR* (Fig. 3). Compared with the sequence of *EcDHDPR* (Scapin *et al.*, 1995), the NADPH- and substrate-binding site in *AbDHDPR* may roughly consist of the segments Gly14–Met16, Phe77–Ala79, Ile99–Gly103, Tyr123–Tyr127, Val153–Gly173, Ile214–Glu217 and His233–Asn240. There are notable sequence differences in these segments when compared with those from other species (Fig. 3). These

differences may cause differences in the interactions with ligands. In view of these facts, structure determinations of the *AbdHDP*R enzyme in the unbound state and in bound states with NADH and NADPH as well as with designed inhibitors are very much required so that the precise modes of substrate and inhibitor binding can be determined. The preliminary crystallographic data of *AbdHDP*R indicate that there are four crystallographically independent molecules in the asymmetric unit, suggesting the formation of a possible tetramer as reported in the cases of *MtDHDPR* (Cirilli *et al.*, 2003) and *SaDHDPR* (Girish *et al.*, 2011).

TPS thanks the Department of Biotechnology (DBT) for the award of a Distinguished Biotechnology Research Professorship. MS thanks the Department of Science and Technology (DST) for grants under the Fast-Track Program in Life Sciences.

References

- Cirilli, M., Zheng, R., Scapin, G. & Blanchard, J. S. (2003). *Biochemistry*, **42**, 10644–10650.
- Dijkshoorn, L., Nemec, A. & Seifert, H. (2007). *Nature Rev. Microbiol.* **12**, 939–951.
- Girish, T. S., Navratna, V. & Gopal, B. (2011). *FEBS Lett.* **16**, 2561–2567.
- Kobayashi, K. *et al.* (2003). *Proc. Natl Acad. Sci. USA*, **100**, 4678–4683.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Oncolül, O., Keskin, O., Acar, H. V., Küçükardali, Y., Evrenkaya, R., Atasoyu, E. M., Top, C., Nalbant, S., Ozkan, S., Emekdaş, G., Cavaşlu, S., Us, M. H., Pahsa, A. & Gökben, M. (2002). *J. Hosp. Infect.* **51**, 47–51.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Pearce, F. G., Sprissler, C. & Gerrard, J. A. (2008). *J. Biochem.* **143**, 617–623.
- Reddy, S. G., Scapin, G. & Blanchard, J. S. (1996). *Biochemistry*, **35**, 13294–13302.
- Scapin, G., Blanchard, J. S. & Sacchettini, J. C. (1995). *Biochemistry*, **34**, 3502–3512.
- Somerville, D. A. & Noble, W. C. (1970). *Rev. Eur. Etud. Clin. Biol.* **15**, 669–671.
- Takahashi, A., Yomoda, S., Kobayashi, I., Okubo, T., Tsunoda, M. & Iyobe, S. (2000). *J. Clin. Microbiol.* **38**, 526–529.
- Towner, K. J. (2009). *J. Hosp. Infect.* **73**, 355–363.
- Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E. & Pitt, T. L. (2006). *J. Clin. Microbiol.* **44**, 2974–2976.
- Zeana, C., Larson, E., Sahni, J., Bayuga, S. J., Wu, F. & Della-Latta, P. (2003). *Infect. Control Hosp. Epidemiol.* **24**, 275–279.