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The purification, crystallization and preliminary X-ray diffraction analysis of dihydrodipicolinate synthase from *Clostridium botulinum*

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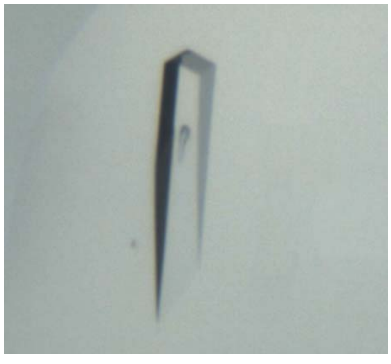

In recent years, dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52) has received considerable attention from both mechanistic and structural viewpoints. This enzyme, which is part of the diaminopimelate pathway leading to lysine, couples (*S*)-aspartate-β-semialdehyde with pyruvate *via* a Schiff base to a conserved active-site lysine. In this paper, the expression, purification, crystallization and preliminary X-ray diffraction analysis of DHDPS from *Clostridium botulinum*, an important bacterial pathogen, are presented. The enzyme was crystallized in a number of forms, predominantly using PEG precipitants, with the best crystal diffracting to beyond 1.9 Å resolution and displaying $P4_22_12$ symmetry. The unit-cell parameters were $a = b = 92.9$, $c = 60.4$ Å. The crystal volume per protein weight (V_M) was 2.07 Å^3 Da^{-1}, with an estimated solvent content of 41%. The structure of the enzyme will help guide the design of novel therapeutics against the *C. botulinum* pathogen.

1. Introduction

Dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52) catalyses the branch-point reaction in the biosynthetic pathway leading to *meso*-diaminopimelate and (*S*)-lysine in plants and bacteria. The catalysed reaction couples pyruvate and (*S*)-aspartate-β-semialdehyde in an aldol-like condensation to form (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid. Since (*S*)-lysine biosynthesis does not occur in animals, pathway members such as DHDPS are attractive targets for rational antibiotic and herbicide design (Coulter *et al.*, 1999; Hutton *et al.*, 2003, 2007). However, a potent inhibitor of DHDPS has yet to be found. As the purported rate-determining step in the (*S*)-lysine-biosynthetic pathway, DHDPS is also of interest to those aiming to engineer plants rich in (*S*)-lysine, which is often the limiting nutrient in staple crops (Miflin *et al.*, 1999).

Although the catalytic mechanism of DHDPS has been extensively studied (Blickling *et al.*, 1997; Dobson, Gerrard *et al.*, 2004; Dobson, Griffin *et al.*, 2004; Dobson, Valegård *et al.*, 2004; Dobson, Devenish *et al.*, 2005), the mechanism of inhibition by the allosteric feedback effector (*S*)-lysine remains poorly understood (Yugari & Gilvarg, 1965; Stahly, 1969; Kumpaisal *et al.*, 1989; Laber *et al.*, 1992; Blickling *et al.*, 1997, 1998; Dobson, Griffin *et al.*, 2004).

The structure of DHDPS from a variety of organisms has been solved (Mirwaldt *et al.*, 1995; Blickling *et al.*, 1998; Dobson, Griffin *et al.*, 2005; Blagova *et al.*, 2006; Pearce *et al.*, 2006). In most cases, DHDPS is a tetrameric protein existing as a dimer of tight dimers. There are four active sites per tetramer, all of which are located near the central cavity of an $(\alpha/\beta)_8$-barrel and each of which is made up of residues from the two peptide chains of a tight dimer. The two allosteric binding sites are located at the tight-dimer interface, although at a distance of approximately 20 Å from the active sites (Dobson, Griffin *et al.*, 2004). Although the active site is well conserved in DHDPS enzymes, it appears that the dimer–dimer interface is not and this represents a target for drugs to achieve organism specificity. Recent work has shown that the dimeric species of *Escherichia coli* DHDPS has drastically lower activity (Perugini *et al.*, 2005). Thus, we have been engaged in a study of DHDPS enzymes from a variety of organisms in order to probe the various interfaces

and to design molecules that disrupt the quaternary structure of DHDPS.

Here, we present preliminary crystallographic studies of DHDPS from *Clostridium botulinum* (*Cbot*-DHDPS) as a first step towards the development of novel therapeutics against *C. botulinum*.

2. Materials and methods

2.1. Expression, purification and crystallization of *Cbot*-DHDPS

E. coli CodonPlus BL21 (DE3) (pETSA1) cells which carry the *Cbot*-DHDPS gene (*Dap*A) were cultured at 310 K in Luria broth containing chloramphenicol (25 µg ml^{-1}) and ampicillin (75 µg ml^{-1}) to an OD_{600} of 0.8. Expression of the native *Cbot*-DHDPS gene product was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 m*M* before incubation at 310 K for 1 h. Cells were harvested by centrifugation at 10 000*g* for 15 min. The cell pellet was stored at 253 K prior to use.

Cell pellets were thawed on ice and resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0) before lysis by sonication with an MSE Soniprep 150 sonicator at 14 µm amplitude, following a 10 min cycle of 3 s bursts and 10 s rest. Centrifugation (10 000*g*, 17 min) at 277 K was then performed and the supernatant was retained as the crude cell lysate. Following 0.45 µm filtration, the cell lysate was applied onto a Q-Sepharose column (50 ml bed volume, 10 cm) pre-equilibrated with buffer *A* at 277 K. The column was then washed with buffer *A* until a stable baseline was reached. Elution of bound protein was performed over five column volumes using a 0–1 *M* NaCl gradient in buffer *A* and 10 ml fractions were collected. Following Q-Sepharose anion-exchange liquid chromatography, ammonium sulfate was added to the pooled DHDPS fractions to a final concentration of 1.0 *M* before loading onto a Phenyl Sepharose column (70 ml bed volume, 10 cm) pre-equilibrated in buffer *B* (20 m*M* Tris–HCl, 1.0 *M* ammonium sulfate pH 8.0) at 277 K. The protein was eluted *via* a five-column-volume gradient of 1.0–0 *M* ammonium sulfate in buffer *B*. Peak fractions were concentrated with a 30 kDa molecular-weight cutoff Centricon before buffer exchange into buffer *A* using a 5 ml HiTrap desalting column. Following buffer exchange, peak fractions were concentrated to 11.3 mg ml^{-1} using a 10 kDa molecular-weight cutoff Centricon at 3000*g* and 277 K. Protein purification steps were monitored using the qualitative *o*-aminobenzaldehyde assay (Dobson, Devenish *et al.*, 2005) and SDS–PAGE. A detailed account of the expression and purification results will be published elsewhere.

Initial protein crystallization experiments were performed at the CSIRO node of the Bio21 Collaborative Crystallization Centre (C3) using the PACT Suite and the JCSG+ Suite crystal screens (Qiagen) at 281 and 293 K. The initial screens, which yielded a variety of small crystal forms, were set up using the sitting-drop vapour-diffusion method with droplets consisting of 100 nl protein solution and 100 nl reservoir solution. The crystal shown in Fig. 1 was obtained from a 400 nl drop made up of 200 nl protein solution (11.2 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0) and 200 nl precipitant [15.7%(*w*/*v*) polyethylene glycol 3350, 100 m*M* malic acid pH 5.66]. The drop was incubated at 281 K. The reservoir (80 µl) contained 15.7%(*w*/*v*) polyethylene glycol 3350 and 100 m*M* malic acid pH 5.66. We note that only freshly purified protein readily produced crystals.

2.2. Data collection and processing

For X-ray data collection, the crystal was soaked in reservoir liquor containing 20%(*v*/*v*) glycerol and directly flash-frozen in liquid nitrogen. Intensity data were collected at 100 K (Oxford Instruments Cryojet) using an R-AXIS IV^{++} image-plate detector coupled to a Rigaku Micromax 007 X-ray generator operating at 40 kV and 30 mA with AXCO capillary optics. The crystal-to-detector distance was 200 mm and each frame was exposed for 5 min with a 0.5° oscillation. The diffraction data were processed using the programs *MOSFLM* (Leslie, 1991) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Cbot-DHDPS crystals were found in a number of conditions from the PACT screen, suggesting that PEGs (in particular PEG 3350) were a

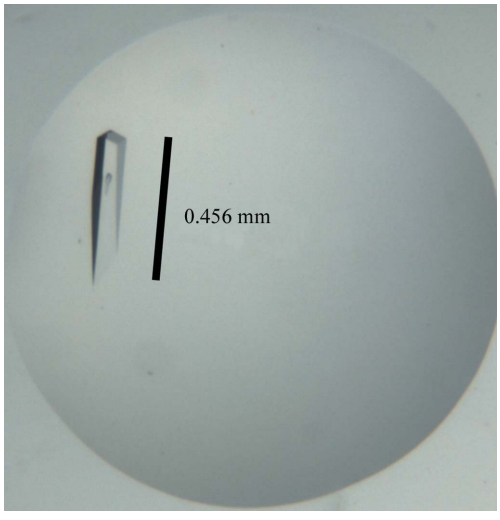

Figure 1
Crystal of recombinant *Cbot*-DHDPS. A small satellite crystal was observed about halfway along the length of the crystal.

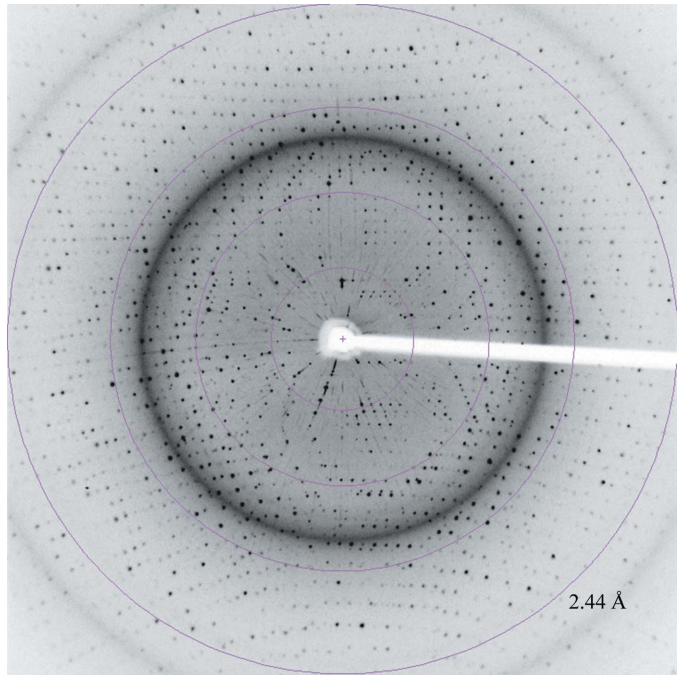

Figure 2
An X-ray diffraction frame of a cryocooled crystal of *Cbot*-DHDPS. The crystal diffracted to beyond 1.95 Å (corners).

Table 1
X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell. The Matthews coefficient and solvent content are based on a molecular weight of 31 527 Da in the asymmetric unit (one molecule of *Cbot*-DHDPS).

Wavelength (Å)	1.5418
No. of images	178
Oscillation (°)	0.5
Space group	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 92.9$, $c = 60.4$
Resolution (Å)	36.81–2.34 (2.53–2.34)
Observed reflections	75339 (13164)
Unique reflections	11412 (2175)
Completeness (%)	98.5 (92.9)
$R_{\rm merge}$†	0.042 (0.086)
$R_{\rm p.i.m.}$‡	0.020 (0.037)
Mean $I/\sigma(I)$	40.6 (18.1)
Redundancy	6.6 (6.1)
Wilson B value (Å^2)	18.6
Molecules per ASU	1
$V_{\rm M}$ (Å^3 Da^{-1})	2.07
Solvent content (%)	40.5

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

general precipitant for *Cbot*-DHDPS. Most crystals diffracted weakly using a variety of cryogenic conditions. For the crystal shown in Fig. 1, the presence of malic acid, an analogue of the *Cbot*-DHDPS substrate pyruvate, may have helped to stabilize the crystal during growth. It has been noted that pyruvate stabilizes DHDPS from *E. coli* (R. C. J. Dobson, unpublished work) and is sometimes added during purification. The crystal appeared after 10 d and continued to grow to a length of ∼0.45 mm (in the longest dimension) over a further 7 d.

The crystal showed excellent diffraction (beyond 1.95 Å; Fig. 2), although the potential for overlapping spots precluded the collection of the highest resolution data (beyond 1.95 Å). Data collection was continued until the crystal diffraction deteriorated significantly (after 176 images at 0.5° oscillation). Nevertheless, 98.5% of the data were collected with 92.9% completeness in the highest resolution shell (Table 1). The diffraction patterns showed evidence of secondary diffraction, perhaps arising from the satellite crystal that can be seen in Fig. 1; however, *MOSFLM* was able to distinguish the patterns and readily integrated the reflections to yield good statistics, including those reflections in the highest resolution shell (Table 1). The space group was assigned as $P4_22_12$, with unit-cell parameters $a = b = 92.9$, $c = 60.4$ Å. $R_{\rm merge}$ was 0.042, $R_{\rm p.i.m.}$ was 0.020, $V_{\rm M}$ was 2.07 Å^3 Da^{-1} and the estimated solvent content was 40.5%.

Molecular replacement using DHDPS from *Thermotoga maritima* as a search model (PDB code 1o5k; 41% sequence identity) confirms the presence of one molecule in the asymmetric unit and model building continues. Future studies will include *Cbot*-DHDPS complexed with substrates and/or inhibitors in order to understand the function of this enzyme and to aid the development of potent inhibitors against dihydrodipicolinate synthase from *C. botulinum*.

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