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Crystallization and preliminary X-ray analysis of dihydrodipicolinate synthase from *Clostridium botulinum* in the presence of its substrate pyruvate

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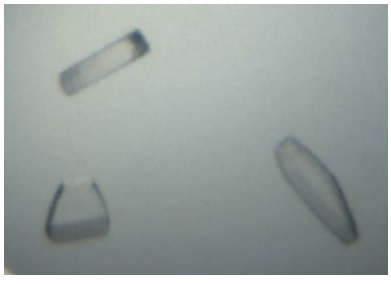

In this paper, the crystallization and preliminary X-ray diffraction analysis to near-atomic resolution of DHDPS from *Clostridium botulinum* crystallized in the presence of its substrate pyruvate are presented. The enzyme crystallized in a number of forms using a variety of PEG precipitants, with the best crystal diffracting to 1.2 Å resolution and belonging to space group *C*2, in contrast to the unbound form, which had trigonal symmetry. The unit-cell parameters were $a = 143.4$, $b = 54.8$, $c = 94.3$ Å, $\beta = 126.3^\circ$. The crystal volume per protein weight (V_M) was 2.3 Å^3 Da^{-1} (based on the presence of two monomers in the asymmetric unit), with an estimated solvent content of 46%. The high-resolution structure of the pyruvate-bound form of *C. botulinum* DHDPS will provide insight into the function and stability of this essential bacterial enzyme.

1. Introduction

Dihydrodipicolinate synthase (DHDPS; EC 4.2.1.52) catalyzes the first committed step in the lysine-biosynthetic pathway in plants, bacteria and some fungi. The products of the pathway, lysine and its precursor *meso*-diaminopimelate, are important in protein and cell-wall peptidoglycan synthesis, respectively. DHDPS catalyzes the condensation of pyruvate and (*S*)-aspartate semialdehyde (ASA) to form (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (HTPA; Blickling *et al.*, 1997). The reaction proceeds *via* a ping-pong mechanism, in which pyruvate binds as a Schiff base to an active-site lysine residue, resulting in the formation of an imine. Tautomerization of the Schiff base to an enamine then occurs, although the details of this process are unknown. ASA then reacts with this enamine and following cyclization forms the product HTPA (Blickling *et al.*, 1997).

The structures of DHDPS from a variety of bacterial species have been determined (Dobson *et al.*, 2005; Blagova *et al.*, 2006; Pearce *et al.*, 2006; Wolterink-van Loo *et al.*, 2008; Kefala *et al.*, 2008; Phenix *et al.*, 2008; Burgess *et al.*, 2008; Girish *et al.*, 2008). The enzyme usually assembles as a tetrameric protein, best described as a dimer of tight dimers, although an active dimer has recently been characterized (Burgess *et al.*, 2008; Girish *et al.*, 2008). Each monomer is an $(\alpha/\beta)_8$-barrel, with the active site situated near the central cavity of the barrel. Residues at the tight-dimer interface contribute to each of the four active sites of the tetramer. In those enzymes that are inhibited by (*S*)-lysine, two allosteric binding sites are located at the tight-dimer interface, displaced from the active site (Dobson *et al.*, 2004). In the *Escherichia coli* DHDPS enzyme the lysine-binding site is ∼15 Å from the active site.

The end product of the lysine-biosynthetic pathway has been observed to feedback-inhibit DHDPS enzymes from plants (Frisch *et al.*, 1991; Dereppe *et al.*, 1992) and some Gram-negative bacteria (Laber *et al.*, 1992). However, allosteric regulation of DHDPS does not occur at biologically relevant lysine concentrations in Gram-positive species (Cremer *et al.*, 1988; Burgess *et al.*, 2008; Girish *et al.*, 2008) and thus the regulatory mechanism of these enzymes is not yet known. We have observed substrate stabilization of *Clostridium botulinum* DHDPS (*Cbot*-DHDPS) by pyruvate (details to be published elsewhere) and it was thus of interest for us to study the

Table 1
X-ray data-collection statistics.

Wavelength (Å)	0.9536
No. of images	1440
Oscillation (°)	0.5
Space group	*C*2
Unit-cell parameters (Å, °)	a = 143.4, b = 54.8, c = 94.3, β = 126.3
Resolution (Å)	25.8–1.2 (1.25–1.2)
Observed reflections	969810
Unique reflections	188454
Completeness (%)	99.9 (98.6)
$R_{\rm merge}$	0.069 (0.512)
$R_{\rm r.i.m.}$	0.077 (0.574)
$R_{\rm p.i.m.}$	0.033 (0.252)
Mean $I/\sigma(I)$	13.6 (2.8)
Redundancy	5.1 (5.0)
Wilson B value (Å^2)	9.4
Molecules per ASU	2
$V_{\rm M}$ (Å^3 Da^{-1})	2.3
Solvent content (%)	46

crystal structure of the enzyme in both the absence and presence of this metabolite.

We have already crystallized ligand-free *Cbot*-DHDPS (Dobson *et al.*, 2008) and its X-ray structure has been determined (unpublished results). Here, we present preliminary near-atomic resolution crystallographic studies of DHDPS from *C. botulinum* with pyruvate in a novel crystal form in order to assist in determining the mechanism of pyruvate tautomerization in the active site and as a first step towards understanding the stability and potential mode of regulation of DHDPS from a Gram-positive bacterial pathogen.

2. Methods and materials

2.1. Expression and purification of *Cbot*-DHDPS

The *C. botulinum dapA* gene, which encodes a native 292-amino-acid enzyme, was cloned, inserted into the vector pET11a and transformed into *E. coli* strain CodonPlus BL21 (DE3) (Dobson *et al.*, 2008). The expressed native *Cbot*-DHDPS enzyme (without cloning artifacts or purification tags) was purified as described previously (Dobson *et al.*, 2008) *via* a three-step purification process consisting of anion-exchange, hydrophobic interaction and size-exclusion chromatography. Typical yields were 20 mg homogeneous *Cbot*-DHDPS from 1.0 l culture. Using mass spectrometry, the N-terminal methonine of the purified enzyme was found to have been cleaved. The predicted native molecular mass was 31 651.04 Da, whereas the mass of the purified enzyme was found to be 31 528.65 Da. This result was confirmed by digestion with the protease GluC followed by tandem mass-spectrometric analysis. The enzyme was routinely stored in 20 m*M* Tris–HCl pH 8.0 at 252 K for crystallization trials.

For crystallization trials, a solution consisting of 12 mg ml^{-1} *Cbot*-DHDPS and 20 m*M* sodium pyruvate was prepared. Initial protein-crystallization experiments were performed at the CSIRO node of the Bio21 Collaborative Crystallization Centre (C3) using the PACT Suite and JCSG+ Suite crystal screens (Qiagen) at 281 and 293 K. These initial screens were set up using the sitting-drop vapour-diffusion method with droplets consisting of 100 nl protein solution and 100 nl reservoir solution. Reagents containing a variety of polyethylene glycols as precipitants gave a number of crystal forms at 293 K. These conditions were repeated using the hanging-drop vapour-diffusion method with drops consisting of 2 µl protein solution and 2 µl reservoir solution at 293 K. A variety of crystal forms were again observed from a number of different conditions. The best diffracting crystal grew from a reservoir solution containing 0.2 *M* sodium chloride, 20%(*w*/*v*) PEG 3350.

2.2. Data collection and processing

For X-ray data collection, the crystal was briefly soaked in reservoir liquor containing 20%(*v*/*v*) glycerol with 20 m*M* pyruvate and directly flash-cooled in liquid nitrogen. Intensity data were collected at 100 K at the Australian Synchrotron using the 3-BM1 beamline (McPhillips *et al.*, 2002; Evans & Pettifer, 2001) producing X-rays at a wavelength of 0.9536 Å. Data were collected in two 360° passes: the first with the ADSC Q210r image-plate detector positioned 85 mm from the crystal with an exposure time of 10 s (low-resolution overloads were rejected during data processing) and the second with the detector positioned 150 mm from the crystal and an exposure time of 3 s. Both data sets were collected in 0.5° steps. Diffraction data sets were processed and scaled using *MOSFLM* (Leslie, 1991) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

As observed for the pyruvate-free form of *Cbot*-DHDPS (Dobson *et al.*, 2008), the enzyme crystallized in a remarkable number of conditions using the PACT screen in the presence of pyruvate. These crystals exhibited a wide variety of diffraction quality in diffraction trials (from <3 to 1.2 Å resolution). Crystals of *Cbot*-DHDPS with pyruvate were also obtained under the same conditions as used for the pyruvate-free enzyme form (Dobson *et al.*, 2008); however, neither of the crystals mounted diffracted beyond 2.5 Å resolution at the Australian Synchrotron. The pyruvate-free form crystallized in the presence of malic acid (100 m*M*) whereas the pyruvate-bound form contained NaCl (0.2 *M*) and the crystal morphologies appeared to be quite different. The increased resolution and alternate crystal forms evident in the pyruvate-bound form may be a result of altered protein dynamics when the substrate is present in the active site, making this high-resolution structure of particular interest. The crystals shown in Fig. 1 appeared after 5 d and continued to grow to a length of 0.2 mm over a further 20 d. The crystal showed the highest diffraction of a DHDPS crystal to date (1.2 Å).

In contrast to the previously reported unliganded form (Dobson *et al.*, 2008), the space group was determined to be *C*2, with unit-cell parameters a = 143.4, b = 54.8, c = 94.3 Å, β = 126.3°. The Matthews coefficient ($V_{\rm M}$) was calculated to be 2.3 Å^3 Da^{-1} for two monomers

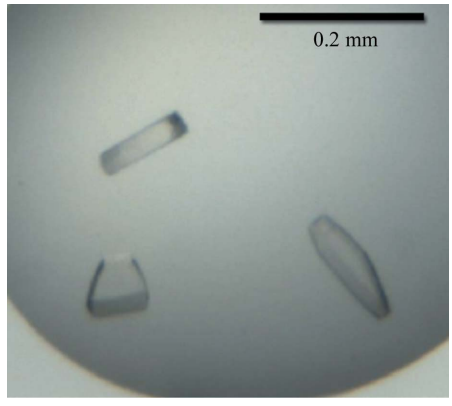

Figure 1
Crystal of recombinant *Cbot*-DHDPS in the presence of pyruvate. The largest dimension of the crystals shown was 0.2 mm.

in the asymmetric unit, with an estimated solvent content of 46%. Scaling and merging of the crystallographic data resulted in an overall $R_{\rm merge}$ of 0.069, with an $R_{\rm merge}$ of 0.512 in the highest resolution shell. Complete data-collection statistics are given in Table 1.

Molecular replacement using the structure of unliganded *Cbot*-DHDPS as a search model (PDB code 3bi8) shows the presence of two monomers in the asymmetric unit (from which a tetramer similar to that of 3bi8 can be generated by crystallographic symmetry) and model building continues. The first round of refinement gave an $R_{\rm free}$ of 26.4% and an R factor of 25.0%. Inspection of the crystal lattice revealed the crystal packing of the pyruvate-bound and unbound (Dobson *et al.*, 2008) forms to be quite different. In addition, clear electron density links the N^{ε} atom of the active-site lysine (Lys161) to a moiety in the active site that has the expected shape and size that would correspond to a bound pyruvate molecule. We anticipate that this structure will assist in our understanding of the mode of pyruvate stabilization of *Cbot*-DHDPS, providing a potential mechanism of regulation of lysine-insensitive DHDPS enzymes from Gram-positive bacteria. The near-atomic resolution of the structure should also assist in determining the exact nature of the Schiff base, helping us to elucidate the mechanism of imine-to-enamine conversion and whether this step requires the presence of the second substrate ASA.

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