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Cloning, expression, purification and crystallization of dihydrodipicolinate synthase from *Agrobacterium tumefaciens*

Dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step of the lysine-biosynthesis pathway in bacteria, plants and some fungi. This study describes the cloning, expression, purification and crystallization of DHDPS (NP_354047.1) from the plant pathogen *Agrobacterium tumefaciens* (AgT-DHDPS). Enzyme-kinetics studies demonstrate that AgT-DHDPS possesses DHDPS activity *in vitro*. Crystals of AgT-DHDPS were grown in the unliganded form and in forms with substrate bound and with substrate plus allosteric inhibitor (lysine) bound. X-ray diffraction data sets were subsequently collected to a maximum resolution of 1.40 Å. Determination of the structure with and without substrate and inhibitor will offer insight into the design of novel pesticide agents.

1. Introduction

Crown gall disease is a significant plant disease that affects a large range of agriculturally important species, including deciduous fruits, vines and vegetables (De Cleene & De Ley, 1976). The aetiological agent of the disease, the bacterium *Agrobacterium tumefaciens* (AgT), infects healthy plants and causes crop decline or mortality (Agrios, 2004; Burr *et al.*, 1998). The disease is of genuine concern because of its worldwide distribution and its potential to cause considerable damage to the agricultural industry. At risk are several important commodities including grapes, the most cultivated fruit in the world (Giribaldi & Giuffrida, 2010), and stone fruits (Kerr, 1969). Therefore, it is surprising that there is still no universally effective treatment for crown gall disease (Anand *et al.*, 2008). Existing strategies for pest control have significant limitations (Burr & Otten, 1999; Escobar *et al.*, 2001; Lee *et al.*, 2003; Viss *et al.*, 2003) and it is clear that there is a real need to develop novel pesticides targeting essential metabolic processes for the treatment of this disease. One such target is the lysine-biosynthesis pathway in *A. tumefaciens*.

Lysine biosynthesis occurs in bacteria, plants and some fungi (Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012). The lysine-biosynthesis pathway yields lysine, which is essential for protein synthesis, and *meso*-DAP, which is a vital constituent of the peptidoglycan layer of the bacterial cell wall (Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012). The pathway is thus of interest for the development of novel antimicrobial agents, given that several enzymes that function in lysine biosynthesis are essential to bacteria (Hutton *et al.*, 2007; Dogovski *et al.*, 2009). These essential enzymes include dihydrodipicolinate synthase (DHDPS), which catalyses the first committed step in the pathway (Mirwaldt *et al.*, 1995; Dobson *et al.*, 2005; Perugini *et al.*, 2005; Burgess *et al.*, 2008; Dogovski *et al.*, 2009, 2012; Voss *et al.*, 2010). DHDPS has been shown to be one of only 271 essential genes in *Bacillus subtilis* (Kobayashi *et al.*, 2003) and is thus considered to be an attractive target for rational drug design (Hutton *et al.*, 2003, 2007; Mitsakos *et al.*, 2011). However, no potent inhibitors have been identified to date. Interestingly, several compounds have displayed clear differentiation in the inhibition of DHDPS enzymes from different species (Mitsakos *et al.*, 2008), suggesting the potential for targeting the enzyme from specific pathogens. Determination of the crystal structure of DHDPS from *A. tumefaciens* will thus offer insight into the design of specific crown gall disease inhibitors.

DHDPS catalyzes the condensation of pyruvate and (*S*)-aspartate semialdehyde [(*S*)-ASA] to form (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (HTPA) (Blickling, Renner *et al.*, 1997; Hutton *et al.*, 2007; Dogovski *et al.*, 2009; Fig. 1). The reaction proceeds via a ping-pong kinetic mechanism in which pyruvate binds as a Schiff base to an active-site lysine residue (Lys162 in AgT-DHDPS; Blickling, Renner *et al.*, 1997; Dobson, Gerrard *et al.*, 2004).

Figure 1
The reaction catalyzed by DHDPS.

The structures of DHDPS from a number of bacterial species have been determined (Table 1). Structures of DHDPS from the plant species *Nicotiana sylvestris* (Blickling, Beisel *et al.*, 1997), *Vitis vinifera* (Atkinson *et al.*, 2012) and *Arabidopsis thaliana* (Griffin *et al.*, 2012) have also been described.

In bacteria, the enzyme is typically comprised of a homotetramer described as a 'head-to-head' dimer of dimers, as depicted in Fig. 2(*a*). Each monomer adopts a (β/α)₈ or TIM-barrel fold. Both the active and allosteric sites are located at the so-called 'tight-dimer interface', with the allosteric cleft, which binds lysine, at the top and bottom of the tetramer (Dobson *et al.*, 2005; Fig. 2*a*). More recently, studies of DHDPS from a number of species have shown that the enzyme can adopt alternative oligomeric states and quaternary architectures. For instance, DHDPS from *Pseudomonas aeruginosa* and *Staphylococcus aureus* exists as dimers (Burgess *et al.*, 2008; Girish *et al.*, 2008; Kaur *et al.*, 2011). Although the enzymes from plant species are homotetrameric (Blickling, Beisel *et al.*, 1997; Atkinson *et al.*, 2012; Griffin

Figure 2
(*a*) DHDPS from *E. coli* (PDB entry 1dhp; Mirwaldt *et al.*, 1995) and (*b*) DHDPS from *V. vinifera* (PDB entry 3tuu; Atkinson *et al.*, 2012), with the location of the active site marked. (*c–e*) DHDPS from *A. tumefaciens*: (*c*) PDB entry 2r8w, (*d*) PDB entry 3b4u, (*e*) PDB entry 2hmc (Midwest Center for Structural Genomics, unpublished work).

et al., 2012), they adopt an alternative ‘back-to-back’ quaternary architecture (Fig. 2*b*). Of interest to this study, there are three structures in the PDB of putative DHDPS enzymes from *A. tumefaciens* that adopt noncanonical quaternary forms, namely dimers [PDB entries 2r8w (NP_353919.2; Fig. 2*c*) and 3b4u (NP_355449.1;

Fig. 2*d*)] and a hexamer [PDB entry 2hmc (NP_355989.1; Fig. 2*e*)] (Midwest Center for Structural Genomics, unpublished work). In addition, comparative sequence analyses of 2r8w, 3b4u and 2hmc show that all three structures lack one or more of the key active-site residues shown to be important for DHDPS catalytic function (Fig. 3).

Table 1
Examples of bacterial AgT-DHDPS orthologues with known crystal structures.

Organism	Ligands	Identity to AgT-DHDPS (%)	Reference
<i>Escherichia coli</i>	Unliganded, lysine, pyruvate	44	Mirwaldt <i>et al.</i> (1995); Dobson <i>et al.</i> (2005); Devenish <i>et al.</i> (2008)
<i>Thermotoga maritima</i>	Unliganded	38	Pearce <i>et al.</i> (2006)
<i>Bacillus anthracis</i>	Unliganded, pyruvate	40	Blagova <i>et al.</i> (2006); Voss <i>et al.</i> (2010)
<i>Mycobacterium tuberculosis</i>	Unliganded	41	Kefala <i>et al.</i> (2008)
<i>Hahella chejuensis</i>	Unliganded	57	Kang <i>et al.</i> (2010)
<i>Staphylococcus aureus</i>	Unliganded, pyruvate	40	Burgess <i>et al.</i> (2008); Girish <i>et al.</i> (2008)
<i>Corynebacterium glutamicum</i>	Unliganded	39	Rice <i>et al.</i> (2008)
<i>Neisseria meningitidis</i>	Unliganded	45	Devenish <i>et al.</i> (2009)
<i>Pseudomonas aeruginosa</i>	Unliganded, lysine	45	Kaur <i>et al.</i> (2011)
<i>Methanococcus jannaschii</i>	Unliganded	46	Padmanabhan <i>et al.</i> (2009)

Figure 3
Sequence alignment of *E. coli* DHDPS and ten putative *dapA* gene products in the annotated *A. tumefaciens* strain C58 genome. Residues of the active site (green), including the catalytic triad (yellow) and the catalytic lysine (red), are highlighted.

However, the product of the putative *dapA* gene from *A. tumefaciens* (NP_354047; designated here as AgT-DHDPS) contains all of the key catalytic residues and also shows high conservation of the allosteric site residues, suggesting that this protein functions as a DHDPS enzyme (Fig. 3). This study describes the cloning, expression, purification, crystallization and preliminary X-ray diffraction analyses of a promising antimicrobial target from *A. tumefaciens*, namely DHDPS. Determination of the structure of AgT-DHDPS will thus offer insight into the design of novel pesticide agents for the treatment of crown gall disease.

2. Materials and methods

2.1. Construction of an AgT-DHDPS expression vector

The gene encoding AgT-DHDPS (NP_354047.1) was amplified by PCR from genomic DNA derived from *A. tumefaciens* strain C58 (forward primer 5'-GACTGTCACACTTCCGACAA-3' and reverse primer 5'-TCTCGCTTGTTCACAGCAG-3'). Amplified DNA was cloned into pCR-Blunt II-TOPO (Invitrogen) to produce the vector pTOPO-NP_354047.1. The presence of the gene encoding AgT-DHDPS was confirmed by restriction-endonuclease analysis and dideoxynucleotide sequencing. The forward and reverse primers 5'-GACGACGACAAGATGTTCAAGGGATCAATTCCCGC-3' and 5'-GAGGAGAAGCCCGGTTTCAGTTCATCAGGCCGCGCATG-3', respectively, facilitated PCR amplification and subcloning of the gene into the expression vector pET-46 Ek/LIC (Novagen). The sequence of the resulting vector (pET-His-NP_354047.1) was confirmed *via* dideoxynucleotide sequencing and allows expression of AgT-DHDPS

Figure 4
SDS-PAGE analysis showing the purification of AgT-DHDPS. Lane 1, molecular-weight markers (labelled in kDa); lane 2, non-induced whole cell lysate; lane 3, IPTG-treated whole cell lysate; lane 4, post IMAC chromatography; lane 5, post size-exclusion chromatography.

Figure 5
o-Aminobenzaldehyde assay showing the activity of samples derived from each stage of purification of AgT-DHDPS. Well 1, IPTG-treated whole cell lysate; well 2, post IMAC chromatography; well 3, post size-exclusion chromatography; well 4, negative control (*E. coli* DHDPR); well 5, positive control (*E. coli* DHDPS). The purple colour indicates DHDPS activity (Mitsakos *et al.*, 2011).

Table 2

Purification of recombinant His-tagged AgT-DHDPS.

	Total protein (mg)	Total activity (U†)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (fold)
Lysate	188	4238	23	—	—
IMAC	19	3326	175	8	8
Size exclusion	18	3125	174	74	8

† 1 U is defined as the consumption of 1 μ mol NADPH per minute.

with an amino-terminal hexahistidine tag (MAHHHHHHVDDD-DEK).

2.2. Expression and purification of AgT-DHDPS

Escherichia coli BL21 (DE3) cells were transformed with pET-His-NP_354047.1 and plated onto Luria–Bertani (LB) agar containing 25 μ g ml⁻¹ kanamycin. Following overnight incubation at 310 K, single colonies were transferred into 10 ml LB broth containing 25 μ g ml⁻¹ kanamycin and grown overnight at 310 K with shaking (180 rev min⁻¹). The cells were diluted 1/100 in flasks containing 1 l LB broth (25 μ g ml⁻¹ kanamycin) and cultivated at 303 K with continuous shaking (180 rev min⁻¹) until an OD_{600 nm} of 0.6 was attained. The cells were subsequently treated with 1.0 mM isopropyl β -D-1-thiogalactopyranoside to induce the expression of recombinant AgT-DHDPS. The cultures were incubated for a further 3 h at 303 K with continuous shaking (180 rev min⁻¹). The cells were immediately harvested by centrifugation at 16 000g for 20 min at 277 K.

The cell pellet was resuspended in 30 ml buffer I (20 mM Tris, 500 mM NaCl, 20 mM imidazole pH 8.0) and the cells were lysed by sonication using an MSE Soniprep 150 sonicator with an 18 mm diameter titanium probe at a power output of 10–14 μ m. Cellular debris was pelleted by centrifugation (30 min, 48 000g) and the soluble fraction was filtered using a 0.45 μ m filter (Millipore) and applied onto a 5 ml His-Trap HP column (GE Healthcare) pre-equilibrated with three column volumes of buffer I. The column was washed in buffer I until a steady baseline absorbance ($A_{280 nm}$) was observed. AgT-DHDPS was eluted *via* a gradient of 100–0% buffer I/0–100% buffer II (20 mM Tris, 500 mM NaCl, 500 mM imidazole pH 8.0) applied over ten column volumes. AgT-DHDPS eluted at an imidazole concentration of approximately 200 mM.

The eluate was dialysed overnight against buffer III (20 mM Tris, 150 mM NaCl pH 8.0) before storage at 193 K. Prior to crystallization, the protein was thawed overnight at 277 K before size-exclusion liquid chromatography using an XK 16/20 column packed with Superdex 200 resin (30 ml bed volume; GE Healthcare) pre-equilibrated with buffer III. If required, the protein solution was concentrated to approximately 10 mg ml⁻¹ using a 10 kDa cutoff Centricon (Millipore) prior to gel-filtration chromatography. All purification steps were carried out at 277 K. The results of each purification step are shown in Fig. 4. Fractions following liquid chromatography were also assessed for DHDPS enzymatic activity using the *o*-aminobenzaldehyde assay (Yugari & Gilvarg, 1965), which demonstrated that recombinant AgT-DHDPS possesses DHDPS enzymatic activity (Fig. 5). For the purification table (Table 2), kinetic analyses were performed using the DHDPS–DHDPR coupled assay as described previously (Dobson, Gerrard *et al.*, 2004) using *E. coli* DHDPR as the coupling enzyme.

2.3. Crystallization of unliganded and ligand-bound AgT-DHDPS

Crystallization studies were initially conducted using a 10 mg ml⁻¹ protein preparation of AgT-DHDPS with the full-length hexahisti-

Table 3

Data-collection and processing statistics.

Values in parentheses are for the highest resolution bin.

	AgT-DHDPS	AgT-DHDPS + pyruvate	AgT-DHDPS + pyruvate and lysine
Wavelength (Å)	0.9536	0.9536	0.9536
No. of images	673	720	212
Step range (°)	0.5	0.5	1
Space group	$P2_12_12_1$	$C2$	$C222_1$
Unit-cell parameters (Å, °)	$a = 94.8, b = 131.7,$ $c = 162.6$	$a = 182.8, b = 77.0,$ $c = 98.0, \beta = 121.2$	$a = 89.1, b = 122.8,$ $c = 129.6$
Resolution (Å)	38.70–1.55 (1.63–1.55)	39.10–1.45 (1.51–1.43)	26.37–1.40 (1.48–1.40)
Observed reflections	2812538 (169697)	1491073 (162951)	457924 (39292)
Unique reflections	265747 (38083)	207498 (25640)	135072 (17665)
Completeness (%)	98.4 (89.9)	96.0 (81.6)	97.2 (88.1)
$R_{\text{merge}}^{\dagger}$	0.077 (0.415)	0.070 (0.344)	0.044 (0.289)
$R_{\text{r.i.m.}}^{\ddagger}$	0.081 (0.470)	0.075 (0.374)	0.052 (0.359)
$R_{\text{p.i.m.}}^{\S}$	0.023 (0.212)	0.028 (0.144)	0.026 (0.207)
Mean $I/\sigma(I)$	17.9 (2.9)	16.5 (4.7)	15.8 (3.1)
Multiplicity	9.7 (4.5)	7.2 (6.4)	3.4 (2.2)
Wilson B value (Å ²)	15.4	11.7	10.6
Matthews coefficient (Å ³ Da ^{−1})	3.84	2.19	2.73
Molecules per asymmetric unit	4	4	2
Solvent content (%)	68	44	54

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.
 $\ddagger R_{\text{r.i.m.}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.
 $\S R_{\text{p.i.m.}} = \sum_{hkl} \{1 / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $I_i(hkl)$ is the i th intensity measurement of reflection hkl , $\langle I(hkl) \rangle$ is its average and N is the redundancy of a given reflection.

dine tag in 20 mM Tris pH 8.0. Protein crystallization trials were performed at the CSIRO node of the Bio21 Collaborative Crystallization Centre (C3; <http://www.csiro.au/c3/>) using the PACT Suite and JCSG+ Suite crystal screens (Qiagen; Newman *et al.*, 2005) at 281 and 293 K as described previously (Dobson, Atkinson *et al.*, 2008; Dommaraju *et al.*, 2010; Hor *et al.*, 2010; Sibarani *et al.*, 2010; Wubben *et al.*, 2010; Atkinson *et al.*, 2011). Screens were performed by the sitting-drop vapour-diffusion method with droplets consisting of 150 nl protein solution (10 mg ml^{−1}) and 150 nl reservoir solution. Several screen conditions produced crystals with varying morphology at 293 K. In addition, trials with Crystal Screen, Crystal Screen 2 and Crystal Screen Cryo (Hampton Research) were also performed in-house using the hanging-drop vapour-diffusion method. 2 µl protein solution and 2 µl precipitant solution were equilibrated against 1 ml reservoir solution in 24-well Linbro plates at 293 K. Optimization of the initial hits by varying the pH and the concentration of the precipitant was carried out as for the Hampton Research screens in 24-well Linbro plates. The best diffracting crystals grew overnight in 0.1 M Tris pH 8.5, 2 M ammonium sulfate. Pyruvate cocrystals obtained using 20 mM pyruvate (1 µl 1 M pyruvate added to 49 µl protein solution just prior to setup of crystal trays) also grew in this condition. The Hampton Research screens described above were also used to obtain lysine and pyruvate cocrystals (1 µl 1 M pyruvate and 1 µl 1 M lysine added to 48 µl protein solution just prior to setup of crystal trays), with the best crystals growing in 2 d from a reservoir solution consisting of 0.17 M lithium sulfate monohydrate, 0.085 M Tris pH 8.5, 25.5% (w/v) PEG 4000, 15% (v/v) glycerol.

2.4. Data collection and processing

For each condition, diffraction data were collected using a single crystal (Fig. 6) on either the MX1 or the MX2 beamline (Evans & Pettifer, 2001; McPhillips *et al.*, 2002) at the Australian Synchrotron. MX1 uses a 03BM1 (dipole/bending-magnet) source and an ADSC Q210r CCD detector. MX2 uses a 03ID1 (3 m in-vacuum undulator)

source and an ADSC Q315r CCD detector. Crystals that had been flash-cooled in liquid nitrogen were mounted on the beamline in a cold nitrogen stream at 110 K. For the unliganded crystal, data were collected on the MX2 beamline in 0.5° steps for one 360° pass with 90% attenuation and an exposure time of 2 s with the detector positioned 160 mm from the crystal. The pyruvate crystal was mounted on the MX1 beamline at 160 mm from the detector and data

(a)

(b)

(c)

Figure 6

Crystals of recombinant AgT-DHDPS: (a) unliganded form, (b) cocrystallized with 20 mM pyruvate and (c) cocrystallized with 20 mM lysine and 20 mM pyruvate. The bar in each image indicates 100 µm.

were collected in 0.5° steps for one 360° pass with an exposure time of 2 s. The lysine/pyruvate crystal was mounted on the MX2 beamline at 140 mm from the detector and data were collected in 1° steps for one 220° pass with 80% attenuation and an exposure time of 2 s. Indexing and integration of the data was performed using *MOSFLM* (Leslie & Powell, 2007). *POINTLESS* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011) was run to verify the space group, and scaling and data reduction were performed using *SCALA* (Evans, 2006), also from *CCP4*. All relevant data-collection and processing parameters are given in Table 3. Data were processed to a resolution at which $I/\sigma(I)$ was >2 but with reasonable values for both R_{merge} and

completeness. Images will be made available *via* the *TARDIS* server (Androulakis *et al.*, 2008) once the structures have been solved and published.

3. Results and discussion

A search for *dapA* genes in the annotated *A. tumefaciens* strain C58 genome (NCBI references NC_003062.2, NC_003063.2, NC_003064.2 and NC_003065.3) identified ten putative DHDPS sequences (Goodner *et al.*, 2001; Wood *et al.*, 2001). A *ClustalW2* (Larkin *et al.*, 2007; Goujon *et al.*, 2010) alignment of *E. coli* DHDPS (BAA16355.1) and the ten putative DHDPS sequences was performed (Fig. 3). Inspection of the primary amino-acid sequences revealed that only one DHDPS sequence (NCBI reference NP_354047.1; AgT-DHDPS) contained all of the key conserved residues known to be essential for catalysis: the catalytic triad (Thr44, Tyr107 and Tyr133), Lys161, which forms a Schiff base with pyruvate, Arg138, which is important for (S)-ASA binding, and Ile203 (Dobson, Valegård *et al.*, 2004; Dobson *et al.*, 2005, 2009; Dobson, Griffin *et al.*, 2008). In order to study the structure and function of AgT-DHDPS, the gene encoding this protein was cloned as described in §2.1. Significant quantities of soluble recombinant protein were isolated as described in §2.2.

Approximately 18 mg pure protein was obtained from 1 l bacterial cell culture following the two-step purification procedure (Table 2). The purity of the sample was at least 95% as estimated by SDS-PAGE (Fig. 4). The *o*-aminobenzaldehyde enzyme assay (Yugari & Gilvarg, 1965) confirmed that the purified recombinant enzyme was active (Fig. 5). AgT-DHDPS was initially screened for crystallization as described in §2.3. Larger crystals were then grown in-house under optimized conditions selected from the successful high-throughput screening trials (Fig. 6). Given that cocrystallization yielded diffracting crystals, ligand soaks were not performed. The crystals shown in Fig. 6 were obtained by mixing 2 μl protein solution (10 mg ml^{-1}) with 2 μl reservoir solution at 293 K.

Figure 7
X-ray diffraction images from crystals of AgT-DHDPS (Fig. 6): (a) unliganded, (b) cocrystallized with pyruvate and (c) cocrystallized with lysine and pyruvate.

In preparation for diffraction data collection, unliganded crystals and crystals containing pyruvate were cryoprotected using 10% (v/v) glycerol in reservoir solution (containing the appropriate ligand) and then immediately mounted in a nylon loop and flash-cooled in liquid nitrogen. Crystals containing lysine and/or pyruvate were grown in 15% (v/v) glycerol and thus were directly mounted and cooled. Diffraction data were collected from the unliganded, pyruvate and pyruvate + lysine crystals to resolutions of 1.55, 1.43 and 1.40 Å, respectively (Fig. 7). The relevant data-collection and processing parameters are provided in Table 3.

The unliganded AgT-DHDPS crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 94.8$, $b = 131.7$, $c = 162.6$ Å. Based on the deduced molecular weight of 32 833 Da, calculation of the Matthews coefficient suggested the presence of four subunits per asymmetric unit, with an estimated solvent content of 68% ($V_M = 3.84$ Å³ Da⁻¹). Analysis of average Matthews coefficient values for protein structures of molecular weight 20–40 kDa in the PDB showed that a significant proportion have V_M values of 3.5 Å³ Da⁻¹ or higher (Kantardjieff & Rupp, 2003). Scaling and merging of the crystallographic data resulted in an overall R_{merge} of 0.077, with an R_{merge} of 0.350 in the highest resolution shell. The diffraction of the crystal was observed to significantly reduce towards the end of the data set and thus only the first 673 images were processed. No evidence of twinning was observed. Molecular replacement using the program *Phaser* (McCoy *et al.*, 2007) employing an *E. coli* DHDPS monomer (PDB entry 1ycx; Dobson *et al.*, 2005; 44% identity; Table 1) as a search model showed an unambiguous solution with four monomers in the asymmetric unit with a translation-function Z-score (TFZ) of 25.9 and a final log-likelihood gain (LLG) of 8379. Given the high solvent content calculated for four monomers, a *Phaser* search for eight monomers was also performed, but again yielded four monomers in the asymmetric unit.

The crystal from the drop containing pyruvate belonged to space group $C22_1$, with four molecules in the asymmetric unit ($V_M = 2.19$ Å³ Da⁻¹). Scaling and merging of the crystallographic data resulted in an overall R_{merge} of 0.069, with an R_{merge} of 0.314 in the highest resolution shell. No evidence of twinning was observed. Molecular replacement employing the unliganded AgT-DHDPS structure as a search model showed an unambiguous solution with four monomers in the asymmetric unit with a translation-function Z-score (TFZ) of 29.5 and a final log-likelihood gain (LLG) of 14 979.

In contrast, the pyruvate and lysine cocrystal belonged to space group $C22_1$, with two molecules in the asymmetric unit ($V_M = 2.73$ Å³ Da⁻¹). Crystal diffraction significantly decreased at the end of the data set and thus only 212 images were included in processing. Scaling and merging of the crystallographic data resulted in an overall R_{merge} of 0.044, with an R_{merge} of 0.256 in the highest resolution shell. No evidence of twinning was observed. Molecular replacement employing the unliganded AgT-DHDPS structure as a search model showed an unambiguous solution with two monomers in the asymmetric unit with a translation-function Z-score (TFZ) of 34.6 and a final log-likelihood gain (LLG) of 9156.

Further model building for each of these structures is under way, although we have yet to confirm that the ligands are present in the relevant models. Determination of these crystal structures will offer insight into the design of novel pesticide agents.

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