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Purification, crystallization and preliminary crystallographic analysis of human dihydrodipicolinate synthase-like protein (DHDPSL)

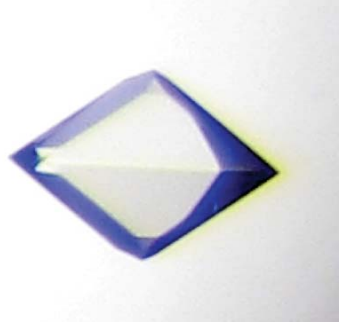
Human dihydrodipicolinate synthase-like protein (DHDPSL) is a gene product of unknown function. It is homologous to bacterial pyruvate-dependent aldolases such as dihydrodipicolinate synthase (DHDPS), which functions in lysine biosynthesis. However, it cannot have this function and instead is implicated in a genetic disorder that leads to excessive production of oxalate and kidney-stone formation. In order to better understand its function, DHDPSL was expressed as an MBP-fusion protein and crystallized using an *in situ* proteolysis protocol. Two crystal forms were obtained, both of which diffracted X-rays to approximately 2.0 Å resolution. One of these, belonging to space group $P6_222$ or $P6_422$ with unit-cell parameters $a = b = 142.9$, $c = 109.8$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$, was highly reproducible and suitable for structure determination by X-ray crystallography.

1. Introduction

Human dihydrodipicolinate synthase-like protein (DHDPSL; Swiss-Prot Q86XE5.1) is an enzyme of unknown function. Based on its distant relationship to the bacterial pyruvate-dependent aldolases (Barbosa *et al.*, 2000), it has been classified as a probable class I (lysine-type) aldolase with a $(\beta/\alpha)_8$-barrel fold (Mirwaldt *et al.*, 1995; Lawrence *et al.*, 1997). Its physiological substrates are unknown, but it shares conserved catalytic residues found in bacterial dihydrodipicolinate synthase (DHDPS) and *N*-acetylneuraminate lyase (NAL) (Barbosa *et al.*, 2000), both of which utilize pyruvate as a substrate. However, DHDPSL must be functionally unlike these two enzymes as DHDPS participates in lysine biosynthesis, a metabolic pathway not found in humans, and human NAL is distinct from DHDPSL.

DHDPSL comprises 327 amino acids (35.2 kDa) and contains a predicted mitochondrial targeting sequence encompassing residues 1–25. An insight into a possible function has come from a recent study in which mutations in the DHDPSL gene were found to be responsible for primary hyperoxaluria type III (Belostotsky *et al.*, 2010), a rare autosomal disorder of unknown aetiology characterized by excessive production of oxalate. The other two types of primary hyperoxalurias result from defective function in two other enzymes that catalyse the catabolism of glyoxylate. If untreated, overproduction of oxalate can lead to a decline in kidney function and the ultimate deposition of calcium oxalate in organs (Bobrowski & Langman, 2008).

These findings implicate DHDPSL in oxalate production. However, the relationship between its activity and how it is regulated in primary hyperoxaluria type III is unknown. Determination of the crystal structure of DHDPSL would allow elucidation of its active-site architecture, possible identification of its physiological substrates and insights into how the naturally occurring mutations affect enzyme function in primary hyperoxaluria type III. Here, we describe the expression, purification and crystallization of human DHDPSL together with preliminary crystallographic data from two crystal forms.



2. Methods

2.1. Cloning, expression and purification

Full-length cDNA encoding the human DHDPSL coding sequence (NCBI reference NM_138413.3) was purchased from Invitrogen (clone identifier IOH26880). The DHDPSL coding sequence was then inserted into the pDEST-hisMBP plasmid used for this study (Addgene plasmid 11085; Nallamsetty *et al.*, 2005) by recombinant cloning using the Gateway cloning system (Invitrogen). This pDEST-hisMBP^{DHDPSL} construct enabled the expression of DHDPSL as a fusion protein with maltose-binding protein (MBP) from *Escherichia coli*, with its periplasm-targeting signal sequence replaced by an N-terminal hexahistidine tag. The linker region between MBP and DHDPSL did not contain a specific protease-cleavage site and the DHDPSL component included the putative mitochondrial targeting sequence. The expressed fusion protein was termed hisMBP-DHDPSL.

An *E. coli* BL21 (DE3) cell line harbouring the tRNA supplementing plasmid, pRP (Novagen), was used for expression. A starter culture was grown from these cells transformed overnight at 310 K with the pDEST-hisMBP^{DHDPSL} plasmid in non-inducing MDG medium (0.5% glucose, 0.25% L-aspartate, 50 m*M* NH_4Cl, 25 m*M* Na_2HPO_4, 25 m*M* KH_2PO4, 5 m*M* Na_2SO_4, 2 m*M* $MgSO_4$) supplemented with ampicillin (100 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}). Expression cultures were inoculated 1:100 with starter culture and grown at 310 K until the OD_{600} reached approximately 0.8 (3–6 h) and were then transferred to 291 K. Protein expression was induced by the addition of 1 m*M* isopropyl β-D-1-thiogalactopyranoside. Cells were harvested after 18 h by centrifugation for 10 min at 3500*g* (277 K) and the cell pellets were washed by resuspension in 20–40 ml ice-cold wash buffer (50 m*M* Tris–HCl, 150 m*M* NaCl pH 8.0) per litre of original culture. Cells were re-collected by centrifugation at 3000*g* for 20 min at 277 K, the supernatant was removed and the cells were processed immediately or stored at 253 K.

Typically, cells from 1 l culture (5–7 g wet cell mass) were resuspended in lysis buffer (40 m*M* EDTA, 50 m*M* Tris–HCl, 150 m*M* NaCl, 0.5 m*M* TCEP pH 8.0 with 2 mg ml^{-1} hen egg-white lysozyme), stirred on ice for 30 min and lysed by sonication with a Sonicator 3000 unit (Misonix) operated at 30 W using 0.5 s pulses on a 50% duty cycle for 10 min. Viable quantities of DHDPSL protein were only obtained when DHDPSL was expressed as a fusion protein with His_6-tagged MBP. Soluble expression was not detected when DHDPSL was previously expressed as a simple poly-His-tagged protein from a pDEST17^{DHDPSL} expression construct (data not shown). Substantial proteolysis of hisMBP-DHDPSL occurred after cell lysis when less than 5 ml of 40 m*M* EDTA per gram of original wet cell mass was included as a protease inhibitor in the lysis buffer.

DHDPSL was purified using a two-step procedure. The first step utilized the MBP moiety to purify the fusion protein using a dextrin-affinity step. Following clarification by centrifugation (30 000*g*, 30 min, 277 K), the lysate was applied onto a 5 ml MBPTrap immobilized dextrin column (GE Healthcare) at 1.7 ml min^{-1}. The column was washed with 25 ml affinity buffer (1 m*M* EDTA, 50 m*M* Tris–HCl, 150 m*M* NaCl, 0.5 m*M* TCEP pH 8.0) and the DHDPSL fusion protein was then eluted from the column with 10 ml elution buffer (1 m*M* EDTA, 3.3 m*M* maltose, 50 m*M* Tris–HCl, 150 m*M* NaCl, 0.5 m*M* TCEP pH 8.0). The eluted hisMBP-DHDPSL was then concentrated to 5 ml using ultrafiltration (30 kDa molecular-weight cutoff concentrator; Vivaspin, GE Healthcare).

In the second step, the fusion protein in 50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* TCEP pH 8.0 was further purified by size-exclusion chromatography on a HiLoad Superdex 16/60 200 preparative-grade size-exclusion column (GE Healthcare) run at 1.2 ml min^{-1} and 277 K. Approximately half of the hisMBP-DHDPSL was found to be present as soluble aggregate, as indicated by an absorption peak close to the void volume of the size-exclusion column (Fig. 1). The soluble aggregate was separated from the second monodisperse hisMBP-DHDPSL absorption peak and discarded. The molecular weight of the species in the second absorption peak was estimated by dynamic light scattering (DLS) to be 291 kDa (R_h 6.7 nm, 15% polydispersity). This molecular weight was consistent with a hisMBP-DHDPSL tetramer. The intact mass of hisMBP-DHDPSL was found to be 77 658.3 Da by mass spectrometry, which was the expected mass for the complete amino-acid sequence. The three most monodisperse fractions as judged by DLS were concentrated to 20 mg ml^{-1}, flash-cooled in liquid nitrogen and stored as 25 µl aliquots at 193 K.

2.2. Crystallization

Initial crystallization experiments with the hisMBP-DHDPSL fusion protein were carried out by sitting-drop vapour diffusion at 291 K using a Cartesian nanolitre dispensing robot and an in-house 480-component crystallization screen (Moreland *et al.*, 2005), but gave only fine needles or heavily overgrown thin plates that could not be successfully optimized. However, analysis of dissolved crystals using SDS–PAGE and mass-spectrometry fingerprint analysis revealed that they contained both intact DHDPSL and fragments of MBP, suggesting that crystallization of hisMBP-DHDPSL might be dependent on proteolysis. An *in situ* proteolysis strategy, based on the approaches of Wernimont & Edwards (2009), was therefore developed and is described below.

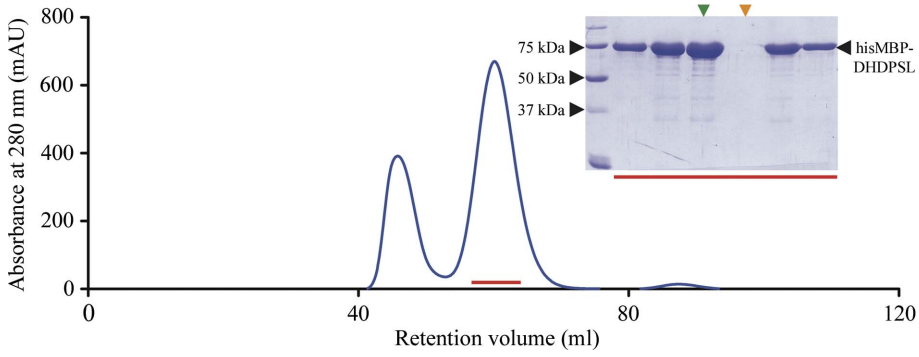
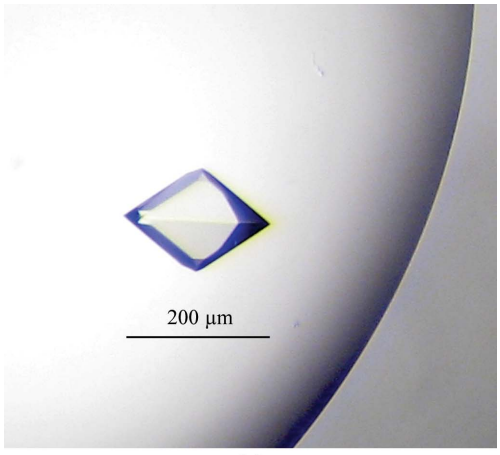
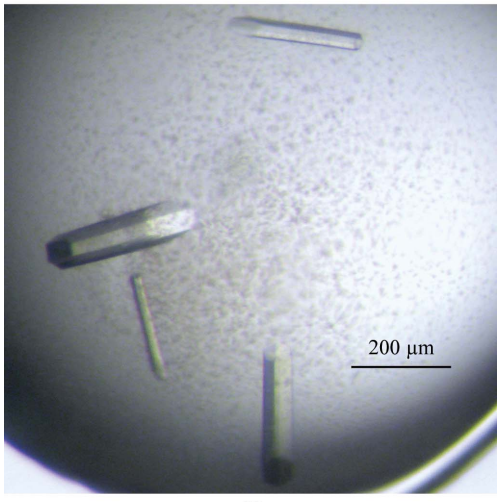


Figure 1
A representative hisMBP-DHDPSL size-exclusion chromatogram and the accompanying SDS–PAGE gel (inset). The red line indicates the fractions (2 ml each) analysed by SDS–PAGE. The fractions indicated by the triangles at the top of the gel were retained for later experiments including crystallization. The sample intended for the empty lane indicated by the orange triangle was not loaded. A HiLoad Superdex 16/60 200 preparative-grade size-exclusion column (GE Healthcare) was used for this purification.

Initial tests with bovine α-chymotrypsin, in which the stock solution of protease (concentration of 1.82 mg ml^{-1} in water) was diluted 1:10, 1:100 and 1:1000, led to the following strategy. The protease solution (1.82 µg ml^{-1} α-chymotrypsin in water) was mixed with protein solution (20 mg ml^{-1} hisMBP-DHDPSL in its storage buffer, 50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* TCEP pH 8.0) at a ratio of 2.5 µl protease solution to 25 µl protein solution. Mixing was carried out on ice immediately before the crystallization experiments were set up. After mixing, crystallization experiments were routinely set up at 291 K using hanging-drop vapour diffusion by manual addition of 1 µl aliquots of precipitant solution to 1 µl drops of the protein/protease mixture described above. Crystals had been obtained from the original 480-component screen using 100 m*M* Tris–HCl pH 8.0, 200 m*M* $MgSO_4$, 17–19% PEG 3350 as a precipitant. For the *in situ* proteolysis crystallization experiments, this precipitant solution was taken as a starting point and was optimized by systematic variation of the pH, the PEG 3350 concentration and the $MgSO_4$ concentration and by testing the effects of additives (Additive Screen, Hampton Research). The optimum precipitant solution comprised 1.0 ml 100 m*M* Tris–HCl pH 8.0, 200 m*M* $MgSO_4$, 19% PEG 3350 supplemented with 50 µl 4 *M* sodium thiocyanate and 50 µl water (1.1 ml total volume). The final crystals were grown in hanging drops at 291 K by adding 1 µl of this precipitant to 1 µl of the protease/protein mixture.



(*a*)



(*b*)

Figure 2
DHDPSL crystals. (*a*) An optimized crystal prepared such that one crystal grew per drop utilizing *in situ* proteolysis with 182 m*M* sodium thiocyanate as an additive to the optimization screen. (*b*) Hexagonal crystals fortuitously grown on one occasion in an optimization screen incubated at 310 K for one week and then at 291 K for six weeks.

2.3. Data collection and processing

DHDPSL crystals were transferred into a cryoprotectant solution comprising 200 m*M* $MgSO_4$, 17.5% PEG 3350, 100 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 22.5% glycerol. When sodium thiocyanate was used as an additive for crystal growth, as in the optimized protocol described above, this was supplemented with 182 m*M* sodium thiocyanate added 1:20 from a 4 *M* stock solution with 1:20 volume of water. Small crystals (approximately 0.1 × 0.1 × 0.2 mm) were best suited to X-ray analysis. For optimal cryoprotection, the crystals were immersed in cryoprotectant solution for 30–60 s before being flash-cooled in liquid nitrogen.

X-ray diffraction data were collected at 100 K on beamline MX2 of the Australian Synchrotron using an ADSC Quantum 315r detector. Data were indexed, integrated and scaled using the *XDS* suite (Kabsch, 2010). The *POINTLESS* package (Evans, 2006) was used for initial space-group prediction and data-format conversion. The averaged intensities were converted to structure-factor amplitudes by *TRUNCATE* (French & Wilson, 1978).

3. Results and discussion

The hisMBP-DHDPSL fusion protein had no specific protease-recognition site, but analysis of the first crystals prepared showed that the protein had the same proclivity to cleave in the crystallization drop as was observed in the initial large-scale purification attempts in the absence of EDTA and during storage at 277 K. Analysis by mass spectrometry (data not shown) showed that the crystals contained intact DHDPSL and MBP fragments. A standardized *in situ* proteolysis protocol was therefore developed using bovine α-chymotrypsin as the protease; the success with this protease made it unnecessary to test any others. This approach, together with the inclusion of sodium thiocyanate as an additive in the crystallization solution, which was essential for reproducibility, yielded high-quality crystals, referred to here as form 1 crystals, overnight.

Crystallization was successful across the pH range 7.5–8.5 and was rapid and reproducible under these experimental conditions, yielding crystals with a pyramidal morphology (Fig. 2*a*). SDS–PAGE analysis of these crystals showed the same pattern of bands that had been observed in the earlier crystallization trials prior to adoption of the *in situ* proteolysis procedure and shown to correspond to intact DHDPSL and fragments of MBP. These form 1 crystals were typically ∼0.2 mm in the long dimension. A crystal was flash-cooled as described above and a complete diffraction data set to 2.0 Å resolution was collected. The crystals were hexagonal, space group $P6_222$ or $P6_422$, with unit-cell parameters $a = b = 142.9$, $c = 109.8$ Å, giving a value for the Matthews coefficient (Matthews, 1968) of 4.60 Å^3 Da^{-1} and a solvent content of 73%, assuming one DHDPSL molecule of 35.2 kDa in the asymmetric unit.

A second crystal form of DHDPSL (form 2) was obtained on only one occasion. Four crystals with rod-like morphology (Fig. 2*b*) grew in one drop containing protein that had been treated *in situ* with α-chymotrypsin and incubated with a precipitant that lacked sodium thiocyanate. In testing the effects of temperature, a crystallization experiment had been incubated at 310 K for one week, in which time heavily overgrown thin plate-like crystals had formed in most conditions. This experiment appeared to be unlikely to yield crystals suitable for crystallographic analysis and had been set aside. The crystallization plate was transferred to 291 K for storage, after which

Table 1
Data-collection and processing statistics for DHDPSL.

Values in parentheses are for the outermost resolution shell.

	Form 1	Form 2
X-ray source	Australian Synchrotron PX2	Cu $K\alpha$ anode
Wavelength (Å)	0.9537	1.5418
Space group	$P6_222$ or $P6_422$	$P6_222$ or $P6_422$
Unit-cell parameters (Å, °)	$a = b = 142.9$, $c = 109.8$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 153.0$, $c = 50.2$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range (Å)	25.0–1.99 (2.10–1.99)	25.0–2.10 (2.21–2.10)
No. of measured reflections	481866 (71182)	366614 (52021)
No. of unique reflections	45562 (6557)	20724 (2959)
R_{sym}† (%)	9.2 (58.9)	7.3 (58.0)
R_{meas}† (%)	10.8 (67.4)	7.7 (61.3)
Completeness (%)	99.9 (100.0)	99.9 (99.9)
Multiplicity	10.6 (10.9)	17.6 (17.6)
Mean $I/\sigma(I)$	17.2 (3.9)	32.6 (5.4)

† R_{sym} and R_{meas} are defined as in Diederichs & Karplus (1997).

it was only occasionally inspected. The crystals were discovered after seven weeks in a drop comprising 1 µl 100 m*M* Tris–HCl pH 7.5, 200 m*M* $MgSO_4$ and 1 µl protein/protease mixture (as described above). A complete diffraction data set was collected to 2.1 Å resolution from one of these form 2 crystals, a rod of dimensions 0.2 × 0.05 × 0.05 mm (Fig. 2*b*). Like the form 1 crystal, this crystal was hexagonal, space group $P6_222$ or $P6_422$, but with a smaller unit cell with $a = b = 153.0$, $c = 50.2$ Å. The shortening of the c axis gives an asymmetric unit with a much lower solvent content; for this crystal, $V_M = 2.41$ Å^3 Da^{-1} and the solvent content was 49%. Data-collection and processing statistics for both crystal forms are shown in Table 1.

Both crystal forms diffracted to high resolution, but the form 1 crystals have excellent reproducibility and are being used for structure determination. DHDPSL is of potential biomedical interest owing to its association with primary hyperoxaluria type III. Elucidation of its crystal structure will provide a detailed description and structural insights into the active site and possible function of the enzyme. The preparation of diffraction-quality crystals represents an important step towards this goal.

X-ray diffraction data were collected at the Australian Synchrotron and we thank Dr Tom Caradoc-Davies for his help and the New Zealand Synchrotron Program for travel support. RDB gratefully acknowledges the award of a Doctoral Scholarship from the University of Auckland and we thank the Maurice Wilkins Centre for Molecular Biodiscovery for their financial support of this project.

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