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Preliminary crystallographic analysis of L-2-keto-3-deoxyarabonate dehydratase, an enzyme involved in an alternative bacterial pathway of L-arabinose metabolism

L-2-Keto-3-deoxyarabonate (L-KDA) dehydratase is a novel member of the dihydrodipicolinate synthase (DHDPS)/*N*-acetylneuraminate lyase (NAL) protein family and catalyzes the hydration of L-KDA to α -ketoglutaric semialdehyde. L-KDA dehydratase was overexpressed, purified and crystallized at 291 K using the hanging-drop vapour-diffusion method. The crystal diffracts to 2.0 Å resolution using synchrotron radiation and belongs to the trigonal space group $P3_121$ or its enantiomorph $P3_221$, with unit-cell parameters $a = b = 78.91$, $c = 207.71$ Å.

1. Introduction

Azospirillum brasilense, a nitrogen-fixing bacterium, metabolizes L-arabinose through a hypothetical pathway *via* nonphosphorylated intermediates, which differs from the well known bacterial and fungal pathways. We are in the process of characterizing this alternative L-arabinose pathway enzymatically and genetically and have recently identified a set of five metabolic genes (Watanabe, Kodaki *et al.*, 2006*a,b*; Watanabe, Shimada *et al.*, 2006; Watanabe *et al.*, 2007). L-2-Keto-3-deoxyarabonate (L-KDA) dehydratase (EC 4.2.1.43; encoded by the *AraD* gene; GenBank accession No. BAE94270) catalyzes the fourth reaction step in this pathway, converting L-KDA to α -ketoglutaric semialdehyde (Fig. 1). This enzyme consists of 309 amino-acid residues and belongs to the dihydrodipicolinate synthase (DHDPS)/*N*-acetylneuraminate lyase (NAL) protein family, which includes the archetypal DHDPS and NAL and also D-5-keto-4-deoxyglucarate dehydratase, D-2-keto-deoxygluconate aldolase, *trans*-*o*-hydroxybenzylidenepyruvate hydrolase-aldolase and *trans*-2'-carboxybenzalpyruvate hydratase-aldolase (Watanabe, Shimada *et al.*, 2006). Although these enzymes form single clusters in the phylogenetic tree, L-KDA dehydratase shows a poor relationship to all of the subclasses. Many crystallographic analyses of DHDPS/NAL proteins, including DHDPS (Blagova *et al.*, 2006; Blickling *et al.*, 1997; Mirwaldt *et al.*, 1995; Pearce *et al.*, 2006; Tam *et al.*, 2004), NAL (Barbosa *et al.*, 2000; Izard *et al.*, 1994) and D-2-keto-deoxygluconate aldolase (Theodossis *et al.*, 2004), have revealed a common ($\beta\alpha$)₈-barrel fold and a common reaction step in their reactions, namely the formation of a Schiff-base intermediate between a strictly conserved lysine residue and the C₂ carbon of a common α -keto acid moiety of the substrate. In the case of L-KDA dehydratase, the structurally equivalent lysine residue has been assigned in the amino-acid sequence (Lys171), but the enzyme reaction does not include cleavage of the C–C or C=C bond of the substrate, in contrast to the reactions catalyzed by other DHDPS/NAL enzymes; therefore, three-dimensional structure analysis should provide a novel insight

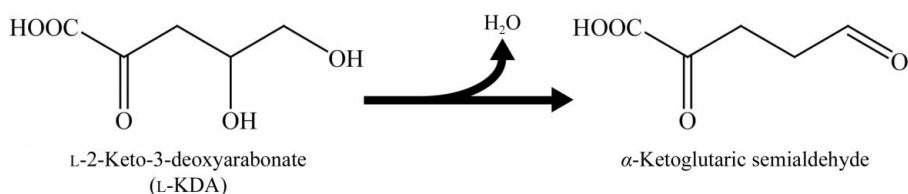
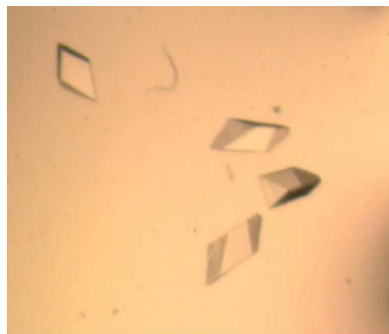


Figure 1
The enzyme reaction catalyzed by L-KDA dehydratase.

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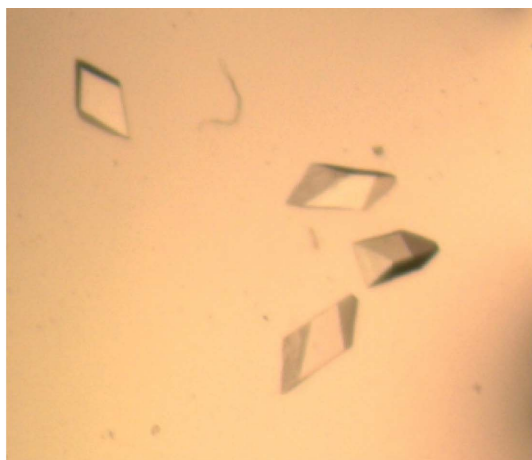
into the catalytic mechanism of this unique enzyme. Here, we describe the crystallization and preliminary X-ray crystallographic analysis of L-KDA dehydratase.

2. Materials and results

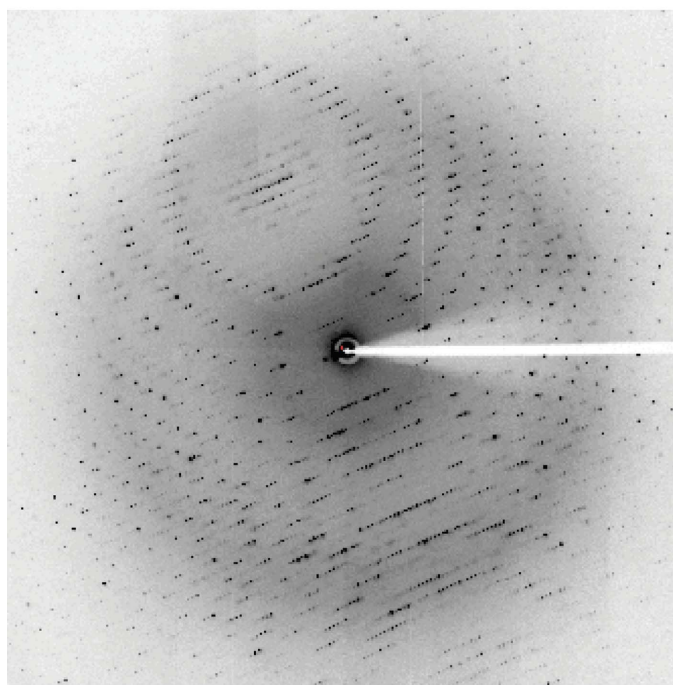
2.1. Protein expression and purification

The L-KDA dehydratase gene from *A. brasiliense* was cloned into pQE-80L (Qiagen), a vector that adds an N-terminal His₆ tag to expressed proteins. The gene with 13 additional residues (MRGSHHHHHHGST) at its N-terminus was transformed into *Escherichia coli* DH5 α cells (Watanabe, Shimada *et al.*, 2006). This recombinant *E. coli* was grown in Super Broth medium (pH 7.0; 12 g peptone, 24 g yeast extract, 5 ml glycerol, 3.81 g K₂H₂PO₄ and 12.5 g of K₂HPO₄ per litre) containing 50 mg l⁻¹ ampicillin at 310 K for 3 h. After addition of 1 mM isopropyl β -D-thiogalactopyranoside, the

culture was grown for a further 6 h to induce the expression of His₆-tagged protein. Cells were harvested, resuspended in buffer A (50 mM sodium phosphate pH 7.5 containing 300 mM NaCl, 10 mM imidazole and 1 mM sodium pyruvate) and lysed by sonication. After centrifugation, the supernatant was loaded onto a Ni-NTA Superflow column (Qiagen) equilibrated with buffer A linked to an ÄKTA Purifier system (Amersham Biosciences). The column was washed with buffer B [buffer A containing 10%(v/v) glycerol and 50 mM instead of 10 mM imidazole] and the proteins were eluted with buffer C (buffer B containing 250 mM instead of 50 mM imidazole). The solution was concentrated by ultrafiltration with Centriplus YM-30 (Millipore) and loaded onto a HiLoad 16/60 Superdex 200 column (1.6 \times 60 cm, Amersham Biosciences) equilibrated with buffer D (50 mM Tris-HCl pH 7.5 containing 1 mM sodium pyruvate). The main single-peak fractions were collected and concentrated to 80 mg ml⁻¹. These purification procedures gave an enzyme preparation that showed a single band on SDS-PAGE which corresponded to polypeptide with a subunit molecular weight of \sim 35 kDa. The native molecular weight was estimated to be \sim 85 kDa by gel filtration, suggesting a dimeric enzyme structure. The specific activity of the recombinant enzyme for L-KDA was 33.3 units per milligram of protein, which was similar to that of the native enzyme (19.8 units per milligram of protein; Watanabe, Shimada *et al.*, 2006). This enzyme sample was dialyzed against buffer D containing 50%(v/v) glycerol and stored at 238 K until use.



(a)



(b)

Figure 2

(a) Crystal of L-KDA dehydratase. The longest dimension is 0.3 mm. (b) X-ray diffraction pattern.

2.2. Crystallization

The stock solution of L-KDA dehydratase was dialyzed at 277 K against distilled water for 2 h without significant inactivation and adjusted to an appropriate concentration with distilled water by the method of Lowry *et al.* (Lowry *et al.*, 1951). When Tris-HCl buffer was used as a dialyzing solvent instead of distilled water, no further improvement was observed. All crystallization experiments were carried out by the hanging-drop vapour-diffusion method in 24-well Linbro tissue-culture plates (ICN Inc.) at 291 K. Each drop was formed by mixing equal volumes (5 μ l) of protein solution and reservoir solution. The initial trial was carried out using Crystal Screens I and II (Hampton Research), Wizard I and II (Emerald BioSystems) and JB Screen Classic (Jena Bioscience). The best crystal of L-KDA dehydratase was obtained within 2 d using a reservoir solution consisting of 50 mM Tris-HCl pH 7.5, 0.4 M NH₄H₂PO₄ and 5%(v/v) 2-propanol and a protein concentration of 4–10 mg ml⁻¹ (Fig. 2a).

2.3. X-ray analysis

A crystal of the enzyme picked up from a droplet was transferred to reservoir solution containing 30%(v/v) 2-methyl-2,4-pentanediol as a cryoprotectant. The crystal was mounted on a nylon loop (Hampton Research, Laguna Niguel, CA, USA) and placed directly in a cold nitrogen-gas stream at 100 K. X-ray diffraction images were collected at 100 K from crystals in the nitrogen-gas stream using a Quantum 4R CCD detector and synchrotron radiation of wavelength 1.0 Å at station BL-38B1 of SPring-8 (Hyogo, Japan; Fig. 2b). The distance between the crystal and detector was set at 22.5 cm and 0.8° oscillation images were recorded with 10 s exposure. Diffraction data were obtained from the crystal in the resolution range 48.8–2.0 Å and were processed using the HKL-2000 program package (Otwinowski & Minor, 1997). The space group of the crystal was determined to be *P*₃₁₂1 or its enantiomorph *P*₃₂₁ (trigonal), with unit-cell parameters *a* = *b* = 78.91, *c* = 207.71 Å. Table 1 summarizes the preliminary X-ray crystallographic properties of L-KDA dehydratase. There may be the

Table 1

Data-collection statistics for a crystal of L-KDA dehydratase.

Values in parentheses are for the highest resolution shell.

Beamline	BL-38B1, SPring-8
Wavelength (Å)	1.0
Resolution range (Å)	48.8–2.00 (2.07–2.00)
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å, °)	$a = b = 78.91$, $c = 207.71$
Measured reflections	257135 (11786)
Unique reflections	48899 (3683)
Multiplicity	5.3 (3.2)
Completeness (%)	94.6 (71.7)
$R_{\text{merge}}^{\dagger}$ (%)	5.6 (12.1)
$I/\sigma(I)$	29.2 (14.7)

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

possibility of collecting higher resolution data; however, the crystal-to-detector distance was limited in order to separate the spots during these measurements. The V_M value (crystal volume per unit protein molecular weight; Matthews, 1968) was calculated to be $2.7 \text{ \AA}^3 \text{ Da}^{-1}$ assuming the presence of two molecules of the enzyme in the asymmetric unit; the solvent content was 53.5%. The V_M value and solvent content lie within the ranges usually found for protein crystals.

To date, several crystal structures of DHDPS/NAL family enzymes have been reported, as described in §1. Of these, DHDPS from *Thermotoga maritima* (PDB code 1o5k) shows the highest sequence similarity to L-KDA dehydratase (29% identity). We attempted molecular replacement using CNS v.1.1 (Brünger *et al.*, 1998) with various parts of the *T. maritima* DHDPS structure as the search probe, but were unsuccessful. This is probably because sequence homology between L-KDA dehydratase and other DHDPS/NAL proteins is limited within the N-terminal half of the protein. As an alternative, the preparation of heavy-atom derivatives, including iodine, platinum and mercury derivatives, for use in the multiple-wavelength anomalous dispersion method is currently in progress.

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