Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

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

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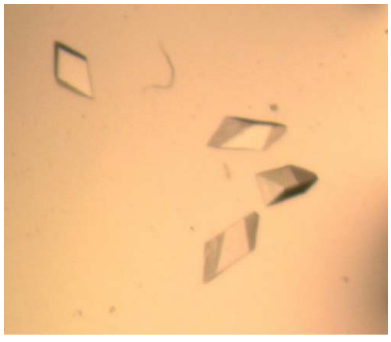
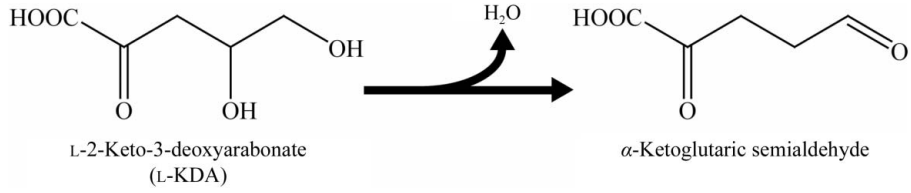
Received 20 February 2007
Accepted 28 March 2007

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 C_2 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





L-2-Keto-3-deoxyarabonate (L-KDA)

α-Ketoglutaric semialdehyde

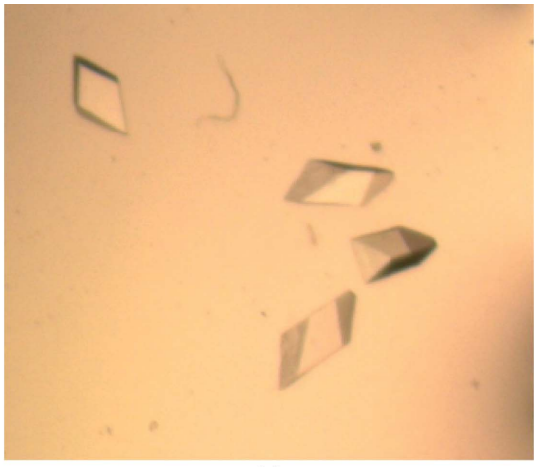
Figure 1
The enzyme reaction catalyzed by L-KDA dehydratase.

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

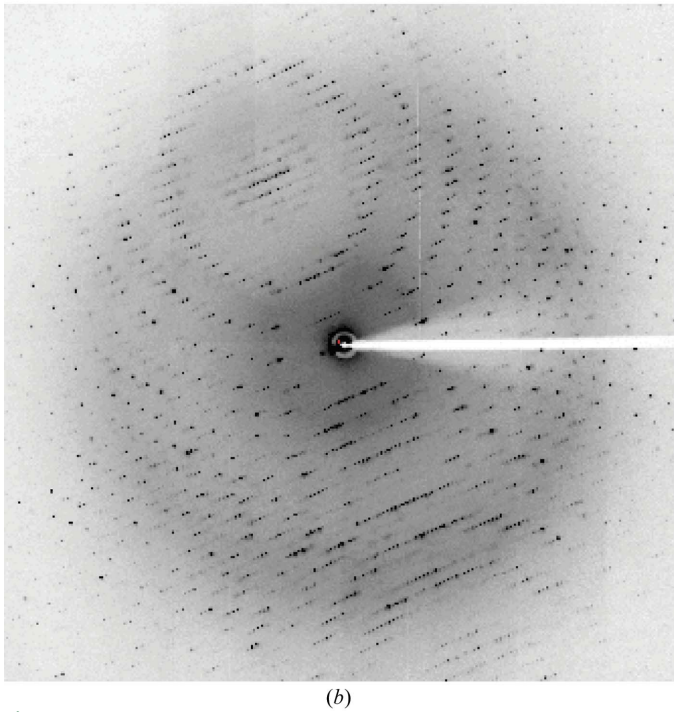
2. Materials and results

2.1. Protein expression and purification

The L-KDA dehydratase gene from *A. brasilense* was cloned into pQE-80L (Qiagen), a vector that adds an N-terminal His_6 tag to expressed proteins. The gene with 13 additional residues (MRGSHHHHHHGST) at its N-terminus was transformed into *Escherichia coli* DH5α 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 $K_2H_2PO_4$ and 12.5 g of K_2HPO_4 per litre) containing 50 mg l^{-1} ampicillin at 310 K for 3 h. After addition of 1 m*M* isopropyl β-D-thiogalactopyranoside, the culture was grown for a further 6 h to induce the expression of His_6-tagged protein. Cells were harvested, resuspended in buffer *A* (50 m*M* sodium phosphate pH 7.5 containing 300 m*M* NaCl, 10 m*M* imidazole and 1 m*M* sodium pyruvate) and lysed by sonication. After centrifugation, the supernatant was loaded onto an 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 m*M* instead of 10 m*M* imidazole] and the proteins were eluted with buffer *C* (buffer *B* containing 250 m*M* instead of 50 m*M* imidazole). The solution was concentrated by ultrafiltration with Centriplus YM-30 (Millipore) and loaded onto a HiLoad 16/60 Superdex 200 column (1.6 × 60 cm, Amersham Biosciences) equilibrated with buffer *D* (50 m*M* Tris–HCl pH 7.5 containing 1 m*M* 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 ∼35 kDa. The native molecular weight was estimated to be ∼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 µl) 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 m*M* Tris–HCl pH 7.5, 0.4 *M* $NH_4H_2PO_4$ and 5%(*v*/*v*) 2-propanol and a protein concentration of 4–10 mg ml^{-1} (Fig. 2*a*).

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. 2*b*). 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 $P3_121$ or its enantiomorph $P3_221$ (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)
$R_{\rm merge}$† (%)	5.6 (12.1)
$I/\sigma(I)$	29.2 (14.7)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I_i\rangle|/\sum_{hkl}\sum_i I_i(hkl)$.

possibility of collecting higher resolution data; however, the crystal-to-detector distance was limited in order to separate the spots during these measurements. The $V_{\rm M}$ value (crystal volume per unit protein molecular weight; Matthews, 1968) was calculated to be 2.7 Å^3 Da^{-1} assuming the presence of two molecules of the enzyme in the asymmetric unit; the solvent content was 53.5%. The $V_{\rm M}$ 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.

We thank Drs H. Sakai and K. Hasegawa of the Japan Synchrotron Radiation Research Institute (JASRI) for their kind help during data collection. X-ray data were collected at BL38B1 of SPring-8 with the approval of the organizing committee of SPring-8 (proposal No. 2006B1652). This work was supported by a Grant-in-Aid for Young Scientists (B) (No. 18760592 to SW), the Center of Excellence (COE) program for the 'Establishment of COE on Sustainable Energy System', a Grant-in-Aid for Scientific Research, a Grant-in-Aid for the National Project on Protein Structural and Functional Analyses (to BM) and Grants for Regional Science and Technology Promotion 'Kyoto Nanotechnology Cluster' project from the Ministry of Education, Science, Sports and Culture, Japan. This work was also supported by CREST and 'Research for Promoting Technological Seeds' of the Japan Science and Technology Corporation.

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