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Received 4 July 2013

Accepted 26 August 2013

Crystallization and preliminary X-ray diffraction studies of D-threo-3-hydroxyaspartate dehydratase isolated from *Delftia* sp. HT23

D-threo-3-Hydroxyaspartate dehydratase (D-THA DH) isolated from the soil bacterium *Delftia* sp. HT23 is a novel enzyme consisting of 380 amino-acid residues which catalyzes the conversion of D-threo-3-hydroxyaspartate to oxaloacetate and ammonia. D-THA DH also catalyzes the dehydration of L-threo-3-hydroxyaspartate, L-erythro-3-hydroxyaspartate and D-serine. The amino-acid sequence of D-THA DH shows significant similarity to that of two eukaryotic D-serine dehydratases derived from *Saccharomyces cerevisiae* and chicken kidney. D-THA DH is classified into the fold-type III group of pyridoxal enzymes and is the first example of a fold-type III dehydratase derived from a prokaryote. Overexpression of recombinant D-THA DH was carried out using a *Rhodococcus erythropolis* expression system and the obtained protein was subsequently purified and crystallized. The crystals of D-THA DH belonged to space group *I*4₁22, with unit-cell parameters $a = b = 157.3$, $c = 157.9$ Å. Single-wavelength anomalous diffraction data were collected to a resolution of 2.0 Å using synchrotron radiation at the wavelength of the Br *K* absorption edge.

1. Introduction

3-Hydroxyaspartate and its derivatives have biological activity as competitive blockers of excitatory glutamate/aspartate transporters of the mammalian nervous system (Balcar *et al.*, 1977; Shigeri *et al.*, 2001). Thus, pharmacological aspects of 3-hydroxyaspartate have attracted the attention of biochemists. 3-Hydroxyaspartate exists as four stereoisomers because it has two chiral centres, *i.e.* D-threo-3-hydroxyaspartate (2*R*,3*R*; D-THA), L-threo-3-hydroxyaspartate (2*S*,3*S*; L-THA), D-erythro-3-hydroxyaspartate (2*R*,3*S*; D-EHA) and L-erythro-3-hydroxyaspartate (2*S*,3*R*; L-EHA), which are difficult to synthesize individually (Kaneko & Katsura, 1963). For this reason, we considered that an enzyme that degrades 3-hydroxyaspartate might be useful for the enzymatic optical resolution of DL-racemic 3-hydroxyaspartate to produce optically pure 3-hydroxyaspartate isomers. However, there have been very few studies on enzymes that act on 3-hydroxyaspartate, although two bacterial enzymes, erythro-3-hydroxyaspartate aldolase (EC 4.1.3.14; Gibbs & Morris, 1964; Kornberg & Morris, 1965) and erythro-3-hydroxyaspartate dehydratase (EC 4.3.1.20; Gibbs & Morris, 1965), were identified many years ago.

In order to obtain amino-acid sequence information for 3-hydroxyaspartate dehydratase, we screened soil microorganisms that can utilize 3-hydroxyaspartate as a sole carbon source and recently found two novel enzymes in a newly isolated bacterium. One enzyme is an L-threo-3-hydroxyaspartate dehydratase (L-THA DH; EC 4.3.1.16; Wada *et al.*, 1999; Murakami *et al.*, 2009) isolated from *Pseudomonas* sp. T62, which only exhibits dehydratase activity towards L-THA. The other enzyme is a D-threo-3-hydroxyaspartate dehydratase (D-THA DH; EC 4.3.1.27; Maeda *et al.*, 2010) isolated from *Delftia* sp. HT23, which exhibits dehydratase activity towards D-THA, L-THA, L-EHA and D-serine.

Interestingly, although L-THA DH and D-THA DH catalyze the same reaction from threo-3-hydroxyaspartate to oxaloacetate and

Figure 1

Scheme of the reactions catalyzed by D-threo-3-hydroxyaspartate dehydratase (D-THA DH).

ammonia, the primary structures of these two enzymes are completely different. Biochemical analyses suggested that both enzymes contain pyridoxal 5'-phosphate (PLP) as a cofactor. Based on their folding patterns, PLP-dependent enzymes are classified into five groups, designated fold-types I–V (Schneider *et al.*, 2000). A sequence-similarity search revealed that L-THA DH belongs to the fold-type II group, which contains most bacterial dehydratases. On the other hand, despite catalyzing the same dehydratase reactions, D-THA DH belongs to the fold-type III group, which contains most bacterial alanine racemases (Eliot & Kirsch, 2004). However, any alanine, serine or aspartate racemase activity of D-THA DH was below the detection limit. The amino-acid sequence of D-THA DH shows 26–36% similarity to two eukaryotic D-serine dehydratases derived from *Saccharomyces cerevisiae* (scDSD; Ito *et al.*, 2008) and chicken kidney (chDSD; Tanaka *et al.*, 2008), which are also classified into the fold-type III group. To the best of our knowledge, D-THA DH is the first example of a fold-type III dehydratase derived from a prokaryote.

Recently, the crystal structure of chDSD has been reported. This is the first report of the three-dimensional structure of a fold-type III enzyme that catalyzes a dehydratase reaction (Tanaka *et al.*, 2011). The primary structural similarity between D-THA DH and chDSD suggests that their active-site structures are similar. Nonetheless, D-THA DH shows unique stereospecificity towards 3-hydroxyaspartate isomers. The main substrate of chDSD is D-serine or D-threonine, which is the D-form of β -hydroxy amino acids. In contrast, the main substrate of D-THA DH is D-THA or L-EHA, *i.e.* not only the D-form but also the L-form of β -hydroxy amino acids act as a substrate for D-THA DH (Fig. 1). In other words, D-THA DH exhibits stringent stereoselectivity at C $^{\beta}$ but relaxed selectivity at C $^{\alpha}$, because D-THA and L-EHA have the same configuration around C $^{\beta}$. In the structure of chDSD, Zn $^{2+}$ is located at the active site and is involved in the catalytic reaction (Tanaka *et al.*, 2011). D-THA DH pretreated with ethylenediaminetetraacetic acid (EDTA) also requires divalent cations for its activation, but the activation pattern differs between the two enzymes. Only Zn $^{2+}$ and Mn $^{2+}$ act as activators of chDSD, whereas Co $^{2+}$, Ni $^{2+}$, Ca $^{2+}$ and Fe $^{2+}$ ions are also activators of D-THA DH in addition to Zn $^{2+}$ and Mn $^{2+}$ ions. In particular, D-THA DH is highly activated by Mn $^{2+}$, Co $^{2+}$ and Ni $^{2+}$ ions, although the natural metal ion bound to D-THA DH has not yet been determined. To resolve the unique substrate specificity and to determine the properties contributing to the difference in their metal requirements, crystallographic studies of D-THA DH are required. Here, we report the improvement of the expression level, crystallization and preliminary X-ray diffraction studies of recombinant D-THA DH.

2. Materials and methods

2.1. Overexpression and purification of recombinant D-THA DH

The identification and cloning of the D-THA DH gene have been reported previously (Maeda *et al.*, 2010). Briefly, the gene encoding D-THA DH (GenBank accession code AB433986) was cloned from genomic DNA of *Delftia* sp. HT23 and inserted into the pQE30 expression vector using *Bam*HI and *Hind*III restriction enzymes. Recombinant D-THA DH was obtained by expression in *Escherichia coli* JM109 and used for enzymatic studies. For crystallization, recombinant D-THA DH was produced using *Rhodococcus erythropolis* as a host cell (Mitani *et al.*, 2005). The gene encoding D-THA DH was amplified using polymerase chain reaction (PCR) with a pQE30dthadh plasmid as template DNA, PrimeSTAR HS DNA polymerase (Takara Bio) and synthetic primers, *i.e.* forward (5'-AGTCTCATGAGTATGCAAGACACACTTCTGAC-3') and reverse (5'-ATATCTCGAGTCACCAGCCATGGAGCCGCT-3'). The bold bases indicate the *Bsp*HI and *Xho*I restriction sites, respectively. The PCR conditions were as follows: 30 cycles of amplification at 371 K for 10 s, at 333 K for 5 s and at 345 K for 1.5 min, followed by an elongation step at 345 K for 7 min. PCR fragments were digested with *Bsp*HI and *Xho*I and cloned into the *Nco*I and *Xho*I sites of the pTip-QC2 expression vector for *Rhodococcus* (Nakashima & Tamura, 2004a,b). *Bsp*HI, which is compatible with *Nco*I, was used for the digestion of the PCR fragments because the DNA sequence of the gene encoding D-THA DH contains an *Nco*I site. The resulting plasmid encoded full-length D-THA DH fused with a Met-Gly-His $_6$ -Ala-Met-Ser sequence at the N-terminus. The plasmid was transformed into *R. erythropolis* L88 cells by electroporation.

The transformed cells were grown at 303 K in 50 ml Luria–Bertani (LB) medium containing 17 $\mu\text{g ml}^{-1}$ chloramphenicol. The pre-culture was then inoculated in 500 ml LB medium supplemented with 0.5 $\mu\text{g ml}^{-1}$ thioestrepton for 24 h at 303 K in order to induce D-THA DH expression. The cells were harvested (10 g wet weight) and resuspended in buffer A (10 mM Tris–HCl pH 8.0, 0.01 mM PLP, 0.1 mM dithiothreitol). The cells were disrupted three times using a French pressure cell (Ohtake Works) at 110 MPa after the addition of 2 mg ml $^{-1}$ lysozyme for 30 min. After centrifugation, the resulting supernatant was applied onto a His-Trap HP column (GE Healthcare) pre-equilibrated with buffer A which was supplemented with 20 mM imidazole and 500 mM NaCl. The enzyme was eluted with a linear gradient of 20–500 mM imidazole in buffer A supplemented with 500 mM NaCl. The fractions containing D-THA DH were collected, dialyzed against buffer A and subsequently concentrated to

Figure 2

Typical crystals of *D-threo*-3-hydroxyaspartate dehydratase (D-THA DH). The crystal used for the current study was obtained with 0.1 M Tris pH 8.5, 0.2 M MgCl₂, 13% PEG 3350.

15 mg ml⁻¹ using a centrifugal filtration device (10 000 Da molecular-weight cutoff, Millipore). The protein concentration was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard. The final protein solution consisted of 10 mM Tris-HCl pH 8.0, 0.01 mM PLP, 0.1 mM dithiothreitol and 15 mg ml⁻¹ of the enzyme in a total volume of 0.7 ml.

2.2. Crystallization

Initial crystallization screening was performed in a 96-well crystallization plate by employing the sitting-drop vapour-diffusion technique at 293 K using commercially available sparse-matrix screen kits from Hampton Research and Emerald BioSystems. Each sitting drop was prepared by mixing 0.5 µl sample solution and 0.5 µl reservoir solution and was equilibrated against 100 µl reservoir solution. The most promising condition was subsequently optimized in a 24-well VDX plate (Hampton Research) by using the hanging-drop vapour-diffusion method at 293 K, with variation of the buffer pH and salt and precipitant concentrations. Each hanging drop was prepared by mixing 1.5 µl sample solution and an equal volume of the reservoir solution and was equilibrated against 500 µl reservoir solution.

2.3. X-ray diffraction studies

For X-ray diffraction under cryogenic conditions, crystals were soaked stepwise in cryoprotectant solution composed of crystallization reservoir and 20% glycerol. Subsequently, crystals were further transferred stepwise into cryoprotectant solution supplemented first with 0.5 M NaBr and then with 1 M NaBr, with an overall soaking time of approximately 30 s (Dauter *et al.*, 2000). Prior to X-ray diffraction analysis, the crystal was flash-cooled in a nitrogen-gas stream at 100 K. Single-wavelength anomalous diffraction (SAD) data were collected on beamline NW-12A at the Photon Factory (PF), Tsukuba, Japan using an ADSC Quantum 210 charge-coupled device detector. The wavelength was set at 0.91944 Å considering the results from a fluorescence scan around the Br K absorption edge. Each oscillation frame was taken with a rotation angle of 0.5° and an exposure time of 1.0 s. The total rotation range for the SAD data collection was 360°. The diffraction images were processed with *iMOSFLM/SCALA* (Battye *et al.*, 2011; Winn *et al.*, 2011). SAD phasing was performed with *SHELXC/D/E* (Sheldrick, 2008).

Figure 3

An X-ray diffraction pattern obtained on the NW-12A beamline at PF, Japan from a single crystal of *D-threo*-3-hydroxyaspartate dehydratase (D-THA DH).

2.4. Enzyme assay

3-Hydroxyaspartate dehydratase activity was assayed spectrophotometrically by measuring the change in absorbance of NADH at 340 nm using a DU800 spectrophotometer (Beckman Coulter). The assay was performed using a coupling system with NADH-dependent malate dehydrogenase (MDH) as described previously (Wada *et al.*, 1999). The standard assay mixture consisted of 100 mM Tris-HCl buffer pH 8.0, 0.01 mM PLP, 10 mM D-THA or L-EHA as a substrate, 0.32 mM NADH, 10 units of MDH and an appropriate amount of the enzyme in a total volume of 0.5 ml. The reactions were carried out at 303 K with addition of the substrate. Reaction mixtures without the substrate served as controls. One unit of the enzyme represented 1 µmol of NADH utilized per minute at 303 K on the basis of an absorption coefficient of 6.22 mM⁻¹ cm⁻¹ for NADH at 340 nm.

3. Results and discussion

The expression level of D-THA DH in *E. coli* was low, probably owing to the high GC content (71.9%) of the gene encoding D-THA DH. To address this problem, overexpression of recombinant D-THA DH was carried out using *R. erythropolis*, a Gram-positive bacterium with a high GC content (Actinobacteria), as a heterologous expression host. As shown by SDS-PAGE, we observed high expression of D-THA DH fused with additional Met-Gly-His₆-Ala-Met-Ser residues at the N-terminus in *R. erythropolis* (Supplementary Fig. S1[†]). As seen in lane 1, the molecular weight of recombinant D-THA DH was found to be 41.7 kDa, and that of the lysozyme added before the cell-disruption step was found to be 14.4 kDa. The specific activity of the cell-free extract of *R. erythropolis* expressing the D-THA DH gene was ~2.5 U per milligram of protein, which is about 20-fold higher

[†] Supplementary material has been deposited in the IUCr electronic archive (Reference: NO5025).

Table 1

Crystallographic parameters and SAD phasing statistics.

Values in parentheses are for the outermost resolution shell.

Beamline	NW-12A, PF, Japan
Temperature (K)	100
Wavelength (Å)	0.91944
Resolution range (Å)	50–2.0 (2.11–2.00)
Unit-cell parameters (Å, °)	$a = b = 157.3$, $c = 157.9$, $\alpha = \beta = \gamma = 90$
Space group	$I4_122$
Average mosaicity (°)	0.43
No. of reflections measured	1967835
No. of unique reflections measured	66655
$R_{\text{merge}}^{\dagger}$	0.149 (0.754)
$R_{\text{meas}}^{\ddagger}$ (all $I+$ and $I-$)	0.157 (0.776)
$R_{\text{p.i.m.}}^{\S}$ (all $I+$ and $I-$)	0.029 (0.142)
Completeness (%)	100.0 (100.0)
Anomalous completeness (%)	100.0 (100.0)
Multiplicity	29.5 (29.8)
Anomalous multiplicity	15.4 (15.3)
Average $I/\sigma(I)$	24.4 (6.9)
Phasing information from <i>SHELXC/D/E</i>	
No. of Br sites used for phasing	42
Best CC _{all/CC_{weak}}	39.3/20.3
FOM	0.55
Map contrast	0.51
Map connectivity	0.90

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\ddagger R_{\text{meas}} = \frac{\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\S R_{\text{p.i.m.}} = \frac{\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , $\langle I(hkl) \rangle$ is the mean intensity of reflection hkl and $N(hkl)$ is the number of observations of reflection hkl .

than that of the cell-free extract of *E. coli* expressing the D-THA DH gene. D-THA DH was purified by Ni-affinity chromatography, resulting in a single band on SDS-PAGE (Supplementary Fig. S1). The calculated molecular weight of the protein with the additional residues is determined to be 41 629 Da, which is consistent with the molecular weight determined by SDS-PAGE. The theoretical isoelectric point of the recombinant protein was calculated to be 6.32. This procedure yielded approximately 10 mg purified D-THA DH per 500 ml of culture.

Diffraction-quality crystals were obtained with a reservoir solution consisting of 0.1 M Tris pH 8.5, 0.2 M MgCl₂, 10–14% PEG 3350. Yellow-coloured crystals appeared in the heavy precipitate and grew to approximate dimensions of 0.2 × 0.2 × 0.2 mm within 1 week (Fig. 2). The Br-SAD data set was collected to a resolution of 2.0 Å using synchrotron radiation at a wavelength of 0.91944 Å (Fig. 3). Preliminary data processing showed that the unit-cell parameters were close to $a = b = c = 158$ Å, $\alpha = \beta = \gamma = 90^\circ$. Thus, the data were integrated and scaled assuming that the crystal belonged to a cubic space group. However, abnormally high R_{merge} values were calculated for any cubic system, suggesting that the crystal did not have three-fold symmetry around the cube diagonal. Therefore, we reduced the symmetry and found that the crystal belonged to an I -centred

tetragonal space group with a reliable R_{merge} value. The reflection condition for (00 l) indicated that the true space group of the crystal was $I4_122$ with unit-cell parameters $a = b = 157.3$, $c = 157.9$ Å. Assuming the presence of two D-THA DH monomers in the asymmetric unit, the calculated value of the crystal volume per protein weight (V_M value; Matthews, 1968) was 2.8 Å³ Da⁻¹. This value corresponds to a solvent content of 56.4%. SAD phasing was performed using *SHELXC/D/E* (Sheldrick, 2008). A total of 42 Br sites were found using the data with a resolution range of 20–2.8 Å. SAD phasing and density modification using *SHELXE* resulted in an interpretable electron-density map. X-ray diffraction data collection and phasing statistics are summarized in Table 1. Model building and refinement are currently under way.

The authors would like to thank the beamline scientists at the Photon Factory, Tsukuba, Japan for their kind assistance in the X-ray diffraction experiment. This work was financially supported in part by a grant-in-aid from the Institute for Fermentation, Osaka (IFO).

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