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Expression, purification, crystallization and preliminary X-ray analysis of 4-hydroxy-3-methyl-2-keto-pentanoate aldolase (asHPAL) from *Arthrobacter simplex* strain AKU 626

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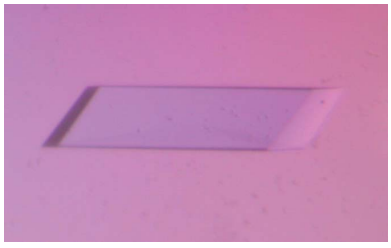
4-Hydroxy-3-methyl-2-keto-pentanoate aldolase (asHPAL), an enzyme used in the synthesis of (2*S*,3*R*,4*S*)-4-hydroxyisoleucine, was crystallized in the absence and the presence of 2-ketobutyrate as one of its substrates by the sitting-drop vapour-diffusion method using PEG 400 as a precipitant. Crystals of asHPAL grown without and with 2-ketobutyrate diffracted to 1.60 and 1.55 Å resolution and belonged to space group *C*2, with unit-cell parameters $a = 116.8$, $b = 88.2$, $c = 85.3$ Å, $\beta = 122.3^\circ$ and $a = 116.2$, $b = 88.1$, $c = 85.0$ Å, $\beta = 122.3^\circ$, respectively.

1. Introduction

(2*S*,3*R*,4*S*)-4-Hydroxyisoleucine (4HIL) is a chiral amino acid extracted from the seeds of fenugreek (*Trigonella foenum-graecum*; Fowden *et al.*, 1973). 4HIL can enhance the production of insulin from pancreatic β-cells. This insulinotropic activity is exhibited by the major (2*S*,3*R*,4*S*) stereoisomer in the micromolar range but not by the minor (2*R*,3*R*,4*S*) stereoisomer (Gieren *et al.*, 1974; Alcock *et al.*, 1989; Jacob *et al.*, 1997; Broca *et al.*, 2000). Unlike other available insulinotropic drugs, 4HIL only potentiates insulin secretion at supranormal concentrations of glucose and is ineffective at low or basal glucose concentrations (Jackson & Bressler, 1981; Jennings *et al.*, 1989; Sauvaire *et al.*, 1998). This unique property of 4HIL is favourable for the avoidance of undesirable side effects such as hypoglycaemia in the treatment of type II diabetes.

The shortest synthetic pathway to 4HIL was described by Smirnov *et al.* (2007). Firstly, 2-ketobutyrate and acetaldehyde are converted to (3*S*,4*S*)-4-hydroxy-3-methyl-2-keto-pentanoate (HMKP) by 4-hydroxy-3-methyl-2-keto-pentanoate aldolase (asHPAL). HMKP is then converted to 4HIL by branched-chain amino-acid amino-transferase (BCAT), which strictly controls the *S*-configuration of the C2 atom in 4HIL (Mamer & Reimer, 1992; Hall *et al.*, 1993). The first reaction can be catalyzed by other aldolases such as YfaU (Rea *et al.*, 2008) and YhaF (Izard & Blackwell, 2000). However, asHPAL is the only enzyme that can catalyze the synthesis of (3*S*,4*S*)-HMKP preferentially at low pH (Smirnov *et al.*, 2007). In order to reveal the structural basis of the stereoselective reaction catalyzed by asHPAL (259 amino-acid residues, 27 kDa), we have prepared and crystallized recombinant asHPAL in the absence and presence of 2-ketobutyrate as one of its substrates. Here, we report the expression, purification, crystallization and preliminary X-ray diffraction analysis of asHPAL.



2. Materials and methods

2.1. Overexpression, purification and crystallization

The gene encoding asHPAL (GenBank EF117323) was amplified by PCR from the genomic DNA of *Arthrobacter simplex* strain AKU 626 and cloned into the *Bam*HI/*Hin*dIII site of the pQE80 plasmid (Qiagen). asHPAL was overexpressed in *Escherichia coli* Rosetta (DE3) cells (Novagen) harbouring the constructed plasmid. The *E. coli* cells were cultivated in Luria–Bertani (LB) medium containing 30 µg ml^{-1} ampicillin at 310 K. Expression of asHPAL was

induced by the addition of 0.5 m*M* (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.6. The cells were then cultivated at 293 K for 16 h and harvested by centrifugation at 5000*g* at 277 K for 20 min.

The harvested cells were suspended in buffer *A* (40 m*M* Tris–HCl pH 7.5, 400 m*M* NaCl, 5 m*M* imidazole) and disrupted by sonication on ice. After centrifugation at 40 000*g* and 277 K for 30 min, the supernatant was loaded onto an Ni-Sepharose 6 Fast Flow (GE Healthcare) column (3 ml gel bed) at 298 K. After washing with buffer *B* (40 m*M* Tris–HCl pH 7.5, 400 m*M* NaCl, 50 m*M* imidazole), the asHPAL protein was eluted with buffer *C* (40 m*M* Tris–HCl pH 7.5, 400 m*M* NaCl, 200 m*M* imidazole) at 298 K. The eluted protein was dialyzed against 40 m*M* Tris–HCl pH 7.5, 1 m*M* dithiothreitol (DTT) at 277 K for 12 h and added to a 6 ml Resource Q (GE Healthcare) column pre-equilibrated with 40 m*M* Tris–HCl pH 7.5 at 277 K. The protein was eluted with a 0–1 *M* NaCl gradient in 40 m*M* Tris–HCl pH 7.5 at 277 K. The purified asHPAL was dialyzed against 20 m*M* Tris–HCl pH 7.5 at 277 K and concentrated to 10 mg ml^{-1} using a Vivaspin 20 concentrator (Sartorius) at 277 K.

The purified asHPAL (10 mg ml^{-1}, corresponding to 0.35 m*M*) was used for crystallization experiments alone and supplemented with 200 m*M* sodium 2-ketobutyrate. Initial crystallization trials were performed by the sitting-drop vapour-diffusion method using the sparse-matrix screening kits Crystal Screen HT, Index HT (Hampton Research) and Wizard I and II (Emerald BioSystems). In all of the crystallization experiments each drop was prepared by mixing equal volumes (0.7 µl) of protein solution and reservoir solution and was equilibrated against 70 µl reservoir solution at 293 K.

2.2. Data collection and processing

Each asHPAL crystal obtained was picked up in a nylon loop (Hampton Research), transferred into a cryosolution prepared by mixing 76%(*v*/*v*) reservoir solution and 24%(*v*/*v*) glycerol and flash-cooled in a nitrogen cryostream (95 K). An X-ray diffraction data set (600 images) was collected from an asHPAL crystal obtained without 2-ketobutyrate using an ADSC Quantum 210 detector on beamline BL-5A at the Photon Factory (Ibaraki, Japan) with a wavelength of 1.0000 Å, an oscillation angle of 0.3° and an exposure time of 0.5 s, while that (360 images) from an asHPAL crystal obtained with 2-ketobutyrate was collected using a MAR Mosaic 225 detector on beamline BL-26B2 at SPring-8 (Hyogo, Japan) with a wavelength of 1.0000 Å, an oscillation angle of 0.5° and an exposure time of 3.7 s. The small oscillation angles (0.3° and 0.5°) were used to avoid partial overlaps of the spots. The diffraction data were indexed, integrated and scaled with *XDS* (Kabsch, 2010). Data-collection statistics are given in Table 1.

3. Results and discussion

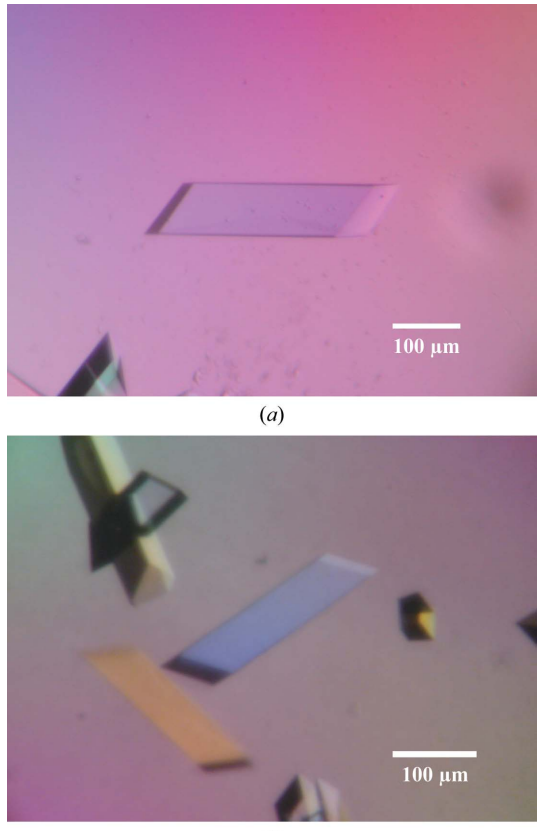
Recombinant asHPAL was expressed in *E. coli* and was purified by immobilized Ni^{2+}-affinity and anion-exchange chromatography. Crystallization experiments on asHPAL were performed by the sitting-drop vapour-diffusion method in the absence and the presence of one of its substrates, 2-ketobutyrate. Crystals of asHPAL both without and with 2-ketobutyrate were first obtained at 293 K using a reservoir solution consisting of 100 m*M* Na HEPES pH 7.5, 30%(*v*/*v*) PEG 400, 200 m*M* $MgCl_2$ (reagent No. 23 of Crystal Screen HT). The crystallization conditions were then optimized at 293 K by a two-dimensional grid screen (pH *versus* PEG 400 concentration). The best crystals of asHPAL without and with 2-ketobutyrate were obtained at 293 K using the same reservoir composition: 100 m*M* Na HEPES pH 7.7, 30%(*v*/*v*) PEG 400, 200 m*M* $MgCl_2$ (Fig. 1). The crystals diffracted X-rays to 1.60 and 1.55 Å resolution, respectively (Fig. 2). The asHPAL crystals obtained without and with 2-ketobutyrate both belonged to space group *C*2, with unit-cell parameters *a* = 116.8, *b* = 88.2, *c* = 85.3 Å, β = 122.3° and *a* = 116.2, *b* = 88.1, *c* = 85.0 Å, β = 122.3°, respectively. Their Matthews coefficients (V_M = 2.17 and 2.15 Å^3 Da^{-1}, respectively; Matthews, 1968) indicated that the crystals were most likely to contain three asHPAL molecules per asymmetric unit, with solvent contents of 43.4 and 42.9%, respectively. Structure determination of asHPAL in the absence and presence of 2-ketobutyrate is currently under way by the molecular-replacement method using the atomic coordinates of 4-hydroxy-2-oxo-heptane-

Table 1
Summary of data-collection statistics of asHPAL crystals obtained in the absence and the presence of 2-ketobutyrate.

Values in parentheses are for the highest resolution shell.

	Without 2-ketobutyrate	With 2-ketobutyrate
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	*a* = 116.8, *b* = 88.2, *c* = 85.3, β = 122.3	*a* = 116.2, *b* = 88.1, *c* = 85.0, β = 122.3
Resolution (Å)	20.0–1.60 (1.64–1.60)	20.0–1.55 (1.59–1.55)
No. of observations	353606 (24291)	383993 (22211)
No. of unique reflections	95854 (6982)	104056 (7303)
Completeness (%)	99.4 (98.0)	99.1 (95.1)
Multiplicity	3.7 (3.5)	3.7 (3.0)
R_{merge}† (%)	4.2 (22.9)	2.8 (12.6)
$\langle I/\sigma(I)\rangle$	19.7 (5.0)	29.7 (8.3)
Mosaicity (°)	0.245	0.180

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the average of individual measurements $I_i(hkl)$.



(*a*)

(*b*)

Figure 1
Crystals of asHPAL obtained in the absence (*a*) and the presence (*b*) of 2-ketobutyrate.

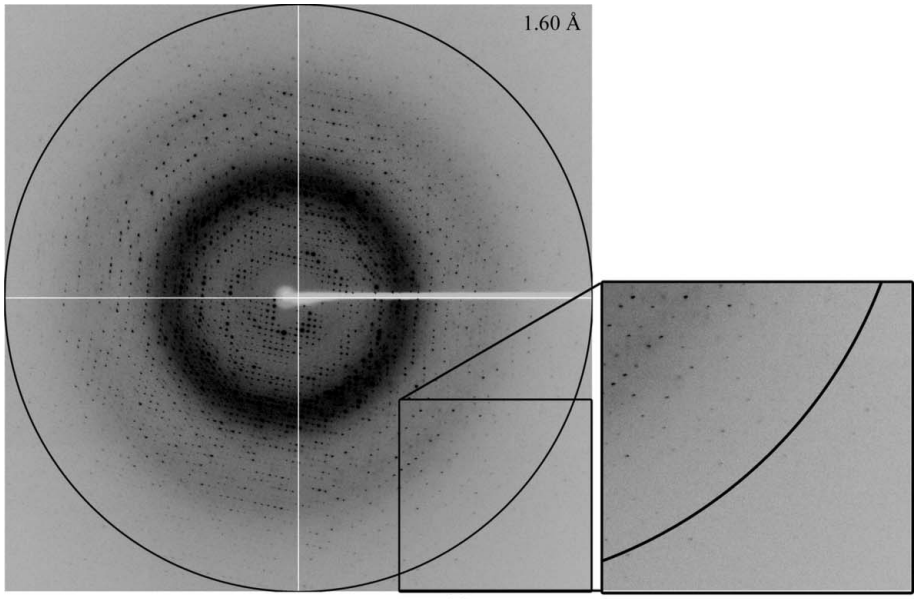


Figure 2
X-ray diffraction images of asHPAL crystals obtained in the absence (*a*) and the presence (*b*) of 2-ketobutyrate. The edges of the diffraction images correspond to 1.60 Å (*a*) and 1.58 Å (*b*) resolution. X-ray diffraction data to 1.60 Å (*a*) and 1.55 Å (*b*) resolution were used for indexing, integration and scaling.

1,7-dioate aldolase (HpcH) from *E. coli* (45% sequence identity to asHPAL; PDB entry 2v5j; Rea *et al.*, 2007) as a search model. HpcH, a member of the divalent metal ion-dependent class II aldolases, catalyzes the conversion of 4-hydroxy-2-oxo-heptane-1,7-dioate to pyruvate and succinic semialdehyde in the 4-hydroxyphenylacetic acid degradation pathway, which is one of the most well characterized aromatic catabolic pathways, in *E. coli*.

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