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Overproduction and preliminary crystallographic study of a human kynurenine aminotransferase II homologue from *Pyrococcus horikoshii* OT3

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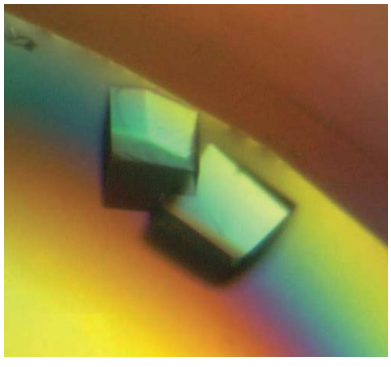

The *Pyrococcus horikoshii* OT3 genome contains a gene encoding a human kynurenine aminotransferase II (KAT II) homologue, which consists of 428 amino-acid residues and shows an amino-acid sequence identity of 30% to human KAT II. This gene was overexpressed in *Escherichia coli* and the recombinant protein (Ph-KAT II) was purified. Gel-filtration chromatography showed that Ph-KAT II exists as a homodimer. Ph-KAT II exhibited enzymatic activity that catalyzes the transamination of L-kynurenine to produce kynurenic acid. Crystals of Ph-KAT II were grown using the sitting-drop vapour-diffusion method and native X-ray diffraction data were collected to 2.2 Å resolution using synchrotron radiation from station BL44XU at SPring-8. The crystals belong to the centred orthorhombic space group $C222_1$, with unit-cell parameters a = 71.75, b = 86.84, c = 137.30 Å. Assuming one molecule per asymmetric unit, the V_M value was 2.19 Å^3 Da^{-1} and the solvent content was 43.3%.

1. Introduction

In mammals, the kynurenine pathway is the major route for tryptophan catabolism. Abnormality in this pathway can cause several pathological consequences such as amyotrophic lateral sclerosis (Ilzecka *et al.*, 2003), neonatal asphyxia (Ceresoli-Borroni & Schwarcz, 2001), Huntington's disease (Guidetti & Schwarcz, 2003) and schizophrenia (Schwarcz *et al.*, 2001). Therefore, structural studies of the enzymes in this pathway are important.

Kynurenine aminotransferase (KAT; EC. 2.6.1.7) is the key enzyme that catalyzes the irreversible transamination of L-kynurenine to produce kynurenic acid in the kynurenine pathway. Two KAT isozymes, KAT I and KAT II, have been found in human brain (Okuno *et al.*, 1991). They share low amino-acid sequence identity. KAT I and KAT II are also referred to as glutamine transaminase K (Cooper, 2004) and α-aminoadipate aminotransferase (AAAT; Tobes & Mason, 1977), respectively.

Crystal structures of human KAT I (Rossi *et al.*, 2004) and its homologues, such as glutamine:phenylpyruvate aminotransferase (Goto *et al.*, 2004) and aspartate aminotransferase (Nakai *et al.*, 1999) from *Thermus thermophilus* HB8, have been solved and substrate-recognition mechanisms have been proposed. However, no information on the tertiary structure of KAT II is currently available.

A human KAT II homologue from *T. thermophilus* HB27 has been identified as an AAAT and suggested to play a role in lysine and branched-chain amino-acid biosynthesis in cell growth (Miyazaki *et al.*, 2004). Likewise, human KAT II homologues from *Pyrococcus furiosus* (Andreotti *et al.*, 1995) and *Saccharomyces cerevisiae* (Iraqui *et al.*, 1998) have been identified as aromatic aminotransferases. However, it remains to be determined whether or not these enzymes exhibit KAT activity.

P. horikoshii OT3 is a hyperthermophilic archaeon with an optimal growth temperature of 371 K and its genomic sequence has been determined (Kawarabayasi *et al.*, 1998). A homology search of the OT3 genome using human KAT II as a query sequence indicates that ORF PH0207 shows the highest amino-acid sequence identity of 30% to human KAT II. It shows an amino-acid sequence identity of 20% to human KAT I. This ORF consists of 428 amino-acid residues. Thus far, the crystal structure of aromatic aminotransferase (Matsui *et al.*,

2000) is the only structure available of the various aminotransferases from *P. horikoshii* OT3. However, this enzyme shows higher sequence identity to human KAT I than to human KAT II.

Here, we report the overproduction, purification and characterization of a human KAT II homologue from *P. horikoshii* OT3 (Ph-KAT II). We also report the crystallization and preliminary X-ray diffraction analysis of Ph-KAT II.

2. Experimental procedures

2.1. Plasmid construction

The plasmid for overproduction of Ph-KAT II (pET-1300Ph) was constructed by ligating the 1.3 kbp DNA fragment containing the Ph-KAT II gene into the *Nde*I–*Eco*RI sites of pET-25b(+) (Novagen). The DNA fragment was amplified by PCR using the genomic DNA of *P. horikoshii* OT3 (Kawarabayashi *et al.*, 1998) as a template. The nucleotide sequences of the primers were 5′-GACGAAACATA-**CATATG**CATGAAGATGTTC-3′ for the 5′-primer and 5′-CGG-TTAGATAT**GAATTC**CTCTCAGAATAGG-3′ for the 3′-primer, where the bases in bold show the positions of the *Nde*I (5′-primer) and *Eco*RI (3′-primer) sites. These primers were synthesized by Hokkaido System Science. It is noted that the initiation codon of the Ph-KAT II gene was changed from GTG to ATG for construction of pET-1300Ph. An overproducing strain for Ph-KAT II was constructed by transforming *Escherichia coli* BL21-codonPlus(DE3)-RIL (Novagen) with this plasmid. The DNA sequence of the Ph-KAT II gene was confirmed using a Prism 310 DNA sequencer (Perkin-Elmer).

2.2. Overproduction and purification

For overproduction of Ph-KAT II, an *E. coli* BL21-codonPlus(DE3)-RIL transformant with pET1300Ph was grown at 310 K in NZCYM medium (Novagen) containing 50 µg ml^{-1} ampicillin. When the absorbance at 660 nm of the culture reached about 0.6, 1 m*M* isopropyl-*β*-D-thiogalactopyranoside was added to the culture medium and cultivation was continued for an additional 4 h. Cells were then harvested by centrifugation at 6000*g* for 10 min and subjected to the following purification procedures at 277 K.

Cells collected from 1 l culture were suspended in 40 ml 20 m*M* Tris–HCl pH 8.0 and disrupted by sonication on ice. The resultant cell lysate was centrifuged at 30 000*g* for 30 min at 277 K to remove insoluble materials. The supernatant was heated at 353 K for 20 min and centrifuged at 30 000*g* for 30 min at 277 K to remove precipitate. The supernatant was dialyzed against 20 m*M* Tris–HCl pH 8.0 and applied onto a column (2.6 × 15 cm) of DE-52 (Whatman) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of 0–0.5 *M* NaCl. Fractions containing Ph-KAT II were collected and applied onto a column (1.6 × 190 cm) of Sephacryl S-300 (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 0.2 *M* NaCl for gel-filtration column chromatography. Fractions containing homogeneous Ph-KAT II were collected, diluted fourfold with 20 m*M* Tris–HCl pH 8.0 and applied onto a column (1.0 × 1.5 cm) of DE-52 equilibrated with the same buffer. Ph-KAT II was eluted from the column with 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl.

The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an A_{280} value of 1.15 for 0.1% solution. This value was calculated using absorption coefficients ε of 1576 M^{-1} cm^{-1} for Tyr and 5225 M^{-1} cm^{-1} for Trp at 280 nm (Goodwin & Morton, 1946). The purity of the protein was confirmed by SDS–PAGE (Laemmli, 1970), followed by staining with Coomassie Brilliant Blue.

2.3. KAT activity assay

KAT activity was determined based on previously described methods (Han *et al.*, 2001). A reaction mixture (50 µl in total) containing 100 m*M* potassium phosphate pH 7.0, 10 m*M* L-kynurenine, 12 m*M* *α*-ketoglutarate, 70 µ*M* 5′-pyridoxal phosphate (PLP) and an appropriate amount of enzyme was incubated at 328 K for 60 min and the reaction was terminated by adding an equal volume of 0.8 *M* formic acid. The amount of kynurenic acid produced upon enzymatic reaction was determined by reverse-phase HPLC. The response factor of kynurenic acid (peak area/µmol) was determined by injecting solutions containing different amounts of this substance onto HPLC. One unit of enzymatic activity was defined as the amount of enzyme that produces 1 µmol of kynurenic acid per minute at 328 K. Specific activity was defined as enzymatic activity per milligram of protein.

2.4. Reverse-phase HPLC

Reverse-phase HPLC was carried out on a column (4.6 × 150 mm) of Aquapore RP-300 (Brownlee Laboratories). Elution was performed by linearly increasing the concentration of solvent *B* in solvent *A* from 0 to 40%(*v*/*v*) over 25 min. Solvent *A* was 4% acetonitrile containing 5 m*M* formic acid and solvent *B* was 100% acetonitrile containing 5 m*M* formic acid. The flow rate was 0.6 ml min^{-1} and L-kynurenine and kynurenic acid were detected with a UV detector set at 330 nm.

2.5. Mass spectrometry

Mass (ESI–MS) spectra were obtained by an LCQ electrospray ionization mass spectrometer (Finnigan Mat). The scan range was 300–4000. The scans were deconvoluted using *FINNIGAN BIOWORKS* software.

2.6. Crystallization

The crystallization condition was initially screened using crystallization kits from Hampton Research (Crystal Screens I, II and Cryo). The conditions were surveyed using the sitting-drop vapour-diffusion method at 293 K. Drops were prepared by mixing 1 µl each of the protein solution (12.6 mg ml^{-1} Ph-KAT II in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl and 10% glycerol) and the reservoir solution and vapour-equilibrated against 100 µl reservoir solution using 96-well Corning CrystalEX Microplates (Hampton Research). Single crystals appeared after 1 d using Crystal Screen I solution No. 6 (0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$, 30% PEG 4000). The crystallization conditions were further optimized and single crystals suitable for X-ray diffraction analysis appeared when the drop was prepared by mixing 2 µl protein solution and 2 µl reservoir solution, which was prepared by mixing solution No. 6 and 60% PEG 4000 in a 9:1 ratio, and vapour-equilibrated against 300 µl reservoir solution at 293 K using CombiClover Plates (B-Bridge).

2.7. Data collection

A crystal of Ph-KAT II was mounted in a CryoLoop (Hampton Research) and then flash-frozen in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected at 100 K on the BL44XU station at SPring-8, Japan using a multiple imaging-plate DIP6040 diffractometer. A total of 180 images were recorded with an exposure time of 10 s per image and an oscillation angle of 1.0°. The intensity data

were processed with the program *DENZO* and merged with the program *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Overproduction, purification and characterization

Recombinant Ph-KAT II was purified to give a single band on SDS–PAGE (Fig. 1) by two column chromatographic steps and concentrated using a small DE-52 column. The amount of the protein purified from 1 l culture was typically 17 mg. The molecular weight of Ph-KAT II was estimated to be 49 kDa by SDS–PAGE, which was nearly identical to that (48 925 Da) calculated from its amino-acid sequence. The molecular weight of this protein in native form was estimated to be 100 kDa by gel-filtration column chromatography, suggesting that Ph-KAT II exists as a homodimer.

When Ph-KAT II was incubated with L-kynurenine as described in §2 and the reaction mixture was analyzed by HPLC, two peaks were detected at around 8 and 20 min (data not shown). The former and latter peaks represent L-kynurenine and kynurenic acid, respectively. The peak at 20 min was identified as kynurenic acid based on its retention time in HPLC and molecular mass determined by mass spectrometry. These results indicate that Ph-KAT II exhibits KAT activity. The specific activity of Ph-KAT II was determined to be 71.7 U mg^{-1} at 328 K.

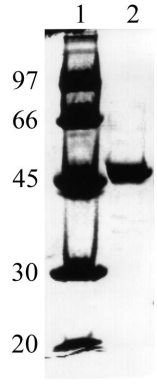

Figure 1
SDS–PAGE of Ph-KAT II. Samples were subjected to electrophoresis on a 12% polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, a low-molecular-weight marker kit (Amersham Biosciences); lane 2, purified Ph-KAT II. Numbers along the gel represent the molecular weights of the standard proteins (in kDa).

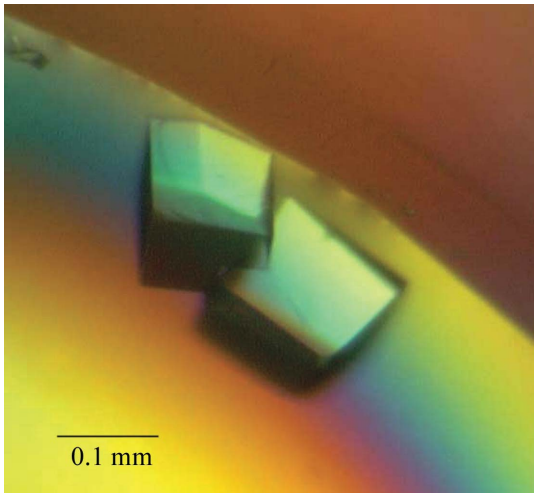

Figure 2
Crystals of Ph-KAT II.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.28–2.20 Å).

X-ray wavelength (Å)	0.9
Temperature (K)	100
Space group	$C222_1$
Unit-cell parameters (Å)	a = 71.75, b = 86.84, c = 137.30
Resolution range (Å)	55.0–2.20
No. measured reflections	157227
No. unique reflections	22148
$R_{\rm merge}$† (%)	4.1 (29.2)
Data completeness (%)	99.9 (99.8)
Average $I/\sigma(I)$	17.5 (6.0)

† $R_{\rm merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum I_{hkl}$, where I_{hkl} is the intensity measurement for reflection with indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity for multiply recorded reflections.

3.2. Preliminary X-ray diffraction analysis

The colourless crystals appeared after 1 d and grew to maximum dimensions of 0.1 × 0.1 × 0.05 mm after a few days (Fig. 2). The crystals diffracted to 2.2 Å resolution. A total of 157 227 measured reflections were merged into 22 148 unique reflections with an $R_{\rm merge}$ of 4.1%. The crystals belong to the centred orthorhombic space group $C222_1$, with unit-cell parameters a = 71.75, b = 86.84, c = 137.30 Å. Table 1 summarizes the data-collection statistics. Based on the molecular weight and the space group, it was assumed that the crystal contains one protein molecule per asymmetric unit, giving a $V_{\rm M}$ value of 2.19 Å^3 Da^{-1} and a solvent content of 43.28%. These values are within the ranges frequently observed for protein crystals (Matthews, 1968), suggesting that this crystal is suitable for structural determination. A search for suitable heavy-atom derivatives is in progress in order to solve the structure by the SIR(AS), MIR(AS) or SAD/MAD method.

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