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Crystallization and preliminary X-ray analysis of AAMS amidohydrolase, the final enzyme in degradation pathway I of pyridoxine

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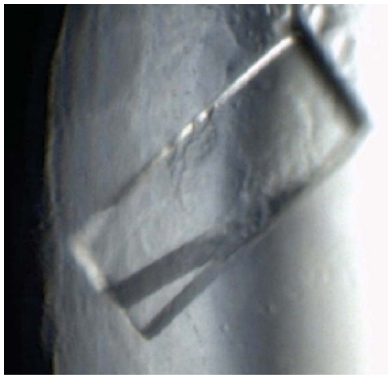

α-(*N*-Acetylaminomethylene)succinic acid (AAMS) amidohydrolase from *Mesorhizobium loti* MAFF303099, which is involved in a degradation pathway of vitamin B_6 and catalyzes the degradation of AAMS to acetic acid, ammonia, carbon dioxide and succinic semialdehyde, has been overexpressed in *Escherichia coli*. To elucidate the reaction mechanism based on the tertiary structure, the recombinant enzyme was purified and crystallized by the sitting-drop vapour-diffusion method using PEG 8000 as precipitant. A crystal of the enzyme belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 393.2$, $b = 58.3$, $c = 98.9$ Å, $\beta = 103.4^\circ$, and diffraction data were collected to 2.7 Å resolution. The V_M value and calculation of the self-rotation function suggested that three dimers with a threefold symmetry were possibly present in the asymmetric unit.

1. Introduction

α-(*N*-Acetylaminomethylene)succinic acid (AAMS) amidohydrolase catalyzes the degradation of AAMS to acetic acid, ammonia, carbon dioxide and succinic semialdehyde as shown in Fig. 1. The enzyme reaction is the final step in degradation pathway I of vitamin B_6 (pyridoxine, pyridoxamine and pyridoxal; Nelson & Snell, 1986). The enzyme has been found in *Pseudomonas* MA-1 (Nyns *et al.*, 1969; Huynh & Snell, 1985) and *Mesorhizobium loti* (Mukherjee *et al.*, 2008; Yuan *et al.*, 2008) cells. The enzyme from *M. loti*, a nitrogen-fixing symbiotic bacterium, has been cloned, sequenced and characterized. The enzyme is a dimer with a subunit molecular mass of 34 kDa. The amino-acid sequence (AB362478; 278 amino-acid residues per subunit) has no possible post-translational modifications. Sequence comparisons suggested that *M. loti* AAMS amidohydrolase does not contain a signature sequence from any defined protein family (Yuan *et al.*, 2008), although the enzyme shows a low but significant (about 27%) identity to fluoroacetate dehalogenase (PDB code 1y37), haloalkane dehalogenase (PDB code 1k5p) and aryl esterase (PDB code 1va4).

AAMS amidohydrolase produces four products from the *E* isomer of AAMS (Fig. 1). The reaction may involve hydrolytic cleavage of the amide bond followed by secondary (possibly nonenzymatic) release of ammonia and decarboxylation (possibly nonenzymatic) of an intermediate. The enzyme shows no reactivity towards the *Z* isomer of AAMS (Mukherjee *et al.*, 2008). Mechanistic analysis of the enzyme has suggested possible alternate routes for the action of the enzyme (Nyns *et al.*, 1969; Mukherjee *et al.*, 2008). However, the catalytic mechanism of the enzyme has not been elucidated because its tertiary and active-site structure have not been determined. Thus, no studies have been perfomed on the action of the residues involved

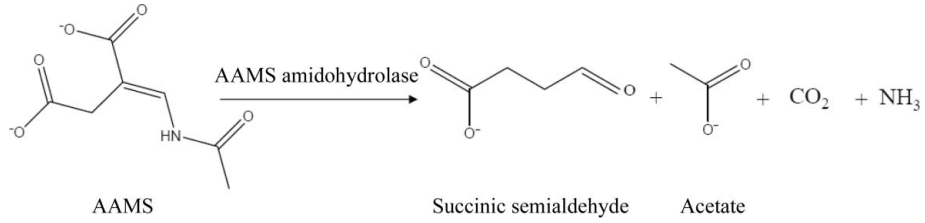

Figure 1
The enzyme reaction catalyzed by AAMS amidohydrolase.

in the catalytic reaction, although the presence of a catalytic triad in the active site of the enzyme has been suggested based on the similarity of its amino-acid sequence and predicted secondary structure to those of aryl esterase (Yuan *et al.*, 2008).

In order to elucidate the catalytic mechanism of AAMS amidohydrolase, determination of the three-dimensional structure of this enzyme is necessary. Here, we describe the crystallization and preliminary X-ray diffraction studies of AAMS amidohydrolase from *M. loti*.

2. Material and methods

2.1. Overexpression and purification of AAMS amidohydrolase

The cloning of a gene encoding AAMS amidohydrolase and the overexpression and purification of the enzyme have been reported previously (Yuan *et al.*, 2008); one additional purification step was used for crystallization as follows. All purification steps were performed at 277–283 K. *Escherichia coli* BL21 (DE3) cells (5.5 g wet weight) harbouring plasmid pET6787 and overexpressing AAMS amidohydrolase were suspended in 25 ml 50 m*M* potassium phosphate buffer pH 7.0 containing 0.1%(*v*/*v*) 2-mercaptoethanol (buffer *A*) and 0.1 m*M* phenylmethylsulfonyl fluoride. The cell suspension was sonicated on ice for 6 min with a Heatsystems-Ultrasonics sonicator W-220. After centrifugation at 10 000g for 20 min at 277 K, the supernatant was stored. The precipitated cells and debris were resuspended in 20 ml buffer *A* containing phenylmethylsulfonyl fluoride, sonicated and centrifuged. The second supernatant was mixed with the first supernatant to make the crude extract. The crude extract (44 ml) was fractionated with ammonium sulfate. The precipitate obtained after centrifugation of a 40–55% saturated solution was dissolved in 15 ml buffer *A* and then dialyzed thoroughly against buffer *A*. 1 *M* (final concentration) ammonium sulfate solution was added to the dialyzed solution and the enzyme solution was then applied onto a Butyl-Toyopearl 650 M column (3.0 × 17.0 cm; Tosoh Corporation, Tokyo, Japan) equilibrated with buffer *A* containing 1 *M* ammonium sulfate. The enzyme was eluted at around 0.4 *M* ammonium sulfate with a linear gradient (1.0–0 *M* ammonium sulfate). The active fractions were pooled and dialyzed thoroughly against buffer *B* (50 m*M* Tris–HCl pH 7.0 containing 0.1% 2-mercaptoethanol). The dialyzed solution was applied onto a hydroxylapatite (Nacalai Tesque, Kyoto, Japan, 100–200 mesh) column (1.5 × 16.0 cm) equilibrated with buffer *B*. The enzyme was eluted at around 10 m*M* potassium phosphate pH 7.0 with a linear gradient of 0–50 m*M* potassium phosphate. Ammonium sulfate (55% saturation) was added to the active fraction and the precipitate was collected by centrifugation (10 000*g* for 20 min at 277 K). The precipitate was dissolved in 1.0 ml 50 m*M* Tris–HCl pH 7.0 containing 0.1%(*v*/*v*) 2-mercaptoethanol and the enzyme solution was applied onto a HPLC gel-filtration column (5-diol-300II; Nacalai Tesque, Kyoto, Japan) equilibrated with buffer *B* as an additional purification step. The enzyme was eluted from the column as a single peak, the elution volume of which corresponded to a dimer. The purified enzyme was dialyzed against 10 m*M* HEPES pH 7.0 containing 0.1%(*v*/*v*) 2-mercaptoethanol and concentrated to 14 mg ml^{-1} with an Amicon Ultra-4 30 kDa Ultracel (Millipore, Billerica, Massachusetts, USA) for crystallization.

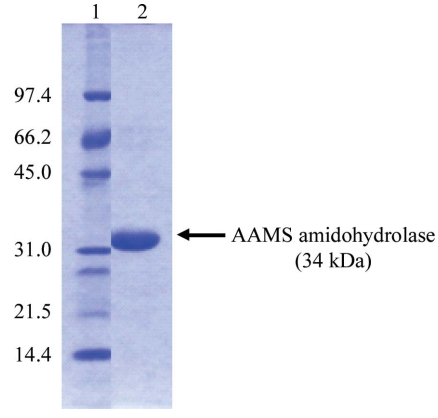

Figure 2
SDS–PAGE analysis of purified AAMS amidohydrolase. Lane 1, molecular-weight markers. Lane 2, purified AAMS amidohydrolase for crystallization.

Table 1
Data-collection statistics of AAMS amidohydrolase.

Values in parentheses are for the highest resolution bin (2.80–2.70 Å).

Temperature (K)	100
Wavelength (Å)	1.0
Resolution range (Å)	50–2.7
No. of measured reflections	162075
No. of unique reflections	61051
Completeness (%)	95.9 (97.5)
Mean $I/\sigma(I)$	9.2 (2.63)
$R_{\rm merge}$† (%)	13 (31.3)
Space group	*C*2
Unit-cell parameters	
a (Å)	393.2
b (Å)	58.3
c (Å)	98.9
β (°)	103.4

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(hkl)$.

2.2. Enzyme assay

AAMS amidohydrolase activity was measured to identify the fractions to be collected during the purification steps. AAMS amidohydrolase activity was determined by measuring the initial decrease in A_{261} of AAMS at 298 K in a reaction mixture consisting of 50 m*M* potassium phosphate buffer pH 7.0, 0.025 m*M* AAMS and the enzyme. One unit of enzyme was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of the substrate per minute. The substrate concentration was near the $K_{\rm m}$ value to make the original absorbance at 261 nm precisely measurable.

2.3. Crystallization and X-ray analysis

The initial crystallization conditions were screened using Crystal Screen I, PEG/Ion Screen (Hampton Research, California, USA) and Wizard Screens I and II (Emerald BioSystems, Washington, USA). The enzyme was crystallized at 293 K by the sitting-drop vapour-diffusion method using CrystalClear Strips from Hampton Research (Laguna Niguel, California, USA). Crystals appeared within three weeks during equilibration of a droplet consisting of a mixture of the same volumes (2 µl) of protein and reservoir solution [Wizard Screen II condition No. 3; 100 m*M* Tris–HCl buffer final pH 8.5, 20%(*w*/*v*) PEG 3000, 200 m*M* $MgCl_2$] against a reservoir containing 100 µl of the latter solution. The drops were set up manually. To optimize the crystallization condition, 40%(*v*/*v*) 2,2,2-trifluoroethanol (solution No. 95 from Additive Screen, Hampton Research, California, USA) was applied. After improvement of the conditions, the reservoir solution was changed to 100 m*M* Tris–HCl buffer (final pH 8.5), 20%(*w*/*v*) PEG 8000, 275 m*M* $MgCl_2$. Thus, a droplet of the enzyme mixture was prepared by mixing 2 µl enzyme solution (14 mg ml^{-1})

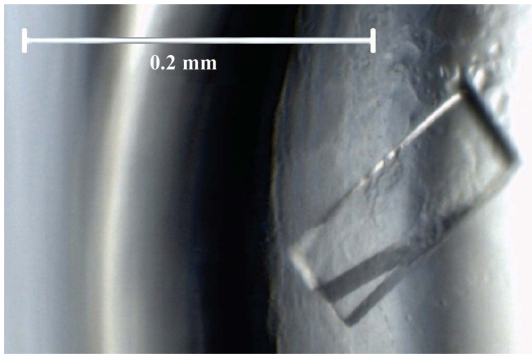

Figure 3
Crystal of *M. loti* AAMS amidohydrolase with dimensions of 0.14 × 0.06 × 0.01 mm.

with 2 µl 80 m*M* Tris–HCl buffer pH 8.5, 16%(*w*/*v*) PEG 8000, 220 m*M* $MgCl_2$, 8%(*v*/*v*) 2,2,2-trifluoroethanol. The optimized crystals grew from the droplet in three weeks at 293 K (Fig. 2).

Data collection was carried out using synchrotron radiation on beamline KEK AR-NW12A at Photon Factory (Tsukuba, Japan). The crystal was soaked for a few seconds in reservoir solution containing 30%(*v*/*v*) glycerol and flash-cooled in a liquid-nitrogen gas stream. Diffraction data were collected at 100 K using an ADSC/CCD detector system. Data processing was carried out using the program *HKL*-2000 (Otwinowski & Minor, 1997) at the beamline station. The data-collection statistics are listed in Table 1.

3. Results and discussion

AAMS amidohydrolase was successfully overexpressed in *E. coli* and purified, maintaining significant enzyme activity. The results of SDS–PAGE analysis of the purified enzyme are shown in Fig. 2.

A crystal suitable for X-ray data collection, which grew to dimensions of 0.14 × 0.06 × 0.01 mm in three weeks after setup, is shown in Fig. 3. The crystal of AAMS amidohydrolase diffracted to a resolution of 2.7 Å, as shown in Fig. 4, and a complete data set was successfully collected to 2.7 Å resolution, as summarized in Table 1. The crystal belonged to the monoclinic space group *C*2, with unit-cell parameters a = 393.2, b = 58.3, c = 98.9 Å, β = 103.4°.

The asymmetric unit is expected to contain 6–8 molecules, with a crystal volume per unit molecular weight V_M of 3.07–2.31 $Å^3$ Da^{-1}, corresponding to a solvent content of 60–47% (Matthews, 1968). Calculation of the self-rotation function using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project 4, Number 4, 1994) revealed a clear local threefold axis, ω = 75.2° (the inclination to the z axis within the yz plane), φ = 0.0° (within the xy plane), κ = 120.2°, peak height = 67.3, and no other local symmetry could be found. Since AAMS amidohydrolase has been reported to form a dimer (Yuan *et al.*, 2008), three dimers are possibly arranged with threefold symmetry in the asymmetric unit. The molecular-replacement method was attempted using the

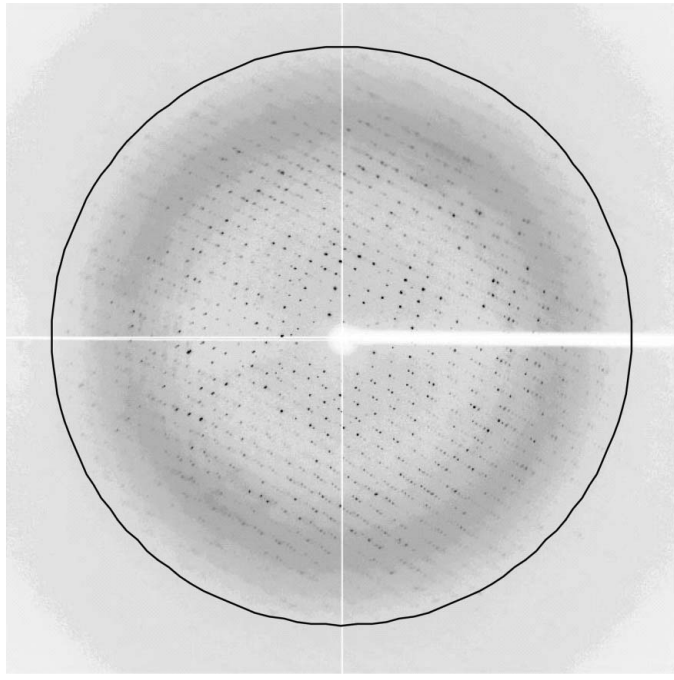

Figure 4
Diffraction image of *M. loti* AAMS amidohydrolase; the ring indicates 2.7 Å resolution.

MOLREP program from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) with the structures of fluoroacetate dehalogenase (PDB code 1y37), haloalkane dehalogenase (1k5p) and aryl esterase (1va4) as models, but a plausible solution could not be obtained.

We are currently refining the crystallization conditions to obtain better diffracting crystals and preparing a selenomethionine derivative of the enzyme for further analysis.

This research was performed with the approval of the Photon Factory Advisory Committee and the National Laboratory for High Energy Physics, Japan.

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