

crystallization communications

Acta Crystallographica Section F
Structural Biology
and Crystallization
Communications

ISSN 1744-3091

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Received 27 April 2009

Accepted 21 July 2009

Crystallization and preliminary X-ray analysis of
4-pyridoxolactonase from *Mesorhizobium loti*4-Pyridoxolactonase from *Mesorhizobium loti* MAFF303099 has been over-expressed in *Escherichia coli*. The recombinant enzyme was purified and was crystallized by the sitting-drop vapour-diffusion method using PEG 4000 and ammonium sulfate as precipitants. Crystals of the free enzyme (form I) and of the 5-pyridoxolactone-bound enzyme (form II) grew under these conditions. Crystals of form I diffracted to 2.0 Å resolution and belonged to the monoclinic space group *C2*, with unit-cell parameters $a = 77.93$, $b = 38.88$, $c = 81.60$ Å, $\beta = 117.33^\circ$. Crystals of form II diffracted to 1.9 Å resolution and belonged to the monoclinic space group *C2*, with unit-cell parameters $a = 86.24$, $b = 39.35$, $c = 82.68$ Å, $\beta = 118.02^\circ$. The calculated V_M values suggested that the asymmetric unit contains one molecule in both crystal forms.

1. Introduction

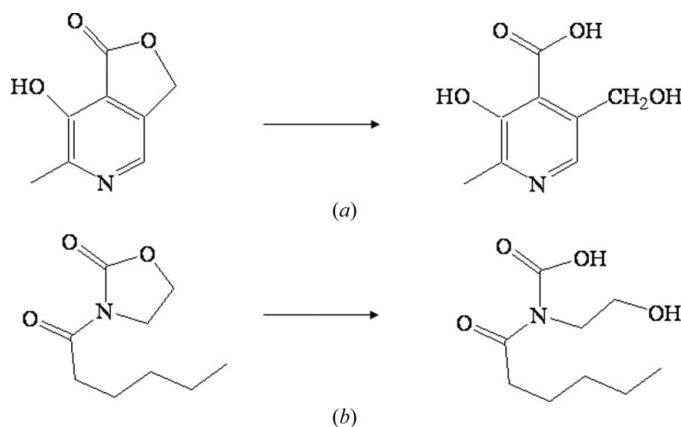
4-Pyridoxolactonase (EC 3.1.1.27) is involved in degradation pathway I of pyridoxine, a free (phosphate-unbound) form of vitamin B₆. The enzyme catalyzes the hydrolysis of 4-pyridoxolactone to 4-pyridoxic acid (Fig. 1a). The enzyme has been purified from *Pseudomonas* MA-1 (Jong & Snell, 1986) and *Mesorhizobium loti* (Funami *et al.*, 2005) cells. The enzyme from *M. loti*, a nitrogen-fixing symbiotic bacterium, has been cloned and sequenced and a recombinant 4-pyridoxolactonase has been characterized. Sequence comparisons suggest that *M. loti* 4-pyridoxolactonase is a member of the metallohydrolase-family proteins with, for example, 33% identity to the AttM/AiiB protein from *Agrobacterium tumefaciens* strain A6 and 30% identity to the acylhomoserine lactonase from *Bacillus thuringiensis* (Altschul *et al.*, 1997). 4-Pyridoxolactonase contains three characteristic histidine residues which are probably involved in the binding of one Zn atom as in other metallohydrolase-family proteins (Funami *et al.*, 2005).Although 4-pyridoxolactonase shows activity towards 4-pyridoxolactone in the degradation pathway, it can also hydrolyze *N*-hexanoyl-*D,L*-homoserine lactone in the degradation pathway, it can also hydrolyze *N*-hexanoyl-

Figure 1
Reactions catalyzed by 4-pyridoxolactonase. (a) Hydrolysis of 4-pyridoxolactone to 4-pyridoxic acid. (b) Hydrolysis of *N*-hexanoyl-*D,L*-homoserine lactone to *N*-hexanoyl-*D,L*-homoserine.

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D,L-homoserine lactone (Fig. 1*b*): its activity towards the latter compound is 41% of that towards the former (Funami *et al.*, 2005). *N*-Hexanoyl-D,L-homoserine lactone interferes with the quorum-sensing systems of Gram-negative bacteria (Dong *et al.*, 2001). These results suggested the possibility that 4-pyridoxolactonase could play a role in the quorum-sensing systems of *M. loti* (a Gram-negative bacterium) cells.

Tertiary structures of *N*-acylhomoserine lactone lactonase have been reported (Liu *et al.*, 2005; Kim *et al.*, 2005) and the mode of interaction between the product and two Zn atoms in the active site (Liu *et al.*, 2008) has also been reported. In contrast, 4-pyridoxolactonase contains only one Zn atom in the active site. Thus, its reaction mechanism should be considerably different from that of *N*-acylhomoserine lactone lactonase and needs to be elucidated based on its tertiary structure and active-site environment. Here, we describe the crystallization and preliminary X-ray diffraction studies of 4-pyridoxolactonase from *M. loti*.

2. Material and methods

2.1. Overexpression and purification of 4-pyridoxolactonase

The overexpression and purification of 4-pyridoxolactonase have previously been reported (Funami *et al.*, 2005); one additional purification step was used for crystallization. Briefly, the enzyme was overexpressed in *Escherichia coli* BL21 (DE3) cells harbouring plasmid pET21a6805 and purified sequentially by Butyl-Toyopearl 650M and QA52 column chromatography. The enzyme fraction eluted from the QA52 column was concentrated by ammonium sulfate precipitation. The precipitate was dissolved in a minute amount of 10 mM HEPES-KOH pH 7.5 containing 0.1% (v/v) 2-mercaptoethanol and 10% (w/v) glycerol (buffer *A*) and the enzyme solution was applied onto a hydroxylapatite column (Wako Pure Chemicals, Osaka, Japan). The enzyme was eluted with buffer *A* containing 20 mM potassium phosphate pH 7.5 and the purified enzyme was dialyzed against 10 mM HEPES pH 7.0 containing 0.1% (v/v) 2-mercaptoethanol. The enzyme was concentrated using an Ultrafree C31GC (Millipore, Billerica, Massachusetts, USA).

2.2. 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 Inc., Washington,

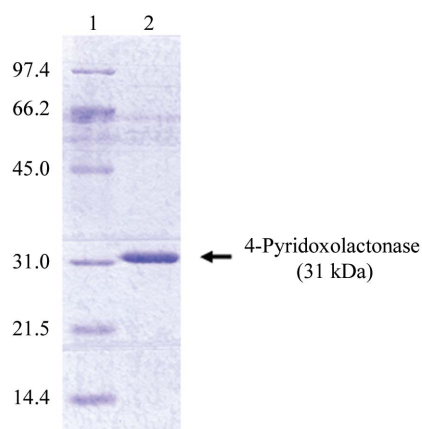
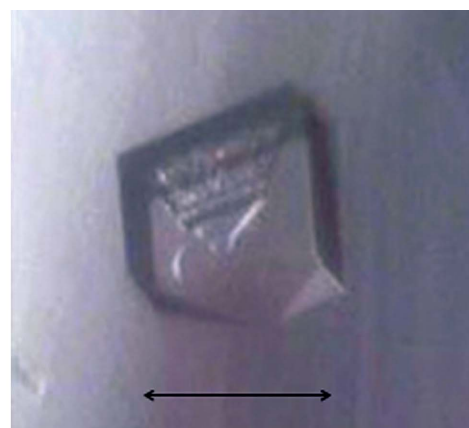


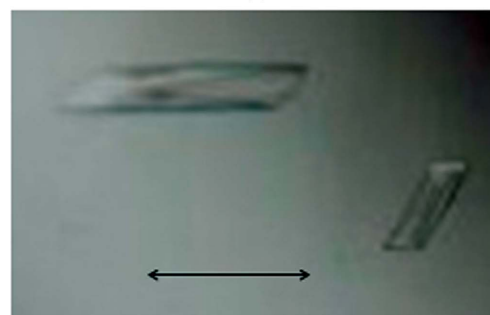
Figure 2
SDS-PAGE analysis of purified 4-pyridoxolactonase. Lane 1, molecular markers (kDa). Lane 2, purified 4-pyridoxolactonase for crystallization.

USA). The enzyme was crystallized at 277 K by the sitting-drop vapour-diffusion method using CrystalClear Strips from Hampton Research (Laguna Niguel, California, USA). Crystals appeared during equilibration of a droplet consisting of a mixture of the same volumes (2 μ l) of protein solution and reservoir solution against a reservoir containing 100 μ l reservoir solution using PEG/Ion Screen condition Nos. 16 and 19. After improvement of the conditions, the reservoir solution was changed to 200 mM ammonium acetate, 100 mM sodium acetate and 30% (w/v) PEG 4000 (final pH 7.5). Thus, the most suitable conditions for crystallization to produce crystals of the free enzyme (form I) were determined to be a mixture of 2 μ l enzyme solution (6.4 mg ml⁻¹) and 2 μ l reservoir solution. Crystals of the enzyme grew from the droplets in two weeks when the enzyme mixture was equilibrated against 100 μ l reservoir solution at 277 K. The enzyme was also crystallized in the presence of 5-pyridoxolactone, a competitive inhibitor (Jong & Snell, 1986), to produce form II crystals. Enzyme solution (2 μ l, 6.4 mg ml⁻¹) containing 0.75 mM 5-pyridoxolactone was mixed with 2 μ l reservoir solution [200 mM ammonium acetate, 100 mM sodium acetate, 30% (w/v) PEG 4000 (final pH 7.0)] and the mixture was equilibrated against a reservoir containing 100 μ l reservoir solution at 277 K. Crystals of the enzyme-inhibitor complex grew in two weeks.

A form I crystal picked up from a droplet was transferred into cryoprotectant solution [25% (v/v) glycerol, 20 mM ammonium acetate, 100 mM sodium acetate and 30% (w/v) PEG 4000 (final pH 7.5)]. The crystal was placed into a cold nitrogen-gas stream at 100 K. The form II crystal was placed into a cold nitrogen-gas stream without cryoprotectant treatment. X-ray diffraction images were collected from the crystals at 100 K in the nitrogen-gas stream with a Rigaku Jupiter 201 CCD detector using synchrotron radiation of wavelength 1.00 Å at station BL38B1 of SPring-8 (Hyogo, Japan).



(a)



(b)

Figure 3
Crystals of 4-pyridoxolactonase. (a) Crystal of form I. (b) Crystal of form II, in which the enzyme binds 5-pyridoxolactone. The scale bars are 0.1 mm in length.

The crystal-to-detector distances for the form I and form II crystals were set to 183.5 and 184.5 mm, respectively. Oscillation images of 0.5° and 1.0° were recorded for form I and form II crystals, respectively, with an exposure time of 6 s.

2.3. Enzyme assay

4-Pyridoxolactonase activity was determined by measuring the initial decrease in the A_{356} of 4-pyridoxolactone ($\epsilon = 8.0 \text{ mM}^{-1} \text{ cm}^{-1}$) at 298 K in a reaction mixture (1.0 ml) consisting of 100 mM potassium phosphate buffer pH 7.5, 0.1 mM 4-pyridoxolactone 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.

3. Results and discussion

4-Pyridoxolactonase 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.

Crystals suitable for X-ray data collection were obtained. Diffraction data were obtained from form I (Fig. 3a) and form II (Fig. 3b) crystals in the resolution ranges 50–2.00 and 50–1.90 Å, respectively, and were processed using the *HKL-2000* program package (*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). Crystal parameters and diffraction data statistics are summarized in Table 1. The space group of the both crystals was determined to be *C2* (monoclinic), with unit-cell parameters $a = 77.93$, $b = 38.88$, $c = 81.60$ Å, $\beta = 117.33^\circ$ for form I and $a = 86.24$, $b = 39.35$, $c = 82.68$ Å, $\beta = 118.02^\circ$ for form II. From the total of 75 700 reflections measured for the form I crystal, 14 565 independent reflections were obtained with an R_{merge} value of 8.2%. The data set was 98.0% complete at the resolution limit of 2.0 Å (Fig. 4a). Similarly, from the total of 103 324 reflections measured for the form II crystal, 19 769 independent reflections were obtained with an R_{merge}

Table 1

Data-collection statistics for crystals of 4-pyridoxolactonase.

Values in parentheses are for the highest resolution shell.

	Form I	Form II
X-ray source	SPring-8 (BL26B1)	SPring-8 (BL38B1)
Wavelength (Å)	1.0000	1.0000
Detector	Jupiter 201 (1 × 1 bin mode)	Jupiter 201 (1 × 1 bin mode)
Crystal system	Monoclinic	Monoclinic
Space group	<i>C2</i>	<i>C2</i>
Unit-cell parameters (Å, °)	$a = 77.93$, $b = 38.88$, $c = 81.60$, $\beta = 117.33$	$a = 86.24$, $b = 39.35$, $c = 82.68$, $\beta = 118.02$
Processing software	<i>HKL-2000</i>	<i>HKL-2000</i>
Resolution limit (Å)	50–2.00 (2.07–2.00)	50–1.90 (1.96–1.90)
Measured reflections	75780	103324
Redundancy	5.2 (4.3)	5.2 (4.6)
Unique reflections	14565 (1389)	19769 (1867)
Completeness (%)	98.0 (93.5)	99.5 (95.7)
$\langle I/\sigma(I) \rangle$	8.5	23.6
R_{merge} (%)	8.2 (34.9)	5.0 (10.0)

value of 5.0%. The data set was 99.5% complete at the resolution limit of 1.90 Å (Fig. 4b). The V_M value (Matthews, 1968), the crystal volume per unit protein molecular weight, for the form I crystal was calculated to be $1.77 \text{ \AA}^3 \text{ Da}^{-1}$ assuming the presence of one molecule of the enzyme in the asymmetric unit, with a solvent content of 30.6%. The V_M value for the form II crystal was $2.00 \text{ \AA}^3 \text{ Da}^{-1}$ assuming the presence of one molecule of the enzyme in the asymmetric unit, with a solvent content of 38.5%. The V_M values and solvent contents lie within the ranges usually found for protein crystals, although they are lower than the mean values.

We are currently preparing a selenomethionine derivative of the enzyme for further analysis.

We thank Dr K. Hasegawa of Japan Synchrotron Radiation Research Institute (JASRI) for his kind help in data collection. X-ray

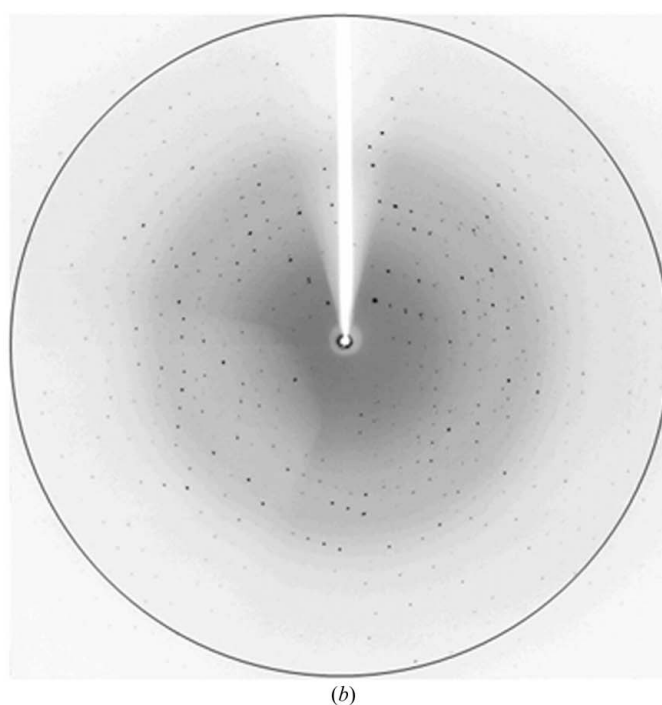
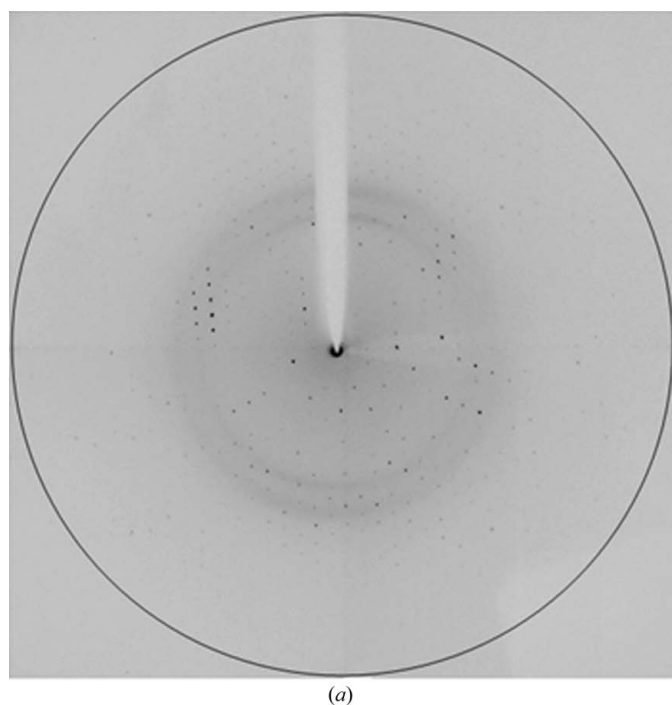


Figure 4

Diffraction images of *M. loti* 4-pyridoxolactonase. (a) Form I crystal with a resolution scale of 1.9 Å indicated by the ring. (b) Form II crystal with a resolution scale of 1.9 Å indicated by the ring.

data collection at BL38B1 of SPring-8 was carried out with the approval of the organizing committee of SPring-8.

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