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Crystallization and preliminary X-ray analysis of 4-pyridoxolactonase from *Mesorhizobium loti*

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4-Pyridoxolactonase from *Mesorhizobium loti* MAFF303099 has been overexpressed 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, β = 117.33°. 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, β = 118.02°. 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 B_6. The enzyme catalyzes the hydrolysis of 4-pyridoxolactone to 4-pyridoxic acid (Fig. 1*a*). 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-

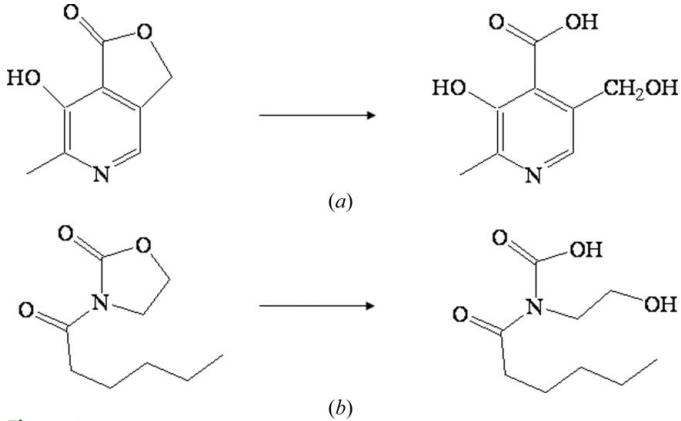

Figure 1
Reactions catalyzed by 4-pyridoxolactonase. (*a*) Hydrolysis of 4-pyridoxolactone to 4-pyridoxic acid. (*b*) Hydrolysis of *N*-hexanoyl-D,L-homoserine lactone to *N*-hexanoyl-D,L-homoserine.

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 m*M* 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 m*M* potassium phosphate pH 7.5 and the purified enzyme was dialyzed against 10 m*M* 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, 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 µl) of protein solution and reservoir solution against a reservoir containing 100 µl reservoir solution using PEG/Ion Screen condition Nos. 16 and 19. After improvement of the conditions, the reservoir solution was changed to 200 m*M* ammonium acetate, 100 m*M* 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 µl enzyme solution (6.4 mg ml^{-1}) and 2 µl reservoir solution. Crystals of the enzyme grew from the droplets in two weeks when the enzyme mixture was equilibrated against 100 µl 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 µl, 6.4 mg ml^{-1}) containing 0.75 m*M* 5-pyridoxolactone was mixed with 2 µl reservoir solution [200 m*M* ammonium acetate, 100 m*M* sodium acetate, 30%(*w*/*v*) PEG 4000 (final pH 7.0)] and the mixture was equilibrated against a reservoir containing 100 µl 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 m*M* ammonium acetate, 100 m*M* 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).

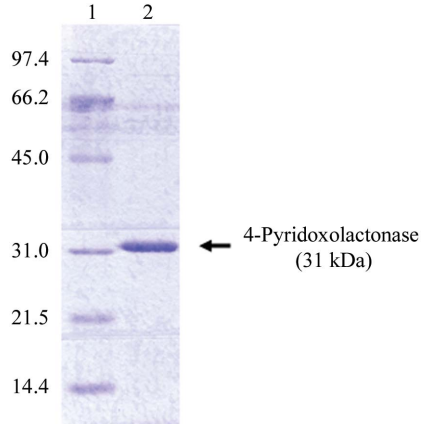

Figure 2
SDS–PAGE analysis of purified 4-pyridoxolactonase. Lane 1, molecular markers (kDa). Lane 2, purified 4-pyridoxolactonase for crystallization.

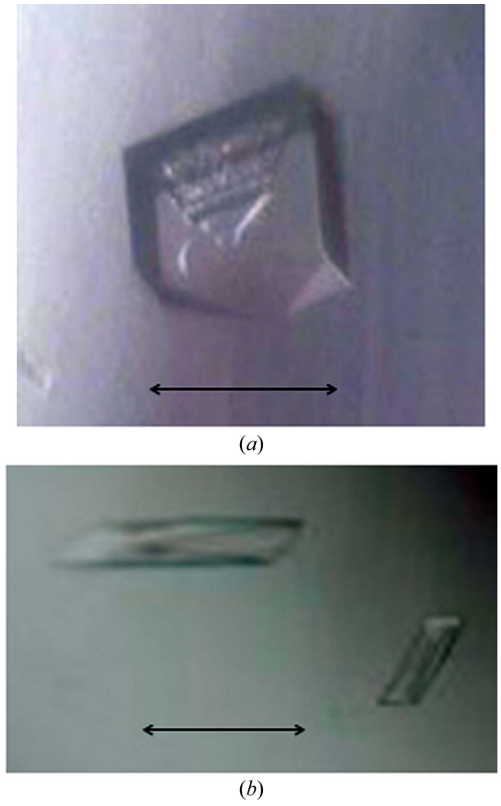

Figure 3
Crystals of 4-pyridoxolactonase. (*a*) Crystal of form I. (*b*) Crystal of form II, in which the enzyme binds 5-pyridoxolactone. The scale bars are 0.1 mm in length.

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 (ε = 8.0 mM^{-1} cm^{-1}) at 298 K in a reaction mixture (1.0 ml) consisting of 100 m*M* potassium phosphate buffer pH 7.5, 0.1 m*M* 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. 3*a*) and form II (Fig. 3*b*) 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 *C*2 (monoclinic), with unit-cell parameters a = 77.93, b = 38.88, c = 81.60 Å, β = 117.33° for form I and a = 86.24, b = 39.35, c = 82.68 Å, β = 118.02° for form II. From the total of 75 700 reflections measured for the form I crystal, 14 565 independent reflections were obtained with an $R_{\rm merge}$ value of 8.2%. The data set was 98.0% complete at the resolution limit of 2.0 Å (Fig. 4*a*). Similarly, from the total of 103 324 reflections measured for the form II crystal, 19 769 independent reflections were obtained with an $R_{\rm merge}$ value of 5.0%. The data set was 99.5% complete at the resolution limit of 1.90 Å (Fig. 4*b*). The $V_{\rm M}$ value (Matthews, 1968), the crystal volume per unit protein molecular weight, for the form I crystal was calculated to be 1.77 Å^3 Da^{-1} assuming the presence of one molecule of the enzyme in the asymmetric unit, with a solvent content of 30.6%. The $V_{\rm M}$ value for the form II crystal was 2.00 Å^3 Da^{-1} assuming the presence of one molecule of the enzyme in the asymmetric unit, with a solvent content of 38.5%. The $V_{\rm M}$ values and solvent contents lie within the ranges usually found for protein crystals, although they are lower than the mean values.

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	*C*2	*C*2
Unit-cell parameters (Å, °)	a = 77.93, b = 38.88, c = 81.60, β = 117.33	a = 86.24, b = 39.35, c = 82.68, β = 118.02
Processing software	*HKL*-2000	*HKL*-2000
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
$R_{\rm merge}$ (%)	8.2 (34.9)	5.0 (10.0)

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

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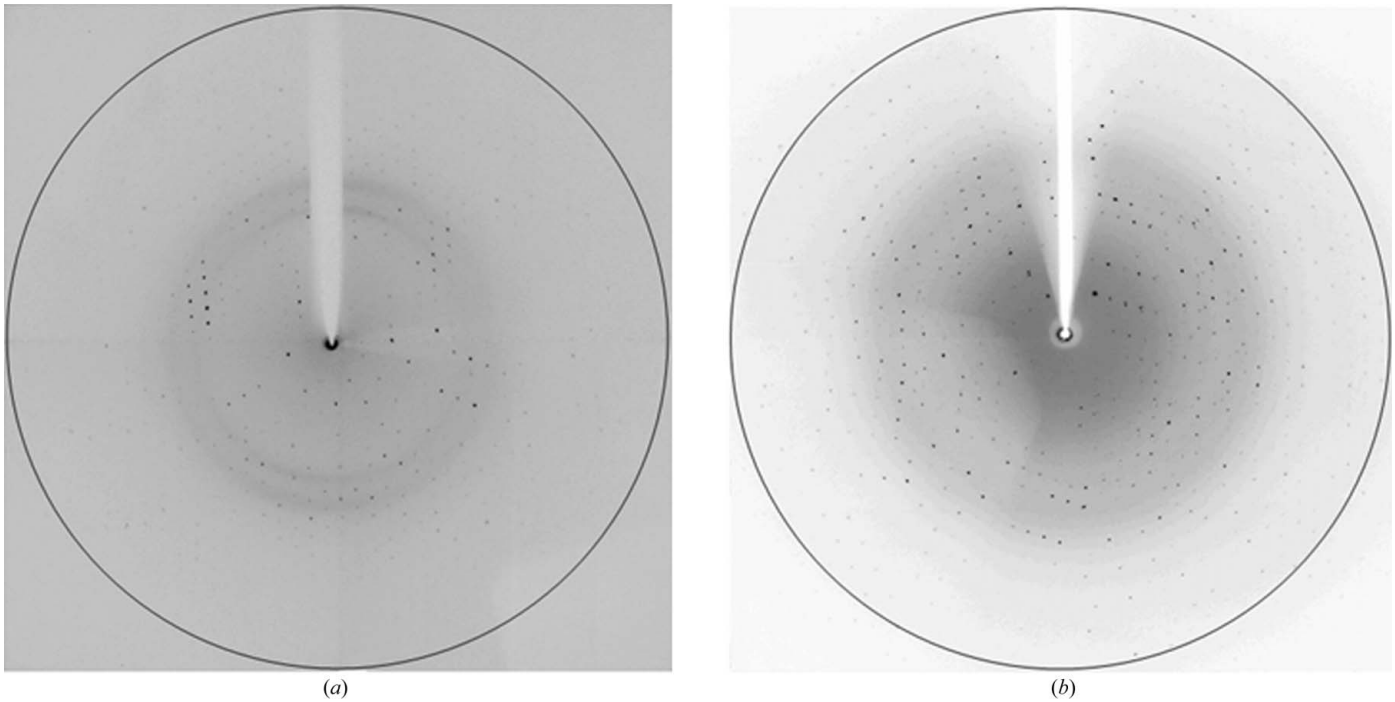

(*a*) (*b*)

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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