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Crystallization and preliminary X-ray analysis of SDR-type pyridoxal dehydrogenase from *Mesorhizobium loti*

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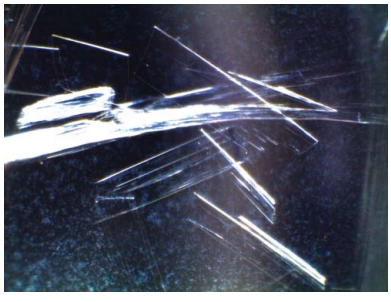
Pyridoxal 4-dehydrogenase from *Mesorhizobium loti* MAFF303099 was overexpressed in *Escherichia coli*. The recombinant selenomethionine-substituted enzyme was purified and crystallized by the sitting-drop vapour-diffusion method using PEG 4000 as precipitant. Crystals grew in the presence of 0.45 m*M* NAD^+. The crystals diffracted to 2.9 Å resolution and belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 86.20, b = 51.11, c = 91.73 Å, β = 89.36°. The calculated V_M values suggested that the asymmetric unit contained four molecules.

1. Introduction

Pyridoxal 4-dehydrogenase (EC 1.1.1.107) is involved in degradation pathway I of pyridoxine, a free (phosphate-unbound) form of vitamin B_6. The enzyme catalyzes the irreversible dehydrogenation of pyridoxal to 4-pyridoxolactone (Fig. 1). To date, the enzyme has been found in three bacteria: *Pseudomonas* MA-1 (Burg & Snell, 1969), *Ochrobactrum* sp. (Yokochi, Yoshikane, Trongpanich *et al.*, 2004) and *Mesorhizobium loti*, a nitrogen-fixing symbiotic bacterium (Yokochi *et al.*, 2006). The *Ochrobactrum* enzyme is a dimeric protein that belongs to the aldo-keto reductase superfamily (Mindnich & Penning, 2009); its diffraction data have been collected to 2.0 Å resolution (Yokochi, Yoshikane, Yagi *et al.*, 2004). In contrast, the *M. loti* enzyme consists of four subunits each with a molecular weight of 26 000 and belongs to the short-chain dehydrogenase/reductase (SDR) superfamily (Persson *et al.*, 2009); no structure of this enzyme has been reported.

The SDR superfamily is one of the largest enzyme superfamilies, with over 46 000 members in sequence databases and over 300 crystal structures deposited in the PDB. The SDR superfamily proteins share common sequence motifs that define the cofactor-binding site (TG*XXX*G*X*G) and the catalytic tetrad (N-S-Y-K) and their tertiary structures are homologous, with a common α/β folding pattern that is characterized by a central β-sheet typical of a Rossmann fold with helices on either side. In contrast to the general homology in catalytic mechanism and overall tertiary structure, the SDR enzymes show an infinite variety of substrate specificity. The substrate specificity of one SDR enzyme cannot be predicted based on homology in primary or tertiary structure because the differences in substrate specificity are largely the consequence of minor differences in the active-site shape and in the surface properties of the active site of the enzyme (Pilka *et al.*, 2009). Indeed, pyridoxal 4-dehydrogenase was annotated as a 3-oxoacyl-(acyl-carrier protein) reductase in RhizoBase (http://



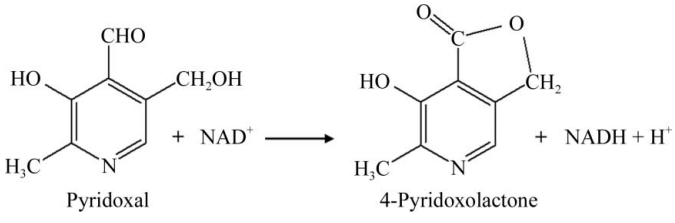


Figure 1
The reaction catalyzed by pyridoxal 4-dehydrogenase. The reaction is irreversible.

genome.kazusa.or.jp/rhizobase/) as it showed the highest identity (40%) to 3-oxoacyl-(acyl-carrier protein) reductase from *Escherichia coli* (PDB code 1q7b; Price *et al.*, 2004). It is the shape and surface properties of the active site of the enzyme that give it a specific high reactivity towards pyridoxal, a pyridine derivative, and make this reaction irreversible. Thus, elucidation of the three-dimensional structure of the enzyme would enhance our understanding of it. Here, we describe the crystallization and preliminary X-ray diffraction studies of tetrameric SDR-type pyridoxal 4-dehydrogenase (SDR-type PLDH) from *M. loti*.

2. Material and methods

2.1. Overexpression and purification of selenomethionine-substituted pyridoxal 4-dehydrogenase

Because the recombinant wild-type enzyme did not provide crystals that were suitable for X-ray data collection, we attempted to crystallize the selenomethionine-substituted enzyme.

E. coli B834 (DE3) cells harbouring plasmid pET6807, which is a pyridoxal 4-dehydrogenase-expressing vector that was prepared as described previously (Yokochi *et al.*, 2006), were cultivated at 310 K with shaking in 200 ml SeMet medium consisting of 2.36%(*w*/*v*) SeMet core medium (Wako Pure Chemicals, Tokyo), 1%(*w*/*v*) glucose, 0.005%(*w*/*v*) L-SeMet, 0.005%(*w*/*v*) ampicillin, 0.025%(*w*/*v*) $MgSO_4.7H_2O$, 0.00042%(*w*/*v*) $FeSO_4.7H_2O$ and 42.5 µ*M* H_2SO_4; when the absorbance of the culture medium at 600 nm reached 0.6, 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside was added to the medium and it was incubated at 310 K for a further 24 h. The cells were harvested by centrifugation at 10 000*g* for 20 min at 277 K, washed thoroughly with 0.9%(*w*/*v*) sodium chloride and stored at 253 K until use. SeMet-substituted pyridoxal 4-dehydrogenase was purified as described previously (Yokochi *et al.*, 2006) but with one additional purification step (an HPLC gel filtration) for crystallization. 5 mg homogeneously purified enzyme was obtained from 1 l medium by sequential column chromatography with Butyl-Toyopearl, QA52 and Shodex KW-803 columns. The flow rate of the final column chromatography step was 0.5 ml min^{-1} and the mobile buffer was 20 m*M* potassium phosphate buffer pH 8.0 containing 0.1%(*v*/*v*) 2-mercaptoethanol, 10% glycerol and 0.1 m*M* NAD^+. The enzyme fractions were collected and dialyzed against 20 m*M* Tris–HCl pH 8.0 containing 0.9 m*M* NAD^+ and 0.1%(*v*/*v*) 2-mercaptoethanol; the concentration of NAD^+ was increased to make the enzyme stable under highly concentrated conditions. The enzyme was concentrated to 10 mg ml^{-1} using an Amicon Ultra concentrator (Millipore, Billerica, USA).

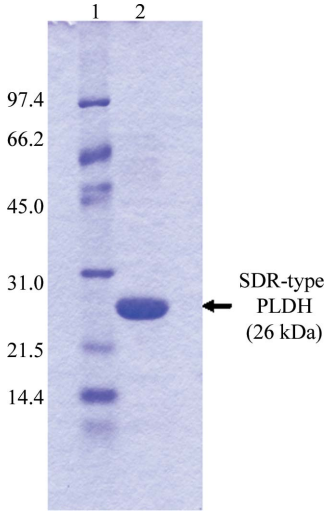


Figure 2
SDS–PAGE analysis of purified SeMet-substituted pyridoxal 4-dehydrogenase. Lane 1, molecular-weight markers (kDa). Lane 2, purified SeMet-substituted pyridoxal 4-dehydrogenase (3 µg). A 10% gel was used.

2.2. Crystallization and X-ray analysis

Initial crystallization conditions were screened using Crystal Screen I, PEG/Ion Screen (Hampton Research, California, USA), Wizard Screens I, II, III and IV (Emerald BioSystems, Washington, USA) and Crystallization Basic Kit for Proteins (Sigma–Aldrich Chemie, Steinheim, Germany). The enzyme was crystallized at 293 K by the sitting-drop vapour-diffusion method using Crystal Clear Strips from Hampton Research. Crystals appeared during equilibration of a drop consisting of a mixture of equal volumes (2 µl) of enzyme solution [10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 containing 0.1%(*v*/*v*) 2-mercaptoethanol and 0.9 m*M* NAD^+] and reservoir solution. The drops were equilibrated against 100 µl reservoir solution [Sigma–Aldrich Crystallization Basic Kit for Proteins condition No. 22; 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000]. After optimization of the conditions, the optimized reservoir solution was 0.1 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000.

The crystal was cryocooled in a nitrogen-gas stream at 100 K directly from the drop (without any cryoprotectant). X-ray diffraction images were collected from the crystals at 100 K in the nitrogen-gas stream on a Rigaku ADSC Q210 CCD detector using synchrotron radiation of wavelength 0.9790 Å at station BL38B1 of SPring-8 (Hyogo, Japan). The crystal-to-detector distance was set to 280.0 mm. Oscillation images of 1.0 ° was recorded with an exposure time of 15 s.

2.3. Enzyme assay

PLDH activity was determined as described previously (Yokochi *et al.*, 2006) by measuring the initial rate of increase in absorbance at 340 nm of NADH plus 4-pyridoxolactone (the product) at 303 K in 1 ml of a reaction mixture consisting of 50 m*M* CHES–NaOH pH 9.2, 1 m*M* pyridoxal, 3 m*M* NAD^+ and the enzyme. The molecular extinction coefficients of NADH and 4-pyridoxolactone at 340 nm are 6220 and 7500 M^{-1} cm^{-1}, respectively. Thus, a molecular extinction coefficient value of 13 720 M^{-1} cm^{-1} was used for the calculation of a unit of enzyme. One unit of enzyme was defined as the amount that catalyzed the formation of 1 mol 4-pyridoxolactone per minute.

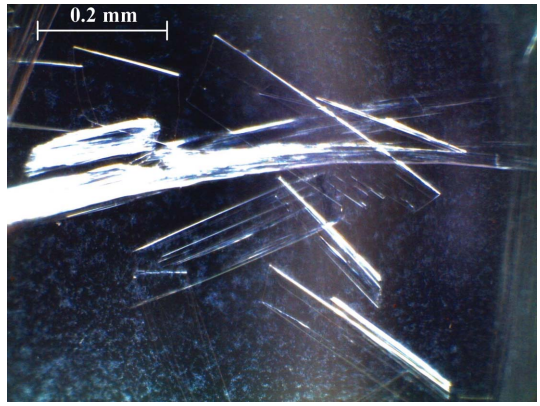


Figure 3
Crystals of SeMet-substituted pyridoxal 4-dehydrogenase.

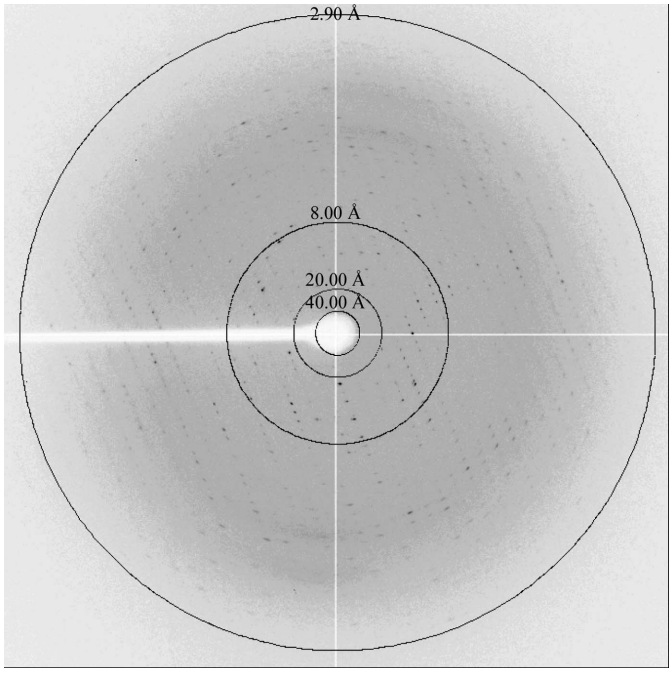


Figure 4
Diffraction image of SeMet-substituted pyridoxal 4-dehydrogenase.

3. Results and discussion

SeMet-substituted SDR-type PLDH was active and was successfully overexpressed in *E. coli* and purified to homogeneity, maintaining significant enzyme activity. The results of SDS–PAGE analysis of the purified enzyme are shown in Fig. 2. The SeMet-substituted enzyme showed slightly higher specific activity (103.9 U mg^{-1}) than recombinant native enzyme (Yokochi *et al.*, 2006).

Crystals suitable for X-ray data collection were obtained (Fig. 3). Interestingly, the wild-type enzyme did not produce crystals suitable for X-ray data collection; they diffracted to only 8 Å resolution. The results suggest that SeMet substitution aids in the stabilization of SDR-type PLDH, as has also been reported for lysozyme (Gassner *et al.*, 1999). Diffraction data were obtained from the crystals in the resolution range 50–2.9 Å (Fig. 4) 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 crystals was determined to be $P2_1$ (monoclinic), with unit-cell parameters a = 86.20, b = 51.11, c = 91.73 Å, β = 89.36°. From the 69 684 total reflections measured for the crystal, 17 931 independent reflections were obtained with an $R_{\rm merge}$ value of 12.6%. The data set was 97.4% complete at the resolution limit of 2.9 Å. The $V_{\rm M}$ value (crystal volume per unit protein molecular weight; Matthews, 1968) for the crystal was calculated to be 2.02 Å^3 Da^{-1} assuming the presence of four molecules of the enzyme in the asymmetric unit, with a solvent content of 39.2%.

Table 1
Data-collection statistics for crystals of SDR-type PLDH.

Values in parentheses are for the highest resolution shell.

X-ray source	Spring-8 (BL38B1)
Wavelength (Å)	0.9790
Detector	ADSC Q210 CCD
Crystal system	Monoclinic
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 86.20, b = 51.11, c = 91.73, β = 89.36
Processing software	*HKL*-2000
Resolution limits (Å)	50–2.90 (3.00–2.90)
Measured reflections	69684
Redundancy	3.9 (3.4)
Unique reflections	17931 (1771)
Completeness (%)	97.4 (97.1)
$\langle I/\sigma(I)\rangle$	12.4 (3.46)
$R_{\rm merge}$† (%)	12.6 (48.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of observed reflection hkl and $\langle I(hkl)\rangle$ is the average intensity for multiple measurements.

The structure determination of SDR-type PLDH is currently under way using the anomalous signal from selenium. In parallel, the molecular-replacement method is being attempted using (*S*)-1-phenylethanol dehydrogenase from the denitrifying bacterium strain EbN1 (PDB code 2ew8; Hoeffken *et al.*, 2006) as the search model.

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