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Crystallization and preliminary crystallographic analysis of D-aspartate oxidase from porcine kidney

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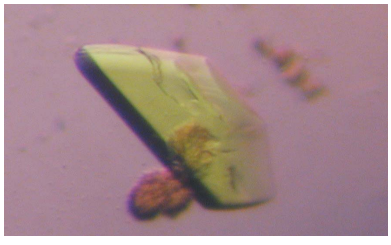

D-Aspartate oxidase (DDO) from porcine kidney was crystallized by the sitting-drop vapour-diffusion method using PEG 8000 as a precipitant. The crystal belonged to space group $P2_1$, with unit-cell parameters a = 79.38, b = 144.0, c = 80.46 Å, β = 101.1°, and diffracted to 1.80 Å resolution. Molecular-replacement trials using the structure of human D-amino-acid oxidase, which is 42% identical in sequence to DDO, as a search model provided a satisfactory solution.

1. Introduction

D-Aspartate exists abundantly as an endogenous substance in the nervous and neuroendocrine systems in mammals (Hashimoto, Kumashiro *et al.*, 1993; Hashimoto, Nikishawa *et al.*, 1993). Recent biological evidence has demonstrated that D-aspartate is an endogenous neurotransmitter (D'Aniello *et al.*, 2011) and is also involved in the synthesis and secretion of hormones in endocrine tissues (D'Aniello *et al.*, 2000). Although two D-amino-acid-degrading oxidases, D-amino-acid oxidase (DAO; EC 1.4.3.3) and D-aspartate oxidase (DDO; EC 1.4.3.1), are found in mammals, endogenous D-aspartate is only degraded by DDO. In DDO-deficient mice, a significant increase in D-aspartate content was observed selectively in tissues in which DDO is highly expressed in wild-type mice (Huang *et al.*, 2006).

DDO requires FAD as a cofactor to catalyze the dehydrogenation of D-aspartate to iminoaspartate, which is nonenzymatically hydrolyzed to oxaloacetate and ammonia. The reduced FAD is reoxidized by O_2 to produce hydrogen peroxide. DDO only degrades acidic amino acids such as D-aspartate, *N*-methyl-D-aspartate and D-glutamate, whereas in mammals neutral and basic D-amino acids are substrates of DAO (Nasu *et al.*, 1982; Negri *et al.*, 1988).

Recently, we purified DDO from porcine kidney, cloned the cDNA and expressed the enzyme in *Escherichia coli* (Yamamoto *et al.*, 2007). The purified enzyme (pDDO) was a homotetramer and showed significant substrate activition of D-aspartate at physiological concentrations of D-aspartate and O_2. Our kinetics study suggested that the oxidized and the reduced forms of pDDO show comparable affinities for D-aspartate and that the reduced enzyme–D-aspartate complex can react with O_2 to produce a catalytically competent oxidized enzyme–D-aspartate complex.

To elucidate the mechanism of substrate activation by pDDO, crystal structure analysis of pDDO is indispensable. Although crystal structures of DAOs from various species have been reported (Mattevi *et al.*, 1996; Umhau *et al.*, 2000; Kawazoe *et al.*, 2006), no crystal structures of DDO have been reported. Here, we report the crystallization and preliminary X-ray crystallographic data of pDDO.

2. Methods and results

2.1. Protein expression, purification and crystallization

pDDO was overexpressed using *E. coli* BL21 (DE3) and purified as described previously (Yamamoto *et al.*, 2007). *E. coli* BL21 (DE3) cells containing a His_6-tagged porcine *ddo* expression plasmid were

Table 1
Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

	meso-Tartrate complex	D-Aspartate complex
Beamline	BL-17A, PF	NW-12A, PF-AR
Oscillation angle (°)	0.5	0.5
Crystal-to-detector distance (mm)	213.9	189.6
Exposure time (s)	10	10
Wavelength (Å)	1.0000	1.0000
Temperature (K)	95	95
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 79.38, b = 144.0, c = 80.46, β = 101.1	a = 79.32, b = 143.8, c = 80.01, β = 100.9
Resolution (Å)	17.0–1.80 (1.89–1.80)	17.0–2.05 (2.15–2.05)
Observations	815711 (109097)	514738 (62167)
Unique reflections	163191 (22081)	108738 (14125)
Completeness (%)	99.7 (99.4)	98.8 (96.7)
Multiplicity	5.0 (4.9)	4.7 (4.4)
Average $I/\sigma(I)$	23.5 (4.2)	18.3 (4.0)
R_{merge} (%)	0.040 (0.470)	0.055 (0.466)
Mosaicity (°)	0.139	0.325

cultured at 310 K for 4–6 h after IPTG induction. After obtaining the cell extracts, the recombinant pDDO was fractionated by ammonium sulfate precipitation. Precipitated recombinant pDDO was dissolved in 20 m*M* sodium phosphate pH 7.4 containing 10 µ*M* FAD, 10 m*M* sodium/potassium L-tartrate, 0.5 *M* sodium chloride and 20 m*M* imidazole and applied onto a HisTrap HP column (Amersham). His_6-tagged pDDO was eluted with 20 m*M* sodium phosphate pH 7.4 containing 10 µ*M* FAD, 10 m*M* sodium/potassium L-tartrate, 0.5 *M* sodium chloride and 0.5 *M* imidazole. Fractions containing His_6-tagged pDDO were concentrated and applied onto a gel-filtration column (TSKgel G3000SW_{XL}, Tosoh) pre-equilibrated with 200 m*M* potassium phosphate pH 6.75 containing 10 µ*M* FAD and 0.3 m*M* EDTA. The obtained His_6-tagged pDDO was digested with enterokinase using an EK Max kit (Invitrogen) for 24 h at 310 K. The resultant protein solution was applied onto a gel-filtration column (TSKgel SuperSW3000, Tosoh) again using the same buffer. SDS–PAGE analysis of the purified recombinant pDDO revealed a single band of 38 kDa, indicating that recombinant pDDO was purified to near-homogeneity. The purified enzyme showed an absorption spectrum with peaks at 374 and 452 nm that were characteristic of the oxidized form of pDDO. The obtained pDDO was concentrated to 18.7 mg ml^{-1} in 10 m*M* sodium phosphate buffer pH 7.4 containing 10 µ*M* FAD and 10 m*M* *meso*-tartrate.

Initial crystallization screening was performed by the hanging-drop vapour-diffusion method using Crystal Screen and Crystal Screen II (Hampton Research, Laguna Niguel, California, USA; Jancarik & Kim, 1991) at 293 K. A hanging drop was prepared by mixing 1.0 µl each of the protein solution and the reservoir solution and was equilibrated against 500 µl reservoir solution. Although no single crystals were obtained in the initial screening, yellow spherulites appeared under two conditions: Crystal Screen condition No. 42 [20%(*w*/*v*) PEG 8000, 50 m*M* potassium phosphate] and Crystal Screen condition No. 36 [8%(*w*/*v*) PEG 8000, 100 m*M* Tris–HCl pH 8.5]. On the basis of these two conditions, the pH value (4.6–8.5) and the concentration of PEG 8000 [5–20%(*w*/*v*)] were systematically optimized, resulting in the appearance of clusters of thin yellow plate-shaped crystals in 10%(*w*/*v*) PEG 8000, 100 m*M* sodium acetate pH 4.6. In order to obtain single crystals, the effects of various salts were examined. Potassium dihydrogen phosphate and sodium dihydrogen phosphate were found to be effective in solving the problem. After several trials, the optimal reservoir solution was finally determined to be 5–10%(*w*/*v*) PEG 8000, 100 m*M* sodium dihydrogen phosphate (or 100 m*M* potassium dihydrogen phosphate), 100 m*M* sodium acetate pH 4.7 using 6.7–18.7 mg ml^{-1} protein solution. The sitting-drop vapour-diffusion method gave larger crystals (approximately 0.5 × 0.2 × 0.02 mm) than the hanging-drop vapour-diffusion method. In addition, anaerobic crystallization in a 'HARD' anaerobic chamber (Hirasawa; Senda *et al.*, 2007) reduced the amount of precipitate, resulting in larger crystals. The largest plate-shaped crystal obtained under anaerobic conditions was used for diffraction data collection (Fig. 1).

Crystallization of the pDDO–D-aspartate complex was performed by the cocrystallization method under anaerobic conditions. pDDO concentrated to 13.7 mg ml^{-1} was mixed with an excess amount of D-aspartate; the molar ratio of pDDO to D-aspartate was approximately 1:20 in the solution. Plate-shaped crystals appeared in 5–10%(*w*/*v*) PEG 8000, 100 m*M* sodium dihydrogen phosphate (or 100 m*M* potassium dihydrogen phosphate), 100 m*M* sodium acetate pH 4.7. The crystals obtained were colourless, suggesting that the pDDO in the crystals was in the reduced form.

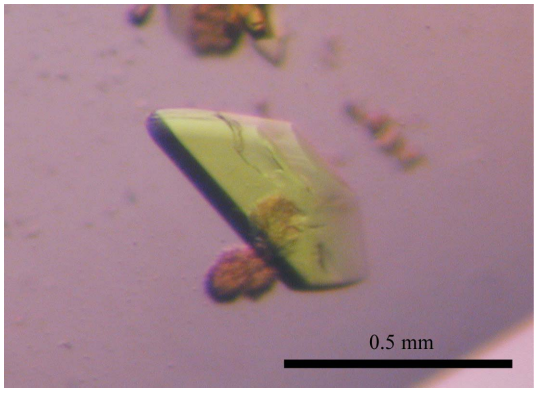

Figure 1
A crystal of the pDDO–*meso*-tartrate complex.

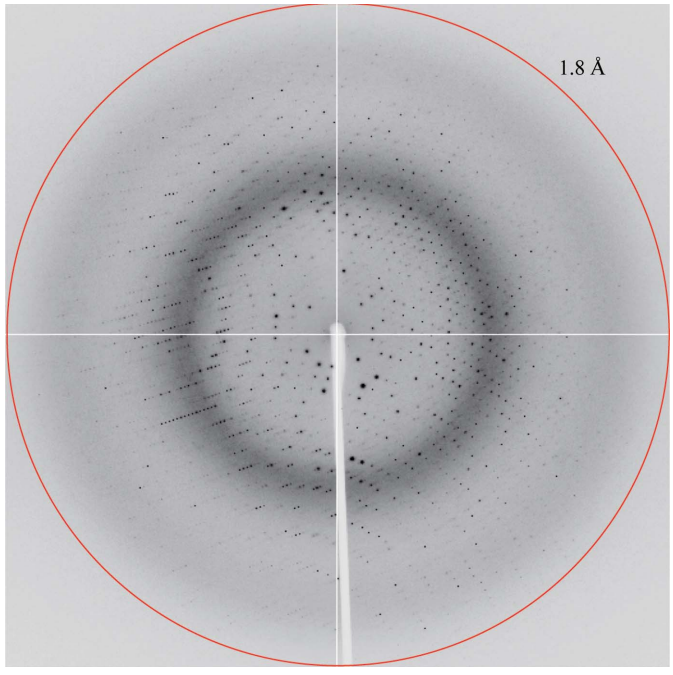

Figure 2
An X-ray diffraction pattern of a pDDO–*meso*-tartrate crystal. The diffraction data were collected on beamline BL-17A of PF using an ADSC Quantum 270 CCD detector.

2.2. X-ray diffraction analysis

Diffraction data were collected from pDDO–*meso*-tartrate and pDDO–D-aspartate crystals at 95 K using an ADSC CCD detector on beamline BL-17A of the Photon Factory (PF; Tsukuba, Japan; Fig. 2) and beamline NW-12A of PF-AR, respectively. Prior to data collection, the pDDO–*meso*-tartrate crystals were soaked in a cryoprotectant solution consisting of 20%(*v*/*v*) glycerol, 20%(*w*/*v*) PEG 8000, 100 m*M* sodium dihydrogen phosphate, 100 m*M* sodium acetate pH 4.7 for 60 s and flash-cooled in an N_2 stream. In order to maintain the reduced state of the pDDO–D-aspartate crystals, they were soaked in a cryoprotectant solution and then cooled using liquid nitrogen under anaerobic conditions.

The diffraction data were processed and scaled using the programs *XDS* and *XSCALE* (Kabsch, 2010). The crystals belonged to space group $P2_1$ (Table 1). Assuming four subunits of pDDO (37.3 kDa) per asymmetric unit of the crystal, the Matthews coefficient (V_M) was calculated to be 3.03 $Å^3$ Da^{-1}, which corresponds to a solvent content of 59.4% (Matthews, 1968).

Molecular-replacement calculations were performed with the program *MOLREP* (Vagin & Teplyakov, 2010) in the *CCP*4 program suite (Winn *et al.*, 2011) using the structure of human D-amino-acid oxidase (PDB entry 2du8; Kawazoe *et al.*, 2006) as a search model. The molecular-replacement calculation yielded a satisfactory solution. Packing analysis showed that there were no close contacts between the subunits. As predicted from the V_M value, the asymmetric unit contains four pDDO molecules. Crystallographic refinement of pDDO–*meso*-tartrate and pDDO–D-aspartate is in progress.

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