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Crystallization and preliminary crystallographic studies of pyridoxal kinase from sheep brain

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Pyridoxal kinase (ATP:pyridoxal 5′-phosphotransferase; EC 2.7.1.35) is a key enzyme in the transformation of vitamin B_6 to pyridoxal-5′-phosphate. Pyridoxal-5′-phosphate is the crucial cofactor required by numerous enzymes involved in the metabolism of amino acids and the synthesis of many neurotransmitters. Pyridoxal kinase from sheep brain was crystallized in an orthorhombic form using the hanging-drop vapour-diffusion method with sodium citrate as the precipitant. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 59.8$, $b = 94.4$, $c = 128.2$ Å, and diffract to a resolution of 2.1 Å. Crystals were transferred into a soaking liquid without citrate and two heavy-atom derivatives were prepared.

1. Introduction

Pyridoxal-5′-phosphate (PLP) is an essential cofactor required by numerous enzymes to catalyze a large variety of reactions in the metabolism of amino acids (Kerry *et al*., 1986). Many neurotransmitters such as dopamine, norepinephrine, serotonin and γ-aminobutyric acid are synthesized by PLP-dependent enzymes (Dakshinamurti *et al.*, 1990). Normal concentration of PLP is a prerequisite for the stability of the nervous system.

Mammals cannot synthesize PLP *de novo*; instead, they require the dietary precursor vitamin B_6, which includes pyridoxal, pyridoxine and pyridoxamine. Pyridoxal kinase catalyzes the phosphorylation of vitamin B_6, which is a key step in the synthesis of PLP. PLP is synthesized in the liver and released into the bloodstream. The circulating PLP must be dephosphorylated before it diffuses through the target-cell membranes. It is then phosphorylated again in the target cell by intracellular pyridoxal kinase; at the same time it is trapped in the cell (Hanna *et al.*, 1997; McCormick & Chen, 1999).

Ubiquitous expression of pyridoxal kinase has been detected in mammalian tissues (Hanna *et al.*, 1997) and has also been found in other species. For example, two genes were identified in the PLP-salvage pathway of *Escherichia coli*, even though bacteria are able to synthesize PLP *de novo* (Yang *et al.*, 1996, 1998).

Many biochemical studies on pyridoxal kinase have been performed (Kwok & Churchich, 1979; Tagaya *et al.*, 1989; Kwok *et al.*, 1987; Scholz & Kwok, 1989; Scholz *et al.*, 1990; Dominici *et al.*, 1988; Wolkers *et al.*, 1991); however, its three-dimensional structure has never been reported. In 1989, the crystallization and preliminary X-ray studies of pyridoxal kinase were described (Arnone *et al.*, 1989). Trigonal crystals could be obtained by this method, but there were defects in the crystals and no suitable heavy-atom derivatives were obtained. Pyridoxal kinase from sheep brain is a homodimer with 312 residues in each monomer and its total molecular weight is 70 kDa. Sequence-homology searches using the *BLAST* program (Madden *et al.*, 1996) on the NCBI website failed to find any other protein in the PDB similar to PLK in sequence (the highest homology found was less than 20%). This indicates that the structure of pyridoxal kinase cannot be solved by the molecular-replacement method.

Recently, we crystallized pyridoxal kinase in an orthorhombic form using sodium citrate as a precipitant. The crystals were transferred into a soaking liquid without citrate and two heavy-atom derivatives were prepared.

2. Materials and methods

2.1. Purification and crystallization

Pyridoxal kinase was purified from sheep brain as described by Kerry *et al.* (1986). Crystallization was performed by the hanging-drop vapour-diffusion method. The concentration of pyridoxal kinase used was 10 mg ml^{-1} in 0.1 *M* sodium citrate pH 5.8. 2 µl of protein solution was mixed with an equal volume of 1.1 *M* sodium citrate pH 5.8 and then equilibrated against 1.1 *M* sodium citrate solution. After incubation for about one month at 290 K, suitable crystals were obtained.

2.2. Transfer of crystals

To prepare heavy-atom derivatives, crystals were transferred into a non-citrate-containing soaking solution containing 20% PEG 8000, 0.1 *M* Na_2SO_4 and 50 m*M* MES buffer pH 5.8 according to the method described by Ray *et al.* (1991). Equal volumes of 1.1 *M* sodium citrate pH 5.8 and 45% PEG 8000 were mixed and vibrated thoroughly to produce a biphasic mixture. After leaving overnight, the salt-rich phase and PEG-rich phase were clarified and then separated. A series of solutions were prepared by mixing the PEG-rich phase and the soaking solution in different proportions. The crystals of pyridoxal kinase were transferred into the salt-rich phase first and then into the PEG-rich phase. They were then transferred in succession into solutions composed of decreasing proportions of PEG-rich phase and increasing proportions of soaking solution and finally into the soaking solution.

2.3. Preparation of heavy-atom derivatives

Heavy-atom reagents were dissolved in the soaking solution. Crystals that had been transferred into the previously mentioned soaking solution were soaked in the heavy-atom solution. Two derivative data sets were obtained from a crystal soaked for 20 h in 0.5 m*M* K_2PtCl_6 and from a crystal soaked for 40 h in 1 m*M* *p*-chloromercuriphenylsulfonic acid (PCMS). Prior to data collection, the crystals were soaked in the soaking solution without heavy atoms for 1 h.

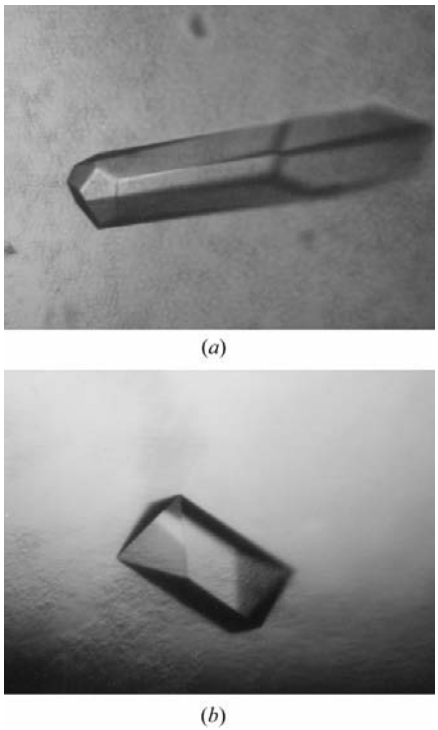

Figure 1
(*a*) Typical trigonal crystal of pyridoxal kinase (0.12 × 0.12 × 0.5 mm); (*b*) typical orthorhombic crystal of pyridoxal kinase (0.15 × 0.15 × 0.4 mm).

2.4. Data collection and preliminary analysis

All data were collected at room temperature. Native data were collected with a Weissenberg camera at beamline BL6B of the Photon Factory (Tsukuba, Japan). The X-ray wavelength was 1.000 Å and the crystal-to-detector distance was 573 mm. A total of 23 image-plate frames were collected with 4.5° oscillations each. Data for the heavy-atom derivatives were collected using the MAR345 image plate of the National Laboratory of Biomacromolecules (Beijing, China). The X-ray wavelength was 1.5418 Å. The crystal-to-detector distance was 140 mm and 1° oscillation images were taken with an exposure time of 7 min each.

All data were processed using the *DENZO*/*SCALEPACK* programs (Otwinowski & Minor, 1997). The self-rotation function of the native data was calculated using the *POLARRFN* program (Collaborative Computational Project, Number 4, 1994). Isomorphous difference Patterson maps and isomorphous difference Fourier maps were calculated with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The heavy-atom parameters were refined with the *MLPHARE* program (Otwinowski, 1991).

Table 1
Data-collection statistics.

Data statistics for the last shell are given in parentheses.

Data set	Native	PCMS	K_2PtCl_6
Resolution (Å)	20–2.1 (2.15–2.10)	20–2.9 (2.95–2.90)	20–2.9 (2.95–2.90)
Total reflections	182984	117789	107721
Unique reflections	42820	16605	16461
Completeness (%)	99.1 (98.7)	99.9 (100.0)	100.0 (100.0)
R_{merge}† (%)	6.6 (35.3)	13.5 (47.4)	13.5 (48.4)
Mean $\langle I/\sigma(I)\rangle$	20.4 (4.1)	15.7 (4.5)	14.2 (3.8)
No. of heavy-atom sites		4	4
Figure of merit		0.31	0.30

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of the reflection h and $\langle I(h)\rangle$ is the mean value of the $I(h,i)$ for all i measurements.

3. Results and discussion

Pyridoxal kinase was purified from sheep brain using a procedure involving ammonium sulfate fractional precipitation and three chromatographic separations. The final preparation presented a single band on both denaturing and non-denaturing PAGE gels. According to the method described by Arnone *et al.* (1989), trigonal crystals with excellent morphology were obtained (Fig. 1*a*). The crystals diffract to 2.2 Å, but the diffraction intensities are dispersed along distinct directions, indicating that the crystal packing was somewhat disordered. Attempts to prepare suitable heavy-atom derivatives using these crystals were unsuccessful.

Crystallization screenings were then carried out. The results of these screenings indicated that a microcrystal-growth environment occurred using sodium citrate as a buffer and PEG as a precipitant. Extensive optimization of this environment resulted in long thin crystals only. Using a high concentration of sodium citrate instead of PEG as a precipitant, shorter and thicker crystals appeared. After careful refinement of the conditions by adjusting the pH and the concentration of sodium citrate, crystals grew to dimensions of 0.15 × 0.15 × 0.4 mm (Fig. 1*b*). These crystals gave a good diffraction pattern.

Crystals of pyridoxal kinase grown using the above conditions belong to the $P2_12_12_1$ space group with unit-cell parameters a = 59.8, b = 94.4, c = 128.2 Å, with a resultant unit-cell volume of 723 700 $Å^3$. The native crystals diffracted to 2.1 Å using a synchrotron X-ray source. The resolution of the heavy-atom derivative data was 2.9 Å. Statistics for the data are shown in Table 1. The number of monomers per asymmetric unit was predicted to be two, corresponding to a V_M (Matthews, 1968) of 2.58 $Å^3$ Da^{-1} and a solvent content of 52%. The peak on $\kappa = 180°$ section of the self-rotation function of pyridoxal kinase indicates that the two monomers are related by a non-crystallographic twofold axis.

The high concentration of sodium citrate in the crystallization condition may hamper the binding of heavy atoms, so the crystals were transferred to the soaking solution without sodium citrate. During the transfer, the resolution of the crystals hardly changed. Two heavy-atom derivatives were prepared in the soaking liquor containing PEG. Inspection of the Harker section of the isomorphous difference Patterson synthesis map of PCMS revealed the position of two Hg atoms (Fig. 2). The other two Hg atoms were located using difference maps. Simi-

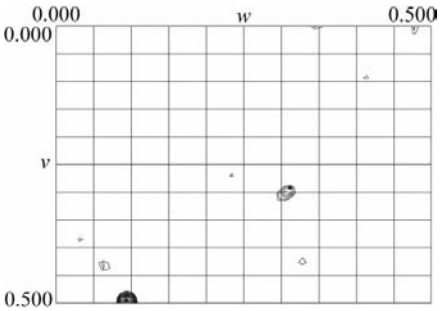

Figure 2
Harker section (0.5, *v*, *w*) of the difference Patterson map for the *p*-chloromercuriphenylsulfonic acid derivative. The black dots in the section show the positions of the Harker peaks predicted from the refined positions of two Hg sites.

larly, the positions of four Pt atoms of the K_2PtCl_6 derivative were determined. The heavy-atom positions were confirmed by cross peaks in the difference Patterson maps. The heavy-atom parameters were refined and the figure of merit was calculated (Table 1). More derivatives will be prepared in order to solve the three-dimensional structure of pyridoxal kinase.

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