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Crystallization and preliminary X-ray crystallographic analysis of PdxJ, the pyridoxine 5′-phosphate synthesizing enzyme

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The enzyme PdxJ catalyzes the condensation of 1-deoxy-D-xylulose-5-phosphate (DXP) and 1-amino-3-oxo-4-(phosphohydroxy)propan-2-one to form pyridoxine 5′-phosphate (PNP). The protein from *Escherichia coli* has been crystallized in several forms under different conditions. The best diffracting crystals were obtained by a combination of the hanging-drop vapour-diffusion and microseeding techniques. Using an in-house image plate, the PdxJ crystals diffracted under cryo-conditions to 2.6 Å resolution. The space group has been determined as $C222_1$, with unit-cell parameters $a = 132.5$, $b = 154.4$, $c = 131.4$ Å, corresponding to four monomers per asymmetric unit. In the search for heavy-atom derivatives, a mercury derivative has been interpreted. The 12 mercury sites located are related by 222 symmetry and, in combination with self-rotation search analyses and gel-filtration experiments, indicate the quaternary assembly of PdxJ into octamers with 422 symmetry.

1. Introduction

Pyridoxal 5′-phosphate (PLP), the biocatalytically active form of vitamin B_6 (pyridoxine, pyridoxol; PN), acts as a central coenzyme in amino-acid metabolism. Pyridoxine is converted to PNP by the kinase PdxK (Yang *et al.*, 1996). PNP and also pyridoxamine 5′-phosphate are then oxidized by PdxH to PLP, which in turn can be transaminated to PNP. In addition to these salvage reactions, there is a biosynthetic pathway to yield an initial substrate that can be recycled. While bacteria, plants and fungi contain the enzymatic machinery to synthesize PN and PNP (Dempsey, 1987; Hill & Spenser, 1986; Tryfiates, 1986), mammals lack such a biosynthetic pathway and are limited to transforming vitamin B_6 obtained in the diet into the other five vitamers.

In *E. coli*, the products of the three genes *pdxA*, *pdxJ* and *pdxH* have been reported to be responsible for PLP biosynthesis (Lam & Winkler, 1992; Notheis *et al.*, 1995; Zhao & Winkler, 1994). PdxA catalyzes the oxidation of 4-(phosphohydroxy)-L-threonine to 1-amino-3-oxo-4-(phosphohydroxy)propan-2-one (Cane *et al.*, 1998) in an NAD^+-dependent reaction. The condensation and subsequent ring-closure reaction of 1-deoxy-D-xylulose-5-phosphate and 1-amino-3-oxo-4-(phosphohydroxy)propan-2-one to yield PNP is carried out by PdxJ (Laber *et al.*, 1999). The PNP oxidation to PLP, the last step in the biosynthesis pathway, is catalyzed by PdxH oxidase (Dempsey, 1980; Hill & Spenser, 1986). Recent studies of PLP biosynthesis and the established roles of PdxA and PdxJ confirm that the B_6 vitamers are synthesized *de novo* and are not only interconverted into each other (Dempsey, 1966; Hockney & Scott, 1979).

PdxJ consists of a single polypeptide chain of 242 amino acids (27.5 kDa) which appears to form a single domain. Cane *et al.* (1998) have reported that the enzyme is a monomer in solution. From a mechanistic point of view, PdxJ is the most interesting of the three enzymes involved in PLP biosynthesis because it catalyzes the complicated ring-closure reaction yielding PNP. Furthermore, PdxJ is a potential target for the development of new antibiotics as its occurrence is restricted to bacteria.

However, none of the *E. coli* enzymes involved in PLP biosynthesis has yet been structurally characterized and no apparent homology to any other protein has been reported for any of them. Therefore, the determination of the PdxJ crystal structure and the analysis of its active-site architecture should be extremely helpful in gaining insight into the chemically demanding steps that take place during PNP biosynthesis. Here, we present the crystallization and preliminary X-ray diffraction data of PdxJ.

2. Materials and methods

2.1. General methods

The PdxJ protein from *E. coli* was cloned, overexpressed and purified as reported previously (Laber *et al.*, 1999). Briefly, the *pdxJ* gene was inserted into the vector pASK-IBA3

Table 1
Crystal characteristics.

		Class II	
	Class I	Native	Thiomersal
Space group	*P*2	$C222_1$	$C222_1$
Unit-cell parameters (Å, °)	a = 87.7	a = 132.5	a = 131.4
	b = 184.6	b = 154.4	b = 155.1
	c = 146.0	c = 131.4	c = 130.1
	β = 104.0		
Diffraction limit (Å)	3.3	2.6	2.6
Mosaicity (°)	0.6	0.3	0.4

Table 2
Data-collection statistics.

	Class II, native	Class II, thiomersal
Temperature (K)	100	100
Resolution range (Å)	25.0–2.6	25.0–2.6
Observed reflections [$I > 0\sigma(I)$]	307023	194829
Unique reflections [$I > 0\sigma(I)$]	40732	40605
Completeness (%)		
Overall	97.3	95.4
Outer shell†	97.3	95.4
$I/\sigma(I)$		
Overall	8.2	5.6
Outer shell†	3.7	2.0
R_{sym}‡ (%)		
Overall	8.4	12.1
Outer shell†	19.2	32.4
Multiplicity		
Overall	4.5	2.8
Outer shell†	4.1	2.3

† Outer-shell data is in the resolution range 2.73–2.60 Å. ‡ $R_{sym} = \sum|I - (I)|/\sum(I)$.

(IBA-Institüt für Bioanalytik GmbH, Göttingen), resulting in the construct pPDXJ1. This plasmid has a C-terminal Strep-tag II (Schmidt *et al.*, 1996) affinity peptide. The gene was expressed in the *E. coli* JM83 strain in Luria–Bertani (LB) medium containing 100 µg ml^{-1} ampicillin. Purification on an affinity Strep Tactin column (IBA) yielded approximately 20 mg protein per liter of cell culture.

2.2. Crystallization

The Hampton Research Crystal Screens I and II and our in-house factorial solutions were used to carry out initial crystallization trials. For this purpose, purified PdxJ (6 mg ml^{-1} in 2 m*M* Tris–HCl pH 8.0) was mixed and equilibrated against a 500 µl reservoir in a sitting-drop vapour-diffusion setup in a 2:1 ratio (3 µl protein solution and 1.5 µl crystallization solution). Two conditions at 293 K yielded diffracting crystals with well defined morphologies, reported here as different classes.

2.2.1. Class I. Triangular-shaped crystals were obtained using 0.1 *M* sodium acetate pH 4.6, 8% PEG 4000 as the precipitant and 0.1 *M* L-cysteine as an additive. Crystals appeared after 10 d (Fig. 1*a*).

2.2.2. Class II. Well diffracting rod-shaped crystals were grown using 10% PEG 6000 and 2 *M* NaCl as reservoir solution (Fig. 1*b*). Unfortunately, two problems were encountered: (i) slow crystal growth that took around six weeks and (ii) reproducibility of the crystals. Even intensive screening of crystallization parameters such as temperature, ratio of protein and reservoir, pH, protein and precipitant concentration and additives did not improve the reproducibility or the rate of crystal growth.

Crystallization could be improved using the microseeding technique. For this purpose, small crystals were crushed into small fragments. Using a rabbit hair, seeds were placed into fresh drops consisting of an equal volume of precipitant and a more concentrated protein solution (13.5 mg ml^{-1} in 2 m*M* Tris–HCl pH 8.0) and were equilibrated for 1 d. Different crystallization setups were tried, but only the hanging-drop method yielded suitable crystals. Large single crystals with a new morphology started to grow after 1 d and reached their maximum size after one week.

2.3. Data collection

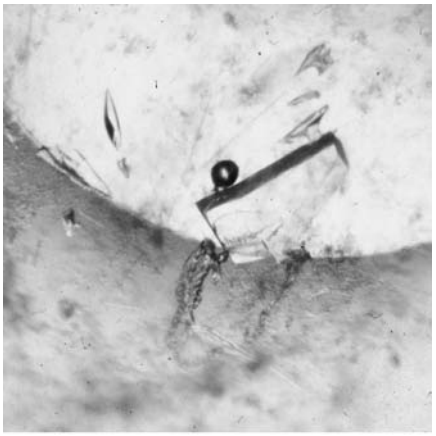
2.3.1. Class I. The diffraction quality of this crystal form allowed us to collect a complete data set of 90 frames (1° oscillation range, 1500 s exposure time) to a resolution of 4.5 Å. A single crystal was mounted in a siliconized thin-wall glass capillary. For all experiments reported here the data was collected with our in-house MAR Research (Hamburg, Germany) image-plate system mounted on a Rigaku (Tokyo, Japan) rotating-anode generator operating at 50 kV and 100 mA (Cu $K\alpha$ radiation, λ = 1.5418 Å). After several hours of exposure, the diffraction power of the crystal had decreased from 3.3 to 5.0 Å resolution (Table 1).

2.3.2. Class II. Preliminary X-ray diffraction studies at room temperature showed that this crystal class belongs to a different space group. Initially, the resolution was 3.0 Å but, as was the case with class I crystals, it decreases rapidly upon X-ray exposure. As an attempt to avoid excessive radiation damage, a cryocooling condition was established. A cryobuffer consisting of the same precipitant supplemented with 10%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD) turned out to be suitable. Crystals were soaked for 10 s in the cryobuffer and were frozen in a nitrogen stream at 100 K (Oxford Cryosystems Cryostream). Under these conditions, a complete data set to 2.6 Å resolution was collected using a 1° oscillation range with an exposure time of 1200 s. Crystals of this class were used for further soaking attempts and a heavy-atom search.

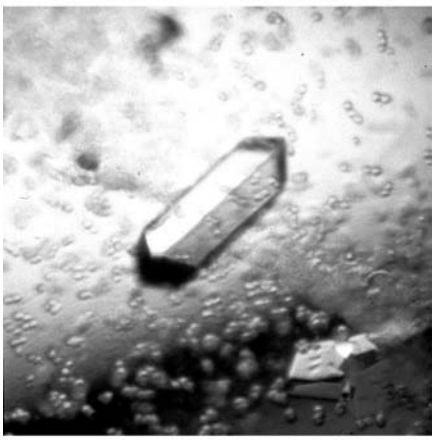
Indexing and integration of diffraction data from both crystal forms was performed using *DENZO* (Otwinowski & Minor, 1997). The data were scaled and merged using the *SCALA* program (Evans, 1991) and were placed on an absolute scale using *TRUNCATE* (French & Wilson, 1978).

2.4. Soaking with PLP

Owing to the similarity of PLP to PNP, the product of the PdxJ-catalyzed reaction, PLP, probably acts as a feedback inhibitor of its own synthesis. In order to determine whether PLP binds to PdxJ, crystals were soaked in solutions of different PLP concentrations (at 293 K in the dark). After 2 h, crystals soaked at 1–10 m*M* PLP



(*a*)



(*b*)

Figure 1
(*a*) Class I and (*b*) class II crystals of the PdxJ enzyme.

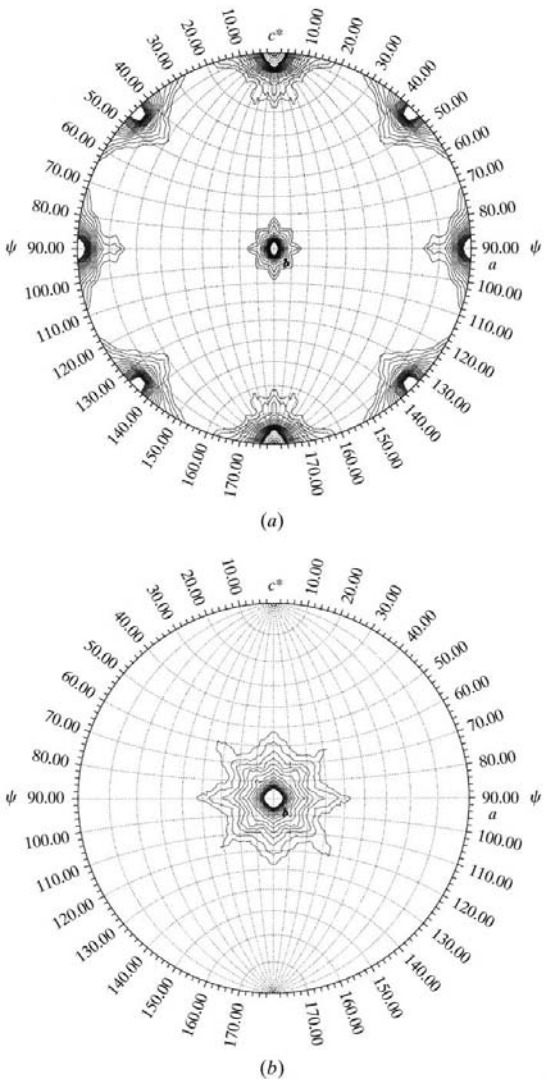


Figure 2
Stereographic projection of the self-rotation function in spherical polar angles. Diffraction data in the resolution range 15.0–2.6 Å were used, with a Patterson integration radii of 30 Å. (*a*) $\kappa = 180^\circ$, with a peak high of 23.8 and 12.5σ for the crystallographic and the non-crystallographic dyad axes, respectively. (*b*) $\kappa = 90^\circ$, with a maximum peak height of 15.7σ.

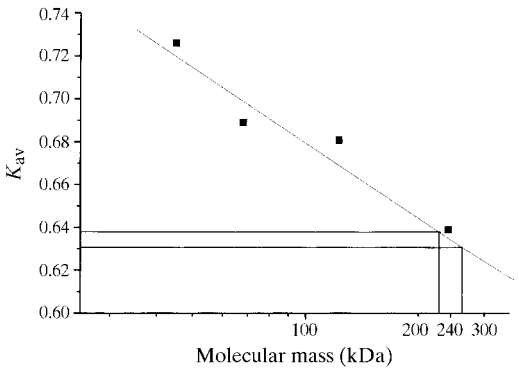


Figure 3
Native molecular mass estimation of PdxJ as performed by gel filtration (Superose12, Pharmacia).

acquired a yellow colour. Crystals in 10 m*M* PLP started to develop cracks, while crystals in 1 m*M* seemed to be unaffected. However, all PLP-treated crystals (2 h soaking and a few seconds backsoaking in cryobuffer) completely lost their diffraction power. Even with synchrotron radiation (beamline BW6, DESY, Hamburg) no reflections could be observed. The crystal cracking and associated loss of crystal order indicated that PLP is inducing spatial rearrangements of the PdxJ which are not tolerated by the class II crystal form. Further co-crystallization experiments are under way.

2.5. Heavy-atom derivatives

One heavy-atom derivative has been interpreted succesfully. After soaking native protein crystals in 20 µl of an appropriately buffered solution containing 1 m*M* thiomersal (ethyl mercury thiosalicylate; EMTS; $C_9H_9HgO_2SNa$) for one week, we collected a complete derivative data set to 2.6 Å resolution.

2.6. Gel filtration

In order to estimate the oligomeric state of PdxJ in solution, 6 µl of sample was loaded on a Superose12 SMART column (Pharmacia) equilibrated with 100 m*M* Tris–HCl pH 8.0 at room temperature. As a control for the size of the separated peaks, four proteins were used to calibrate the column under the same pH and temperature conditions. These markers (Boehringer-Mannheim) were albumin (45 and 68 kDa), aldolase (158 kDa) and katalase (240 kDa), which covered the range expected for monomers to octamers of PdxJ.

3. Results and discussion

The space group for class I crystals was found to be *P*2, with unit-cell parameters $a = 87.7$, $b = 184.6$, $c = 146.0$ Å, $\beta = 104.0^\circ$. The class II crystals belongs to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 134.5$, $b = 154.6$, $c = 133.4$ Å at 293 K. The cryocooled crystals have contracted unit-cell parameters: $a = 132.5$, $b = 154.4$, $c = 131.4$ Å. Thiomersal-soaked crystals were indexed with the same space group and unit-cell parameters $a = 131.4$, $b = 155.1$, $c = 130.1$ Å, and are isomorphous to the natives (Table 1; Fig. 1). Data-collection statistics are summarized in Table 2.

The data from the native and the possible derivative were merged using *CAD* and scaled with *SCALEIT* (Collaborative Computational Project, Number 4, 1994). *SOLVE* (Terwilliger & Berendzen, 1999) was used to find and refine eight heavy-atom positions. Afterwards, four new sites were found using the program *SHARP* (La Fortelle *et al.*, 1997) and all 12 positions, related by 222 symmetry, were refined. The phases were then calculated, resulting in an overall phasing power of 1.9 and a figure of merit of 0.36 for the whole resolution range (20.0–2.6 Å).

Based either on four (or five) molecules per asymmetric unit (ASU), the solvent content is calculated to be 49% (36%), corresponding to a Matthews coefficient V_M of 2.39 Å^3 Da^{-1} (1.91 Å^3 Da^{-1}) for class I (Matthews, 1968). Assuming four molecules per ASU ($V_M = 2.77$ Å^3 Da^{-1}), class II crystals contain approximately 56% solvent.

Self-rotation functions were calculated on the scaled data from class II native crystals (Fig. 2) using the program *GLRF* (Tong & Rossmann, 1997). In the $\kappa = 180^\circ$ section (Fig. 2*a*), in addition to the peaks corresponding to the crystallographic twofold axis, a local dyad in the direction of the *ac* diagonal is obvious. The $\kappa = 90^\circ$ section (Fig. 2*b*) presents a strong peak arising from a local fourfold axis parallel to *b*. Assuming four PdxJ molecules per ASU, these calculations, together with the 222 symmetry deduced from the heavy-atom positions, suggest a 422 symmetric octamer of PdxJ. It had been previously reported by Cane *et al.* (1998) that PdxJ is active as a monomer. In order to confirm our suggestion that PdxJ is an octamer in solution, we performed a gel-filtration experiment (Superose12, Pharmacia). In several runs, PdxJ eluted between K_{av} values of 0.630 and 0.638, corresponding to a molecular mass of 240 ± 10 kDa (Fig. 3). With the molecular weight of 27.5 kDa calculated from its sequence, the gel filtration confirms that PdxJ is an octamer.

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