Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

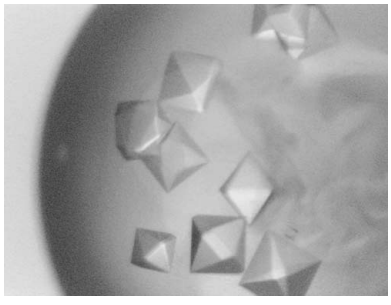
Jun Yong Ha,‡ Ji Hyun Lee,‡ Kyoung Hoon Kim, Do Jin Kim, Hyung Ho Lee, Hye-Kyung Kim, Hye-Jin Yoon and Se Won Suh*

Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea

‡ These authors contributed equally to this work.

Correspondence e-mail: sewonsuh@snu.ac.kr

Received 13 August 2005
Accepted 6 January 2006



Overexpression, crystallization and preliminary X-ray crystallographic analysis of erythronate-4-phosphate dehydrogenase from *Pseudomonas aeruginosa*

The enzyme erythronate-4-phosphate dehydrogenase catalyses the conversion of erythronate-4-phosphate to 3-hydroxy-4-phospho-hydroxy-α-ketobutyrate. It belongs to the D-isomer-specific 2-hydroxyacid dehydrogenase family. It is essential for *de novo* biosynthesis of vitamin B_6 (pyridoxine). Erythronate-4-phosphate dehydrogenase from *Pseudomonas aeruginosa*, a homodimeric enzyme consisting of two identical 380-residue subunits, has been overexpressed in *Escherichia coli* with a C-terminal purification tag and crystallized at 297 K using 0.7 *M* ammonium dihydrogen phosphate, 0.4 *M* ammonium tartrate, 0.1 *M* sodium citrate pH 5.6 and 10 m*M* cupric chloride. X-ray diffraction data were collected to 2.20 Å from a crystal grown in the presence of NADH. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 84.77, b = 101.28, c = 142.58 Å. A dimeric molecule is present in the asymmetric unit, giving a crystal volume per protein weight (V_M) of 3.64 Å^3 Da^{-1} and a solvent content of 66%.

1. Introduction

Pyridoxal-5′-phosphate, the biologically active form of vitamin B_6 (pyridoxine), is an essential cofactor for a large number of metabolic enzymes. Mammals are unable to synthesize vitamin B_6 *de novo* and thus vitamin B_6 is an essential component of human diet. However, bacteria, fungi, parasites and plants possess functional vitamin B_6 synthesis machinery (Tanaka *et al.*, 2005). Two distinct biosynthetic pathways for *de novo* synthesis of vitamin B_6 have been recognized: the *Escherichia coli*-like PdxA/PdxJ pathway and the fungi-like PDX1/PDX2 pathway. The former pathway consists of the *pdx* family enzymes (PdxA, B, C, F, H, J and GapA) and seems to be restricted to the γ subdivision of proteobacteria (di Salvo *et al.*, 2003). The second pathway requires two proteins (PDX1/PdxS/SNZ/SOR1/PYROA and PDX2/PdxT/SNO). Genes coding for similar proteins are highly conserved in plants, sponges, plasmodia, archaea, yeast and many bacteria (Garrido-Franco, 2003; Banks & Cane, 2004; Seack *et al.*, 2001; Wrenger *et al.*, 2005; Belitsky, 2004).

The Pdx family is best characterized in *E. coli*. Biosynthesis of vitamin B_6 starts with D-erythrose-4-phosphate, which is oxidized by erythrose-4-phosphate dehydrogenase (Epd, GapB) to 4-phospho-erythronate. 4-Phosphoerythronate is further oxidized by erythronate-4-phosphate dehydrogenase (PdxB) and transaminated by the PdxF (SerC) enzyme to form 4-phosphohydroxy-L-threonine, which is then converted to pyridoxine 5′-phosphate by 4-hydroxythreonine-4-phosphate dehydrogenase (PdxA) and pyridoxine-5-phosphate synthase (PdxJ) (Yang *et al.*, 1998; Garrido-Franco, 2003). Four of the enzymes, erythrose-4-phosphate dehydrogenase (Epd, GapB), erythronate-4-phosphate dehydrogenase (PdxB), 4-hydroxy-threonine-4-phosphate dehydrogenase (PdxA) and pyridoxine-5-phosphate synthase (PdxJ), are unique to this pathway. While none of these enzymes are present in mammals, they are essential for eubacteria that employ this Pdx pathway. Thus, each of the four proteins represents an attractive target for the design of new antibiotics (Banks & Cane, 2004).

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacterium that belongs to the γ subdivision of proteobacteria. It is one of the top three causes of opportunistic human infections. Infection of this pathogen is life-threatening in cystic

fibrosis patients. The *pdxB* gene of *P. aeruginosa* encodes erythronate-4-phosphate dehydrogenase (EC 1.1.1.–), a 380-residue protein (41 002 Da). *P. aeruginosa* PdxB shows an amino-acid sequence identity of 48% to the *E. coli* enzyme. As the first step toward structure determination of *P. aeruginosa* PdxB, we overexpressed it in *E. coli* and crystallized it in the presence of its cofactor NADH. Its crystallization conditions and preliminary X-ray crystallographic data are reported here.

2. Experimental

2.1. Protein expression and purification

The erythronate-4-phosphate dehydrogenase gene (*pdxB*; PA1375) was amplified by the polymerase chain reaction using the genomic DNA of *P. aeruginosa* strain PAO1 as template. The forward and reverse oligonucleotide primers designed using the published genome sequence were 5′-G GAA TTC **CAT ATG** CGT ATT CTC GCC GAT GAA AA-3′ and 5′-CCG CCG **CTC GAG** GAC CAA CTG CGC CCC CAG C-3′, respectively, where the bases in bold represent the *Nde*I and *Xho*I restriction-enzyme cleavage sites. The amplified DNA was inserted into the *Nde*I/*Xho*I-digested expression vector pET-21a(+) (Novagen). This vector construction adds an eight-residue tag (LEHHHHHH) to the carboxyl-terminus of the gene product to facilitate protein purification. The protein was overexpressed in *E. coli* C41(DE3) cells (Miroux & Walker, 1996). The cells were grown at 310 K to an OD_{600} of 0.5 in Terrific Broth medium containing 50 µg ml^{-1} ampicillin and protein expression was induced by 1.0 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). Cells growth continued at 288 K for 24 h after IPTG induction and cells were harvested by centrifugation at 4200*g* (6000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* sodium chloride, 5 m*M* imidazole) and was then homogenized with an ultrasonic processor. The crude cell extract was centrifuged at 36 000*g* (18 000 rev min^{-1}; Hanil Supra 21K rotor) for 1 h at 277 K and the recombinant protein in the supernatant fraction was purified in two chromatographic steps. The first step utilized the hexahistidine tags by metal-chelate chromatography on Ni–NTA resin (Qiagen). Next, gel filtration was performed on a HiLoad XK 16 Superdex 200 prep-grade column (Amersham Pharmacia), which was previously equilibrated with buffer *A* (20 m*M* Tris–HCl pH 7.5) containing 100 m*M* sodium chloride. The homogeneity of the purified protein was assessed by SDS–PAGE (Laemmli, 1970). The protein solution was concentrated using a YM10 ultrafiltration membrane (Millipore, Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 50 070 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

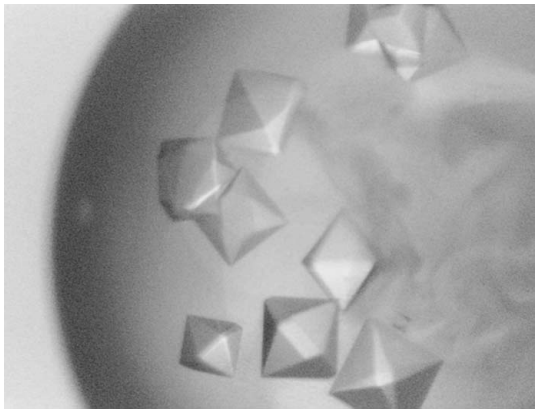


Figure 1
Crystals of erythronate 4-phosphate from *P. aeruginosa*. Their approximate dimensions are 0.20 × 0.20 × 0.20 mm.

2.2. Crystallization and dynamic light scattering

Crystallization was performed at 297 K by the hanging-drop vapour-diffusion method using 24-well VDX plates (Hampton Research). Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I and II, SaltRX, Index I and II, PEG/Ion and MembFac) and from deCODE Biostructures Group (Wizard I and II). To grow the best crystals, each hanging drop was prepared on a siliconized cover slip by mixing 2 µl each of the protein solution (at 90–100 mg ml^{-1} concentration in a buffer consisting of 20 m*M* Tris–HCl pH 7.5, 100 m*M* sodium chloride, 10 m*M* dithiothreitol, 10 m*M* NADH) and the reservoir solution (0.7 *M* ammonium dihydrogen phosphate, 0.4 *M* ammonium tartrate, 0.1 *M* sodium citrate pH 5.6, 10 m*M* cupric chloride). Each hanging drop was placed over 0.48 ml reservoir solution. Dynamic light-scattering experiments were performed using a DynaPro-801 instrument from Wyatt (Santa Barbara, CA, USA). The data were measured at 297 K with the protein at 1 mg ml^{-1} concentration in 20 m*M* Tris–HCl pH 7.5, 100 m*M* sodium chloride.

2.3. X-ray diffraction experiment

The crystals were flash-cooled using a cryoprotectant solution consisting of 0.7 *M* ammonium dihydrogen phosphate, 0.4 *M* ammonium tartrate, 0.1 *M* sodium citrate pH 5.6, 10 m*M* cupric chloride, 30%(*v*/*v*) glycerol. Crystals were soaked in 5 µl cryoprotectant solution for 10 s before being flash-cooled in liquid nitrogen. X-ray diffraction data were collected at 100 K at three different wavelengths using an Area Detector System Corporation Quantum 210 CCD detector at experimental station NW12A of the Photon Factory, Japan. The crystal was rotated through a total of 180°, with a 1.0° oscillation range per frame. The raw data were processed and scaled using the *HKL*2000 program package (Otwinowski & Minor, 1997).

3. Results

When we expressed erythronate-4-phosphate dehydrogenase from *P. aeruginosa* in *E. coli* as a fusion with both the N-terminal 20-residue tag (MGSSHHHHHHSSGLVPRGSH) and the C-terminal eight-residue tag (LEHHHHHH), it was highly expressed but the protein tended to aggregate easily. When we expressed it with only the C-terminal tag, it was also highly overexpressed but aggregation was less severe. Therefore, we screened crystallization conditions using this construct. The yield was ∼100 mg purified enzyme per litre of culture. The native molecular weight (∼83 kDa) of the recombinant *P. aeruginosa* erythronate-4-phosphate dehydrogenase, as estimated by dynamic light-scattering analysis, indicates that the enzyme exists as a homodimer in solution (the calculated monomer weight including the C-terminal tag is 42 067 Da). This is the first report on the oligomeric state of *P. aeruginosa* erythronate-4-phosphate dehydrogenase.

Despite the presence of the C-terminal tag, the recombinant enzyme readily formed well diffracting crystals. The best crystals were grown with reservoir solution consisting of 0.7 *M* ammonium dihydrogen phosphate, 0.4 *M* ammonium tartrate, 0.1 *M* sodium citrate pH 5.6, 10 m*M* cupric chloride. Primitive orthorhombic crystals grew to dimensions of 0.2 × 0.2 × 0.2 mm within a few days (Fig. 1). The

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.33–2.20 Å).

X-ray wavelength (Å)	1.0000
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 84.77, b = 101.28, c = 142.58
Resolution range (Å)	30.0–2.20
Total/unique reflections	1201321/63477
$R_{\rm merge}$† (%)	3.9 (33.4)
Data completeness (%)	99.9 (99.9)
Average $I/\sigma(I)$	36.5 (4.3)

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

presence of NADH in the hanging drop improved the reproducibility of crystallization, but similar crystals could also be grown in its absence. A set of diffraction data was collected to 2.20 Å from a flash-cooled crystal. Table 1 summarizes the statistics of data collection. If it is assumed that a dimeric molecule is present in the crystallographic asymmetric unit, the crystal volume per protein weight ($V_{\rm M}$) is 3.64 Å^3 Da^{-1} and the solvent content is 66% by volume. We have solved the structure using the multiwavelength data and the refined model shows a molecule of NADH in each subunit.

This work was supported by a grant from the Korea Ministry of Science and Technology (NRL-2001). JYH, JHL, KHK, DJK and HHL are supported by BK21 Fellowships.

References

Banks, J. & Cane, D. E. (2004). *Bioorg. Med. Chem. Lett.* **14**, 1633–1636.
Belitsky, B. R. (2004). *J. Bacteriol.* **186**, 1191–1196.
di Salvo, M. L., Safo, M. K., Musayev, F. N., Bossa, F. & Schirch, V. (2003). *Biochim. Biophys. Acta*, **1647**, 76–82.
Garrido-Franco, M. (2003). *Biochim. Biophys. Acta*, **1647**, 92–97.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Miroux, B. & Walker, J. E. (1996). *J. Mol. Biol.* **260**, 289–298.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Seack, J., Perovic, S., Gamulin, V., Schroder, H. C., Beutelmann, P., Muller, I. M. & Muller, W. E. (2001). *Biochim. Biophys. Acta*, **1520**, 21–34.
Tanaka, T., Tateno, Y. & Gojobori, T. (2005). *Mol. Biol. Evol.* **22**, 243–250.
Wrenger, C., Eschbach, M. L., Muller, I. B., Warnecke, D. & Walter, R. D. (2005). *J. Biol. Chem.* **280**, 5242–5248.
Yang, Y., Zhao, G., Man, T. K. & Winkler, M. E. (1998). *J. Bacteriol.* **180**, 4294–4299.