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Crystallization and preliminary X-ray analysis of a novel dye-linked L-proline dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix*

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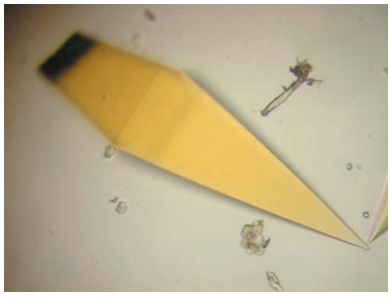

A novel dye-linked L-proline dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* was crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol 8000 as the precipitant. The crystals belonged to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$, with unit-cell parameters $a = b = 61.1$, $c = 276.3$ Å, and diffracted to 2.87 Å resolution using a Cu $K\alpha$ rotating-anode generator with an R-AXIS VII detector. The asymmetric unit contained one protein molecule, giving a crystal volume per enzyme mass (V_M) of 2.75 Å^3 Da^{-1} and a solvent content of 55.3%.

1. Introduction

Dye-linked dehydrogenases catalyze the oxidation of various amino acids, organic acids, amines and alcohols in the presence of artificial electron accepters such as ferricyanide and 2,6-dichloroindophenol. Many types of dye-linked dehydrogenases have been identified in mesophilic microorganisms and it has been suggested that they have potential for use as specific elements in biosensors (Frew & Hill, 1987). However, their low stability has thus far precluded their use in practical applications and has limited our ability to obtain detailed information about their structures and functions. In contrast, hyperthermophilic archaea represent a source of extremely stable enzymes. Indeed, we have identified several novel dye-linked dehydrogenases in hyperthermophilic archaea, including dye-linked L-proline dehydrogenase (LPDH; Sakuraba *et al.*, 2001; Kawakami *et al.*, 2005), dye-linked D-proline dehydrogenase (Satomura *et al.*, 2002) and dye-linked D-lactate dehydrogenase (Satomura *et al.*, 2008), and have found these enzymes to be highly stable and to exhibit great potential for application.

LPDH catalyzes the oxidation of L-proline to Δ^1-pyrroline-5-carboxylate in the presence of an artificial electron acceptor. Two different types of LPDH, PDH1 and PDH2, have been identified in the anaerobic hyperthermophile *Pyrococcus horikoshii* OT-3 (Kawakami *et al.*, 2005). PDH1 is a heterooctameric complex ($\alpha_4\beta_4$; molecular mass 440 kDa) containing FAD, FMN, Fe and ATP (Kawakami *et al.*, 2005), while PDH2 is a tetrameric complex ($\alpha\beta\gamma\delta$; molecular mass 120 kDa). Structural analysis of the PDH1 complex showed the enzyme to be a unique diflavin dehydrogenase containing a novel electron-transfer system that is completely different from that of the PDH2 complex, which contains four components: L-proline dehydrogenase, NADH dehydrogenase, a ferredoxin-like protein and a protein of unknown function (Kawakami *et al.*, 2004; Tsuge *et al.*, 2005). Proteins homologous to PDH1 and PDH2 are widely distributed among the hyperthermophilic archaea that belong to the phylum Euryarchaeota (Kawakami *et al.*, 2005).

We recently identified LPDHs from two hyperthermophilic archaea, *Pyrobaculum calidifontis* (Satomura *et al.*, submitted) and *Aeropyrum pernix* (described in this paper), belonging to the phylum Crenarchaeota. The newly identified LPDHs are composed of two identical subunits (2 × 46 kDa) and thus have a much simpler subunit structure than PDH1 or PDH2. Although the amino-acid sequences of the two-subunit enzymes share 42.7% identity, they are considerably less homologous (24–28%) to the PDH1 and PDH2 β-subunits,

which are directly responsible for L-proline dehydrogenation. This suggests these two LPDHs are inherently different from PDH1 and PDH2 and may be representative of a new family of LPDHs. In this paper, we describe the crystallization and preliminary X-ray analysis of *A. pernix* LPDH, as well as the expression of the gene in *Escherichia coli*, as a first step in the structural analysis of this novel type of LPDH.

2. Methods and results

2.1. Construction of the expression system and purification of recombinant protein

To construct the expression plasmid for the putative *A. pernix* LPDH homologue, a 1.2 kbp gene fragment comprised of the gene encoding the enzyme (APE_1267.1) and *Nde*I and *Bam*HI linkers was amplified by PCR with the following primers. The primer 5′-GTGCTCGTGCATATGCCGCGTTTCGACTAT-3′ was designed to contain the N-terminal region of the LPDH gene homologue and an *Nde*I digestion sequence, while the primer 5′-CCGCAGGATC-CTCGGCGTCTAGAGGACTAG-3′ was designed to contain the C-terminal region and a *Bam*HI digestion sequence. The genomic DNA was prepared using a Genomic DNA Isolation kit for Bacteria (Nexttec GmbH Biotechnologie, Leverkusen, Germany). The amplified 1.2 kbp fragment was digested using *Nde*I and *Bam*HI and then ligated with the expression vector pET11a (Novagen, Madison, Wisconsin, USA), previously linearized using *Nde*I and *Bam*HI, to generate pLPDH. Thereafter, *E. coli* strain BL21 (DE3) Codon Plus RILP (Stratagene, La Jolla, California, USA) was transformed with pLPDH and the transformants were cultivated at 310 K in 1 l SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K_2HPO_4, 0.38% KH_2PO_4 and 0.5% glycerol) containing 50 µg ml^{-1} ampicillin until the optical density at 600 nm reached 0.4. Expression was then induced by adding 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside to the medium and cultivation was continued for an additional 4 h. The cells were then harvested by centrifugation, suspended in 10 m*M* potassium phosphate buffer pH 7.2 supplemented with 100 m*M* NaCl (buffer *A*) and disrupted by ultrasonication. The LPDH activity in the crude extract was assayed as described previously (Sakuraba *et al.*, 2001).

To isolate the *A. pernix* LPDH, the crude extract was heated at 353 K for 15 min and the denatured protein was removed by centrifugation (10 000*g* for 10 min). The resultant supernatant was loaded onto a Q-Sepharose column (1.8 × 5 cm; GE Healthcare Bioscience UK Ltd, Buckinghamshire, England) equilibrated with buffer *A*, after which the column was washed with three bed volumes of the same buffer. The enzyme was eluted with 80 ml of a linear gradient of 100–500 m*M* NaCl in 10 m*M* potassium phosphate buffer pH 7.2. The active fractions were pooled and loaded onto a Sephacryl S-300 gel-filtration column (26 mm × 80 cm) equilibrated with 10 m*M* potassium phosphate buffer pH 7.2.

We found that the transformant cells exhibited a high level of LPDH activity and the enzyme was readily purified from the crude extract of the transformants in three simple steps: heat treatment, Q-Sepharose ion-exchange column chromatography and Sephacryl S-300 gel-filtration column chromatography. About 8 mg purified enzyme was obtained from 1 l *E. coli* culture.

2.2. Molecular-mass determination

The molecular mass of the recombinant enzyme was determined using a gel-filtration column (Sephacryl S-300; 26 mm × 80 cm). The column was equilibrated with buffer *A* and the following standard proteins (Bio-Rad, Hercules, California, USA) were used to produce the calibration curve: bovine thyroglobulin (molecular mass 670 kDa), bovine *γ*-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1350 Da).

2.3. Crystallization

Crystallization of *A. pernix* LPDH was accomplished using the sitting-drop vapour-diffusion method. Drops (1 µl) of protein solution (10 mg ml^{-1}) were mixed with an equal volume of reservoir solution containing 9% PEG 8000 and 0.1 *M* Tris–HCl pH 8.6 and were equilibrated against 0.1 ml reservoir solution at 293 K. Crystals appeared within 3 d and reached maximum dimensions of ∼0.1 × 0.1 × 0.3 mm within one week (Fig. 1).

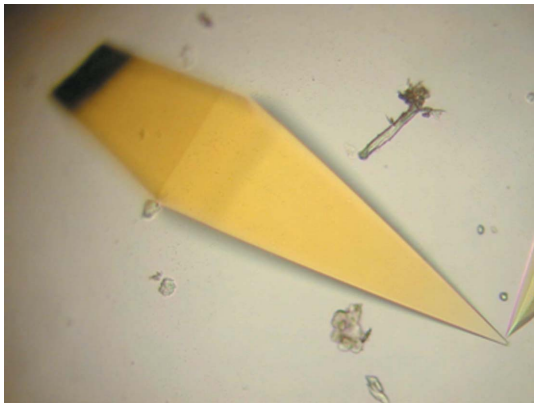

Figure 1
Photograph of an *A. pernix* LPDH crystal. The dimensions of the crystal are 0.1 × 0.1 × 0.3 mm.

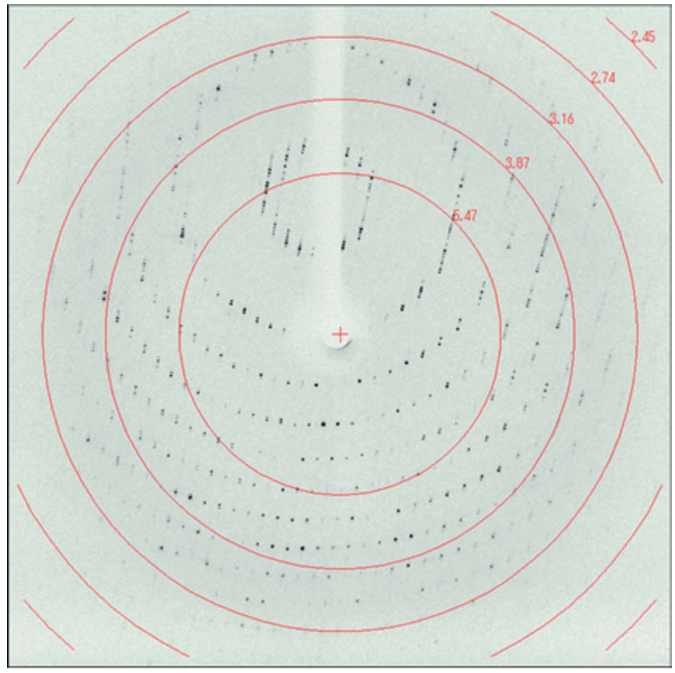

Figure 2
X-ray diffraction of an *A. pernix* LPDH crystal.

Table 1
Data-collection and processing statistics for *A. pernix* LPDH.

Values in parentheses are for the highest resolution shell.

Source	Cu $K\alpha$
Wavelength (Å)	1.5418
Temperature (K)	100
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	a = 61.1, b = 61.1, c = 276.3
Resolution range (Å)	50–2.87 (2.97–2.87)
No. of measured reflections	173174
No. of unique reflections	12905
Redundancy	13.4
Completeness (%)	99.8 (97.9)
$R_{\rm merge}$† (%)	7.7 (29.1)
$\langle I/\sigma(I)\rangle$	10.7 (6.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

2.4. Data collection and preliminary X-ray analysis

The *A. pernix* LPDH crystals were flash-frozen in liquid nitrogen at 100 K. Reservoir solution supplemented with 20%(*v*/*v*) ethylene glycol was used as the cryoprotectant solution, but when we placed the crystals in this cryoprotectant solution the quality of the X-ray diffraction quickly deteriorated. To overcome this problem, we twice added 2 µl of the cryoprotectant solution to the 2 µl crystallization drops, after which the crystals were directly flash-frozen. Since the number of crystals with high diffraction quality was very limited, the selection of a good crystal was required. Diffraction data were collected at 2.87 Å resolution on an R-AXIS VII imaging-plate detector using a rotating copper-anode in-house generator with confocal mirrors (MicroMax-007, Rigaku, Japan) operating at 40 kV and 20 mA (Fig. 2). The crystal-to-detector distance was 250 mm. The oscillation angle per image was set to 1°. The data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

The crystals belonged to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$. A summary of the data statistics is presented in Table 1. Based on the Sephacryl S-300 gel-filtration chromatography, the molecular mass of the native enzyme was estimated to be about 88 kDa (data not shown), indicating that the oligomeric state of this enzyme was a homodimer in solution. Assuming one subunit in the asymmetric unit, the crystal volume per enzyme mass ($V_{\rm M}$) and the solvent content were calculated to be 2.75 Å^3 Da^{-1} and 55.3%, respectively. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). An attempt was made to solve the structure using the heavy-atom isomorphous replacement method by soaking the crystals in reservoir solutions containing several Hg, Au or Pt compounds. However, we have not yet obtained an adequate heavy-atom derivative. Therefore, we are now trying to prepare selenomethionine-substituted *A. pernix* LPDH.

In this study, the first diffraction-quality crystals were obtained for a novel type of LPDH whose subunit structure (a homodimer) is much simpler than those of previously described LPDHs: *i.e.* PDH1 (an $\alpha_4\beta_4$ heterooctamer) and PDH2 (an $\alpha\beta\gamma\delta$ heterotetramer). We expect that the elucidation of the three-dimensional structure of this enzyme will provide new insights into its oligomeric state and further our understanding of the structure–function relationships in hyperthermophilic LPDHs.

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