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

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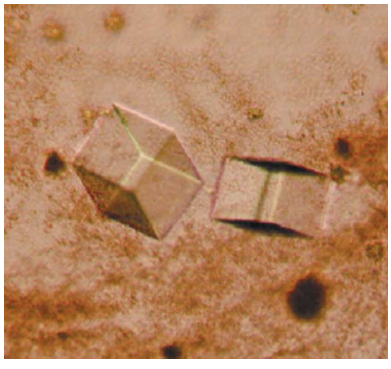

A dye-linked D-lactate dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* was crystallized using the hanging-drop vapour-diffusion method with polyethylene glycol 8000 as the precipitant. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 63.4, b = 119.4, c = 70.2 Å, β = 112.0°, and diffracted to 2.0 Å resolution on the BL26B1 beamline at SPring-8. The overall $R_{\rm merge}$ was 4.5% and the completeness was 99.8%.

1. Introduction

Dye-linked dehydrogenases catalyze the oxidation of various amino acids, organic acids, amines and alcohols in the presence of artificial electron acceptors such as ferricyanide and 2,6-dichloroindophenol (Cl2Ind). Many types of dye-linked dehydrogenases have been identified in mesophilic microorganisms and it has been suggested that they have the potential to serve as specific elements in biosensors (Frew & Hill, 1987). However, their poor 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 can be a good source of extremely stable enzymes. Indeed, we have identified four types of dye-linked amino-acid dehydrogenases in hyperthermophiles, including three different L-proline dehydrogenases (Sakuraba *et al.*, 2001; Kawakami *et al.*, 2004, 2005; Satomura *et al.*, 2011) and a D-proline dehydrogenase (Satomura *et al.*, 2002), all of which are highly stable and totally novel flavin-containing enzymes. In addition, we have determined the crystal structure of a dye-linked L-proline dehydrogenase from *Pyrococcus horikoshii* (Tsuge *et al.*, 2005).

While screening for dye-linked dehydrogenases in hyperthermophiles, we identified dye-linked D-lactate dehydrogenases (DLDHs) from two hyperthermophilic archaea, *Sulfolobus tokodaii* (Satomura *et al.*, 2008) and *Aeropyrum pernix* (described in this paper), which catalyze the oxidation of D-lactate with reduction of Cl2Ind. To date, the presence of DLDH in hyperthermophilic archaea has only been reported for *Archaeoglobus fulgidus* (Reed & Hartzell, 1999). This organism can use D-lactate, L-lactate and pyruvate as carbon and electron sources for dissimilatory sulfate reduction and DLDH is known to be involved in the transfer of electrons from D-lactate to the anaerobic respiratory chain in the membrane (Pagala *et al.*, 2002). In contrast, *S. tokodaii* and *A. pernix* are aerobic archaea and their energy-production system is postulated to be substantially different from that of *Ar. fulgidus*, which is an anaerobic sulfur-requiring archaeon (Stetter, 1988). This suggests that the DLDHs from *S. tokodaii* and *A. pernix* are likely to differ from the *Ar. fulgidus* enzyme both structurally and functionally. The physiological function of the aerobic archaeal DLDHs is currently unknown. Structural analysis of these enzymes may cast light on the features and diversity of the DLDHs from hyperthermophilic archaea. In contrast to the enzyme from *Ar. fulgidus*, which is unstable under aerobic conditions, the newly identified DLDHs from *S. tokodaii* and *A. pernix* are highly stable, which increases our ability to obtain useful information about their structures and functions.

The crystal structure of DLDH has only been reported for the enzyme from *Escherichia coli* (Dym *et al.*, 2000), which is located on the cytoplasmic side of the inner membrane and functions as a peripheral membrane respiratory component involved in electron transfer. It is thought that the membrane-binding domain of the *E. coli* enzyme is present on an electropositive surface containing basic amino acids that interact with the negatively charged phospholipid head groups in the membrane. The amino-acid residues in the membrane-associated region of the *E. coli* enzyme are not conserved in the sequences of DLDHs from hyperthermophilic archaea, suggesting that these archaeal DLDHs are inherently different from the *E. coli* enzyme. In this paper, we describe the crystallization and preliminary X-ray analysis of *A. pernix* DLDH, as well as the expression of the gene in *E. coli*, as a first step in the structural analysis of DLDHs from hyperthermophilic archaea.

2. Methods and results

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

To construct the expression plasmid for the putative *A. pernix* DLDH homologue, a 1.4 kbp gene fragment composed of the gene encoding the enzyme (APE_0487) plus *Nde*I and *Bam*HI restriction sites was amplified by PCR with the following two primers. The primer 5′-AACATATGGCTCGTATAGCTGAGGAGCTTG-3′ was designed to contain the N-terminal region of the DLDH gene homologue and an *Nde*I digestion sequence, while the primer 5′-TTGGATCCTCACTCAGCCGCTACAACCTTC-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 1.4 kbp fragment was digested with *Nde*I and *Bam*HI and then ligated with the expression vector pET15b (Novagen, Madison, Wisconsin, USA) previously linearized using *Nde*I and *Bam*HI, yielding pDLDH. *E. coli* strain Rosetta-gami 2 (DE3) (Stratagene, La Jolla, California, USA) was then transformed with the vector 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 and 50 µg ml^{-1} chloramphenicol until the optical density at 600 nm reached 0.6. Expression was then induced by adding 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside to the medium and cultivation was continued for an additional 21 h at 293 K. The cells were then harvested by centrifugation, suspended in 50 m*M* sodium phosphate buffer pH 8.0 supplemented with 300 m*M* NaCl (buffer *A*) and disrupted by ultrasonication. DLDH activity in the crude extract was assayed as described previously (Satomura *et al.*, 2008).

To isolate the *A. pernix* DLDH, the crude extract was heated at 353 K for 15 min, after which denatured protein was removed by centrifugation (10 000*g* for 10 min). The resultant supernatant was loaded onto a Protino Ni–IDA resin column (1.5 × 4 cm, MACHERY-NAGEL), which was then equilibrated with buffer *A* and washed with three bed volumes of the same buffer. The enzyme was eluted with 80 ml of a linear gradient of 0–250 m*M* imidazole in the same buffer. The active fractions were then pooled and loaded onto a HiLoad 26/60 Superdex 200 gel-filtration column (GE Healthcare) equilibrated with 50 m*M* sodium phosphate buffer pH 8.0 supplemented with 200 m*M* NaCl. After elution, the active fractions of the eluate were pooled and used as the purified enzyme preparation.

We found that the transformant cells exhibited a high level of DLDH activity and the enzyme was readily purified from the crude cell extract in three simple steps: heat treatment, Ni–IDA resin column chromatography and Superdex 200 gel-filtration column chromatography. About 9 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 Superose 6 10/300 GL column (GE Healthcare) with 10 m*M* potassium phosphate buffer pH 7.0 containing 200 m*M* NaCl as the elution buffer. Gel-filtration calibration kits (GE Healthcare) were used as molecular-mass standards. The subunit molecular mass was determined by SDS–PAGE using eight marker proteins (6–175 kDa; New England Biolabs Inc.). The native and subunit molecular masses of the enzyme were determined to be about 61 and 59 kDa, respectively, suggesting that the enzyme is a monomer in solution.

2.3. Crystallization

The purified enzyme was dialyzed against 10 m*M* potassium phosphate buffer pH 7.0 and concentrated to 15 mg ml^{-1} for crystallization trials. Initial screening was carried out with Crystal Screen, Crystal Screen 2 (Hampton Research, USA) and Wizard I, II and III (Emerald Biosystems, USA) at 293 K using the sitting-drop vapour-diffusion method, in which 1 µl drops of protein solution were mixed with equal volumes of reservoir solution and equilibrated against 0.1 ml reservoir solution using CompactClover Crystallization Plates (Emerald BioSystems). Initially, microcrystals were grown from reagent No. 35 (14.4% PEG 8000, 80 m*M* cacodylate pH 6.5, 160 m*M* calcium acetate and 20% glycerol) of Wizard III, but the crystal size could not be improved by changing the precipitant concentration or buffer conditions. Thus, we optimized the vapour-diffusion method using the same reagent condition. Finally, a few diffraction-quality crystals (maximum dimensions of ∼0.1 × 0.1 × 0.1 mm; Fig. 1) were obtained within two weeks at 298 K using the hanging-drop vapour-diffusion method, in which 2 µl drops of protein solution were mixed with equal volumes of reservoir solution and equilibrated against 0.1 ml reservoir solution.

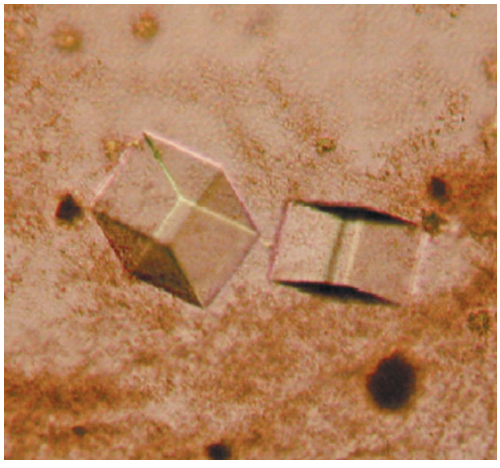

Figure 1
Photograph of an *A. pernix* DLDH crystal. The dimensions of the crystal are 0.1 × 0.1 × 0.1 mm.

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

Values in parentheses are for the highest resolution shell.

Source	BL26B1, SPring-8
Wavelength (Å)	1.00
Temperature (K)	100
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 63.4, b = 119.4, c = 70.2, β = 112.0
Resolution range (Å)	50–2.0 (2.03–2.00)
No. of measured reflections	456330
No. of unique reflections	65248
Multiplicity	7.0 (6.9)
Completeness (%)	99.8 (99.4)
$R_{\rm merge}$†	0.045 (0.244)
$\langle I/\sigma(I)\rangle$	17.6 (6.8)

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

2.4. Data collection and preliminary X-ray analysis

An *A. pernix* DLDH crystal was flash-cooled in liquid nitrogen at 100 K. The crystal was cryoprotected with reservoir solution supplemented with 30%(*v*/*v*) glycerol, replacing the water in the buffer with cryoprotectant. Diffraction data were collected at 2.0 Å resolution using monochromated radiation of wavelength 1.0 Å and a Saturn A200 CCD detector system on beamline BL26B1 at SPring-8 (Harima, Japan). The crystal-to-detector distance was 180 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 monoclinic space group $P2_1$. A summary of the data statistics is presented in Table 1. Assuming two protein molecules in the asymmetric unit, the crystal volume per enzyme mass ($V_{\rm M}$) and the solvent content were calculated to be 2.5 Å^3 Da^{-1} and 51.1%, respectively, which are within the frequently observed ranges for protein crystals (Matthews, 1968).

The amino-acid sequence identity between *A. pernix* DLDH and the *E. coli* enzyme is 20.8%, suggesting that these two enzymes are inherently different from each other. When we compared the amino-acid sequences of the DLDH homologues for which crystal structures have previously been determined, we found that *A. pernix* DLDH exhibits highest identity (31.3%) to a putative dehydrogenase (RPA1076) from *Rhodopseudomonas palustris* CGA009 (PDB entry 3pm9; Joint Center for Structural Genomics, unpublished work). Based on this structure, we used the molecular-replacement method for phase calculation, but could not obtain useful data. We are now trying to solve the phase by soaking the crystals in reservoir solution containing several Hg, Au or Pt compounds and using the heavy-atom isomorphous replacement method.

To date, no DLDHs from archaea (the third domain of life) or from hyperthermophiles have been structurally characterized. In the present study, the first diffraction-quality crystals were obtained for a DLDH from a hyperthermophilic archaeon. We expect that elucidation of the three-dimensional structure of this enzyme will further increase our understanding of structure–function relationships in DLDHs.

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