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Crystallization and preliminary X-ray diffraction analysis of the hyperthermostable NAD-dependent glutamate dehydrogenase from *Pyrobaculum islandicum*

Mohammad W. Bhuiya,[a] Haruhiko Sakuraba,[a] Kazunari Yoneda,[a] Toshihisa Ohshima,[a]* Takahito Imagawa,[b] Nobuhiko Katunuma[b] and Hideaki Tsuge[b,c]*

[a]Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan, [b]Institute for Health Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan, and [c]Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

Correspondence e-mail: ohshima@bio.tokushima-u.ac.jp, tsuge@tokushima.bunri-u.ac.jp

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NAD-dependent glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum* was crystallized in the apo- and holoenzyme forms. Crystals were obtained using 2-propanol and polyethylene glycol MME 550 as precipitants for the apoenzyme and holoenzyme, respectively. The apoenzyme crystals belong to the trigonal space group $P3_121$ or its enantiomorph $P3_221$. The asymmetric unit contains three subunits; the values of the Matthews coefficient (V_M) and the solvent content are 2.9 Å^3 Da^{-1} and 57%, respectively. A native data set was collected to a highest resolution limit of 4.0 Å on an in-house X-ray source using a rotating-anode generator (overall R_{sym} of 12.3% and completeness of 97%). The holoenzyme crystals belong to the orthorhombic space group $P2_12_12_1$; the asymmetric unit contains one hexamer, giving a V_M of 2.79 Å^3 Da^{-1} and a solvent content of 55%. Native and derivative data sets were collected. The crystals diffract to a maximum resolution of 2.8 Å on the KEK-NW12 beamline at the Photon Factory and gave a data set with an overall R_{sym} of 7.9% and a completeness of 91%. Attempts are being made to solve the structure by the SIRAS method.

1. Introduction

More than 60 strains of hyperthermophiles have been isolated from marine and continental volcanic environments over the last two decades (Hutchins *et al.*, 2001). Of the enzymes from these organisms, GluDH is the one for which the most abundant information concerning enzymological properties and the relationship between structure and function has been obtained. Studies of GluDHs from hyperthermophilic archaea have mainly focused on those from marine hyperthermophilic species of the order *Thermococcales*, such as the *Pyrococcus* (*Pc.*) and *Thermococcus* geni. They belong to the Euryarchaeota, one of the two phyla in the Archaea. Almost all the GluDHs from these organisms are NADP-specific enzymes (EC 1.4.1.4; DiRuggiero *et al.*, 1993; Ma *et al.*, 1994; Ohshima & Nishida, 1993, 1994; Kobayashi *et al.*, 1995; Rahman *et al.*, 1998). Although it has been shown that the enzyme from *Pc. furiosus* exhibits a dual coenzyme specificity (EC 1.4.1.3; Consalvi *et al.*, 1991; Robb *et al.*, 1992), its relative activity with NAD compared with that with NADP was significantly low (1.1%; Ohshima & Nishida, 1993). We have reported the presence of an NAD-specific GluDH (EC 1.4.1.2) in the continental hyperthermophilic archaeon *Pyrobaculum* (*Pb.*) *islandicum* (Kujo & Ohshima, 1998), which belongs to the Crenarchaeota, the second phylum in the Archaea. This is the only NAD-specific GluDH described to date in the hyperthermophilic Archaea.

In general, GluDHs fall into three classes based on their coenzyme specificity and their physiological function (Ohshima & Soda, 1990). NAD-dependent GluDHs are mainly involved in the production of 2-oxoglutarate (glutamate catabolism) and NADP-dependent GluDHs are involved in glutamate production (ammonia assimilation) in microorganisms (Smith *et al.*, 1975). GluDHs with dual coenzyme specificity are present in mammalian tissues and are subject to extensive allosteric control (Smith *et al.*, 1975; Hudson *et al.*, 1993). The presence of a functional citric acid cycle in the anaerobic *Thermococcales* has not yet been reported and the principal function of the NADP-dependent GluDHs from *Thermococcales* has been proposed to be L-glutamate biosynthesis coupled with L-alanine production (Ohshima & Nishida, 1993; Kengen & Stams, 1994; Kobayashi *et al.*, 1995). However, Selig & Schönheit (1994) have suggested the presence of the citric acid cycle in *Pb. islandicum* and have proposed its function to be the oxidation of organic compounds to CO_2, with elemental sulfur or thiosulfate as the electron acceptor. Thus, we have proposed that the physiological role of the NAD-dependent GluDH from *Pb. islandicum* is distinct from that of the

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Thermococcales enzymes and may be linked to the citric acid cycle *via* 2-oxoglutarate (Kujo & Ohshima, 1998).

The three-dimensional structure of several NADP-dependent GluDHs from *Thermococcales* have already been determined [those from *Pc. furiosus* (Yip *et al.*, 1995), *T. litoralis* (Britton *et al.*, 1999) and *T. profundus* (Nakasako *et al.*, 2001)]. However, no information is available as yet regarding the three-dimensional structure of the NAD-dependent GluDH in hyperthermophilic archaea. The NAD-dependent GluDH from *Pb. islandicum* has a sequence identity of 43.5, 46.0 and 46.1% to the NADP-dependent GluDHs from *T. profundus*, *T. litoralis* and *Pc. furiosus*, respectively. Comparison of the structure of the *Pb. islandicum* GluDH with those of the *Thermococcales* enzymes may help to elucidate the relationship between the physiological function and the differences in the coenzyme specificity of the hyperthermophilic GluDHs. In addition, no three-dimensional structure of a GluDH from a crenarchaeon has been reported so far. In this report, we describe the crystallization and preliminary X-ray diffraction analysis of the GluDH from *Pb. islandicum* in the apo and holo (NAD-bound) forms.

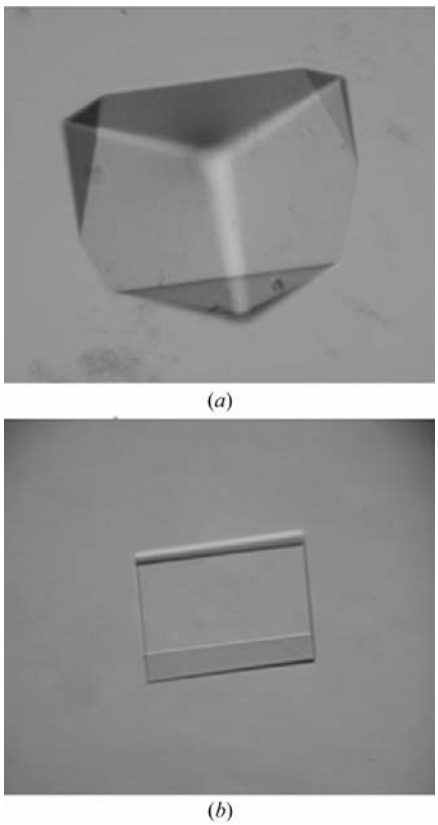

Figure 1
Photographs of *Pb. islandicum* GluDH crystals: (*a*) apoenzyme and (*b*) holoenzyme. The dimensions of the largest apo- and holoenzyme crystals are approximately 0.3 × 0.3 × 0.3 and 0.3 × 0.25 × 0.05 mm, respectively.

Table 1
Data-collection and processing statistics.

The apoenzyme crystal belongs to space group $P3_121$ or its enantiomorph $P3_221$, with unit-cell parameters $a = b = 134.46$, $c = 135.47$ Å. The holoenzyme crystal belongs to space group $P2_12_12_1$, with $a = 104.29$, $b = 165.71$, $c = 180.97$ Å. Values in parentheses correspond to the highest resolution shell.

	Apoenzyme	Holoenzyme		
	Native	Native	Hg	Native
Source	Cu *Kα*	Cu *Kα*	Cu *Kα*	NW12 (PF)
λ (Å)	1.5418	1.5418	1.5418	1.00
Distance (mm)	250	250	250	200
Resolution range (Å)	50–4.0 (4.21–4.0)	40–3.5 (3.68–3.5)	40–4.2 (4.42–4.2)	50–2.8 (2.9–2.8)
Total No. observations	84143	89401	69588	287706
No. unique reflections	12279 (1725)	35581 (5255)	18159 (2650)	70811 (7155)
Redundancy	6.9	2.5	3.7	3.9
Completeness (%)	99.6 (99.9)	90 (95)	77 (80)	91 (93)
R_{sym}† (%)	12.3 (29.6)	14.8 (36.2)	24.8 (36.3)	7.9 (42.3)
$\langle I/\sigma(I)\rangle$	5.7 (3.1)	4.0 (2.7)	2.5 (3.3)	8.9 (2.6)

† $R_{sym}(I) = \sum_i |I_i - \langle I\rangle| / \sum_i I_i$.

2. Materials and methods

2.1. Construction of the expression system and purification of GluDH

The plasmid vector pKGDH1 was constructed as previously described (Kujo *et al.*, 1999). To obtain an efficient expression vector, the *gludh* gene on pKGDH1 was subcloned into a pET11a vector (Novagen, USA). The DNA fragment containing the *gludh* gene was amplified by PCR using two primers which contain *Nde*I and *Bam*HI restriction sites (5′-CCG GAA CAT ATG GAG AGG ACA GGG-3′ and 5′-CCC GGA TCC TTA GAT CCA CCC TC-3′). The pEGDH2 plasmid was constructed by inserting the PCR product into the *Nde*I and *Bam*HI sites of pET11a. *Escherichia coli* strain BL21 (DE3) Codon Plus–RIL cells (Stratagene, USA) were transformed with pEGDH2. The transformants were cultivated at 310 K in Luria–Bertani medium containing ampicillin (50 µg ml^{-1}) until the absorbance reached 0.6 at 600 nm. Induction was carried out by the addition of 1 m*M* isopropyl-*β*-D-thiogalactopyranoside to the medium (3.0 l) and cultivation was continued for 3 h. Cells (11.6 g wet weight) were harvested by centrifugation, suspended in 10 m*M* potassium phosphate buffer pH 7.2 containing 10% glycerol, 1 m*M* EDTA and 0.1 m*M* DTT (buffer *A*) and disrupted using a French press (Ohtake, Japan). After centrifugation (15 000*g*, 15 min), the soluble fraction of the extract was heated at 358 K for 1 h. The denatured proteins were removed by centrifugation and the supernatant was applied to a 4 × 10 cm Red Sepharose CL-4B column (Ohshima & Sakuraba, 1986) equilibrated with buffer *A*. After washing with the same buffer, the enzyme was eluted with the same buffer containing 0.5 *M* NaCl. The active fractions were pooled. The enzyme solution was dialyzed against buffer *A* and applied to a 2 × 10 cm Red Sepharose CL-4B column equilibrated with the same buffer. The column was washed with one column volume of buffer *A* and subsequently equilibrated with the same buffer supplemented with 5 m*M* L-glutamate pH 7.2. The enzyme was eluted with a linear gradient of NAD (0–1.0 m*M*) in the presence of 5 m*M* L-glutamate. The active fractions containing the pure enzyme (38 mg) were pooled and dialyzed against buffer *A*.

2.2. Crystallization

The purified enzyme was dialyzed against 10 m*M* potassium phosphate buffer pH 7.0 containing 10% glycerol, 1 m*M* EDTA and 0.1 m*M* DTT for crystallization trials. Initial crystallization screening was carried out using Crystal Screens I and II (Hampton Research, USA) with the hanging-drop vapour-diffusion method. Two types of crystals having different morphologies were obtained. The apoenzyme crystals (type I; Fig. 1*a*) were obtained in hanging drops made by mixing 2 µl of 10 mg ml^{-1} enzyme solution with an equal volume of a reservoir solution consisting of 28%(*v*/*v*) 2-propanol, 200 m*M* $MgCl_2$ and 100 m*M* HEPES–KOH pH 6.5 at 293 K. Crystals appeared within 2 d and reached maximum dimensions of ∼0.3 × 0.3 × 0.3 mm within one week. Holoenzyme crystals (type II; Fig. 1*b*) appeared in hanging drops when 5 µl of 10 mg ml^{-1} enzyme solution containing 5 m*M* NAD was mixed with an equal

volume of mother liquor consisting of 24%(*v*/*v*) PEG MME 550, 100 m*M* NaCl and 100 m*M* Tris–HCl pH 8.0 at 277 K. Well diffracting crystals appeared within 3 d and reached maximum dimensions of ~0.3 × 0.25 × 0.05 mm within one week.

2.3. Preliminary X-ray diffraction analysis

All data were collected at room temperature because of the large mosaicity obtained using cryoconditions. The crystals were mounted in a thin-walled glass capillary tube. Diffraction data from the apoenzyme crystals were collected at 4.0 Å resolution on a Bruker–Nonius DIP 2030 image-plate detector using an in-house rotating copper-anode generator operating at 40 kV and 90 mA. The oscillation angle per image was set to 1°. The data were processed with *HKL* 2000 (Otwinowski & Minor, 1997). The apoenzyme crystals belong to the trigonal space group $P3_121$ or its enantiomorph $P3_221$. Data-collection and processing statistics are summarized in Table 1. High-resolution native data from the holoenzyme crystal were collected at 2.8 Å on the KEK-NW12 beamline at the Photon Factory (Tsukuba, Japan) with an $R_{\rm sym}$ of 7.9%. The holoenzyme crystals belong to the orthorhombic space group $P2_12_12_1$ (Table 1). One hexamer per asymmetric unit is found in the GluDHs from *T. profundus* (PDB code 1euz; Nakasako *et al.*, 2001) and *T. litoralis* (PDB code 1bvu; Britton *et al.*, 1999). In *Pc. furiosus* GluDH, the asymmetric unit contains a trimer (PDB code 1gtm; Yip *et al.*, 1995). For the apoenzyme crystals, assuming three subunits of GluDH in the asymmetric unit, the value of the Matthews coefficient ($V_{\rm M}$) and the solvent content are calculated to be 2.9 Å^3 Da^{-1} and 57%, respectively. In the case of the holoenzyme crystals, assuming six subunits each of molecular weight 47 kDa in the asymmetric unit, the value of the Matthews coefficient ($V_{\rm M}$) and the solvent content are calculated to be 2.79 Å^3 Da^{-1} and 55%, respectively. These values are within the range frequently observed in protein crystals (Matthews, 1968).

A heavy-atom derivative was prepared by soaking the holoenzyme crystals in mother liquor containing 1 m*M* $HgCl_2$ for 12 h. Data were collected in-house to a resolution limit of 4.0 Å. SIRAS (single isomorphous replacement with anomalous scattering) phases were calculated using *SOLVE* (Terwilliger & Berendzen, 1999). Six mercury-binding sites per asymmetric unit were found by interpreting the difference and anomalous Patterson maps, which indicate that one mercury is bound per monomer. Heavy-atom refinement and phasing are under way using the programs *MLPHARE* (Collaborative Computational Project, Number 4, 1994) and *SOLVE*.

As far as we are aware, *Pb. islandicum* GluDH is the most thermostable NAD-specific GluDH; the activity was not lost after incubation at 373 K for 2 h (Kujo & Ohshima, 1998). The enzyme is highly resistant to denaturants, organic solvents and detergents (Kujo & Ohshima, 1998) and is distinct from other hyperthermostable GluDHs based on its coenzyme specificity and physiological function. We expect that the elucidation of the three-dimensional structure of *Pb. islandicum* GluDH will provide new insights into its unique coenzyme specificity and lead to further understanding of the structure–function relationships in this hyperthermostable enzyme.

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