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Crystallization and preliminary X-ray diffraction studies of hyperthermostable glutamate dehydrogenase from *Thermococcus profundus*

Sadaharu Higuchi,[a] Masayoshi Nakasako[b]* and Toshiaki Kudo[a]

[a]The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-0198, Japan, and [b]PRESTO, Japan Science and Technology Corporation and The Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi 1-1-1, Bunkyoku, Tokyo 113-0032, Japan

Correspondence e-mail: nakasako@iam.u-tokyo.ac.jp

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Recombinant glutamate dehydrogenase from a hyperthermophilic archaeon, *Thermococcus profundus*, was crystallized in the presence of both polyethylene glycol 8000 and lithium sulfate. Four types of crystals having different morphologies appeared in the crystallization trials; however, only one type was suitable for X-ray crystal structure analysis. The crystal belonged to the monoclinic space group $P2_1$ and the unit-cell parameters were a = 112.99, b = 163.70, c = 133.07 Å, β = 113.46° at 110 K. The calculated V_M value of 3.42 Å^3 Da^{-1} was acceptable when one hexamer of the enzyme, which was the physiological functional unit, occupied a crystallographic asymmetric unit. X-ray diffraction intensity data were collected to a resolution of 2.25 Å with good statistics at the BL44B2 beamline of SPring-8.

1. Introduction

Hyperthermophile microorganisms grow optimally at extremely high temperatures (at or near 373 K; Adams, 1995; Madigan *et al.*, 1997). Most of the enzymes produced by those organisms show exceptional thermostability: the enzymes are active for several hours at or above 373 K (Laderman *et al.*, 1993). Therefore, attention has been focused on such enzymes in an effort to identify the key structural determinants causing hyperthermostability. Elucidation of the determinants may provide new insight into the physical mechanisms involved in protein stability and provide helpful clues for designing thermostable enzymes to be utilized in various industrial fields.

Among the enzymes in the cytoplasm of hyperthermophilic microorganisms, glutamate dehydrogenase (GluDH) is the most abundant protein so far known (Consalvi *et al.*, 1991; Robb *et al.*, 1992; Ohshima & Nishida, 1993; Kobayashi *et al.*, 1995). This enzyme forms a hexamer as its functional unit and is involved in the reversible interconversion of glutamate to 2-oxoglutarate and ammonia, using either NAD(H) or NADP(H) as the coenzyme (Frieden, 1963). Because of its high abundance, GluDH has been studied as a typical target in investigations focusing on the structural determinants for hyperthermostability (DiRuggiero *et al.*, 1993; Eggen *et al.*, 1993; Britton *et al.*, 1995; Higuchi *et al.*, 1997). The three-dimensional structure of GluDH from a hyperthermophile, *Pyrococcus furiosus*, has been determined (Yip *et al.*, 1995) and compared with that of the GluDH from *Clostridium symbiosum* (Baker *et al.*, 1992; Stillman *et al.*, 1993). A series of ion-pair networks was identified to exist only on the surface of the hyperthermostable GluDH, suggesting a significant contribution of these networks to hyperthermostability (Yip *et al.*, 1995). It is believed that the formation of such extensive networks may represent a major stabilizing feature associated with adaptation to extremely high temperatures (Knapp *et al.*, 1997; Rahman *et al.*, 1998; Vertiani *et al.*, 1998; Yip *et al.*, 1998).

The hyperthermophilic archaeon *Thermococcus profundus* was isolated from a deep-sea hydrothermal vent and grows optimally at 353 K (Kobayashi *et al.*, 1994). The GluDH produced by this organism consists of 419 amino-acid residues (M_W = 46696 Da) and forms a hexamer (M_W = 280176 Da) as its physiological functional unit (Kobayashi *et al.*, 1995). The gene encoding this enzyme was cloned and expressed in order to investigate the key structural determinants causing its hyperthermostability (Higuchi *et al.*, 1997). The thermostability of this enzyme is lower than that of GluDHs from *P. furiosus* and *T. litoralis* (Higuchi *et al.*, 1997). Non-homologous amino-acid substitutions compared with the other GluDHs are considered to be responsible for the different thermostability. In addition, the enzyme exhibits temperature-dependent characteristics with respect to both the structure and the activity; while the content of α-helices decreases as temperature increases, the enzymatic activity gradually increases up to a temperature of 358 K, (Kobayashi *et al.*, 1995). In particular, the activity and the helix content show discontinuity near 334 K indicating that a structural transition in the enzyme is required in order to

realise its full enzymatic activity (Kobayashi *et al.*, 1995).

The structural basis of the temperature-dependent structural change and the structural features responsible for the different thermostability compared with other GluDHs are still unknown. Thus, we have started crystal structure analysis of the GluDH from *T. profundus* in an effort to elucidate the structural determinants causing the unique characteristics of this enzyme. Here, we report the results of the crystallization and the preliminary X-ray diffraction experiments.

2. Experimental and results

2.1. Crystallization

Recombinant GluDH of *T. profundus* was expressed using an overexpression system constructed in *Escherichia coli* (Higuchi *et al.*, 1997). The enzyme was roughly purified by heat treatment at 343 K for 60 min and was further purified using affinity column chromatography followed by gel-filtration chromatography as described previously (Higuchi *et al.*, 1997). The purified enzyme was concentrated to 6.4 mg ml^{-1} by ultrafiltration.

Crystallization trials were carried out by the hanging-drop vapor-diffusion method at 293 K. In these trials, 2 µl of protein solution was mixed with 2 µl of a precipitant solution and equilibrated against 1 ml of the precipitant solution. Hampton Crystal Screens I and II (Hampton Research) were used to determine initial precipitation conditions.

Extremely thin needle-like crystals appeared when a precipitant solution containing 15%(*w*/*v*) polyethylene glycol (PEG) 8000 and 0.5 *M* lithium sulfate (pH 3.5) was used. Because the crystals (∼0.01 × 0.01 × 0.08 mm) were not of suitable dimensions for diffraction experiments at our laboratory, optimal crystallization conditions were sought by varying the concentrations of the two precipitant reagents, the pH and the protein concentration. Through this factorial optimization of crystallization conditions, four types of crystals having different morphologies were obtained.

A thin plate-like crystal (type I; Fig. 1*a*) usually appeared in a polycrystalline state within a week when the precipitant solution contained 15–20%(*w*/*v*) PEG 8000 and 0.5 *M* lithium sulfate (pH 3.5–6.5). The polycrystalline tendency of the type I crystal was not improved by adding various organic solvents or salts. Under nearly identical conditions, a tetrahedral-shaped crystal (type II; Fig. 1*b*) was obtained and grew to dimensions of more than 0.1 × 0.1 × 0.1 mm within a month. A type III crystal, bipyramidal in shape (Fig. 1*c*), was optimally formed when the precipitant solution contained 1.5%(*w*/*v*) PEG 8000 and 1.5 *M* lithium sulfate (pH 4.5). The type III crystal grew to dimensions of 0.25 × 0.25 × 0.25 mm in two months.

The type IV plate-shaped crystal (Fig. 1*d*) appeared under nearly identical crystallization conditions to those employed to obtain the type III crystal. The precipitant solution was composed of 1.0%(*w*/*v*) PEG 8000, 1.5 *M* lithium sulfate and 50 m*M* sodium acetate (pH 5.0). The reproducibility of the type IV crystal was very low, even when the precipitant solution was carefully prepared. The crystal grew to dimensions of 0.45 × 0.15 × 0.04 mm in three months.

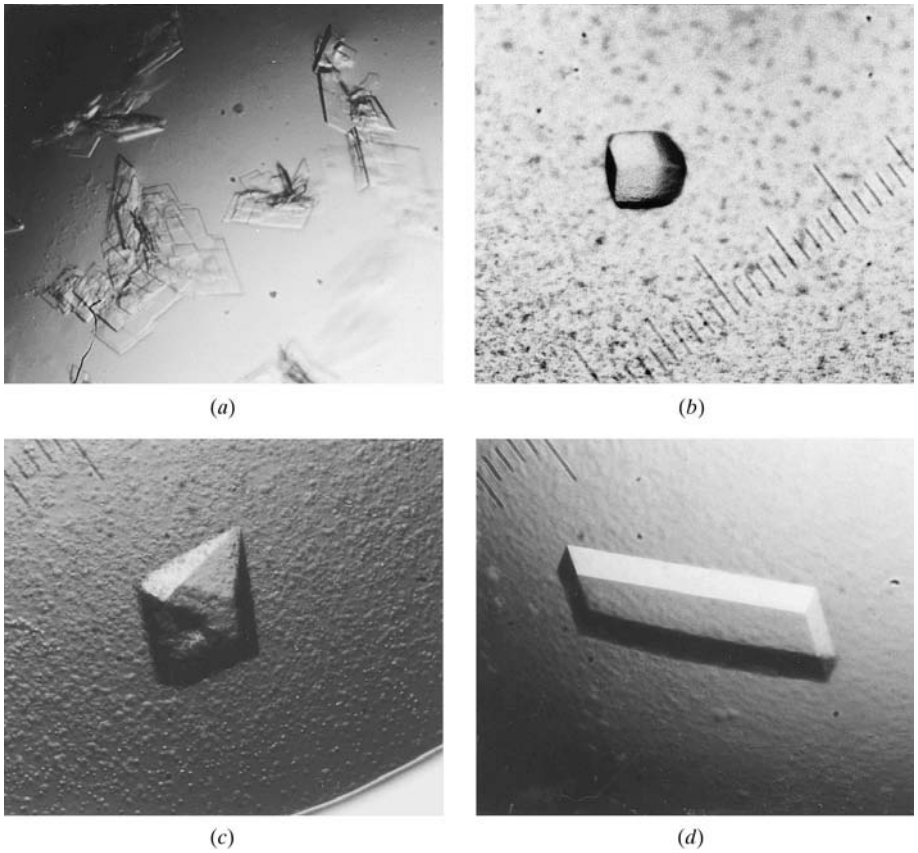

Figure 1
Photographs of GluDH crystals: (*a*) type I, (*b*) type II, (*c*) type III and (*d*) type IV crystals.

2.2. X-ray diffraction experiments at the laboratory

The crystals obtained were examined by X-ray diffraction experiments at both 293 and 110 K using an R-AXIS IV system (Rigaku) with an X-ray generator (Ultrax18, Rigaku) and double focusing-mirror optics (Rigaku). The generator was operated at a load of 4.1 kW (45 kV, 90 mA) and Cu $K\alpha$ radiation was selected with a nickel foil. Diffraction patterns were recorded as a series of 0.6° oscillations with an exposure time of 20 min at a crystal-to-detector distance of 200 mm. In cryogenic experiments with all crystals, glycerol was successfully used as a cryo-protectant reagent at a concentration of 10–20%(*w*/*v*). Exchange of the mother liquor for that containing the antifreeze reagent was carried out using micro-dialysis cells. The initial cooling of the crystals was carried out using nitrogen gas at 100 K produced by a cold nitrogen-gas generator (Rigaku).

The type I–III crystals of GluDH diffracted X-rays poorly to a resolution of less than 8 Å at both 293 and 110 K, indicating that these three types of crystals were not suitable for crystal structure analyses. In contrast, the type IV crystal diffracted X-rays beyond a resolution of 2.5 Å. The mosaic spread of the crystal was estimated to be less than 0.6° even at 110 K. Therefore, further experiments were carried out on the type IV crystal.

The collection of diffraction intensity data for the GluDH type IV crystal was carried out at the BL44B2 beamline (Adachi *et al.*,

1996) of SPring-8. The detector used was the R-AXIS IV system (Rigaku). The X-ray wavelength was adjusted to 1.0000 Å using a double-crystal monochrometer of fixed exit geometry, and the crystal-to-detector distance was set at 300 mm. The temperature of the crystal was maintained at 110 K using cold nitrogen gas produced by a Cryostream Cooler (Oxford Cryosystems).

The type IV crystal used in the diffraction experiment had approximate dimensions 0.45 × 0.15 × 0.04 mm. The crystal was cooled at our laboratory and transferred to the synchrotron facility using a SC4/2V dry-shipper (MVE). The mother liquor used for the cryogenic experiment contained 1.7 *M* lithium sulfate, 10%(*w*/*v*) glycerol and 50 m*M* sodium acetate (pH 5.0). Prior to the initial cooling, the crystal was dialyzed against the cryo-mother liquor for 16 h. The data were collected as a series of 0.9° oscillations per 4 min exposure. The GluDH type IV crystal diffracted X-rays beyond a resolution of 2.0 Å and exhibited no radiation damage during 18 h exposure. An example of the diffraction pattern is shown in Fig. 2.

The indexing, the calculation of integrated intensity of reflections and the evaluation of the diffraction data, including the relative scaling and the post-refinement, were performed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). In total, 1488939 reflections with $I \geq \sigma(I)$ were collected to a resolution of 2.0 Å and 206380 unique reflections in the resolution range 40.00–2.25 Å were prepared for the subsequent structural analysis. The completeness of the data was 99.7% against the expected number of reflections to a resolution of 2.25 Å. Even in the highest resolution shell (2.33 to 2.25 Å), the completeness was 99.5%. The average ratios of I/σ for all reflections and in the highest resolution shell were 18.0 and 3.3, respectively. The merging R factor $[R^{I}_{\rm merge} \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)]$ of this diffraction data was 0.072 for all reflections with $I \geq \sigma(I)$ and 0.31 for the highest resolution shell.

From the data processing, the unit-cell parameters of the GluDH type IV crystal were determined to be $a = 112.99$, $b = 163.70$, $c = 133.07$ Å, $\beta = 113.46°$. The space group of this crystal was $P2_1$, according to the systematic absence of $(0k0)$ reflections. When a hexamer of GluDH molecules (M_W = 280176 Da) occupied an asymmetric unit, the calculated V_M value was 3.42 Å^3 Da^{-1}. This value is within the reasonable range for crystals of soluble proteins (Matthews, 1968). The solvent content of this crystal was calculated to be 63.9%.

This low molecular-packing density is advantageous for examination of the structure of the surface region of the enzyme without the constraints of crystallographic symmetry: each subunit is more free from crystal contacts than in the crystals of GluDHs from various other hyperthermophilic microorganisms (Yip *et al.*, 1995; Knapp *et al.*, 1997).

Because the amino-acid sequence of this GluDH is highly homologous to that of GluDH from *P. furiosus* (Yip *et al.*, 1995), crystal structure analysis is under way by the molecular-replacement method.

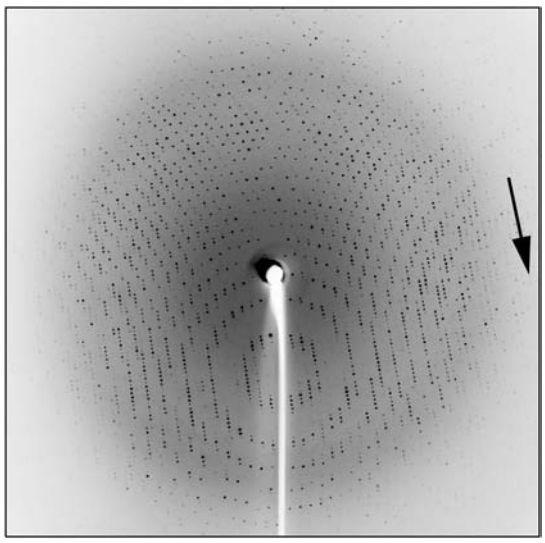

Figure 2
An X-ray diffraction pattern of the type IV crystal of GluDH recorded at the BL44B2 beamline of SPring-8. The arrow indicates a resolution of 2.5 Å.

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