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Crystallization of the NAD(P)-dependent glutamate dehydrogenase from the hyperthermophile *Pyrococcus furiosus*.

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Abstract

The NAD(P)-dependent glutamate dehydrogenase from *Pyrococcus furiosus* has been crystallized by the hanging-drop method of vapour diffusion using lithium sulfate as the precipitant. The crystals belong to the tetragonal system and are in space group $P4_22_12$ with unit-cell dimensions of $a = b = 167.2$, $c = 172.9$ Å. Consideration of the values of V_m and possible packing of the molecules within the cell suggest that the asymmetric unit contains a trimer. *P. furiosus* belongs to the family of Archaea and is one of the most thermostable organisms known, having an optimal growth temperature of 376 K. The glutamate dehydrogenase isolated from this organism has a half-life of 12 h at 373 K and, therefore, the determination of the structure of this enzyme will be important in advancing our understanding of how proteins are adapted to enable them to survive at such extreme temperatures.

Introduction

The reversible oxidative deamination catalysed by amino-acid dehydrogenases provides a major route for incorporation or elimination of ammonia. In most organisms the enzyme fulfilling this crucial role is L-glutamate dehydrogenase (GluDH) which catalyses the oxidation of L-glutamate to 2-oxoglutarate and ammonia with concomitant reduction of $NAD(P)^+$. Owing to their widespread occurrence GluDH's have been isolated from a wide range of species including members of the Bacteria, Archaea and Eukarya kingdoms. GluDH's from different species generally lie in one of two metabolic classes, involved with ammonia assimilation [generally $NADP^+$-dependent (E.C. 1.4.1.4)], or glutamate catabolism [generally NAD^+-dependent (E.C. 1.4.1.2)]. The dual coenzyme specificity GluDH (E.C. 1.4.1.3) found in vertebrates and in archaea exhibit similar affinities for NAD^+ and $NADP^+$ which may reflect an amphibolic role for this enzyme or that Archaea and Eukarya shared a common evolutionary history (Benachenhou-Lahfa, Forterre & Labedan, 1993). Many of these thermophilic archaea, are strictly anaerobic heterotrophs that utilize either carbohydrates or complex peptide mixtures as carbon and energy sources. They appear to obtain energy for growth from a fermentative metabolism (Fiala & Stetter, 1986; Neuner, Jannasch, Belkin & Stetter, 1990).

Recently, significant interest has developed in exploiting the biotechnological potential of extremophilic archaea which can commonly be found in submarine hydrothermal areas and volcanic fields and which are able to grow above the temperature of boiling water. The analysis of the structure/function relationships of enzymes from such hyperthermophiles is expected to provide insights into the molecular mechanisms which nature employs to permit such extreme temperature stability to be maintained. Such insights might suggest how proteins might be redesigned for novel commercial applications. In this regard, studies of GluDH have become a model system for the analysis of thermostability since this enzyme is abundant in these organisms and has been widely studied from a variety of species (Consalvi *et al.*, 1991, Maras *et al.*, 1992, DiRuggiero *et al.*, 1993, Maras *et al.*, 1994).

Biochemical studies on GluDH's have shown that the vast majority of these enzymes are based on a hexameric oligomer of identical subunits with an M_r of around 50 000 (McPherson & Wootton, 1983; Smith, Austen, Blumenthal & Nyc, 1975; Rice, Hornby & Engel, 1985; Maras *et al.*, 1994). Sequence comparisons for GluDH's from a diverse range of sources show that the hexameric enzymes are structurally homologous whatever their coenzyme specificity (Lilley *et al.*, 1991; Baker *et al.*, 1992). Specifically, the homology is poor for the N-terminal 50 residues, with homologous sequences being found for the next 350 residues. The highest level of sequence homology is found within the N-terminal half of the enzyme sequences. Structural studies on the GluDH from *Clostridium symbiosum* have shown that this region of the polypeptide chain is involved in glutamate/oxoglutarate binding and catalytic activity (Baker *et al.*, 1992; Stillman, Baker, Britton & Rice, 1993).

To date, the sequences of six GluDH's from archaea have been published and these show significant homology to the bacterial GluDH's and in particular to the GluDH from *C. symbiosum*. The most well studied of these hyperthermophilic microorganisms is *Pyrococcus furiosus*, which grows at temperatures up to 376 K (Klump *et al.*, 1992). The GluDH from this organism is extremely stable with a half-life at 373 K of 12 h (Consalvi *et al.*, 1991; Robb, Park & Adams, 1992). In order to enhance our understanding of the molecular basis of thermal stability we have initiated a structural study of the GluDH from *P. furiosus*.

Experimental

P. furiosus cells were grown on peptone and yeast extract (Raven, Ladwa, Cossar & Sharp, 1992) and all the purification steps were carried out at 277 K, using a modified procedure based on Consalvi *et al.* (1991). The cells were resuspended in 2 volumes of 20 m*M* Tris–HCl at pH 8 and ground in a mortar with sea sand. The mixture was centrifuged at 1500*g* for 45 min to remove the sand, the supernatant was diluted with 2 volumes of buffer *A* (20 m*M* phosphate buffer pH 6.5, 7% glycerol and 7 m*M* 2-mercaptoethanol) and centrifuged for a further 45 min at 30 000*g* to remove the cell debris. The crude extract was then

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loaded onto a column packed with Matrex gel red A (Amicon) previously equilibrated with the same buffer. The column was washed stepwise with 0.1 *M* and then 0.25 *M* NaCl in buffer *A* and the enzyme was eluted with buffer *A* containing 0.35 *M* NaCl. The elutant containing GluDH activity was concentrated through a YM-30 Diaflo membrane in an ultrafiltration cell (Amicon) and dialysed against 400 ml of 0.1 *M* KH_2PO_4 at pH 6. The purified protein was brought to 65% saturation with ammonium sulfate and the resulting precipitate stored at 277 K. The enzyme activity was found to be 340 units mg^{-1} using the method of Consalvi *et al.* (1991) and the purity further checked by sodium dodecyl sulfate and native polyacrylamide gel electrophoresis.

For the crystallization trials, samples of the stored *P. furiosus* GluDH precipitate were centrifuged for 20 min at 13 000 rev min^{-1} and the resulting pellet was dissolved in 50 m*M* potassium phosphate buffer (pH 6) and then dialysed against the same buffer. The concentration of the protein was adjusted to 10 mg ml^{-1} using an Amicon Centricon 30 microconcentrator by centrifugation at 3600 rev min^{-1} in a bench-top centrifuge. Samples (7 µl) of this protein solution were mixed with an equal volume of lithium sulfate solution in 0.1 *M* HEPES at pH 7.5 in the range 0.5–2.7 *M* and slowly concentrated at 290 K using the hanging-drop method of vapour diffusion. Crystals with the morphology of rhombic dodecahedra and with maximum dimensions 1.4 × 0.6 × 0.4 mm were obtained after 2 weeks over a lithium sulfate concentration range of 2.3–2.7 *M*. The crystals were confirmed to be that of *P. furiosus* GluDH by washing a single crystal, which had been subject to X-ray analysis, with 2.7 *M* lithium sulfate followed briefly with water and then determining the amino-acid sequence for the first ten residues on an Applied Biosystems 476A sequencer.

Results and discussion

X-ray precession photographs of these crystals showed that they belong to a primitive tetragonal system (Fig. 1). For the *k* and *l* axes, only even ordered reflections were represented in the diffraction pattern, providing good evidence that these are twofold and fourfold screw axes, respectively, identifying the space group as $P4_22_12$. The cell dimensions are $a = b = 167.2$, $c = 172.9$ Å and the cell volume is 4.83×10^6 Å^3. Gel-filtration studies have shown that this GluDH is hexameric (Consalvi *et al.*, 1991) and since it shows sequence similarity (34% identity over 413 equivalenced positions in the sequence alignment) with that of the hexameric GluDH from *C. symbiosum* this indicates that the subunits are arranged in 32 symmetry. Given the subunit M_r of 47 000, (Maras *et al.*, 1994) if the asymmetric unit of the cell contains a hexamer the corresponding value for the V_m would be 2.14 Å^3 Da^{-1}. Alternatively with a trimer in the asymmetric unit the V_m would be 4.29 Å^3 Da^{-1} and the GluDH hexamer would have to be constructed using the crystallographic twofold which runs across the (110) diagonal. If this were to be the case, the high solvent content of the cell (71%) would lie at the extreme end of the values previously reported for proteins by Matthews (1977).

Preliminary images recorded on station PX9.5 at the DRAL Daresbury Laboratory SRS, indicate that the crystals diffract to beyond 2.0 Å. 45°of data have been collected from two GluDH crystals on this station to a resolution of 2.2 Å using the rotation method of data collection and with rotations of 0.6° per frame. Observations were recorded on a large MAR Research image plate at an X-ray wavelength of 0.92 Å. 416 001 measurements were made of 115 181 independent reflections and the data set processed using the *MOSFLM* (Leslie, 1992) and *CCP*4 (Collaborative Computational Project, Number 4, 1994) software packages. The crystals showed no appreciable radiation damage during data collection and the data were merged to an *R* factor of 4.3% with 93% completeness of the data from 20 to 2.2 Å resolution.

A self-rotation function, calculated with the program *POLARRFN* (Kabsch, unpublished; Collaborative Computational Project, Number 4, 1994) using the strongest 8300 reflections from 10 to 5 Å and a 30 Å radius of integration, gave a peak with 94% of the height of the origin in the $\kappa = 120°$

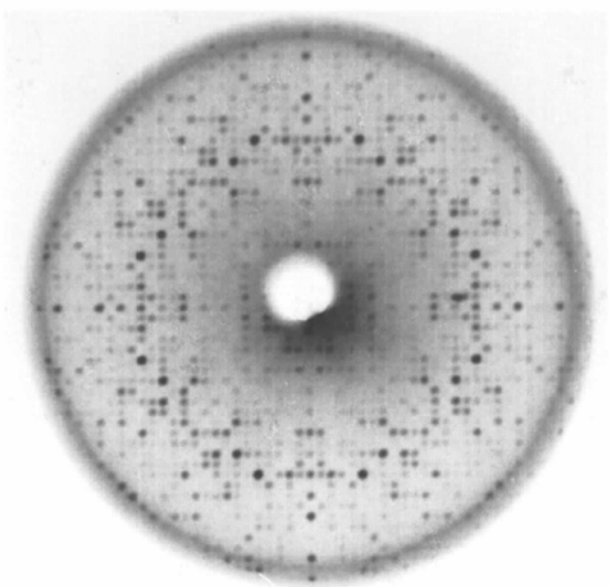

Fig. 1. A screened 7° precession photograph of the *hh*0 zone of the *P. furiosus* GluDH crystals taken with nickel-filtered Cu $K\alpha$ radiation.

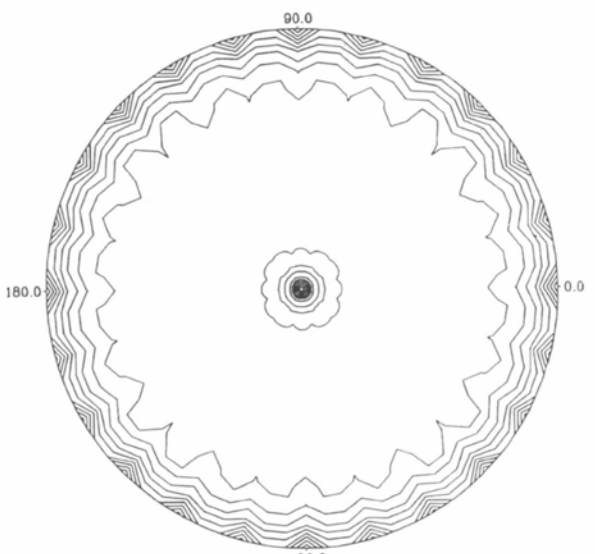

Fig. 2. A stereographic projection of the $\kappa = 180°$ section of the self-rotation function of the *P. furiosus* GluDH native data. The non-crystallographic twofold axes can be seen at the perimeter of the plot ($\omega = 90°$) and occur at φ values of every 15°.

section at $\omega = 0°$. In addition, the non-crystallographic twofolds which occur on the $\kappa = 180°$ section at $\omega = 90°$ and every 15° on φ can be clearly seen in Fig. 2. These results are consistent with the hexamer being aligned in the cell with the molecular threefold very close to the *c* axis, and perpendicular to the crystallographic twofold which bisects *a* and *b*. The structure determination of *C. symbiosum* GluDH has shown that the molecule is approximately 100 Å in length along the direction of the threefold axis. Thus, given the *c*-axis cell dimension of 173 Å, packing considerations strongly suggest that the asymmetric unit contains a trimer and that the crystals have a high solvent content.

An attempt to solve the structure by molecular replacement is now underway using the model of the NAD-linked GluDH from *C. symbiosum*. The results of this analysis may well provide important new insights into the molecular basis of the adaptation of enzymes to life at extreme temperatures.

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