Acta Cryst. (1995). D**51**, 395–398

Preliminary crystallographic analysis of an extremely thermostable glutamate dehydrogenase from the archaeon *Pyrococcus woesei*.

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(*Received* 14 *July* 1994; *accepted* 30 *September* 1994)

Abstract

The extremely heat-stable glutamate dehydrogenase (GluDH) from *Pyrococcus woesei* was crystallized by the hanging-drop vapour-diffusion method. Crystals suitable for X-ray crystallographic investigations were obtained using polyethylene glycol (PEG) 4000 and ammonium acetate as precipitating agents. The crystals obtained diffract to a resolution of 2.8 Å, have a prismatic shape and grow up to 0.5 mm in their maximal dimension. They belong to the triclinic system (space group $P1$; $a = 90.9$, $b = 92.8$, $c = 107.1$ Å, $\alpha = 69.1$, $\beta = 80.7$, $\gamma = 65.0°$) with a unit-cell volume of 765 052 Å^3 which accommodates one GluDH hexamer of 276 kDa. The averaged density of the crystal determined by Ficoll-gradient centrifugation is 1.15 g cm^{-3}, which corresponds to a molecular mass of 255 kDa in the unit cell. A native data set has been collected on an MAR image-plate system using Cu $K\alpha$ radiation. The completeness of the data set in the range 316–3 Å is 74%, and contains 64% of the data in the outer shell (3.4–2.8 Å), with an average R_{merge} value of 9%. Calculation of self-rotation functions revealed the 32 symmetry of the hexamer; 3 non-crystallographic twofold axes were found at a distance of 120° in a plane perpendicular to the non-crystallographic threefold axis.

Introduction

Recently a number of hyperthermophilic microorganisms that grow at or above 283 K have been isolated from geothermally heated environments (Stetter, Fiala, Huber, Huber & Segerer, 1990). All these organisms are prokaryotes and belong to the kingdom of archaea (Woese, Kandler & Wheelis, 1990). One of the most thermophilic organisms isolated so far is *Pyrococcus woesei* (Zillig *et al.*, 1987) a strictly anaerobic heterotrophic archaeon which belongs to the family *Thermococcales*.

Proteins expressed in these organisms are extremely thermostable (Adams, 1993; Koch, Spreinat, Lemke & Antranikian, 1991) and resistant to denaturing chemicals like urea or guanidinium hydrochloride. The phenomenon of thermostability of proteins cannot be explained from sequence information alone. Structural information is needed to understand the strong interactions between polypeptide chains and to analyze non-covalent forces that might be responsible for the stability of these enzymes.

We have chosen glutamate dehydrogenases (GluDH) as a model protein because the enzyme is well studied, evolutionary conserved in all three primary domains eucarya, bacteria and archaea (Teller, Smith, McPherson, Engel & Guest, 1992), and structural information is already available for one mesophilic enzyme from *Clostridium symbiosum* (Baker *et al.*, 1992). The enzyme is remarkably thermostable and has a half life of more than 3 h at 373 K (Eggen, Geerling, Waldkötter, Antranikian & de Vos, 1993). Furthermore it offers a model to study the quaternary assembly and interactions between protein subunits at high temperatures.

GluDH catalyses the oxidative deamination of L-glutamate to 2-oxo-glutarate and ammonia and thereby reduces either NAD^+ or $NADP^+$ (Goldin & Frieden, 1971). The NAD^+-dependent enzymes are part of the glutamate catabolism whereas the predominantly $NADP^+$-dependent glutamate dehydrogensases are involved in ammonia assimilation. Enzymes purified from vertebrates are able to use both coenzymes.

GluDH's from mammals (Julliard & Smith, 1979), *Escherichia coli* (McPherson & Wootton, 1983; Valle *et al.*, 1984) and archaea (Eggen *et al.*, 1993) show significant sequence similarities and form a hexameric structure with subunits of 45–56 kDa. A second class of GluDH's is found in fungi like *Saccharomyces cerevisiae* (Uno, Matsumoto, Adachi & Ishikawa, 1984) and *Neurospora crassa* (Veronese, Nyc, Degani, Brown & Smith, 1974) (the latter expresses also the first class of GluDH's). These glutamate dehydrogenases are NAD^+ dependent and form tetramers of a larger subunit mass (116 kDa).

A high-resolution structure (1.96 Å) has been described for *Clostridium symbiosum* GluDH (Baker *et al.*, 1992). Six subunits are arranged in 32 symmetry. Each subunit contains two domains separated by a deep cleft which binds NAD^+.

Experimental

Pyrococcus woesei (DSM 3773) was grown on a sulfur-free medium as described by Rüdiger, Ogbonna, Märkl & Antranikian (1992). Batch cultivations were performed in a 20 l reactor (Bioengineering, Wald, Switzerland) under continuous gassing at 10%(*v*/*v*) min^{-1} with N_2:CO_2 (80:20). Routinely, GluDH was purified from 3 g cells, which were suspended in 10 ml of 100 m*M* sodium phosphate buffer (pH 5) and disrupted by sonication for 3 min. The cell-free extract was separated by centrifugation and dialyzed against 50 m*M* sodium acetate buffer (pH 5.0). The denatured proteins were separated by centrifugation and the supernatant was loaded onto an epoxy-activated Sepharose 6B column to which maltose was coupled and eluted with 100 m*M* sodium acetate buffer pH 5.0. Fractions containing GluDH activity were further purified by preparative native polyacrylamide electrophoresis (PrepCell, Bio-Rad, München), and concentrated for crystallization trials to a protein concentration of 10 mg ml^{-1}. Screening for optimal crystallization conditions was performed by the vapour-diffusion method using a screening system with 48 different conditions in a pH range $4.5 < \text{pH} < 9.0$. The droplets were prepared by mixing 3 µl of a 10 mg ml^{-1} protein solution in 50 m*M* acetate buffer pH = 5.0, with 3 µl of the reservoir solution.

ISSN 0907-4449

All X-ray diffraction measurements were performed at 285–286 K. X-ray diffraction intensity data were collected with an MAR image-plate detector installed on a MAC Science/Siemens rotating-anode generator which was operated at 45 kV and 90 mA. Graphite-monochromatized Cu $K\alpha$ radiation was used. By the rotation technique 180 images of 1.0° rotation increment were taken in 180 s each at a crystal-to-detector distance of 150 mm. The raw data were indexed and evaluated with the *MARXDS* package (Klein, 1993; Kabsch, 1993) on a VAX 4000-90 workstation with 80 MB RAM main memory. Merging and scaling of the data was performed within the *PROTEIN* program system (Steigemann, 1974) using scale and relative isotropic temperature factors. The conversion of the unsorted reflection intensity output from *MARXDS* was performed with the interactive program *MTP* (Mar to Protein) developed for the purpose of this study. *MTP* assigns scaling group numbers to groups of frames and produces an unmerged output of indices, structure-factor amplitudes, σ values and crystal identifiers in ascending order of the scaling group numbers.

A native Patterson map was calculated on a 1.0 Å grid using intensity data ($F^2 > 2\sigma$) in the resolution range 10–4 Å. The final conditions selected for the correlation calculations were that the first Patterson map was represented by all data with $F^2 > 2\sigma$ between 10 and 4 Å resolution. From this map the 4000 highest peaks were selected to represent the second Patterson function.

Self-rotation functions (Rossmann & Blow, 1962) for twofold and threefold symmetry axes were calculated using the real-space search options and a product target function of both program systems *PROTEIN* (Steigemann, 1974) (not shown) and *X-PLOR* (Brünger, 1992). The Patterson space explored was a hollow sphere. The origin peak of the map was excluded by choosing an inner radius limit of 5 Å. The outer radius limit was set to 25 Å. Results of the calculations were displayed in a ψ, φ, χ polar angle coordinate system. The rotation function was explored in spherical polar coordinates in 5 and 1° intervals for the scans in ψ, φ at constant χ. Furthermore, scans of ψ, φ were performed at variable χ which increased in 10° steps in an interval from 0 to 180° and thus covered all possible symmetry axes. The highest correlation values (σ) of each single scan were plotted against χ.

Results and discussion

Several different crystal shapes were observed in the initial screening. One optimal crystal form, thick skew prisms (Fig. 1), was grown at 293 K from hanging droplets using a reservoir solution containing 15% PEG 4000, 0.1 *M* sodium acetate in 50 m*M* MES/K^+[2-(*N*-morpholino)ethanesulfonic acid] buffer pH 5.4.

Single crystals with a certain tendency to form clusters were obtained within several days. It was easily possible to separate the crystals by a glass needle before mounting them. The crystals are mechanically sensitive, grow to a size of 0.5 mm and showed no inhomogeneities in polarized light. Before sealing them in glass capillaries they were stabilized with a solution of 20% PEG 4000, 0.2 *M* sodium acetate in 50 m*M* MES/K^+buffer (pH = 5.5). The crystals have the shape of skew prisms with a maximum dimension of 0.5 mm and diffracted X-rays to better than 2.8 Å resolution.

The reciprocal lattice shows no specific extinctions and only the Friedel symmetry could be detected. Accordingly, the crystals belong to the triclinic system with space group *P*1. The refined (*MARXDS*) unit-cell constants of the native crystals are $a = 90.9$, $b = 92.8$, $c = 107.1$ Å, $\alpha = 69.1$, $\beta = 80.7$, $\gamma = 65.0°$.

The unit-cell parameters were determined from a complete native data set to 2.8 Å resolution by auto-indexing and refinement in *MARXDS*.

The scaled intensities gave a linear *R* factor of 9% and the completeness of the data set was 74% (316–3 Å) and 64% in the outermost resolution shell from 3.4 to 2.8 Å.

Biochemical data suggest that the enzyme is a hexamer with a molecular mass of 276 kDa composed of identical subunits. The volume of the unit cell ($V = 765\,052$ Å^3) is compatible with the presence of a hexamer in the triclinic cell. This corresponds to a packing density of 2.77 Å^3 Da^{-1}, a value which is among the most probable packing densities found in protein crystals (Matthews, 1968). It has been described, however, that other oligomers like tetramers of this enzyme can also occur in solution (Consalvi *et al.*, 1991). We have, therefore, determined the crystal density by centrifugation in a Ficoll gradient (Bode & Schirmer, 1984). The crystals have an averaged density of 1.15 g cm^{-3}, which corresponds to a molecular mass of 255 kDa in the unit cell. This value is in agreement with the assumption of six subunits in the unit cell.

Self-rotation functions, calculated for twofold ($\chi = 180°$) and threefold ($\chi = 120°$) symmetry axes showed several correlation maxima, which are consistent with molecular 32 symmetry of the glutamate dehydrogenase hexamer. (Fig. 2, Table 1).

By constucting unit vectors coincident with the local axes and calculating the appropriate scalar products it was possible to show that three coplanar local twofold axes are orthogonal (scalar products are zero) to the local threefold axis and repeat at angles of 120°. In addition, scans in χ were performed (Fig. 3), showing high correlation only for the correct repeat periods of two- and threefold local axes. The most clear results were obtained with an integration radius of 25 Å. The local 32 axis system of the glutamate dehydrogenase hexamer is shown in Fig. 2 together with the orientation (ψ, φ) and height of the corresponding correlation maxima. A model of the subunit assembly which is based on the data obtained by the self-

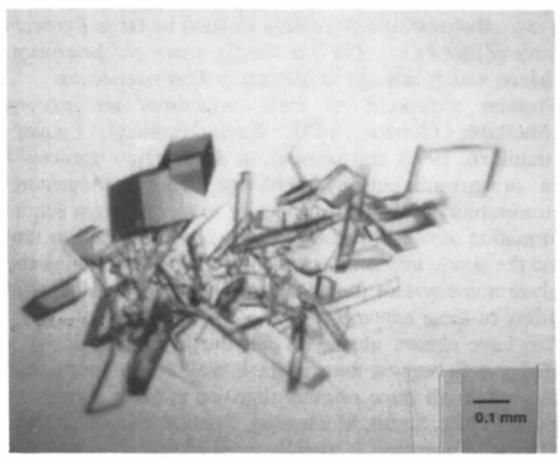

Fig. 1. Crystals of glutamate dehydrogenase from *Pyrococcus woesei* grown in PEG 4000, ammonium acetate and MES/K^+ buffer as described in the text. (Bar = 0.1 mm.)

rotation analysis has been constructed and is shown in Fig. 4. The subunits of the GluDH from *Pyrococcus woesei* are arranged in the same way as the mesophilic protein from *C. symbiosum.*

The data obtained from this preliminary analysis enables us to determine the structure of this extremely thermostable enzyme by multiple-isomorphous or molecular replacement. However, it cannot be expected that the determination of the structure alone will fully explain the phenomenon of thermostability of this glutamate dehydrogenase, but it will certainly initiate further investigations to understand better the stabilizing forces in this protein as well as between its subunits.

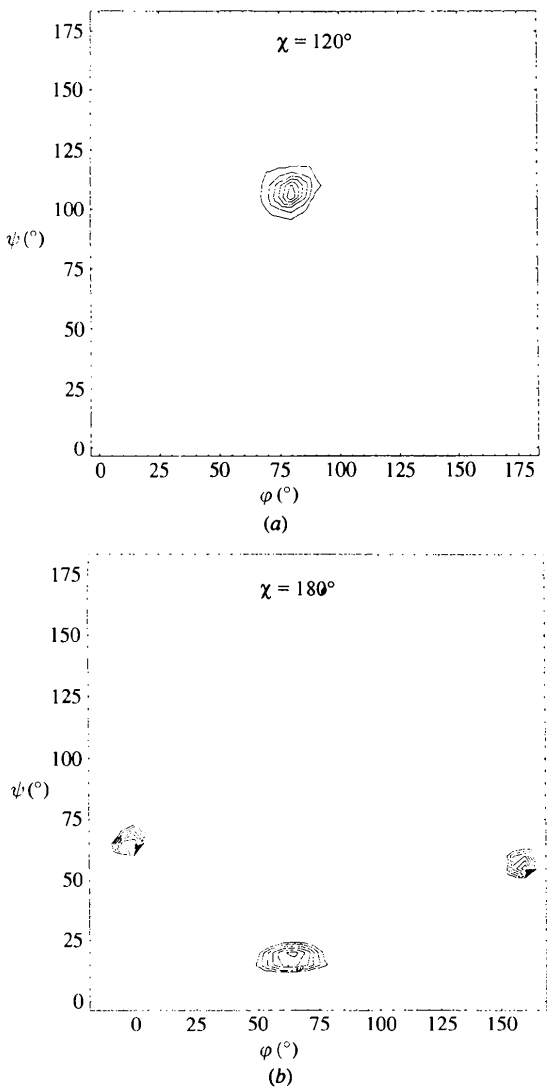

Fig. 2. (*a*) Self-rotation function section ($\chi = 120°$) showing the non-crystallographic threefold axis. The next highest peak was 3.5σ. (*b*) Self-rotation function section ($\chi = 180°$) showing the three non-crystallograhic twofold axes. It can be shown that the twofold axes which repeat in 120° angles lie in a plane which is perpendicular to the non-crystallographic threefold axis. The relative heights and the location of the peaks are shown in Table 1. The next highest peak was 3.9σ and the mean value of the rotation function was 4.8σ.

Table 1. *Relative heights and locations of peaks illustrated in Fig. 2*

Local axes	ψ (°)	φ (°)	Height of correlation maxima (σ)
Threefold	105.0	77.5	6.5
Twofold 2(1)	63.8	0.0	6.5
Twofold 2(2)	17.8	65.0	6.4
Twofold 2(3)	53.0	159.8	6.4

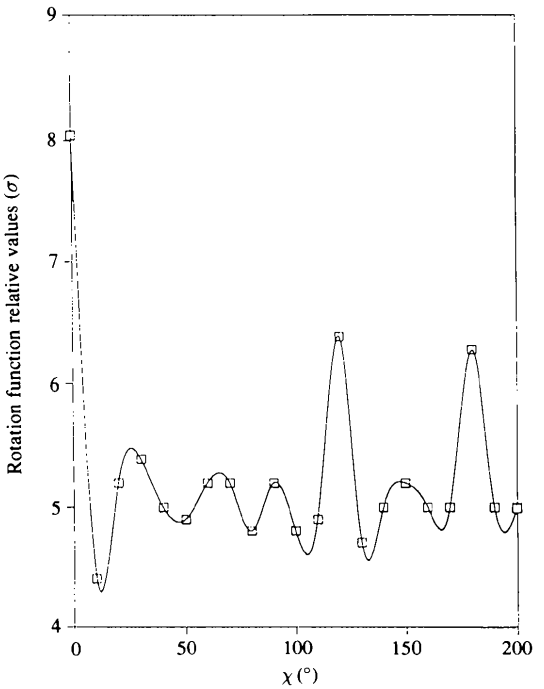

Fig. 3. χ-scan in steps of 10°, showing the relative peak heights of the rotation-function search in relation to the origin peak. The maximal peak height of the ψ,φ search at each χ was plotted against χ. High correlation is obtained only for the proper repeat periods of two- and threefold local axes.

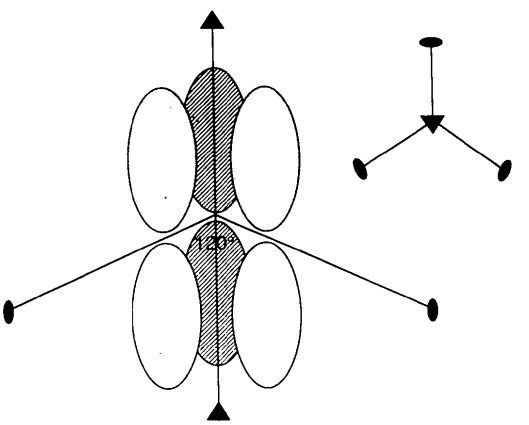

Fig. 4. Schematic drawing of the subunit assembly of glutamate dehydrogenase from *Pyrococcus woesei.* The asymmetry of the subunits is not taken into account.

The authors are grateful to the Deutsche Forschungsgemeinschaft and the Commission of the European Communities (EC Project Biotechnology of Extremophiles, contract number BIO 2-CT93-0274) for financial support.

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