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Preliminary crystallographic studies of triosephosphate isomerase (TIM) from the hyperthermophilic Archaeon *Pyrococcus woesei*

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Abstract

Recombinant triosephosphate isomerase (TIM) from a hyperthermophilic Archaeon, *Pyrococcus woesei*, has been crystallized. Three crystal forms have been obtained: monoclinic, orthorhombic and hexagonal. The monoclinic crystals belong to space group $P2_1$ with cell dimensions a = 79.1, b = 89.2, c = 145.4 Å and β = 92.8°, and diffract to at least 2.6 Å. The orthorhombic crystals belong to space group $P2_12_12$ with a = 89.4, b = 155.9, c = 79.5 Å, and diffract to 2.9 Å. Diffraction from the hexagonal form showed extensive disorder. The monoclinic form contains two tetramers in the asymmetric unit, which are in the same orientation but related by a pseudo-centring. The orthorhombic form contains one tetramer in the asymmetric unit which is in approximately the same orientation as in the monoclinic form. Knowledge of the structure of this hyperthermostable TIM, which is tetrameric in contrast to dimeric forms previously observed, will add to the understanding of protein thermostability.

1. Introduction

Structural analyses of numerous proteins from thermophilic (328–358 K) organisms have revealed many different potential mechanisms of thermal adaptation, such as increased compactness and hydrophobicity (Russell & Taylor, 1995; Vieille & Zeikus, 1996). Analysis of protein structures from hyperthermophilic (>358 K) organisms reveals a potential common feature for thermal stabilization, namely electrostatic interactions, with residues clustered into complex ion-pair networks, often at the intersubunit regions (Yip *et al.*, 1995; Russell *et al.*, 1997), or straddling the entire surface of the molecule (Aguilar *et al.*, 1997). Mutagenesis studies have recently confirmed the importance of such networks in a hyperthermophilic glyceraldehyde-3-phosphate dehydrogenase (Pappenberger *et al.*, 1997). There are also several examples of higher oligomeric states for hyperthermophilic proteins compared to their mesophilic counterparts (*e.g.* Schurig *et al.*, 1995; Hennig *et al.*, 1997; Kengen *et al.*, 1993; Aguilar *et al.*, 1997).

Pyrococus woesei is a hyperthermophilic organism originally isolated from sediment samples obtained from marine solfataric vents (Zillig *et al.*, 1987) and exhibits an optimum growth temperature of ∼373 K. Triosephosphate isomerase (TIM) from *P. woesei* has been chosen as a model enzyme for a study of the structural basis of hyperthermostability, as it is a ubiquitous enzyme of the glycolytic pathway and is one of the most studied enzymes with a large structural database for a comparative study. Seven crystal structures are available from bacterial and eukaryotic sources: chicken muscle (Banner *et al.*, 1975), *Trypanosoma brucei* (Wierenga *et al.*, 1987), yeast (Lolis *et al.*, 1990), *Escherichia coli* (Noble *et al.*, 1993), human (Mande *et al.*, 1994), *Plasmodium falciparum* (Velanker *et al.*, 1997) and the thermophilic bacterium *Bacillus stearothermophilus* (Delboni *et al.*, 1995). All these structures are homodimeric, with monomers exhibiting the highly conserved eight-stranded α/β barrel fold and sharing around 40% sequence identity. The structure of an engineered monomeric TIM has also been reported (Borchert *et al.*, 1993). TIM from *P. woesei* has been cloned, sequenced and partially characterized (Kohlhoff *et al.*, 1996), and at 224 residues per monomer it is the shortest reported TIM sequence. Sucrose density-gradient centrifugation and gel-filtration experiments have shown that *P. woesei* TIM is a tetramer, as is the TIM from *Methanothermus fervidus* (optimum growth temperature 356 K) (Kohlhoff *et al.*, 1996).

The sequence identity of *P. woesei* TIM, compared with the sequences of TIMs whose structures are known, is low at ∼20%; however, the three key catalytic lysine, histidine and glutamate residues are conserved, as are eight of the 14 highly conserved dimer-interface residues. Determination of the structure should add to our understanding of protein hyper-

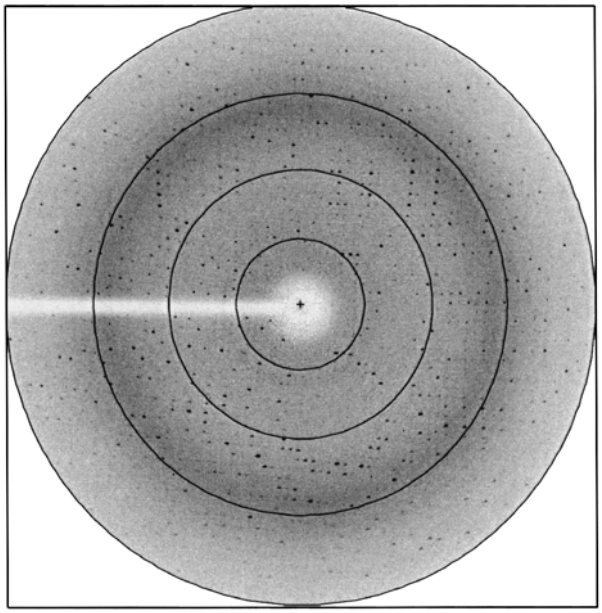

Fig. 1. A 10 min exposure 0.5° oscillation image from a flash-frozen monoclinic crystal of *P. woesei* TIM. Crystal-to-detector distance was 220 mm giving 2.6 Å resolution at the edge.

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Table 1. *Data-collection statistics*

Values in parentheses refer to reflections in the outer resolution shell, 2.71–2.60 Å for the monoclinic data and 4.14–4.0 Å for the orthorhombic data.

	Monoclinic with 2-CP	Orthorhombic with 2-PG
Number of measured reflections	870 586	95 933
Number of unique reflections	60 910	9 907
Resolution (Å)	2.6	4.0
$R_{\rm merge}$ (%)†	10.4 (26.2)	11.0 (14.0)
Completeness (%)	98.1 (90.0)	98.8 (97.7)
$I/\sigma(I)$	17.1 (5.5)	10.4 (8.8)

† $R_{\rm merge} = \sum |I(k) - \langle I\rangle| / \sum I(k)$, where $I(k)$ is the value of the kth measurement of the intensity of a reflection, $\langle I\rangle$ is the mean value of the intensity of that reflection and the summation is over all measurements.

thermostability and to the role of higher oligomeric states in achieving such stability.

2. Expression and purification

The TIM gene from *P. woesei* was cloned into the vector pJF118 EH (Fürste *et al.*, 1986) *via* two new restriction sites (EcoRI and PstI) created by PCR mutagenesis with the primers 5′-GATTGGTGAGAATTCATGGCTAAACTC-3′ and 5′-ACAAAGCCCTTATTAATTGGACGTCTTCGATTTAG-3′. The sequence of the inserted gene was confirmed on both strands. Expression in *E. coli* DH5α cells was performed using standard procedures (Sambrook *et al.*, 1989). Following expression, 6 g of wet *E. coli* cells were resuspended in 25 ml of 50 m*M* Tris–HCl buffer, pH 8.5, containing 25 m*M* NaCl, 2 m*M* edta and 1 m*M* PMSF. After sonication, the cell extract was heated to 358 K for 15 min and denatured protein removed by centrifugation. The supernatant was applied to a gel-filtration column (dimensions 60 × 5 cm, containing Pharmacia S300 matrix). Pooled fractions exhibiting TIM activity were loaded onto an anion-exchange column (three 5 ml Pharmacia High Trap Q were used in series) with the TIM eluting at 0.15 *M* NaCl. Salt was removed by dialysis against the same buffer described above but without PMSF and the protein was concentrated to 10 mg ml^{-1}.

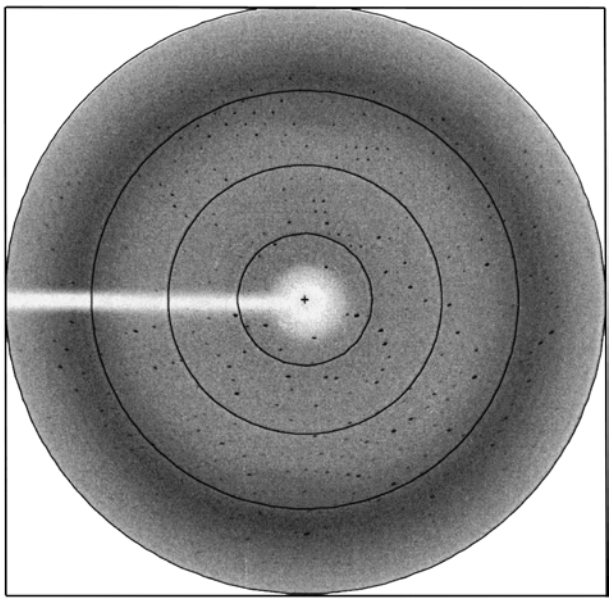

Fig. 2. A 10 min exposure 0.5° oscillation image from a flash-frozen orthorhombic crystal of *P. woesei* TIM. Crystal-to-detector distance was 250 mm giving 2.9 Å resolution at the edge.

3. Crystallization and data collection

Initial crystallization conditions were screened using the sparse-matrix method (Jancarik & Kim, 1991) by the hanging-drop vapour-diffusion method. Crystals of diffraction quality could only be obtained in the presence of inhibitors. A monoclinic crystal form was obtained by co-crystallization with 20 m*M* 2-carboxyethylphosphonic acid (2-CP) with 0.1 *M* sodium acetate, pH 4.0, and 7% PEG 4000 as the precipitant buffer. An orthorhombic crystal form was obtained by co-crystallization with 20 m*M* 2-phosphoglycolic acid (2-PG) with 0.1 *M* sodium acetate, pH 4.2, and 5% PEG 4000 as the precipitant buffer. Occasionally, hexagonal crystal forms were obtained under these same conditions with 2-PG, but their diffraction showed high disorder.

Data were collected at 100 K on an in-house Cu rotating-anode X-ray source (operating at 45 kV, 80 mA) and a 150 mm radius MAR image-plate detector, with both monoclinic and orthorhombic crystals being cryoprotected in the crystal-

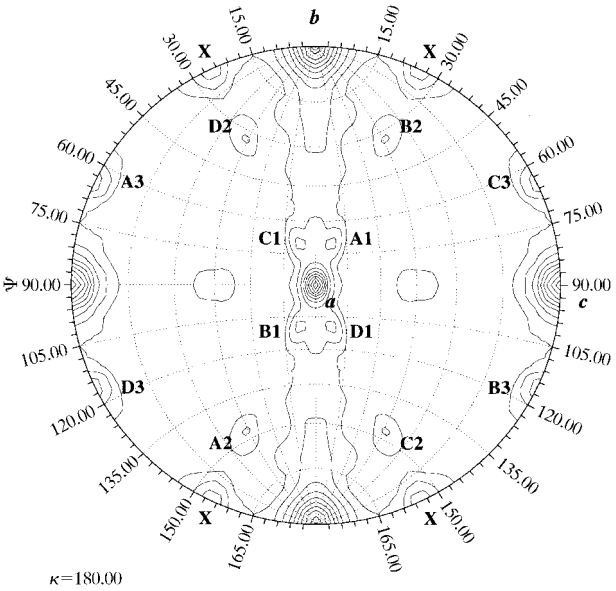

Fig. 3. The κ = 180° section of the self-rotation function for the orthorhombic crystal form, using data from 10 to 4 Å, calculated and drawn using *GLRF* (Tong & Rossmann, 1997). Spherical polar angles are defined as: φ, the angle from the Cartesian x axis (c) in the xy plane; ψ, the angle from the z axis (b); κ, the rotation around the axis defined by $\varphi\psi$. A 222 n.c.s. set is formed by peaks A1, A2 and A3, where the $\varphi\psi$ angles for peaks A1, A2 and A3 are (81, 69°), (135, 147°) and (0, −66°), respectively. Peaks labelled B, C and D form other 222 n.c.s. sets related to the first by the crystallographic twofold axes. A1, A2 and A3 have peak heights of 33, 22 and 36% of height of the origin, respectively. Peaks labelled X, 36% of the origin, arise from interactions between the crystallographic screw axis along a and the n.c.s. twofold axes at A3, B3, C3 and D3.

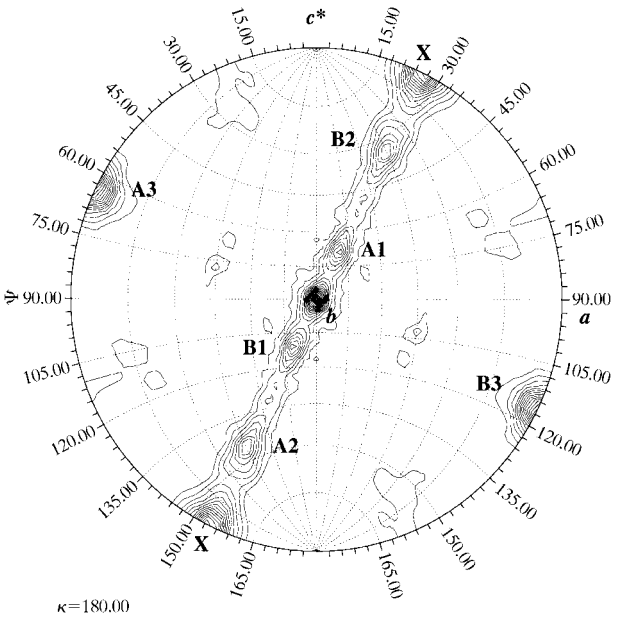

Fig. 4. The κ = 180° section of the self-rotation function for the monoclinic crystal form, using data from 10 to 3 Å, calculated and drawn using *GLRF*. The orthogonalization was chosen to place axes of similar length in the same orientation as in the orthorhombic crystal form in Fig. 3. Spherical polar angles are defined as: φ, the angle from the Cartesian x axis (a) in the xy plane; ψ, the angle from the z axis (c^*); κ, the rotation around the axis defined by $\varphi\psi$. A 222 n.c.s. set is formed by peaks A1, A2 and A3, with $\varphi\psi$ angles (78, 69°), (135, 144°) and (0, −63°), respectively. Peaks B1, B2 and B3 form the n.c.s. set related by the 2_1 screw axis along b. A1, A2 and A3 have peaks heights of 48, 44 and 79% of the height of the origin, respectively. Peaks labelled X arise from the interaction between the crystallographic screw and the n.c.s. twofold axes A3 and B3.

lization buffer containing 35% glycerol. Crystals had to be introduced slowly to this level of glycerol in a stepwise sequence: 10 min in 10% glycerol, 10 min in 20%, 5 min in 30% and 2 min in 35%. The monoclinic unit-cell dimensions were a = 79.1, b = 89.2, c = 145.4 Å and β = 92.8°, in space group $P2_1$, and diffraction was observed to at least 2.6 Å (Fig. 1). The orthorhombic crystals belong to space group $P2_12_12$, with a = 89.4, b = 155.9, c = 79.5 Å, and diffract to 2.9 Å (Fig. 2), but data collection was limited to 4.0 Å. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) (Table 1).

P. woesei TIM is a tetramer of 4 × 24 kDa. Assuming one tetramer per asymmetric unit in the orthorhombic form gives a V_m value of 2.92 Å^3 Da^{-1}, which corresponds to a solvent content of 58% (Matthews, 1968). Given the similarity of the monoclinic cell to the orthorhombic cell, this suggest two tetramers per asymmetric unit for the $P2_1$ form, giving a V_m of 2.71 Å^3 Da^{-1} and a solvent content of 55%.

4. Non-crystallographic symmetry

Since eukaryotic and bacterial TIMs are dimeric, it seems likely that the tetrameric *P. woesei* TIM will possess 222 non-crystallographic symmetry (n.c.s.). This is supported by the self-rotation functions which show orthogonal twofold axes, distinct from the crystallographic axes, and no evidence of fourfold symmetry. Fig. 3 shows the κ = 180° section of the self-rotation function for the orthorhombic crystal form, which reveals the orientation of the 222 tetramer with one twofold n.c.s. axis lying in the *bc* plane. Fig. 4 shows a similar section for the monoclinic crystal form, which clearly reveals the orientation of a set of 222 n.c.s. axes, with one twofold n.c.s. axis lying in the *ac* plane. Crystal packing suggests the presence of two tetramers in the asymmetric unit, yet only one unique set of n.c.s. axes is observed. Calculation of a native Patterson using the monoclinic data reveals a peak at (a/2, b/4, c/2) which is approximately 50% of the height of the origin peak. We conclude that the $P2_1$ asymmetric unit contains two tetramers in the same orientation related by pseudo-centring. Figs. 3 and 4 show that the orientation of the tetramer is very similar with respect to the cell axes of similar length in the two crystal forms. Attempts are being made to determine the structure by molecular replacement and by heavy-atom isomorphous replacement.

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