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Structure of a high-resolution crystal form of human triosephosphate isomerase: improvement of crystals using the gel-tube method

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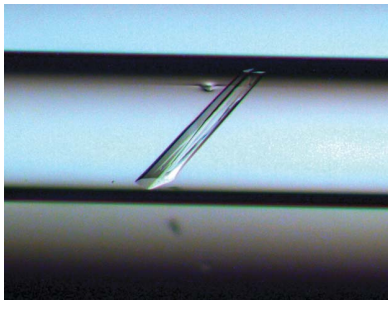

Crystals of human triosephosphate isomerase with two crystal morphologies were obtained using the normal vapour-diffusion technique with identical crystallization conditions. One had a disordered plate shape and the crystals were hollow (crystal form 1). As a result, this form was very fragile, diffracted to 2.8 Å resolution and had similar crystallographic parameters to those of the structure 1hti in the Protein Data Bank. The other had a fine needle shape (crystal form 2) and was formed more abundantly than crystal form 1, but was unsuitable for structure analysis. Since the normal vapour-diffusion method could not control the crystal morphology, gel-tube methods, both on earth and under microgravity, were applied for crystallization in order to control and improve the crystal quality. Whereas crystal form 1 was only slightly improved using this method, crystal form 2 was greatly improved and diffracted to 2.2 Å resolution. Crystal form 2 contained a homodimer in the asymmetric unit, which was biologically essential. Its overall structure was similar to that of 1hti except for the flexible loop, which was located at the active centre Lys13.

1. Introduction

Accurate and reliable protein structures are of significance and help in the understanding of biological reactions and in structure-based rational drug design. The quality of crystals is the most important key factor in acquiring a high-resolution structure. Therefore, a number of methods to improve the quality of protein crystals during the course of crystal growth are being developed. For example, methods to increase the stability of the crystal-growth environment include growth in a high magnetic field (Kinoshita *et al.*, 2003), growth in the presence of cross-linking agents (Pakhomova *et al.*, 2000), growth in gels (Cudney *et al.*, 1994; García-Ruiz & Moreno, 1994; Zhu *et al.*, 2001) and growth in microgravity (Borgstahl *et al.*, 2001; Vergara *et al.*, 2003; Miele *et al.*, 2003).

Crystals of human triosephosphate isomerase (TIM; EC 5.3.1.1) with two crystal morphologies were obtained using the normal vapour-diffusion technique with identical crystallization conditions. One had a disordered plate shape and the crystals were hollow (crystal form 1; Fig. 1*a*) and were very fragile. The crystals diffracted to 2.8 Å resolution and had similar crystallographic parameters to those of the structure 1hti in the Protein Data Bank (Table 1; Mande *et al.*, 1994). The crystals were suitable for structure analysis, but it was difficult to grow crystals suitable for high-resolution data collection. Agarose gel combined with the vapour-diffusion method gave slightly larger crystals, but did not improve the crystal quality. The crystals maintained their disordered shape and the vacant space inside. The other form of human TIM had a fine needle shape (crystal form 2; Fig. 1*b*); these crystals were formed more abundantly than crystal form 1, but were unsuitable for structure analysis. Agarose gel improved crystal growth to some degree; however, the resulting crystals remained unsuitable for X-ray analysis.

TIM is an enzyme that is essential for glycolysis in both prokaryotes and eukaryotes and reversibly catalyses the isomerization of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. A number of TIM structures from various species are available and have been used for structural studies. For example, based upon parasitic structures, drug discovery directed towards high inhibitory activity has been developed (Velanker *et al.*, 1997). In addition to

effectiveness, selectivity against human TIM is necessary because TIM is also essential for human beings. If human TIM was inhibited by drug candidates, severe toxicity would arise.

High-resolution structures of human TIM as well as comprehensive understanding of the enzyme reaction should greatly help in the discovery of drugs with selectivity between the respective TIMs. Therefore, gel-tube methods on earth and under microgravity were applied for TIM crystallization.

2. Materials and methods

2.1. Preparation and purification

Human TIM was PCR-amplified using human liver Marathon-Ready cDNA (Clontech) and cloned into the pTrcHis B vector (Invitrogen). Sequencing of the recombinant DNA, performed with a PRISM310 genetic analyzer (Applied Biosystems), confirmed the integrity of the cloned DNA. Luria Broth medium containing 50 mg ml^{-1} ampicillin was inoculated with pre-cultured DH5α strains (Toyobo) containing the TIM constructs. Bacterial growth was performed for 1.5 h at 310 K. Expression was induced with 1 m*M* isopropyl-1-β-D-1-thiogalactopyranoside for 4 h. The cells were harvested by centrifugation and the pellet was resuspended in buffer *A* (25 m*M* Tris–HCl, 20 m*M* 2-mercaptoethanol, 0.5% Tween-20 and 0.5 mg ml^{-1} lysozyme pH 7.4). The suspension was sonicated and cell debris was removed by centrifugation. After addition of 2.5 *M* NaCl, the supernatant was loaded onto an Ni–NTA column (Amersham Pharmacia) and washed with five column volumes of buffer *B* (25 m*M* Tris–HCl, 250 m*M* NaCl, 20 m*M* 2-mercaptoethanol pH 7.4). The proteins were eluted with buffer *B* containing 200 m*M* imidazole. After dialysis against buffer *C* (25 m*M* Tris–HCl and 5 m*M* dithiothreitol pH 7.4), the N-terminal His-tag portion was removed by the EK Max (Invitrogen) enzyme. The protein was concentrated and loaded onto a Superdex 200 HR column (Amersham Pharmacia). Homogeneous proteins were purified from this column by isocratic elution with buffer *D* (25 m*M* Tris–HCl, 150 m*M* NaCl and 5 m*M* dithiothreitol pH 8.0). Protein purity was determined by SDS–PAGE and dynamic light-scattering analysis.

Table 1
Crystallographic parameters.

	1hti	Form 1†	Form 2
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)			
a	65.81	64.09	46.56
b	75.39	71.72	70.88
c	92.81	91.42	141.41
V_M value (Å^3 Da^{-1})	2.17	1.97	2.19

† Results from preliminary analysis.

2.2. Crystallization

Initial screening was performed by the hanging-drop vapour-diffusion method using the Crystal Screen HT kit (Hampton Research). Crystals were obtained under many conditions containing a variety of polyethylene glycols (PEGs). The most favourable crystals were obtained using condition A6 of the kit, which was refined using the sitting-drop method. Optimized conditions contained 30–35% PEG 4000, 0.1 *M* $MgCl_2$ and 0.1 *M* Tris–HCl pH 8.0. The gel-tube method was performed referring to Tanaka *et al.* (2004). A glass tube filled with protein solution concentrated to 5 mg ml^{-1} in buffer *D* was placed into the 1% agarose gel in the bottom of the outer box. The precipitant solution with a 15-fold volume of the protein solution was poured on top of the agarose

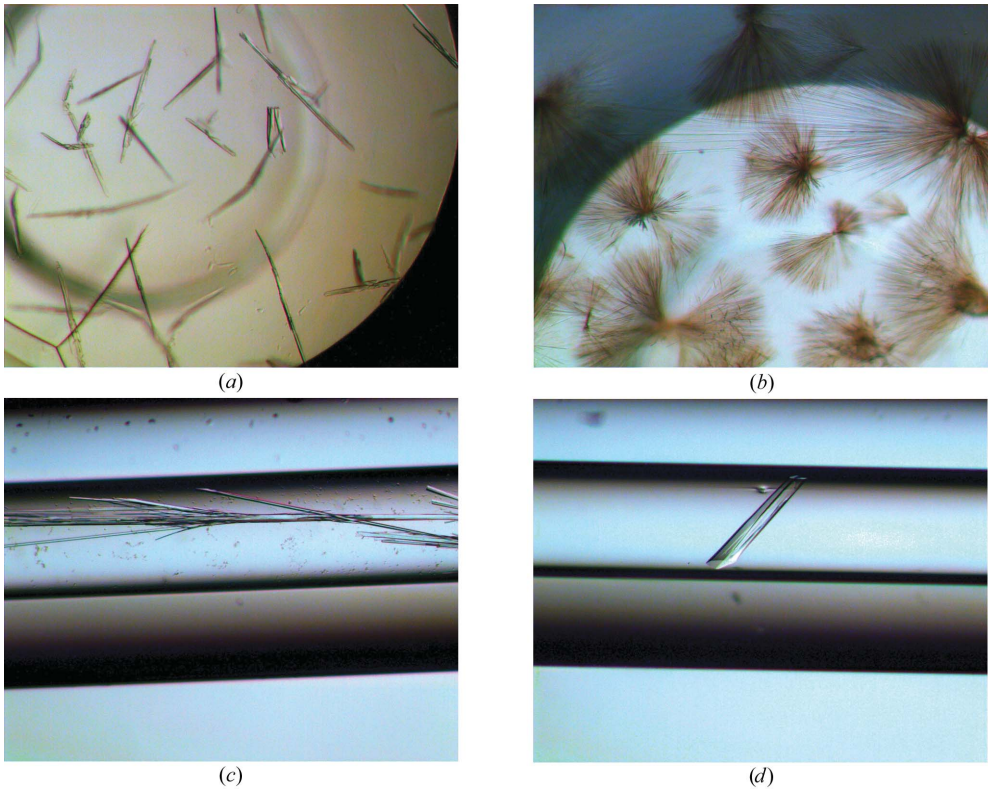

Figure 1
Crystals of human triosephosphate isomerase. (*a*) Crystal form 1 from the sitting-drop vapour-diffusion method. (*b*) Crystal form 2 from the sitting-drop vapour-diffusion method. (*c*) Crystal form 2 from the gel-tube method on earth. (*d*) Crystal form 2 from the gel-tube method in microgravity.

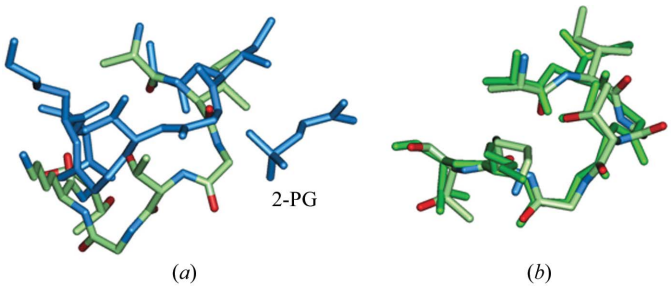

Figure 2
Structures of the flexible loop consisting of Val169 and Ala176. (*a*) Structures of molecule *A* in form 2 and 1hti (blue). (*b*) Structures of molecule *B* in form 2 and 1hti (green).

layer. The precipitant solution contained 42.5% PEG 4000, 0.1 *M* $MgCl_2$ and 0.1 *M* Tris–HCl pH 8.0. The Granada Crystallization Facility (GCF), developed by the European Space Agency and the University of Granada, was purchased from Hampton Research and was used as the outer box of the gel-tube crystallization. Six tubes of 60 mm length and 0.5 mm diameter were placed into a GCF. The Russian Service Module developed by the Russian Federal Space Agency was used for the space experiment. Both experiments using the gel-tube method were started at the same time at 293 K and stopped after three months.

2.3. Data collection and structure analysis

Crystals were mounted in a nylon loop (Hampton Research) and cooled to 100 K in an N_2 gas stream (Rigaku). Diffraction data sets were collected at beamline 32B2 at SPring-8 using an R-AXIS V image-plate detector (Rigaku). A wavelength of 1.00 Å and a crystal-to-detector distance of 200 mm were used. Data integration and scaling were performed with *CrystalClear* (Molecular Structure Corporation). The structure of crystal 2 was solved and refined using this data, the programs *AMoRe* (Navaza, 1993) and *CNX* (Accelrys) and protein model 1hti from the PDB. 5% of the reflections were kept separate throughout for cross-validation. Data-collection and refinement statistics of the complexes are shown in Table 2. Two amino acids from the N-terminus were omitted from the final model because of ambiguous or discontinuous electron density for the corresponding regions.

3. Results and discussion

Our examination of the crystallization conditions of 1hti gave no crystals, but crystal form 1 had similar unit-cell parameters to those reported for 1hti (Table 1). From these preliminary structure analytical results, we found that both crystal forms had the same crystal packing. The differences in the crystallization conditions might originate from the fact that our construct had two residues, glycine and serine, which are longer than those of 1hti.

Gel-tube methods, both on earth and in microgravity, had little effect on crystal form 1. The crystals grew slightly larger with disordered crystal shapes compared with the use of vapour-diffusion methods. They diffracted to 2.8 Å resolution, which was the same resolution as in the case of using the vapour-diffusion method on earth. The crystals grew at unfixed positions in the tubes. Generally, concentration gradients of the proteins and precipitant solutions were generated in the tube. In the experimental period, the tube solution was not in its equilibrium state. Therefore, it seemed that the growth of crystal form 1 was not significantly controlled by the gel-tube method.

Table 2
Data collection, processing and refinement statistics of crystal form 2.

Values in parentheses are for the last resolution shell.

Oscillation angle (°)	1
Exposure time (s)	60
No. of images	180
No. of observations	124578
No. of unique reflections	23600
Resolution range (Å)	28.2–2.2
Completeness (%)	96.3 (99.8)
$R_{\rm merge}$† (%)	13.8 (34.4)
$\langle I/\sigma(I)\rangle$	4.9 (1.5)
$R/R_{\rm free}$‡ (%)	24.7/29.6 (27.6/36.3)
R.m.s. deviations from ideal geometry	
Bond lengths (Å)	0.0007
Bond angles (°)	1.2
Dihedral angles (°)	22.7
Improper angles (°)	0.82

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i I_{hkl}$. ‡ $R = \sum_{hkl}\sum_i |I_{hkl}||F_{\rm obs}| - |F_{\rm calc}||/\sum_{hkl}|F_{\rm obs}|$.

On the other hand, the crystal quality of form 2 was improved using the gel-tube method on earth (Fig. 1*c*). All crystals grew at 50–60 mm from the diffusion surface. Therefore, the gel-tube method should affect crystal growth as the solution diffusion at this position is slower than that near the diffusion interface. Indeed, the procedure increased the crystal dimensions, but the resulting crystals were still unsuitable for X-ray experiments. However, the method under microgravity further improved the crystal quality (Fig. 1*d*). Crystal form 2 diffracted to 2.2 Å resolution and had unit-cell parameters that were different from those of crystal form 1 or 1hti. Microgravity may also provide favourable influences on the crystal growth of form 2.

The overall structure of crystal form 2 was similar to that of 1hti. The asymmetric unit contained a homodimer, which is the essential unit for enzyme activity. The structures of the corresponding monomers of crystal form 2 and 1hti resembled each other except for the flexible loop, which was located near the active centre Lys13. Molecule *A* (Mol-*A*) in 1hti contains an inhibitor (2-phosphoglycolate; 2-PG) in the active site and the flexible loop shows no interaction with adjacent molecules in the crystal. The flexible loop of Mol-*A* of form 2 is also free from crystal-packing interactions and has a different conformation to that of 1hti (Fig. 2*a*). The loop of form 2 moves the active site (the 2-PG-binding site in the 1hti structure) forward. The results indicate that 2-PG induces structural changes in the flexible loop. The flexible loop of molecule *B* (Mol-*B*) in 1hti does not contain the inhibitor in the active site, which may be a consequence of the crystal packing. The flexible loop of Mol-*B* in crystal form 2 has a similar conformation to that of 1hti (Fig. 2*b*). This loop in form 2 also shows interactions with adjacent molecules, but the interaction patterns differ from those of 1hti. The flexible loop of form 2 interacts with Gly140 and Glu186–Gly190 of the adjacent molecules. The flexible loop of 1hti interacts with Lys68, His115–Ala118 and Asp152–Lys155 of the neighbouring molecules. Therefore, the differing contact patterns in the crystal forms may indicate that this loop conformation is one of the local minima.

In summary, we have acquired a high-resolution structure of a new crystal form of human TIM by employing the gel-tube method in microgravity. The results suggest that further refinements of crystallization conditions using the gel-tube method on earth could allow a supply of high-resolution structures of human TIM complexed with various compounds.

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