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Crystallization and preliminary X-ray diffraction analysis of L-threonine dehydrogenase (TDH) from the hyperthermophilic archaeon *Thermococcus kodakaraensis*

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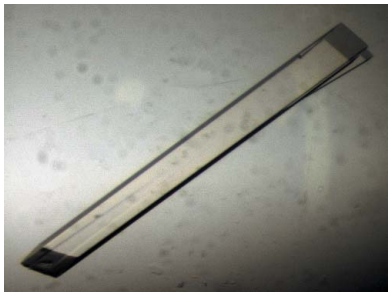

The enzyme L-threonine dehydrogenase catalyses the NAD^+-dependent conversion of L-threonine to 2-amino-3-ketobutyrate, which is the first reaction of a two-step biochemical pathway involved in the metabolism of threonine to glycine. Here, the crystallization and preliminary crystallographic analysis of L-threonine dehydrogenase (Tk-TDH) from the hyperthermophilic organism *Thermococcus kodakaraensis* KOD1 is reported. This threonine dehydrogenase consists of 350 amino acids, with a molecular weight of 38 kDa, and was prepared using an *Escherichia coli* expression system. The purified native protein was crystallized using the hanging-drop vapour-diffusion method and crystals grew in the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 124.5$, $c = 271.1$ Å. Diffraction data were collected to 2.6 Å resolution and preliminary analysis indicates that there are four molecules in the asymmetric unit of the crystal.

1. Introduction

Thermococcales is composed of two major genera *Thermococcus* and *Pyrococcus*. They are strictly anaerobic obligate heterotrophs that grow on complex proteinaceous substrates and their growth is strongly associated with the reduction of elemental sulfur. Alternatively, with a few exceptions, they are capable of gaining energy in the absence of elemental sulfur by the fermentation of peptides, amino acids and sugars, forming acids, CO_2 and H_2 (Fukui *et al.*, 2005). The members of *Thermococcus* are ubiquitous in natural high-temperature environments and are therefore considered to play a major role in the ecology and metabolic activity of microbial consortia within hot-water ecosystems (Fukui *et al.*, 2005).

The hyperthermophilic archaeon *T. kodakaraensis* (KOD1) was isolated from a solfatara on Kodakara Island, Kagoshima, Japan (Atomi *et al.*, 2004; Morikawa *et al.*, 1994). The strain is an obligate anaerobe with optimal growth around 358 K. The genome has been fully sequenced (Fukui *et al.*, 2005) and an orthologue search revealed a DNA sequence corresponding to L-threonine dehydrogenase (EC 1.1.1.103). The half-life of the enzyme is 24 min in boiling water and more than 2 h at 358 K, making it the most stable threonine dehydrogenase known. It has optimal activity at pH 12, which is the highest optimal pH reported to date for enzymes of this family.

The enzyme L-threonine dehydrogenase is involved in the two-step conversion of threonine to glycine by catalysing the NAD^+-dependent conversion of L-threonine to 2-amino-3-ketobutyrate (Fig. 1). The product is then further degraded by 2-amino-3-ketobutyrate coenzyme A ligase in a reaction with coenzyme A that forms glycine and acetyl-CoA (Edgar, 2002). TDH is recognized as the major route for threonine utilization in both prokaryotes (Boylan & Dekker, 1981) and eukaryotes (Dale, 1978). TDH enzymes are multimeric

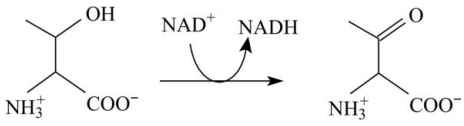

Figure 1
The NAD^+-dependent reaction catalysed by L-threonine dehydrogenase, which yields 2-amino-3-ketobutyrate (right) from the amino acid L-threonine (left).

(typically dimeric or tetrameric) with subunit molecular weights of around 35 kDa and contain zinc, although there is controversy as to whether the metal ion has a catalytic or structural role (Epperly & Dekker, 1991).

The *T. kodakaraensis* TDH enzyme is classified as a member of the medium-chain NAD(H)-dependent and zinc-containing alcohol/polyol dehydrogenase family (Aronson *et al.*, 1989) and is only the second L-threonine dehydrogenase enzyme to be crystallized, the first being that from *Pyrococcus horikoshii* (Ph-TDH; Higashi *et al.*, 2005). Ph-TDH has been shown to have a very similar tertiary structure to alcohol dehydrogenases (ADHs), despite a low sequence homology of no more than 30% (Ishikawa *et al.*, 2007). Given that Tk-TDH exhibits 87% sequence identity to Ph-TDH (*ClustalW*; Larkin *et al.*, 2007), it is expected that Tk-TDH will also exhibit high structural homology to ADHs. Furthermore, the high level of sequence conservation in residues thought to constitute the active site suggests that a similar catalytic mechanism may be preserved between ADHs and TDHs and that one or both of the structural and catalytic zinc ions required by some ADHs (Guy *et al.*, 2003) may be present in Tk-TDH. The Tk-TDH crystal structure will not only provide an informative insight into the structural and functional similarities between TDHs and ADHs, but will also help to further elucidate the mechanism of catalysis and the basis for their substrate specificity. In the broader context, structural information about proteins from hyperthermophilic organisms such as *T. kodakaraensis* will further our understanding of their stability at such extremes of temperature and pH.

2. Expression and purification of native threonine dehydrogenase

The Tk-TDH gene (TK0916) from the genomic DNA of *T. kodakaraensis* was amplified by PCR and ligated into the pTZ57R/T cloning vector using T4 DNA ligase (Fermentas). The recombinant pTZ-tdh plasmid was digested with *Nco*I and *Eco*RI and the Tk-TDH gene ligated with pET-8c, giving the recombinant pET-tdh plasmid that was then used to transform *Escherichia coli* DH5α competent cells. Recombinant pET-tdh plasmid was then purified from *E. coli* DH5α and transformed into the expression strain *E. coli* BL21 (DE3). Expression of the untagged full-length Tk-TDH protein was undertaken on a 1 l scale in LB medium and, following growth of the cultures to mid-log phase, expression of the gene was induced with 0.2 m*M* IPTG at 310 K for a further 4 h. Cells were harvested by centrifugation at 6000*g* for 10 min at 277 K and washed with 50 m*M* Tris–HCl pH 8.0. The cell pellet was resuspended in the same buffer and the protein was purified by a series of steps involving lysis of the cells by sonication, removal of the insoluble material by centrifugation (15 000*g* for 30 min at 277 K) and heating of the soluble fraction to 358 K for 20 min to precipitate further impurities, which were removed by centrifugation at 15 000*g* for 30 min at a temperature of 277 K. This was followed by anion-exchange chromatography using a Resource Q column (Amersham Biosciences, United Kingdom) which was equilibrated with 50 m*M* Tris–HCl buffer pH 8.0. Proteins were eluted with a linear gradient of 0–1.0 *M* sodium chloride in 50 m*M* Tris–HCl buffer pH 8.0. Fractions with TDH activity were dialyzed against 50 m*M* Tris–HCl buffer pH 8.0 containing 2 *M* ammonium sulfate and applied onto a hydrophobic interaction chromatography column (Resource ISO; Amersham Biosciences, United Kingdom) equilibrated with 50 m*M* Tris–HCl buffer pH 8.0 containing 2 *M* ammonium sulfate. The bound proteins were eluted with a linear gradient from 2.0 to 0 *M* ammonium sulfate in 50 m*M* Tris–HCl buffer pH 8.0.

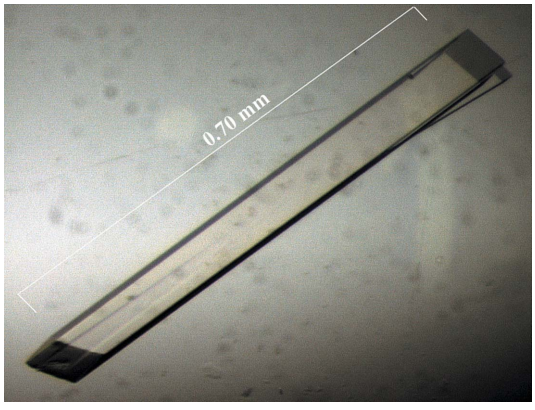

Figure 2
A crystal of Tk-TDH that diffracted to 2.6 Å resolution. The crystal shown here measured approximately 0.70 mm in its longest dimension. Most of the crystals observed were of a similar oblong shape. Those not crystallized in the presence of glycerol were highly fractured in appearance and were often not single crystals.

The purified enzyme was stored in 50 m*M* Tris–HCl pH 8.0 and was found to have a subunit molecular weight of 38 016 Da by electrospray mass spectrometry. It was found to be tetrameric by gel filtration, thereby making it similar to the TDHs from *E. coli*, *P. horikoshii*, *P. furiosus* and *Cytophaga* sp.

3. Crystallization

Crystallization trials were set up at room temperature using the hanging-drop vapour-diffusion method on 24-well plates and conditions were initially screened using both Molecular Dimensions screens MD 1 and 2 and Jena Biosciences JB Screens 1–10. A 1 ml reservoir was used with a 5 µl drop containing 2.5 µl reservoir solution and 2.5 µl protein solution. Crystals of Tk-TDH grew in 0.1 *M* sodium citrate pH 5.6, 2 *M* ammonium sulfate and 0.2 *M* sodium/potassium tartrate (MD2 screen, well 29). Three rounds of optimization screens were set up to refine the crystallization conditions, as it was frequently found that fractured crystals formed, leading to twinned data that proved too difficult to process.

Several of these optimization screens were set up in the presence of 5%(*v*/*v*) glycerol and it was found that in general crystals from these trays were less fractured in appearance and grew as single crystals. It was expected that Tk-TDH requires Zn^{2+} ions for structural reasons and possibly for catalytic purposes based on our knowledge of alcohol dehydrogenase structures (Guy *et al.*, 2003). As it was unclear whether zinc would have remained bound to Tk-TDH throughout the purification, zinc chloride was added to some crystallization trays to a final concentration of 0.1 m*M*. Single crystals of Tk-TDH eventually grew in 0.05 *M* sodium citrate pH 5.6, 2.4 *M* ammonium sulfate, 0.1 *M* sodium/potassium tartrate, 5%(*v*/*v*) glycerol and 0.1 m*M* zinc chloride (Fig. 2). In all cases Tk-TDH at a concentration of 3.4 mg ml^{-1} was used. The crystals were mounted using Litholoops (Molecular Dimensions) and cryoprotected in the mother liquor with 10%(*v*/*v*) glycerol. They were then frozen in a cryostream at 100 K and stored in liquid nitrogen.

4. Preliminary X-ray diffraction analysis of Tk-TDH

The crystals were taken to the European Radiation Synchrotron Facility (ESRF) in Grenoble, France, where a data set was collected to 2.6 Å resolution (Fig. 3) on beamline ID14-3. 190 images were

collected with 1° oscillations and an exposure time of 30 s per image with three passes per image.

The data were processed using *MOSFLM* (Leslie, 2006), *SCALA* (Evans, 2006) and other programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). However, the determination of the space group was ambiguous and varied depending on the number and the angular range of the images chosen for autoindexing. Initially the data were processed in space groups *C*2 and *I*222, but in both cases *MOSFLM* was unable to predict the spot positions correctly. However, when using a different selection of images the preferred space-group assignment was *P*4, which gave much better spot predictions. The data were processed in *P*4 and then scaled, giving an $R_{\rm merge}$ of 0.154. However, the presence of diagonal mirror planes in 'pseudo-precession' images that were generated from the processed data using *HKLVIEW* (Collaborative Computational Project, Number 4, 1994) suggested that the crystal had higher symmetry. Hence, the data set was then processed in all space groups of the 422 point group and the final choice of $P4_32_12$, which was based on the rotation and translation function (see below), gave an $R_{\rm merge}$ of 0.118. The corresponding unit-cell parameters are $a = b = 124.5$, $c = 271.1$ Å (Table 1).

It was expected that Tk-TDH would exist as a homotetramer because several ADHs and TDH from *P. horikoshii* exist in this oligomeric form. The program *MATTHEWS_COEF* (Collaborative Computational Project, Number 4, 1994) was used to determine the likely number of molecules present in the asymmetric unit, from which it was estimated that four monomers per asymmetric unit would give a solvent content of 64%. Molecular replacement was performed using *Phaser* (Read, 2001), which gave a log-likelihood gain (LLG) of 15 485 for $P4_32_12$ (after rigid-body refinement), which was significantly higher than that for any of the other solutions in the 422 point group (the next best solution, for $P4_322$, had a LLG of 780). Although higher LLGs were found with eight molecules in the asymmetric unit for the lower symmetry space groups $P222_1$ (LLG 21 182) and $P2_12_12_1$ (LLG 24 276), the models, when viewed in *Coot* (Emsley & Cowtan, 2004), showed poor electron density for some of the monomers and the *R* factor and $R_{\rm free}$ values would not decrease below 50% during refinement. The model in space group $P4_32_12$, when viewed in *Coot*, packed as a homotetramer as expected, with strong electron density for all four monomers in the asymmetric unit. Refinement using *CNS* (Brünger *et al.*, 1998) and *PHENIX* (Adams *et al.*, 2002) with NCS restraints and model building in *Coot* are in progress.

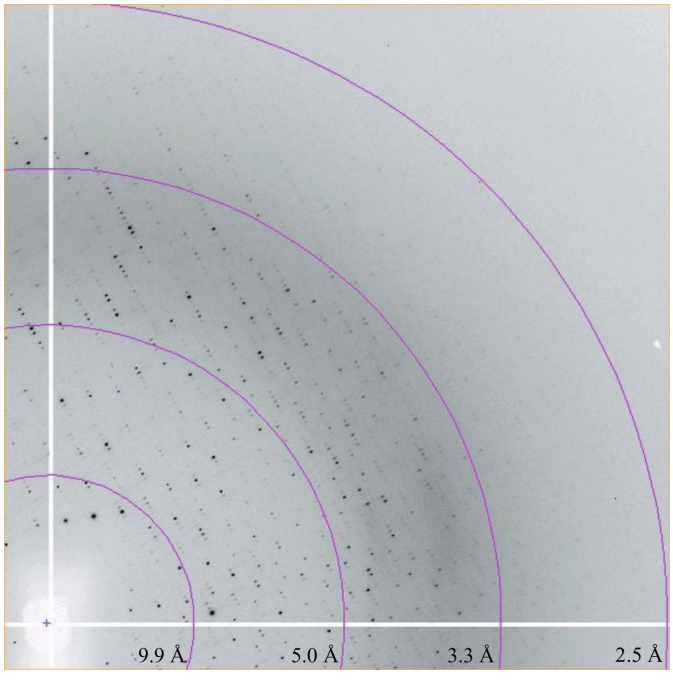

Figure 3
A quadrant of a diffraction image obtained from a crystal of Tk-TDH on beamline ID14-3 at ESRF (Grenoble, France) showing diffraction spots to 2.6 Å resolution.

Table 1
Data-collection and processing statistics for native Tk-TDH.

Values in parentheses are for the outer resolution shell.

Beamline	ID14-3 (ESRF)
Wavelength (Å)	0.931
Space group	$P4_32_12$
Unit-cell parameters	
$a = b$ (Å)	124.5
c (Å)	271.1
Resolution (Å)	73.9–2.6 (2.7–2.6)
$R_{\rm merge}$† (%)	11.4 (60.0)
Completeness (%)	100 (100)
Average $I/\sigma(I)$	18.2 (4.8)
Multiplicity	15.1 (14.8)
No. of observed reflections	1001694 (141093)
No. of unique reflections	66425 (9511)
Wilson *B* factor (Å^2)	51.3
Matthews coefficient (Å^3 Da^{-1})	3.50
Solvent content (%)	64.90
No. of molecules in the ASU	4

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the scaled observations $I_i(hkl)$.

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