Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction study of glycerol kinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis*

Ryota Katsumi,[a] Yuichi Koga,[a] Dong-Ju You,[a] Hiroyoshi Matsumura,[b,c] Kazufumi Takano[a,c] and Shigenori Kanaya[a]*

[a]Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, [b]Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, and [c]CREST (Sosho Project), JST, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Correspondence e-mail: kanaya@mls.eng.osaka-u.ac.jp

Received 19 November 2006
Accepted 11 January 2007

© 2007 International Union of Crystallography
All rights reserved

Glycerol kinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* was crystallized and preliminary crystallographic studies of the crystals were performed. Crystals were grown at 293 K by the sitting-drop vapour-diffusion method. Native X-ray diffraction data were collected to 2.4 Å resolution using synchrotron radiation at station BL44XU of SPring-8. The crystal belongs to the rhombohedral space group *R*3, with unit-cell parameters $a = b = 217.48$, $c = 66.48$ Å. Assuming the presence of two molecules in the asymmetric unit, the V_M value was 2.7 $Å^3\ Da^{-1}$ and the solvent content was 54.1%. The protein was also cocrystallized with substrates and diffraction data were collected to 2.7 Å resolution.

1. Introduction

Glycerol kinase (GK; EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) catalyzes the Mg/ATP-dependent phosphorylation of glycerol to produce glycerol-3-phosphate, an important metabolic intermediate in glycolysis. The enzyme is widely present in all three kingdoms of living organisms: bacteria, archaea and eukaryotes. *Escherichia coli* GK has been the most extensively studied GK with respect to its function and structure. Since *E. coli* GK is subject to several types of regulation involving protein–ligand and protein–protein interactions, the enzyme plays a key role in the regulation of glycerol metabolism in *E. coli* (Zwaig & Lin, 1970). Two allosteric inhibitors, fructose 1,6-bisphosphate (FBP) and IIA^{Glc}, a component of the glucose-specific phosphoenolpyruvate phosphotransferase system (PTS), have been studied with respect to their mechanisms of regulation of GK activity (de Riel & Paulus, 1978*a*,*b*,*c*). The crystal structures of *E. coli* GK in complex with FBP (Ormö *et al.*, 1998) and with IIA^{Glc} (Hurley *et al.*, 1993; Feese *et al.*, 1994, 1998) have been determined. *E. coli* GK exists in a dimer–tetramer equilibrium with an apparent dissociation constant of about 6.1×10^{-8} *M* (Peng & Pettigrew, 2003). FBP selectively binds to the tetramer and inhibits its activity (Liu *et al.*, 1994). The crystal structure of the complex between *E. coli* GK and the substrate analogue indicates that the FBP-bound tetrameric form of GK has an energetic barrier to the conformational change required to open the active site, which is necessary for efficient catalysis (Bystrom *et al.*, 1999). In contrast, another allosteric inhibitor, IIA^{Glc}, binds to both the dimer and the tetramer and noncompetitively inhibits the GK activity for both substrates, glycerol and ATP (Novotony *et al.*, 1985). The IIA^{Glc}-binding site, which is different from the FBP-binding site, is located far from the active site (Hurley *et al.*, 1993).

GK is a member of the ATPase superfamily, which includes hexokinases, actins and heat-shock proteins (Hurley, 1996). These proteins share a common $\beta\beta\beta\alpha\beta\alpha\beta\alpha$ fold. The members of this superfamily are known to change conformation greatly upon substrate binding owing to interdomain motion. The crystal structure of the *E. coli* GK–substrate complex indicates that GK forms an asymmetric dimer with one open-conformation subunit and one closed-conformation subunit. The conformational difference between these two subunits is brought about by interdomain motion, which is supposed to be associated with a catalytic step or allosteric inhibition by FBP or IIA^{Glc} (Bystrom *et al.*, 1999).

The GK from the hyperthermophilic archaeon *Thermococcus kodakaraensis* (*Tk*-GK) has been overproduced in *E. coli*, purified and biochemically characterized (Koga *et al.*, 1998). It shows high amino-acid sequence identity (57%) to *E. coli* GK. Nevertheless, *Tk*-GK is much more highly stable than *E. coli* GK. For example, *Tk*-GK is highly resistant to heat inactivation and fully retains enzymatic activity upon incubation at 353 K for 60 min, while *E. coli* GK loses 90% of its enzymatic activity upon incubation at 323 K for 60 min. Comparison of a model of the *Tk*-GK structure and the crystal structure of *E. coli* GK, as well as mutational studies of these enzymes (Koga *et al.*, 2001), have allowed us to propose that the increased number of ion pairs at the surface of the protein molecule and the stabilization of the subunit interface contribute to the thermal stability of *Tk*-GK. However, the structural basis of the high stability of *Tk*-GK remains to be analyzed. In addition, unlike *E. coli* GK, *Tk*-GK exists only in a dimeric form, is not sensitive to FBP inhibition and does not show negative cooperativity for ATP binding (Koga *et al.*, 1998). To understand the stabilization mechanism, subunit interaction and unique enzymatic properties of *Tk*-GK at the atomic level, it is necessary to determine its crystal structure.

Here, we report the crystallization and preliminary X-ray crystallographic studies of *Tk*-GK and its complex with glycerol and ATP.

2. Experimental procedures

2.1. Purification

Tk-GK was overproduced in *E. coli* BL21 (DE3) cells and purified as described previously (Koga *et al.*, 1998), with slight modifications to the purification procedures. The cells were collected by centrifugation, resuspended in 100 m*M* Tris–HCl pH 7.5, disrupted using a French press and centrifuged at 8000*g* for 30 min. The supernatant was incubated at 363 K for 30 min and centrifuged at 18 000*g* for 30 min in order to remove heat-denatured *E. coli* proteins as a precipitate. Ammonium sulfate was added to the supernatant to a concentration of 80% saturation and the resultant precipitate was collected by centrifugation at 15 000*g* for 30 min. The precipitate was dissolved in 20 m*M* Tris–HCl pH 7.5, dialyzed against the same buffer and applied onto a HiTrapQ HP column (Pharmacia/GE Healthcare) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of 0–1.0 *M* NaCl. The fractions containing the protein were collected, dialyzed against 5 m*M* sodium phosphate pH 6.8 and applied onto a Bio-Scale CHT20-I column (Bio-Rad) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of 5–500 m*M* sodium phosphate. The fractions containing the protein were collected, dialyzed against 10 m*M* Tris–HCl pH 7.5 and concentrated to about 10 mg ml^{-1} using a Centricon ultrafiltration system (Millipore). All purification procedures were carried out at 277 K. The protein concentration was determined from the UV absorption at 280 nm using a cell with an optical path length of 1 cm and an A_{280} value of 2.2 for a 0.1% solution. This value was previously determined by measuring the amount of the protein by amino-acid analysis (Koga *et al.*, 1998). The purity of the protein was analyzed by SDS–PAGE (Laemmli, 1970) followed by staining with Coomassie Brilliant Blue.

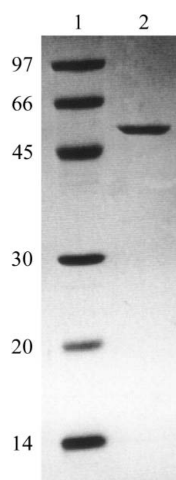

Figure 1
SDS–PAGE of *Tk*-GK. Samples were subjected to electrophoresis on a 15% polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, a low-molecular-weight marker kit (Amersham Biosciences); lane 2, purified *Tk*-GK (56 kDa). Numbers along the gel represent the molecular weights of the standard proteins in kDa.

2.2. Crystallization

The crystallization conditions were initially screened using crystallization kits from Hampton Research (Crystal Screens I and II) and Emerald Biostructures (Wizard I and II). The conditions were surveyed using the sitting-drop vapour-diffusion method at 293 K. Drops were prepared by mixing 1 µl each of the protein solution (approximately 10 mg ml^{-1}) and reservoir solution and were vapour-equilibrated against 100 µl reservoir solution in a sealed chamber. Native *Tk*-GK crystals appeared after a few days using Wizard I solution No. 25 (0.1 *M* Tris pH 8.5, 0.2 *M* $MgCl_2$, 30% PEG 400). The crystallization conditions were further optimized by changing the concentration of the reservoir solution mixed with the protein solution. Single crystals suitable for X-ray diffraction analysis appeared when the drops were prepared by mixing 1 µl each of the protein solution (approximately 10 mg ml^{-1}) and twofold diluted reservoir solution and were vapour-equilibrated against 100 µl reservoir solution. In addition, *Tk*-GK was cocrystallized with its substrates by adding 2.5 m*M* glycerol and 2.5 m*M* ATP to the drops using the same conditions.

2.3. Data collection

Crystals were mounted on a CryoLoop (Hampton Research) with Paratone-N (Hampton Research) as cryoprotectant and then flash-frozen in a nitrogen-gas stream at 100 K. Diffraction data were collected on beamline BL44XU at SPring-8 (Hyogo, Japan) using synchrotron radiation and a DIP6040 imaging-plate detector (MAC Science/Bruker AXS). A total of 132 images of 1.0° oscillation were

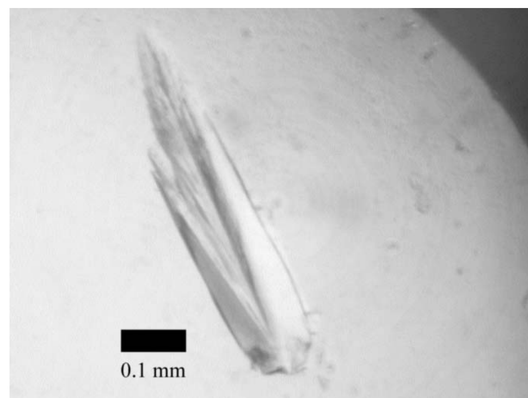

Figure 2
Crystals of *Tk*-GK grown by the sitting-drop vapour-diffusion method.

recorded with an exposure time of 10 s per image. The diffraction data of cocrystals with the substrates were collected on BL38B1 at SPring-8, a bending-magnet beamline. A total of 180 images of 1.0° oscillation were recorded with an exposure time of 15 s per image. The data were processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results

Tk-GK was purified to give a single band on SDS–PAGE by heat treatment and ammonium sulfate precipitation followed by two column-chromatography steps (Fig. 1). Approximately 5 mg purified protein was obtained from 1 l culture.

The crystallization of *Tk*-GK was achieved by the sitting-drop vapour-diffusion method. The crystals appeared in a few days and grew to maximum dimensions of 0.6 × 0.2 × 0.2 mm after one week (Fig. 2). Diffraction spots sufficient for structural determination at high resolution were not obtained when a crystal of *Tk*-GK was mounted on a CryoLoop without cryoprotectant. However, the diffraction patterns of the crystal were improved drastically by using Paratone-N as cryoprotectant (Fig. 3). Diffraction data from *Tk*-GK crystals grown in the absence of substrate were collected to 2.4 Å resolution. A total of 179 553 measured reflections were merged into 45 951 unique reflections with an $R_{\rm merge}$ of 8.1%. The crystals belong to the primitive rhombohedral space group *R*3, with unit-cell parameters $a = b = 217.48$, $c = 66.48$ Å. The data-collection statistics are summarized in Table 1. Based on the molecular weight and space group, the crystal was assumed to contain two molecules in the asymmetric unit. This corresponded to a Matthews coefficient ($V_{\rm M}$) value of 2.7 Å^3 Da^{-1}, with a solvent content of 54.1% (Matthews, 1968). A self-rotation function was calculated on the scaled data from 48.6 to 3.0 Å using *MOLREP* (Collaborative Computational Project, Number 4, 1994; Fig. 4). The $\kappa = 180°$ section shows significant peaks other than those belonging to the crystallographic space group. The result clearly shows the twofold noncrystallographic symmetry and supports the assumption of the presence of two molecules in the asymmetric unit. Since biochemical studies show that *Tk*-GK exists as a dimer (Koga *et al.*, 1998), *Tk*-GK is expected to exist as a dimer in the crystal. Diffraction data were collected to 2.7 Å resolution from the cocrystals with the substrates. The crystal belongs to the same rhombohedral space group *R*3, with unit-cell parameters $a = b = 216.54$, $c = 65.81$ Å. Assuming the presence of two molecules in asymmetric unit, the $V_{\rm M}$ value was 2.7 Å^3 Da^{-1} and the solvent content was 53.3%. Twofold noncrystallographic symmetry was also observed in the self-rotation function map of this crystal. The crystal structures are currently being solved by the molecular-replacement method with *MOLREP* (Collaborative Computational Project, Number 4, 1994) using the structural model of *Tk*-GK (Koga *et al.*, 1998). The two molecules of *Tk*-GK are oriented and positioned at (α = 65.48,

Table 1
Statistics of data collection.

Values in parentheses refer to the highest resolution shell.

	Native	Glycerol/ATP complex
X-ray wavelength (Å)	0.9	1.0
Temperature (K)	100	100
Space group	*R*3	*R*3
Unit-cell parameters (Å)	$a = b = 217.48$, $c = 66.48$	$a = b = 216.54$, $c = 65.81$
Resolution range (Å)	50.0–2.40	50–2.69
No. of measured reflections	179553	153859
No. of unique reflections	45951	31999
$R_{\rm merge}$† (%)	8.1 (47.2)	7.1 (37.7)
Completeness (%)	98.2 (94.0)	99.3 (95.4)
Average $I/\sigma(I)$	17.2 (1.4)	18.4 (2.3)

† $R_{\rm merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum I_{hkl}$, where I_{hkl} is the intensity measurement for the reflection with indices *hkl* and $\langle I_{hkl}\rangle$ is the mean intensity for multiply recorded reflections.

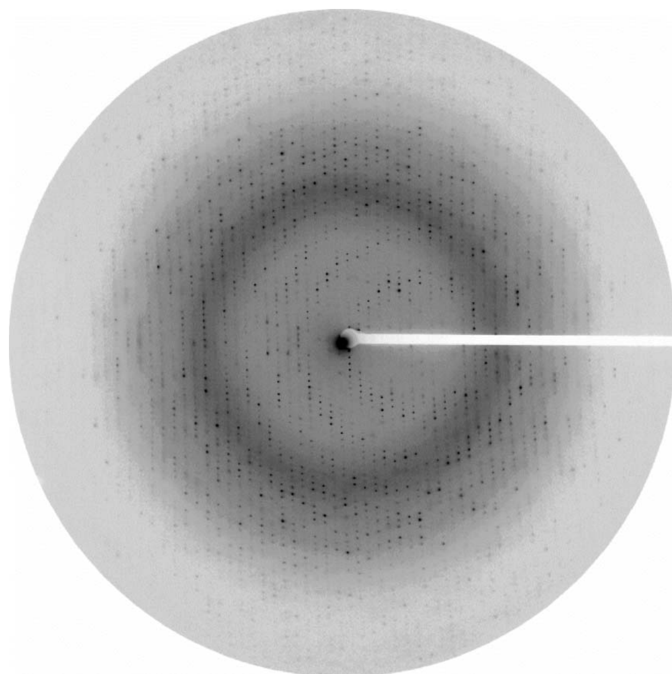

Figure 3
X-ray diffraction pattern of the native *Tk*-GK crystal. The crystal was frozen at 100 K using Paratone-N as cryoprotectant and the diffraction data were collected on BL44XU at this temperature. The photograph shows a single frame of 1.0° oscillation with an exposure time of 10 s per image and a crystal-to-detector distance of 170 mm. The crystal diffracted to 2.2 Å (the edge of the plate).

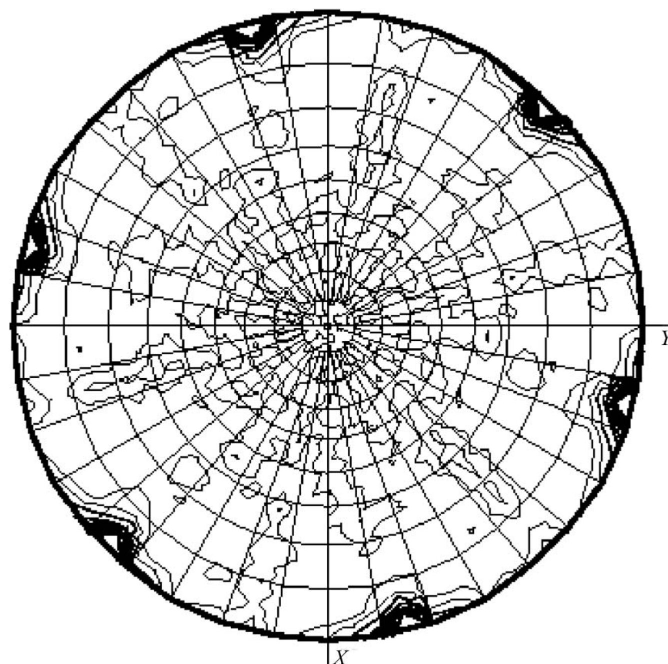

Figure 4
The $\kappa = 180°$ section of the self-rotation function calculated for the *R*3 data set using *MOLREP* (Collaborative Computational Project, Number 4, 1994). The integration radius was 30 Å and data were used in the resolution range 48.6–3.0 Å.

β = 55.56, γ = 79.71°, $X_{\rm frac}$ = 0.861, $Y_{\rm frac}$ = 0.297, $Z_{\rm frac}$ = 0.000) and (α = 82.61, β = 124.25, γ = −99.51°, $X_{\rm frac}$ = 0.516, $Y_{\rm frac}$ = 0.431, $Z_{\rm frac}$ = 0.650) in the crystal symmetry. The initial correlation coefficient and R factor are 0.273 and 57.8% for one molecule and 0.339 and 55.5% for the other molecule, respectively.

The synchrotron-radiation experiments were performed at BL44XU and BL38B1 at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI; Proposal No. 2006A6812, 2006A1733). This work was supported in part by a Grant-in-Aid for the National Project on Protein Structural and Functional Analyses and by an Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

Bystrom, C. E., Pettigrew, D. W., Branchaud, B. P., O'Brien, P. & Remington, S. J. (1999). *Biochemistry*, **38**, 3508–3518.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Feese, M., Faber, H. R., Rystrom, C. E., Pettigrew, D. W. & Remington, S. J. (1998). *Structure*, **6**, 1407–1418.
Feese, M., Pettigrew, D. W., Meadow, N. D., Roseman, S. & Remington, S. J. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 3544–3548.
Hurley, J. H. (1996). *Annu. Rev. Biophys. Biomol. Struct.* **25**, 137–162.
Hurley, J. H., Feber, H. R., Worthylake, D., Meadow, N. D., Roseman, S., Pettigrew, D. W. & Remington, S. J. (1993). *Science*, **259**, 673–677.
Koga, Y., Haruki, M., Morikawa, M. & Kanaya, S. (2001). *J. Biosci. Bioeng.* **91**, 551–556.
Koga, Y., Morikawa, M., Haruki, M., Nakamura, H., Imanaka, T. & Kanaya, S. (1998). *Protein Eng.* **11**, 1219–1227.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Liu, W. Z., Faber, R., Feese, M., Remington, S. J. & Pettigrew, D. W. (1994). *Biochemistry*, **33**, 10120–10126.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Novotony, M. J., Frederickson, W. L., Waygood, E. B. & Saier, M. H. Jr (1985). *J. Bacteriol.* **162**, 810–815.
Ormö, M., Bystrom, C. E. & Remington, S. J. (1998). *Biochemistry*, **37**, 16565–16572.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Peng, Y. & Pettigrew, D. W. (2003). *Biochemistry*, **42**, 4232–4252.
Riel, J. K. de & Paulus, H. (1978*a*). *Biochemistry*, **17**, 5134–5140.
Riel, J. K. de & Paulus, H. (1978*b*). *Biochemistry*, **17**, 5141–5146.
Riel, J. K. de & Paulus, H. (1978*c*). *Biochemistry*, **17**, 5146–5150.
Zwaig, N. & Lin, E. C. C. (1970). *J. Bacteriol.* **102**, 754–759.