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

Cloning, expression, purification, crystallization and preliminary X-ray crystallographic study of molybdopterin synthase from *Thermus thermophilus* HB8

Shankar Prasad Kanaujia,[a] Chellamuthu Vasuki Ranjani,[a] Jeyaraman Jeyakanthan,[b] Miwa Ohmori,[b] Kazuko Agari,[b] Yoshiaki Kitamura,[b] Seiki Baba,[b,c] Akio Ebihara,[b] Akeo Shinkai,[b] Seiki Kuramitsu,[b,c] Yoshitsugu Shiro,[b] Kanagaraj Sekar[a,d]* and Shigeyuki Yokoyama[b,e,f]*

[a]Bioinformatics Centre (Centre of Excellence in Structural Biology and Biocomputing), Indian Institute of Science, Bangalore 560 012, India, [b]RIKEN SPring-8 Center, Harima Institute, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan, [c]Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, [d]Supercomputer Education and Research Centre, Indian Institute of Science, Bangalore 560 012, India, [e]RIKEN Genomic Sciences Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan, and [f]Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Correspondence e-mail: sekar@physics.iisc.ernet.in, yokoyama@biochem.s.u-tokyo.ac.jp

Received 23 December 2006
Accepted 12 March 2007

Thermus thermophilus is a Gram-negative aerobic thermophilic eubacterium which can grow at temperatures ranging from 323 to 355 K. In addition to their importance in thermostability or adaptation strategies for survival at high temperatures, the thermostable enzymes in thermophilic organisms contribute to a wide range of biotechnological applications. The molybdenum cofactor in all three kingdoms consists of a tricyclic pyranopterin termed molybdopterin that bears the *cis*-dithiolene group responsible for molybdenum ligation. The crystals of molybdopterin synthase from *T. thermophilus* HB8 belong to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 33.94, b = 103.32, c = 59.59 Å, β = 101.3°. Preliminary studies and molecular-replacement calculations reveal the presence of three monomers in the asymmetric unit.

1. Introduction

Proteins from thermophilic organisms maintain their biologically active structure even at high temperature. This has kindled curiosity among researchers to study their mechanism and their structural changes under various conditions during the evolutionary process. *Thermus thermophilus* is a Gram-negative aerobic thermophilic eubacterium which can grow at temperatures ranging from 323 to 355 K. Molybdenum-cofactor (MoCo) biosynthesis is a phylogenetically conserved pathway that is present in all three kingdoms, including thermophilic organisms (Rajagopalan, 1991, 1997; Schindelin *et al.*, 2001). MoCo has been found to be essential for the activity of sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase in humans (Reiss, 2000). MoCo contains a tricyclic pyranopterin termed molybdopterin (MPT) that bears the *cis*-dithiolene group responsible for molybdenum ligation. Molybdenum-like cofactors are present in a large family of diverse enzymes involved in electron-transfer reactions (Kisker *et al.*, 1997). In almost all molybdoenzymes, with few exceptions, the active centre is formed by MoCo, which consists of a mononuclear molybdenum ion bound to molybdopterin (Bader *et al.*, 2004). MoCo biosynthesis involves two steps. The first step is not clearly understood; however, it involves the synthesis of precursor Z (Wuebbens & Rajagopalan, 1995; Rieder *et al.*, 1998). The molybdoenzymes MoaABC are thought to be involved in the first step (Pitterle *et al.*, 1993; Rieder *et al.*, 1998; Rivers *et al.*, 1993). The protein MoaA is thought to be involved in the catalysis of this step and has been proposed to act as an oxidoreductase (Menendez *et al.*, 1996). The role of the MoaB protein is not known (Bader *et al.*, 2004); however, it shows significant homology to the MogA protein, which is also involved in MoCo biosynthesis. Furthermore, several proteins such as gephyrin (*Rattus norvegicus*), cinnamon (*Drosophila melanogaster*) and Cnx1 (*Arabidopsis thaliana*) show homology to the MoaB protein (Schwarz *et al.* (1997; Stallmeyer *et al.*, 1999; Wittle *et al.*, 1999). The crystal structure of MoaC from *Escherichia coli* has been reported (Wuebbens *et al.*, 2000). In the second step, precursor Z is converted to molybdopterin. The dithiolene group of molybdopterin is generated by molybdopterin synthase, which consists of large (MoaE) and small (MoaD) subunits along with a small enzyme MoeB (Pitterle & Rajagopalan,

1993; Pitterle *et al.*, 1993). Structural studies of these enzymes from other organisms have shown that the enzymes are heterotetrameric and that the C-terminus of each MoaD subunit is inserted into a MoaE subunit to form the active site (Rudolph *et al.*, 2003). The common step during MPT biosynthesis is the ATP-dependent transfer of sulfur from cysteine to give a thiocarboxylate group on the MoaD C-terminus (Rizzi & Schindelin, 2002; Leimkuhler *et al.*, 2001; Xi *et al.*, 2001).

Thus, the enzymes involved in molybdenum-cofactor biosynthesis pathway play a significant role in the metabolism of bacterial species. Understanding the three-dimensional crystal structure of MPT synthase from *T. thermophilus* will be useful in the comprehensive biochemical, enzymatic and molecular analysis of related proteins and may shed light on mutational studies that may lead to the determination of the factors responsible for thermostability of these proteins. In this paper, we report the preliminary X-ray crystallographic studies of molybdopterin synthase from *T. thermophilus* HB8.

2. Materials and methods

2.1. Cloning, expression and purification

T. thermophilus MoaB (TTHA0341) protein consists of 164 amino-acid residues with a predicted molecular weight of 17.9 kDa. The MoaB gene was amplified by PCR using *T. thermophilus* HB8 genomic DNA as the template. The amplified fragment was cloned under the control of the T7 promoter of the *E. coli* expression vector pET-11a (Novagen, Madison, WI, USA). The expression vector was introduced into *E. coli* BL21(DE3) strain (Novagen) and the recombinant strain was cultured in 6 l LB medium supplemented with 50 µg ml^{-1} ampicillin in shake flasks. The cells (26 g) were collected by centrifugation, washed with 20 ml of 20 m*M* Tris–HCl pH 8.0 containing 50 m*M* NaCl and resuspended in 70 ml of the same buffer. The cells were then disrupted by sonication in a chilled water bath and the cell lysate was incubated at 343 K for 10 min. The sample was centrifuged at 150 000*g* for 1 h at 277 K and ammonium sulfate was then added to the supernatant to a final concentration of 1.5 *M*. The sample was then applied onto a Resource PHE column (GE Healthcare Bioscience Corp., Piscataway, NJ, USA) pre-equilibrated with sodium phosphate buffer pH 7.0 containing 1.5 *M* ammonium sulfate and was eluted with a linear gradient of 1.5–0 *M* ammonium sulfate. The eluted fractions containing the MoaB were collected, desalted by fractionation on a HiPrep 26/10 Desalting column (GE Healthcare Bioscience Corp.) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 and then applied onto a Resource Q column (GE Healthcare Bioscience Corp.) pre-equilibrated with the same buffer. The flow-through fraction was collected and applied onto a Resource S column (GE Healthcare Bioscience Corp.) pre-equilibrated with 20 m*M* MES pH 6.0, which was eluted with a linear gradient of 0–0.5 *M* NaCl. The eluted fractions containing MoaB were pooled, desalted by fractionation on a HiPrep 26/10 Desalting column pre-equilibrated with 10 m*M* sodium phosphate buffer pH 7.0 containing 0.15 *M* NaCl and then applied onto a hydroxyapatite CHT10 column (Bio-Rad Laboratories. Inc., Hercules, CA, USA) pre-equilibrated with the same buffer, which was eluted with a linear gradient of 10–250 m*M* sodium phosphate buffer pH 7.0. The sample containing MoaB was then loaded onto a HiLoad 16/60 Superdex 75pg column (GE Healthcare Bioscience Corp.) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 150 m*M* NaCl. The purified MoaB was concentrated with a VivaSpin 20 concentrator (10 kDa molecular-weight cutoff, Sartorius AG, Goettingen, Germany). The purified protein was homogeneous as determined by SDS–PAGE. The protein concentration was determined by measuring the absorbance at 280 nm (Kuramitsu *et al.*, 1990). The yield of the purified protein was 5.6 mg per litre of culture.

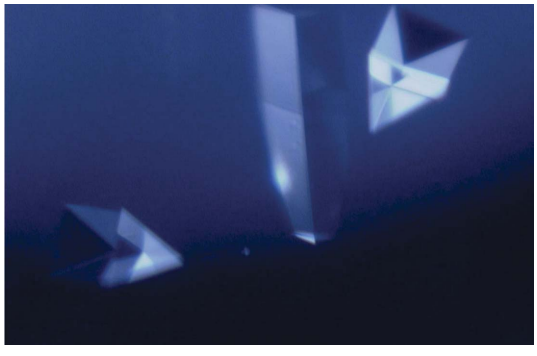

Figure 1
Crystals of molybdopterin synthase from *T. thermophilus* HB8. The dimensions of the crystals were approximately 0.1 × 0.1 × 0.3 mm.

Table 1
Crystal data and X-ray data-collection statistics of molybdopterin synthase from *T. thermophilus* HB8.

Values in parentheses are for the last resolution shell.

Crystal characteristics	Primitive monoclinic
Wavelength (Å)	1.0
Temperature (K)	100
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 33.94, b = 103.32, c = 59.59, β = 101.3
Matthews coefficient (Å^3 Da^{-1})	1.90
Solvent content (%)	35.2
Resolution range (Å)	50.0–1.64 (1.7–1.64)
No. of observed reflections	253272
No. of unique reflections	48481 (4585)
Completeness (%)	98.7 (93.0)
$R_{\rm sym}$ (%)†	6.3 (25.5)
Multiplicity	5.2 (4.0)
Average $I/\sigma(I)$	23.32 (2.53)
Wilson B (Å^2)	21.3

† $R_{\rm sym} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

2.2. Crystallization experiments

The protein concentration was 11 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl. Freshly purified protein was used for crystallization trials using the PEG/Ion kit (Hampton). Crystals were obtained using the sitting-drop vapour-diffusion method by pipetting 1 µl protein solution and 1 µl well solution [20%(*w*/*v*) PEG 3350 and 0.2 *M* tripotassium citrate monohydrate] at 293 K. Crystals (Fig. 1) appeared in about two weeks. 20%(*v*/*v*) PEG 400 was used as a cryoprotectant.

2.3. Data collection and processing

Diffraction data were collected at 100 K using the RIKEN Structural Genomics Beamline II (BL26B2) at SPring-8 (Hyogo, Japan) with a Jupiter 210 CCD detector (Rigaku MSC Co., Tokyo, Japan). The crystal-to-detector distance was maintained at 150 mm. The *HKL* program suite (Otwinowski & Minor, 1997) was used to process the X-ray diffraction data. The crystal dimensions were 0.1 × 0.1 × 0.3 mm and diffraction was recorded to a resolution of 1.64 Å. Details of the diffraction data statistics are given in Table 1.

3. Results and discussion

Analysis of the X-ray diffraction data indicates that the crystal of molybdopterin synthase from *T. thermophilus* HB8 is primitive monoclinic (space group $P2_1$), with unit-cell parameters a = 33.94, b = 103.32, c = 59.59 Å, β = 101.3°. The calculated Matthews coefficient (V_M) is 1.90 Å^3 Da^{-1}, with a solvent content of 35.2% (Matthews, 1968), corresponding to the presence of three monomers in the asymmetric unit. X-ray diffraction data statistics are given in Table 1.

3.1. Molecular replacement

The partial crystal structure of molybdopterin synthase from *T. thermophilus* HB8 was solved by molecular-replacement calculations using the program *CNS* v.1.1 (Brünger *et al.*, 1998). The coordinates of PDB entry 1jlj (Schwarz *et al.*, 2001) were used as the search model. The nonprotein atoms were removed from the search model used in the molecular-replacement calculations. The trimer molecule was used as a search model. The crystal-packing value was 0.7609 for the best solution. The search model had 50% amino-acid sequence identity to that of molybdopterin synthase from *T. thermophilus*. Three monomers in the asymmetric unit were subjected to rigid-body refinement using the program *CNS* v.1.1 (Brünger *et al.*, 1998). A total of 5% of the reflections were used for R_{free} calculation (Brünger, 1992). The crystallographic R_{work} and R_{free} values of the partially refined structure were 39.1% and 41.7%, respectively.

SPK, CVR and KS thank the Department of Biotechnology (DBT), Government of India for funding. We thank Dr Koji Takio and Naoko Takahashi for the N-terminal amino-acid sequence analysis of the recombinant protein. This work was supported by the RIKEN Structural Genomic/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Bader, G., Gomez-Ortiz, M., Haussmann, C., Bacher, A., Huber, R. & Fischer, M. (2004). *Acta Cryst.* D**60**, 1068–1075.
Brünger, A. T. (1992). *Nature (London)*, **355**, 472–474.
Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Kisker, C., Schindelin, H. & Rees, D. C. (1997). *Annu. Rev. Biochem.* **66**, 233–267.
Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y. & Kagamiyama, H. (1990). *Biochemistry*, **29**, 5469–5476.
Leimkuhler, S., Wuebbens, M. M. & Rajagopalan, K. V. (2001). *J. Biol. Chem.* **276**, 34695–34701.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Menendez, C., Siebert, D. & Brandsch, R. (1996). *FEBS Lett.* **391**, 101–103.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pitterle, D. M., Johnson, J. L. & Rajagopalan, K. V. (1993). *J. Biol. Chem.* **268**, 13506–13509.
Pitterle, D. M. & Rajagopalan, K. V. (1993). *J. Biol. Chem.* **268**, 13499–13505.
Rajagopalan, K. V. (1991). *Adv. Enzymol.* **64**, 215–290.
Rajagopalan, K. V. (1997). *Biochem. Soc. Trans.* **25**, 757–761.
Reiss, J. (2000). *Hum. Genet.* **106**, 157–163.
Rieder, C., Eisenreich, W., O'Brien, J., Richter, G., Gotze, E., Boyle, P., Blanchard, S., Bacher, A. & Simon, H. (1998). *Eur. J. Biochem.* **255**, 24–36.
Rivers, S. L., McNairn, E., Blasco, F., Giordano, G. & Boxer, D. H. (1993). *Mol. Microbiol.* **8**, 1071–1081.
Rizzi, M. & Schindelin, H. (2002). *Curr. Opin. Struct. Biol.* **12**, 709–720.
Rudolph, M. J., Wuebbens, M. M., Turque, O., Rajagopalan, K. V. & Schindelin, H. (2003). *J. Biol. Chem.* **278**, 14514–14522.
Schindelin, H., Kisker, C. & Rajagopalan, K. V. (2001). *Adv. Protein Chem.* **58**, 47–94.
Schwarz, G., Boxer, D. H. & Mendel, R. R. (1997). *J. Biol. Chem.* **272**, 26811–26814.
Schwarz, G., Schrader, M., Mendel, R. R., Hecht, H.-J. & Schindelin, H. (2001). *J. Mol. Biol.* **312**, 405–418.
Stallmeyer, B., Schwarz, G., Schulze, J., Nerlich, A., Reiss, J., Kirsch, J. & Mendel, R. R. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 1333–1338.
Wittle, A. E., Kamdar, K. P. & Finnerty, V. (1999). *Mol. Gen. Genet.* **261**, 672–680.
Wuebbens, M. M., Liu, M. T., Rajagopalan, K. & Schindelin, H. (2000). *Structure*, **8**, 709–718.
Wuebbens, M. M. & Rajagopalan, K. V. (1995). *J. Biol. Chem.* **270**, 1082–1087.
Xi, J., Ge, Y., Kinsland, C., McLafferty, F. W. & Begley, T. P. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 8513–8518.