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Crystallization and preliminary crystallographic analysis of molybdenum-cofactor biosynthesis protein C from *Thermus thermophilus*

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The Gram-negative aerobic eubacterium *Thermus thermophilus* is an extremely important thermophilic microorganism that was originally isolated from a thermal vent environment in Japan. The molybdenum cofactor in this organism is considered to be an essential component required by enzymes that catalyze diverse key reactions in the global metabolism of carbon, nitrogen and sulfur. The molybdenum-cofactor biosynthesis protein C derived from *T. thermophilus* was crystallized in two different space groups. Crystals obtained using the first crystallization condition belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 64.81$, $b = 109.84$, $c = 115.19$ Å, $\beta = 104.9^\circ$; the crystal diffracted to a resolution of 1.9 Å. The other crystal form belonged to space group $R32$, with unit-cell parameters $a = b = 106.57$, $c = 59.25$ Å, and diffracted to 1.75 Å resolution. Preliminary calculations reveal that the asymmetric unit contains 12 monomers and one monomer for the crystals belonging to space group $P2_1$ and $R32$, respectively.

1. Introduction

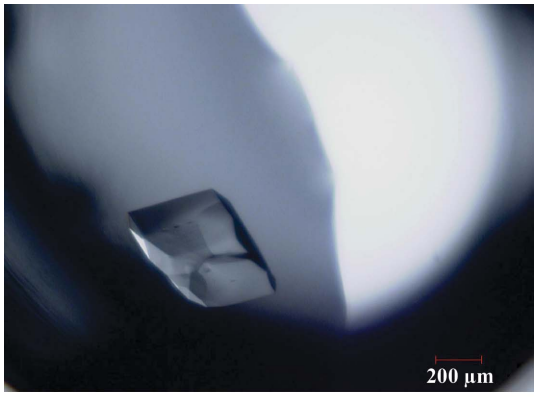
Molybdenum-cofactor (MoCo) biosynthesis is an evolutionarily conserved pathway in archaea, eubacteria and eukaryotes, including humans (Michael *et al.*, 2000). The metal molybdenum is considered to be an essential component that is required by enzymes that catalyze diverse key reactions in the global metabolism of carbon, nitrogen and sulfur (Mendel & Florian, 2006). A genetic deficiency of these enzymes leads to various autosomal recessive diseases with severe neurological symptoms which may even lead to early childhood death (Michael *et al.*, 2000). The biosynthesis of the molybdenum cofactor requires the synthesis of pterin with its 4-carbon phosphorylated side chain, formation of the dithiolene moiety and incorporation of molybdenum (Johnson *et al.*, 1993; Rajagopalan & Johnson, 1992). Five loci are involved in MoCo synthesis and have been identified by chlorate-resistance screening in *Escherichia coli* (Tine *et al.*, 1994). The MoCo-biosynthetic pathway includes one or more enzymes from each locus. The two loci *moa* and *moe* are required for the initial steps of MoCo biosynthesis, which involve the synthesis of molybdopterin (Nohno *et al.*, 1988; Rivers *et al.*, 1993). A literature survey shows that the sequence of the *moa* loci contains five genes in an operon (*moaA–moaE*) and that the gene products of *moaA* and *moaC* are thought to be involved in the production of the precursor of molybdopterin. *moaD–moaE* along with *moeB* are responsible for the conversion of the precursor into molybdopterin (Pitterle & Rajagopalan, 1989, 1993; Rivers *et al.*, 1993).

We have undertaken the work reported in this paper in order to better understand the structural features which are responsible for the functional activity of MoCo in *Thermus thermophilus* (Wuebbens *et al.*, 2000). Molybdenum-cofactor protein C (MoaC) was crystallized in two different space groups, $P2_1$ and $R32$. Here, we report the characterization of the protein crystals and the preliminary X-ray diffraction experiments. The present study should enhance the research interests of structural biologists involved in the catalytic study of thermophilic microorganisms.

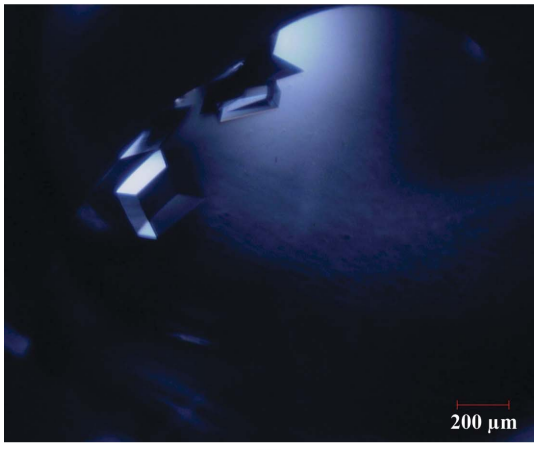
2. Materials and methods

2.1. Cloning, expression and purification of the protein

The *moaC* gene (TTHA1789) 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 the *E. coli* BL21 (DE3) strain (Novagen) and the recombinant strain was cultured in 6 l LB medium supplemented with 50 µg ml^{-1} ampicillin. The cells (20.5 g) were collected by centrifugation, washed with 20 ml buffer *A* (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 $(NH_4)_2SO_4$ 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 50 m*M* sodium phosphate buffer pH 7.0 containing 1.35 *M* $(NH_4)_2SO_4$, which was eluted with a linear gradient of 1.5 *M* $(NH_4)_2SO_4$. The eluted fractions containing the recombinant MoaC were collected, desalted by fractionation on a HiPrep 26/10 desalting column pre-equilibrated with buffer *A* and applied onto a Resource Q column (GE Healthcare Bioscience Corp.) pre-equilibrated with the same buffer, which was eluted with a linear gradient of 0–0.5 *M* NaCl. The eluted fractions containing the recombinant protein were collected and desalted by fractionation on a HiPrep 26/10 desalting column pre-equilibrated with sodium phosphate buffer pH 7.0 and applied onto a hydroxyapatite CHT10 column (Bio-Rad Laboratories Inc., Hercules, CA, USA), which was eluted with a linear gradient of 10–500 m*M* potassium phosphate buffer pH 7.0. The eluted fractions containing the recombinant protein were collected and concentrated with a Vivaspin 20 concentrator (5 kDa molecular-weight cutoff, Sartorius, AG, Goettingen, Germany) and loaded onto a HiLoad 16/60 Superdex 75 pg column (GE Healthcare Bioscience Corp.) pre-equilibrated with buffer *A* containing 150 m*M* NaCl.



(*a*)



(*b*)

Figure 1
Crystals of molybdenum-cofactor biosynthesis protein C from *T. thermophilus*. (*a*) Primitive monoclinic form; (*b*) rhombohedral form.

2.2. Crystallization experiments

The purified protein was concentrated using a Vivaspin 20 concentrator (5 kDa molecular-weight cutoff, Sartorius). The protein concentration was determined by measuring the absorbance at 280 nm (Kuramitsu *et al.*, 1990). The concentration of the purified protein was 11 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl. Crystallization was performed using the sitting-drop vapour-diffusion method under two different conditions. Preliminary screening for the first crystallization condition was performed by the sitting-drop vapour-diffusion method using Emerald Biostructures kits Cryo I and II. Crystals (Fig. 1*a*) were obtained when 1 µl protein solution was mixed with 1 µl well solution and allowed to equilibrate against 100 µl well solution at 293 K. The well solution consisted of 25%(*v*/*v*) 1,2-propanediol, 5%(*w*/*v*) PEG 3000, 0.1 *M* phosphate–citrate buffer pH 4.2 and 10%(*v*/*v*) glycerol. The crystals appeared in about 3 d. The initial screening for the second crystallization condition was carried out by the sitting-drop vapour-diffusion method using the Hampton Research SaltRX kit. The chosen conditions were further optimized using narrow intervals of pH (4.6–5.0). Crystals (Fig. 1*b*) were obtained when 1 µl protein solution was mixed with 1 µl well solution and allowed to equilibrate against 100 µl well solution at 293 K. The well solution contained 0.1 *M* sodium acetate buffer pH 5.0 and 1.0 *M* ammonium dihydrogen phosphate. 100%(*v*/*v*) paraffin oil was used as a cryoprotectant. Diffraction-quality crystals appeared in about 5 d.

2.3. Data collection

Diffraction data were collected from the monoclinic crystal at 100 K using the RIKEN Structural Genomics Beamline II (BL26B2) at SPring-8 (Hyogo, Japan) with a Jupiter210 CCD detector (Rigaku MSC Co., Tokyo, Japan). The crystal-to-detector distance was maintained at 180 mm. In the case of the primitive rhombohedral crystal, diffraction data were collected at 100 K using beamline 22-BM (SER-CAT) at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL, USA) with a MAR 225 CCD detector (MAR Research USA, Evanton, IL, USA). The distance between the crystal and the detector was maintained at 180 mm. The data-processing software used was *DENZO* (Otwinowski, 1993). The monoclinic and the rhombohedral crystals diffracted to 1.9 and 1.75 Å resolution, respectively.

3. Results and discussion

Analysis of the diffraction intensities of the crystal of MoaC obtained from the first crystallization condition shows that it belongs to a primitive monoclinic space group. The space group is $P2_1$, with unit-cell parameters a = 64.81, b = 109.84, c = 115.19 Å, β = 104.9°. The MoaC protein from *T. thermophilus* (NCBI accession No. YP_145055) consists of 157 amino-acid residues with a subunit molecular weight of 17 kDa. The molecular weight of the protein was determined by gel-filtration experiments and showed the presence of a hexamer in solution (data not shown), suggesting the biological

Table 1
X-ray data-collection statistics of molybdenum-cofactor biosynthesis protein C from *T. thermophilus*.

Values in parentheses are for the last resolution shell.

Space group	$P2_1$	$R32$
Wavelength (Å)	1.0	0.9724
Temperature (K)	100	100
Unit-cell parameters (Å, °)	a = 64.81, b = 109.84, c = 115.19, β = 104.9	$a = b$ = 106.57, c = 59.25
Matthews coefficient (Å^3 Da^{-1})	2.0 (12 subunits per ASU)	1.9 (1 subunit per ASU)
Solvent content (%)	36.7	35.6
Resolution range (Å)	50.0–1.90 (1.97–1.90)	50.0–1.75 (1.81–1.75)
No. of observed reflections	667221	288658
No. of unique reflections	121501 (12072)	13115 (1288)
Completeness (%)	99.9 (99.7)	99.9 (100)
$R_{\rm sym}$†	4.5 (17.2)	8 (17.7)
Multiplicity	5.5 (5.1)	22 (21.3)
Average $I/\sigma(I)$	24 (5)	57 (15)

† $R_{\rm sym} = \sum I_{hkl} - \langle I \rangle / \sum I_{hkl}$.

significance of the molecule. However, the calculated crystal-packing coefficient ($V_{\rm M}$ = 2.0 Å^3 Da^{-1}) with a solvent content of 36.7% (Matthews, 1968) indicates the presence of 12 monomers in the asymmetric unit. The relevant parameters are given in Table 1.

The crystal obtained using the second crystallization condition was found to belong to the primitive rhombohedral space group $R32$, with unit-cell parameters $a = b$ = 106.57, c = 59.25 Å. The calculated $V_{\rm M}$ coefficient is 1.9 Å^3 Da^{-1} (corresponding to 35.6% solvent content), indicating the presence of one monomer in the asymmetric unit. The data-collection statistics are given in Table 1.

3.1. Molecular replacement

The crystal structure of MoaC was solved by molecular-replacement calculations using the program *CNS* v.1.1 (Brünger *et al.*, 1998). The search model coordinates were those of MoaC from *E. coli* (PDB code 1ekr; Wuebbens *et al.*, 2000). The search model used in the molecular replacement had 53% sequence identity with the sequence of MoaC from *T. thermophilus*. The molecular-replacement search revealed 12 monomers and one monomer in the asymmetric unit for the crystals belonging to space groups $P2_1$ and $R32$, respectively. Subsequently, the molecules were subjected to rigid-body refinement using *CNS* v.1.1 (Brünger *et al.*, 1998). In both cases, 5% of the reflections were kept aside for the calculation of $R_{\rm free}$ (Brünger, 1992). The $R_{\rm work}$ and $R_{\rm free}$ values of the partially refined structures were 40.2 and 39.5%, respectively, for the crystal belonging to space group $P2_1$. The corresponding values for the crystal belonging to space group $R32$ were 36.6 and 38.3%, respectively.

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