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Crystallization and Preliminary X-Ray Diffraction Analysis of 5,10-Methylenetetrahydrofolate Dehydrogenase/Cyclohydrolase from *Thermoplasma acidophilum DSM 1728*

Kim, Jae-Hee, Min-Woo Sung, Eun Hye Lee, Ki Hyun Nam, and Kwang Yeon Hwang*

Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

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The methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from the thermoacidophilic archaeon *Thermoplasma acidophilum* is a 30.6 kDa molecular-mass enzyme that sequentially catalyzes the conversion of formyltetrahydrofolate to methylenetetrahydrofolate, with a preference for NADP as a cofactor, rather than NAD. In order to elucidate the functional and structural features of MTHFDC from archaeons at a molecular level, it was overexpressed in *Escherichia coli* and crystallized in the presence of its cofactor, NADP, at 295 K using polyethylene glycol (PEG) 4000 as a precipitant. The crystal is a member of the monoclinic space group $P2_1$, with the following unit cell parameters: a=66.333 Å, b=52.868 Å, c=86.099 Å, and β= 97.570°, and diffracts to a resolution of at least 2.40 Å at the synchrotron. Assuming a dimer in the crystallographic asymmetric unit, the calculated Matthews parameter (V_M) was 2.44 Å^3/Da and the solvent content was 49.7%.

Keywords: *Thermoplasma acidophilum*, dehydrogenase, cyclohydrolase, crystallization

Thermoplasma acidophilum is a thermoacidophilic archaeon that inhabits a hot (59°C) and highly acidic environment (pH 2.0), in which few organisms are viable. The genome of *T. acidophilum* is one of the smallest among free-living organisms [4, 19]. Species of the *Thermoplasma* genus do not harbor a rigid cell wall, but are delimited only by a plasma membrane. Many macromolecular assemblies from *Thermoplasma*, principally proteases and chaperones, have been shown to be crucial in elucidating the structure and function of their more complex eukaryotic homologs. Owing to its biological importance, the complete genome sequence of strain DSM 1728 has been previously reported [4, 19].

*Corresponding author
Phone: 82-2-3290-3009; Fax: 82-2-923-3225;
E-mail: chahong@korea.ac.kr

One-carbon metabolism provides the scaffolding for one essential and five major carbon transfer reactions within the cell [6, 12]. Glycine and serine are the major sources of one-carbon metabolites. They yield 5,10-methylenetetrahydrofolate, which may be utilized itself as an intermediate in biosynthetic reactions or it may be enzymatically oxidized or reduced to a one-carbon derivative of tetrahydrofolate (THF) that serves as the intermediate in other biosynthetic reactions. The different one-carbon derivatives of THF are involved in the formation of representatives of various classes of cell metabolites including amino acids and proteins, purine and pyrimidine bases, and a vitamin [6, 7, 12]. This metabolism is founded on the ability of THF to carry one-carbon units at different oxidation states [17]. These reactions include the interconversion of glycine and serine, the breakdown of histidine, and the production of thymidylate, methionine, and purines [11]. During the reaction, in which a single carbon unit is carried in H_4folate, methylene-H_4folate (which is required for thymidylate synthesis) is generated and then reduced to methyl-H_4folate with the participation of dehydrogenase [12]. THF performs a critical role as a molecule for the generation and utilization of monocarbon units in a variety of oxidation states [6]. The bacterial enzymes and cytosolic enzymes of eukaryotes are predominantly NADP-dependent, whereas the majority of mitochondrial enzymes and monofunctional dehydrogenases utilize NAD [21]. These enzymes bind to a single folate substrate [18] and a single NADP coenzyme per subunit [1, 3, 10]. In higher organisms, the activities involved in the modification of the monocarbon, which can be transferred as a methylene, methenyl, or formyl group, are linked together to form a trifunctional enzyme consisting of methylene-THF dehydrogenase (D), methenyl-THF cyclohydrolase (C), and 10-formyl-THF synthetase (S) [15]. Enzymes involved in THF metabolism are of particular pharmaceutical interest, as their function is known to be crucial for both amino acid and DNA biosynthesis. Therefore, the enzyme is an attractive target

for the development of mechanism-based inhibitors, which may prove useful as novel therapeutic drugs [9, 19].

The primary amino acid sequence of MTHFDC from *T. acidophilum* (*Ta*MTH) presents 36%, 29%, and 31% identity with the corresponding domains from *Escherichia coli,* yeast, and humans, respectively. It is composed of 276 amino acids residues (M_r=30,600 Da). Thus far, three-dimensional structures of the bifunctional 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from *Mycobacterium tuberculosis* (PDB ID; 2C2Y), *E. coli* (PDB ID; 1B0A) [18], *Saccharomyces cerevisiae* (PDB ID; 1EDZ) [15], and humans (PDB ID; 1A4I) [20], have been made available in the PDB. However, no crystal structure of MTHFDC from archaeons has yet been obtained. Thus, as an initial step toward the elucidation of its structure [8], we have overexpressed *Ta*MTH and crystallized it.

The full length of the *Ta*MTH gene was amplified by polymerase chain reaction from the *T. acidophilum* cDNA library, using two primers (forward primer: 5'-GGAATTCC-ATATGATGAAGATCCTCAGGGGAGAGGAGATCG-3'; reverse primer: 5'-CCGCTCGAGTTACAGGTTGTTTTT-TTGGAATTCTGCCG-3'). The PCR product was then purified, digested with NdeI and XhoI, and ligated into the pET-28a vector (Novagen) harboring a 6× His-tag at its N-terminus. The resultant plasmid was then transformed into *Escherichia coli* strain BL21 (DE3) (Novagen), which was grown at 37°C in Luria-Bertani medium with kanamycin (50 μg/ml). The cells were grown at 37°C to an OD_{600} of 0.4–0.8, and the expression of the recombinant *Ta*MTH fused to the His tag was induced with 1 mM isopropyl-D-thiogalactopyranoside at an optical density of approximately 0.5 at 600 nm (Fig. 1). After 16 h of induction at 18°C, the cells were then harvested by centrifugation at 6,000 ×*g* for 30 min at 4°C. The pelleted cells were then suspended in buffer A (20 mM Tris-HCl, 2 mM β-mercaptoethanol, 100 mM NaCl, pH 8.5) and sonicated in the same buffer while maintaining the temperature at 4°C. The supernatant was heat-treated at 50°C for 5 min, and then centrifuged at 20,000 ×*g* for 30 min. The supernatant was loaded onto a His-Trap affinity column (Amersham Biosciences) equilibrated with buffer A. The protein was eluted with a linear gradient of 0–0.5 M imidazole, in the same buffer. The concentrated protein solution was then applied to and eluted from a Superdex-S200 column (Amersham Biosciences) for further purification. The product homogeneity of the purified preparation was determined by gel electrophoresis (Fig. 1). Sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions was conducted using Precision Protein Standard (Bio-rad) as a reference protein for the estimation of molecular weight. The fractions containing *Ta*MTH were then pooled and concentrated to 1.8 mg/ml in 10 mM Tris-HCl, 50 mM KCl, and 2 mM DTT. The *Ta*MTH was cocrystallized with NADP as a cofactor. Initial searches for the crystallization of *Ta*MTH was by the sitting-drop vapor-diffusion method using 96-well Intelli plates (Hampton Research) and a Hydra II Plus One (Matrix Technology) at 295 K. The initial search for crystallization conditions was performed using commercially

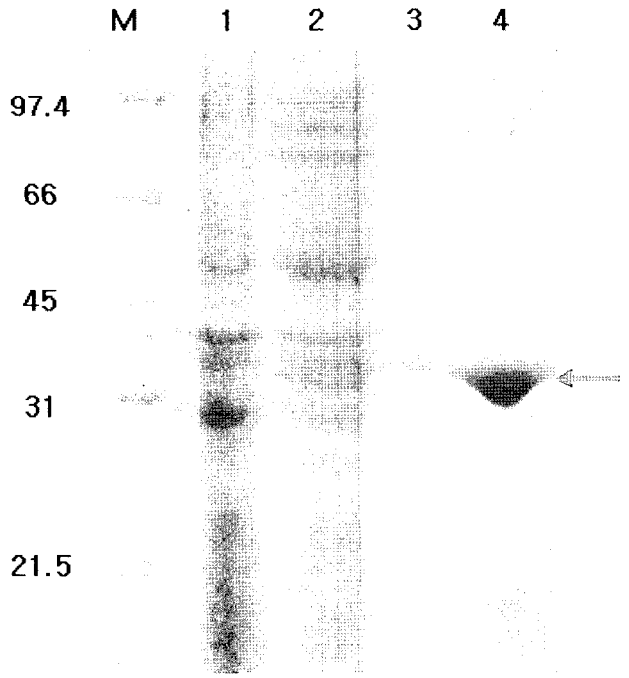

Fig. 1. Expression and purification of the recombinant 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from *Thermoplasma acidophilum* as determined by SDS-PAGE (15% [v/v]).
Lane M, molecular mass protein marker (kDa); lane 1, uninduced cell lysate; lane 2, induced cell lysate; lane 3, after His-tag affinity column chromatography; lane 4, purified bifunctional 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from *Thermoplasma acidophilum*.

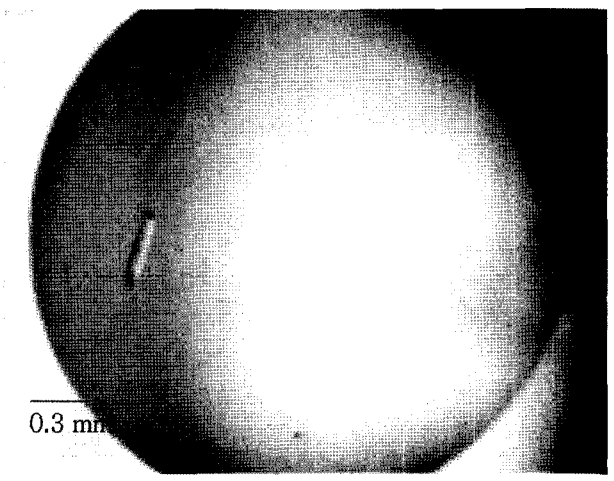

Fig. 2. Crystals of the recombinant 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from *Thermoplasma acidophilum*, used for the collection of X-ray diffraction data.
The crystal size was approximately 0.2 mm×0.1 mm×0.15 mm.

available kits from JB screen 9, 10 NR LBP, and NR LBD. Each hanging drop was placed over 0.5 ml of reservoir solution. Initial crystals were obtained for 3–5 days in a variety of PEG 4000 conditions. Initial optimization was achieved in a solution of 18% PEG 4000, 400 mM NaCl, 100 mM Tris-HCl (pH 8.0), and 5 mM NADP. In this condition, plate-like crystals were grown within 5 days at 22°C. The crystal was transferred into a cryoprotection solution containing 18.5% PEG 4000, 400 mM NaCl, 30% sucrose, and 100 mM Tris-HCl (pH 8.0). It was then scooped up in a cryoloop, frozen in liquid nitrogen, and mounted on the goniometer in a stream of 100 K nitrogen. X-ray diffraction data were obtained from the cooled crystal using an ADSC Quantum CCD 210 detector at 4A MXW in the Pohang Accelerator Laboratory (Pohang, South Korea). The crystal was oscillated by 1.0° per frame over a total range of 330° at a wavelength of 1.000 Å. X-ray diffraction data to 2.40 Å for the NADP-complex were collected with synchrotron radiation. Data were integrated and scaled using the *DENZO* and *SCALEPACK* crystallographic data-reduction routines [15]. The auto-indexing procedure conducted with *DENZO* showed that the crystals belonged to the monoclinic space group $P2_1$ on the basis of systematic absences, with the following unit cell parameters: a= 66.333 Å, b=52.868 Å, c=86.099 Å, and β=97.570° for the NADP-complex. Assuming a dimer in the crystallographic asymmetric unit, the calculated Matthews parameter (V_M) was 2.44 $Å^3$/Da and the solvent content was 49.7% [13]. The data-collection statistics are shown in Table 1. The structure was solved by molecular replacement in Program CCP4 [2]. A monomer of MTHFDC structure from *E. coli* was used as a starting model and we found two clear rotations and translated positions within the asymmetric unit. The structure determination of *Ta*MTH is currently under way, and the structural details will be described in a separate paper.

Table 1. Data collection statistics of a crystal of a bifunctional 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFDC) from *Thermoplasma acidophilum*.

	*Ta*MTH
Space group	*P2*(1)
Wavelength	1.000
Cell dimension (Å)	a=66.333 Å, b=52.868 Å, c=86.099 Å, α=γ=90°, β=97.57°
Resolution range (Å)	50–2.40 (2.49–2.40)
Observed reflections	21,571 (1,370)
Redundancy	4.5 (2.5)
Completeness (%)	91.6 (59.0)
R_{sym} (%)	8.8 (24.2)
I/sigma (I)	16.15 (2.78)

Values in parentheses are for the highest resolution shell.
$R_{sym}=\Sigma|I-(I)|/\Sigma(I)$, where I is the observed intensity and (I) is the average intensity.

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