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# Overexpression, crystallization, and preliminary X-ray crystallographic analysis of shikimate dehydrogenase from *Thermotoga maritima*

**Hyung Ho Lee**

Department of Bio and Nano Chemistry, Kookmin University, Seoul, 136-702, Republic of Korea

Correspondence e-mail: hhlee@kookmin.ac.kr

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Shikimate dehydrogenase (SDH), which catalyses the NADPH-dependent reduction of 3-dehydroshikimate to shikimate in the shikimate pathway, is an attractive target for the development of herbicides and antimicrobial agents. Previous structural studies showed that SDH exists in two conformations, an open form and a closed form, and it is believed that the conformational state is crucial to understanding a catalytic mechanism. To facilitate further structural comparisons among SDHs, structural analysis of an SDH from *Thermotoga maritima* encoded by the *Tm0346* gene has been initiated. SDH from *T. maritima* has been overexpressed in *Escherichia coli* and crystallized at 296 K using ammonium sulfate as a precipitant. Crystals of *T. maritima* SDH diffracted to 1.45 Å resolution and belonged to orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 54.21, $b$ = 62.45 and $c$ = 68.68 Å. The asymmetric unit contains a monomer, with a corresponding $V_M$ of 2.01 Å$^3$ Da$^{-1}$ and a solvent content of 38.9% by volume.

## 1. Introduction

The shikimate pathway is essential in higher plants, bacteria and fungi, but is absent from mammals, so it is an attractive target for the development of herbicides and antimicrobial agents (Davies *et al.*, 1994). The shikimate pathway consists of seven enzymatic steps, the first step of which is the condensation of phosphoenolpyruvate and erythrose-4-phosphate by 3-deoxy-D-*arabino*-heptolosonate 7-phosphate synthase (Singh *et al.*, 2005). Shikimate dehydrogenase (SDH; EC 1.1.1.25), encoded by the *aroE* gene in bacteria, catalyses the NADPH-dependent reduction of 3-dehydroshikimate to shikimate in the fourth reaction of the shikimate pathway (Singh *et al.*, 2005).

The crystal structures of two types of shikimate dehydrogenases from bacteria [SDH (PDB code 1nyt) and YdiB (PDB code 1o9b)] have been determined (Michel *et al.*, 2003). Subsequently, the crystal structure of a novel shikimate dehydrogenase, which shows different kinetic properties from those of SDH and YdiB, has been determined in *Haemophilus influenzae* (Singh *et al.*, 2005). YdiB catalyzes the reversible reductions of dehydroquinate to quinate and dehydroshikimate to shikimate in the presence of either NADH or NADPH, while the SDH of *H. influenzae* catalyzes the oxidation of shikimate but not quinate (Singh *et al.*, 2005). SDHs usually form oligomers in most bacteria (Anton & Coggins, 1988; Chaudhuri & Coggins, 1985), whereas SDH in *Escherichia coli* is present as a monomer. The structure of *H. influenzae* SDH reveals that monomeric SDH is composed of two domains. The catalytic domain shows a novel fold, while the NADPH-binding domain has a typical Rossmann fold and a unique glycine-rich P-loop with a conserved sequence motif of GAGGXX (Ye *et al.*, 2003). In contrast, YdiB of *E. coli* and SDH of *Methanococcus jannaschii* are shown to exist as dimers in solution and in crystals (Michel *et al.*, 2003; Padyana & Burley, 2003). Subsequently, the structures of SDHs from *Staphylococcus epidermidis* (Han *et al.*, 2009), *Thermus thermophilus* (Bagautdinov & Kunishima, 2007), *Aquifex aeolicus* (Gan *et al.*, 2007) and *Arabidopsis* (Singh & Christendat, 2006) have been determined. The crystal structure of *A. aeolicus* SDH in complex with NADP$^+$ and shikimic acid has a closed conformation while a ternary complex of

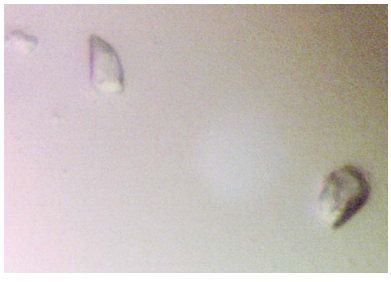

**Table 1**
Data collection and refinement statistics.

Values in parentheses refer to the highest resolution shell (1.50–1.45 Å).

| | |
|---|---|
| X-ray source | BL-6B, Pohang Light Source |
| X-ray wavelength (Å) | 0.9197 |
| Detector | MacScience 2030 |
| No. of crystals | 1 |
| Crystal-to-detector distance (mm) | 200 |
| Rotation range per frame (°) | 1 |
| Total rotation range (°) | 150 |
| Exposure time per image (s) | 10 |
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å) | $a$ = 54.21, $b$ = 62.45, $c$ = 68.68 |
| Resolution range (Å) | 20–1.45 |
| Total/unique reflections | 173010/38992 |
| Completeness (%) | 98.4 (92.8) |
| Mean $I/\sigma(I)$ | 22.3 (7.3) |
| $R_{\rm merge}$† (%) | 2.7 (15.0) |
| Mosaicity (°) | 0.8 |
| Redundancy | 4.4 |

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| \sum_h \sum_i I(h)_i$, 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 $h$.

*T. thermophilus* SDH, $NADP^+$ and shikimic acid exhibits an open conformation (Gan *et al.*, 2007).

In order to facilitate further structural comparisons among SDHs and facilitate the design of inhibitors targeting SDHs, we are interested in determining the three-dimensional structure of an SDH from *T. maritima* (Tm0346) that shares moderate levels of amino-acid sequence identity with the structurally characterized SDHs. The sequence identity is 27% against SDH from *E. coli*, 27% against YdiB from *E. coli*, 27% against SDH from *H. influenzae*, and 31% against SDH from *M. jannaschii*. The SDH from *T. maritima* (Tm0346) has been overexpressed in *E. coli* and crystallized. Its crystallization conditions, X-ray crystallographic data and preliminary structural determination are reported here.

## 2. Experimental

### 2.1. Protein expression and purification

The *aroE* gene encoding the SDH of *T. maritima* (Tm0346) was amplified from the genomic DNA by the polymerase chain reaction. The forward and reverse oligonucleotide primers were 5′-GG GAA TTC **CAT ATG** AAA TTC TGC ATC ATA GGG-3′ and 5′-A TCG **GGA TCC** TCA TTT CAG AAC CTC CCC GAA CAC-3′, respectively. The bases in bold represent the *Nde*I and *Bam*HI restriction enzyme cleavage sites. The amplified DNA was digested with *Nde*I and *Bam*HI and was then inserted into the *Nde*I/*Bam*HI–digested expression vector pET-22*b*(+) (Novagen). The plasmid was transformed into the *E. coli* strain C41(DE3) (Miroux & Walker, 1996) for protein expression. The cells were grown at 310 K up to an $OD_{600}$ of 0.5 in Terrific Broth medium containing 50 µg $ml^{-1}$ ampicillin and the protein expression was induced by 1.0 m*M* isopropyl-*β*-D-thiogalactopyranoside (IPTG). The cells continued to grow at 293 K for 22 h after IPTG induction and were harvested by centrifugation at 4200*g* (6000 rev $min^{-1}$; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer *A* (20 m*M* Tris–HCl pH 9.0, 200 m*M* NaCl, 1 m*M* *β*-mercaptoethanol and 1 m*M* EDTA) containing 1 m*M* phenylmethylsulfonylfluoride and was homogenized with an ultrasonic processor, and then heated for 10 min at 353 K. The crude cell extract was centrifuged at 36 000*g* (18 000 rev $min^{-1}$, Hanil Supra 21 K rotor) for 1 h at 277 K. The supernatant was subjected to ion-exchange chromatography on a Q-Sepharose column (GE Healthcare), which was previously equilibrated with buffer *A*, and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in buffer *A*. Next, purification was performed by gel filtration on a HiLoad XK 16 Superdex 75 prep-grade column (GE Healthcare), which was previously equilibrated with buffer *A* containing 200 m*M* sodium chloride, and was desalted by dialysis with buffer *B* [20 m*M* *N*-(2-acetamido)iminodiacetic acid, pH 6.0, 20 m*M* NaCl, 1 m*M* *β*-mercaptoethanol, and 1 m*M* EDTA]. The protein was subjected to a Mono S column (GE Healthcare), which was previously equilibrated with buffer *B*, and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in buffer *B*. Final purification was performed by gel filtration on a HiLoad XK 16 Superdex 75 prep-grade column (GE Healthcare), which was previously equilibrated with buffer *B* containing 200 m*M* sodium chloride. Homogeneity of the purified protein was assessed by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). The protein solution was concentrated using a YM10 ultrafiltration membrane (Amicon) to about 70 mg $ml^{-1}$. The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated molar extinction coefficient of 35 870 $M^{-1}$ $cm^{-1}$ (SWISS-PROT; http://www.expasy.ch/).

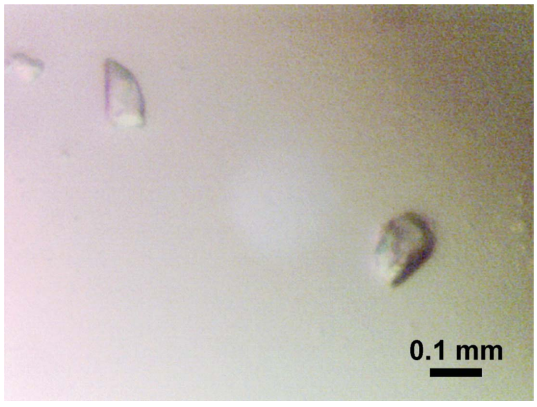


**Figure 1**
Crystals of shikimate dehydrogenase from *T. maritima*. Approximate dimensions are 0.10 × 0.10 × 0.15 mm.

### 2.2. Crystallization, dynamic light scattering and X-ray data collection

Crystallization experiments were carried out using the hanging-drop vapor-diffusion method at 296 K using 24-well tissue-culture plates (Hampton Research). A hanging drop on a siliconized cover slip was prepared by mixing equal volumes (2 µl each) of the protein solution and the reservoir solution. It was placed over 0.5 ml of the reservoir solution. Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screen I, Screen II, and MembFac) and from deCODE Biostructures Group (Wizard I and Wizard II). Dynamic light-scattering experiments were performed on a DynaPro-801 instrument from Wyatt (Santa Barbara, California). The data were measured at 297 K with the protein at 1 mg $ml^{-1}$ concentration, dissolved in buffer *B* containing 200 m*M* sodium chloride.

Crystals of *T. maritima* SDH were obtained after optimization using ammonium sulfate as a precipitant. The crystals were flash-frozen in a liquid nitrogen stream employing 15%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data were collected at 100 K at the

BL-6B experimental station of the Pohang Light Source, Pohang, Korea, with a MacScience 2030 image-plate detector. The wavelength of synchrotron X-rays was 0.9197 Å and a 0.3 mm collimator was used. The crystal was rotated through a total of 150° with 1.0° oscillation range per frame. The raw data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

## 3. Results

*T. maritima* SDH in its intact form has been overexpressed in soluble form with a yield of ∼17.5 mg of homogeneous protein per litre of culture. The optimized reservoir condition for crystallization was 100 m*M* sodium HEPES buffer (pH 7.5), 2.0 *M* ammonium sulfate, and 2%(*w*/*v*) polyethylene glycol (PEG) 400. Crystals grew up to maximum dimensions of 0.10 × 0.10 × 0.15 mm within six months (Fig. 1). A set of X-ray diffraction data was collected to 1.45 Å resolution at 100 K. A total of 173 010 measured reflections were merged into 38 992 unique reflections, giving an $R_{\rm merge}$ of 2.7% and a completeness of 98.4%. The space group was determined to be $P2_12_12_1$ on the basis of systematic absences and symmetry of diffraction intensities. The unit-cell parameters are $a$ = 54.21, $b$ = 62.45, $c$ = 68.68 Å. Table 1 summarizes the statistics for data collection. The molecular mass of the recombinant SDH was estimated to be ∼30 kDa by dynamic light-scattering analysis, indicating that the enzyme exists as a monomer in solution (calculated monomer mass = 28 889 Da). If it is assumed that one monomeric molecule is present in the crystallographic asymmetric unit, the crystal volume per protein mass ($V_{\rm M}$) is 2.01 Å$^3$ Da$^{-1}$ and the solvent content is 38.9% (Matthews, 1968).

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