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The hyperthermophilic cystathionine γ -synthase from the aerobic crenarchaeon *Sulfolobus tokodaii*: expression, purification, crystallization and structural insights

Dan Sato,^a Tomoo Shiba,^a Sae Mizuno,^a Ayaka Kawamura,^a Shoko Hanada,^a Tetsuya Yamada,^b Mai Shinozaki,^b Masahiko Yanagitani,^b Takashi Tamura,^b Kenji Inagaki^b and Shigeharu Harada^{a*}

^aDepartment of Applied Biology, Kyoto Institute of Technology, Goshō Kaidō-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, and ^bDepartment of Biofunctional Chemistry, Okayama University, Tsushima-naka 1-1-1, Kita-ku, Okayama 700-8530, Japan. *Correspondence e-mail: harada@kit.ac.jp

Cystathionine γ -synthase (CGS; EC 2.5.1.48), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyzes the formation of cystathionine from an L-homoserine derivative and L-cysteine in the first step of the transsulfuration pathway. Recombinant CGS from the thermoacidophilic archaeon *Sulfolobus tokodaii* (StCGS) was overexpressed in *Escherichia coli* and purified to homogeneity by heat treatment followed by hydroxyapatite and gel-filtration column chromatography. The purified enzyme shows higher enzymatic activity at 353 K under basic pH conditions compared with that at 293 K. Crystallization trials yielded three crystal forms from different temperature and pH conditions. Form I crystals (space group $P2_1$; unit-cell parameters $a = 58.4$, $b = 149.3$, $c = 90.2$ Å, $\beta = 108.9^\circ$) were obtained at 293 K under acidic pH conditions using 2-methyl-2,4-pentandiol as a precipitant, whereas under basic pH conditions the enzyme crystallized in form II at 293 K (space group $C222_1$; unit-cell parameters $a = 117.7$, $b = 117.8$, $c = 251.3$ Å) and in form II' at 313 K (space group $C222_1$; unit-cell parameters $a = 107.5$, $b = 127.7$, $c = 251.1$ Å) using polyethylene glycol 3350 as a precipitant. X-ray diffraction data were collected to 2.2, 2.9 and 2.7 Å resolution for forms I, II and II', respectively. Structural analysis of these crystal forms shows that the orientation of the bound PLP in form II is significantly different from that in form II', suggesting that the change in orientation of PLP with temperature plays a role in the thermophilic enzymatic activity of StCGS.

1. Introduction

Sulfur-containing amino acids are ubiquitously distributed in all organisms and play biologically important roles in protein synthesis, in the methylation of DNA and proteins, and in the biosynthesis of vitamins, polyamines and antioxidants. The major sulfur-containing amino acids (methionine, cysteine and homocysteine) are interconvertible *via* cystathionine by the transsulfuration pathway (methionine $\leftrightarrow$ homocysteine $\leftrightarrow$ cystathionine $\leftrightarrow$ cysteine; Stipanuk, 2004). The pathway present in plants, bacteria and archaea metabolizes L-cysteine to L-methionine, whereas in mammals the reverse transsulfuration pathway converts L-methionine to L-cysteine (Aitken *et al.*, 2011).

Cystathionine γ -synthase (CGS; EC 2.5.1.48), a pyridoxal 5'-phosphate (PLP) dependent enzyme, catalyzes the γ -replacement reaction that synthesizes L-cystathionine from L-cysteine and activated forms of L-homoserine in the first step of the transsulfuration pathway, as well as α , γ - and

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α,β -elimination reactions yielding α -keto acids, thiols and ammonia. Microbial CGS utilizes *O*-succinyl- and *O*-acetyl-L-homoserine as the activated forms, whereas plant-type CGS, located in the chloroplast, uses *O*-phospho-L-homoserine (Aitken & Kirsch, 2005). Since CGS does not exist in mammals, it is a promising target for the development of novel herbicides and antibiotics. Currently, bacterial CGSs from *Escherichia coli* (Tran *et al.*, 1983), *Salmonella typhimurium* (Kaplan & Flavin, 1966) and *Helicobacter pylori* (Kong *et al.*,

2008), and plant CGSs from *Arabidopsis thaliana* (Ravanel *et al.*, 1998), wheat (Kreft *et al.*, 1994), spinach (Ravanel *et al.*, 1995) and tobacco (Clausen *et al.*, 1999), have been enzymatically characterized. Crystal structures are available for CGSs from *E. coli* (PDB entry 1cs1; Clausen *et al.*, 1998), tobacco (PDB entry 1qgn; Steegborn *et al.*, 1999), *H. pylori* (PDB entry 4l0o; K. F. Tarique, S. A. A. Rehman, E. Ahmed & S. Gourinath, unpublished work) and *Mycobacterium ulcerans* Agy99 (PDB entries 3qi6 and 3qh; Clifton *et al.*, 2011), and

Figure 1

Sequence alignment of StGCS and homologous enzymes with known X-ray structures. Abbreviations used: St, *S. tokodaii*; Ec, *E. coli*; Hp, *H. pylori*; Mu, *M. ulcerans* Agy99; Nt, tobacco; Cf, *C. freundii*. The sequences were aligned using *Clustal Omega* (Sievers *et al.*, 2011). Identical and similar amino acids are shown on black and grey backgrounds, respectively. Residues interacting with PLP by hydrogen bond(s) are indicated by white triangles and Lys192, which forms a Schiff base with the PLP, is indicated by a black triangle. Phe97, which is unique to StGCS, is indicated by a circle.

structures in complex with inhibitors have been determined for tobacco CGS (PDB entries 1I41, 1I43 and 1I48; Steegborn *et al.*, 2001).

Sulfolobus tokodaii, a thermoacidophilic crenarchaeon inhabiting sulfur-rich acidic hot springs, grows optimally at pH 2–3 and 353 K (Kawarabayasi *et al.*, 2001), and its ability to oxidize hydrogen sulfide to sulfate has been utilized for the disposal of industrial waste water (Kawarabayasi *et al.*, 2001). CGSs from *S. tokodaii* (StCGS) and other species are homologous to methionine γ -lyase (MGL; EC 4.4.1.11; Fig. 1). For instance, the amino-acid identity of StCGS to *Citrobacter freundii* MGL (PDB entry 2rfv; Nikulin *et al.*, 2008) is comparable to that to tobacco CGS (40.3 and 39.7% respectively); however, MGL only catalyzes α,γ - and α,β -elimination reactions (Sato & Nozaki, 2009). StCGS is unique in that a catalytically essential Tyr residue at the 97th position, which is highly conserved in CGSs well as in related PLP enzymes, is replaced by Phe (indicated by a circle in Fig. 1). The phenolate anion of the Tyr residue in the vicinity of the PLP cofactor accepts a proton from the α -amino group of a substrate, and the lone pair on the N atom then attacks the PLP (Clausen *et al.*, 1998; Steegborn *et al.*, 1999; Clifton *et al.*, 2011); mutation of Tyr to Phe drastically decreases the activities of *E. coli* CGS (Jaworski *et al.*, 2012) and other PLP enzymes (Inoue *et al.*, 2000; Sato *et al.*, 2008). In this work, recombinant StCGS enzyme was crystallized under different pH and temperature conditions in order to understand the pH and temperature dependence of the activity of StCGS based on its X-ray structures.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Cloning and expression of StCGS. The StCGS gene cloned into pET-11a vector (Novagen) was a kind gift from Professor Seiki Kuramitsu (Osaka University). The F97Y mutant was constructed by site-directed mutagenesis using KOD FX DNA polymerase (Toyobo). PCR was performed using the oligonucleotide primers 5'-GAGATATGTATGGA-AGGACTTACAGATTCTTTACGG-3' and 5'-CCGTAAA-GAATCTGTAAGTCCTTCCATACATATCTC-3' (mutated nucleotides are underlined), with pET11a-StCGS as a template. The PCR product was incubated with DpnI (Toyobo) to digest the template DNA, followed by transformation into JM109 competent cells. The amplified plasmid was purified and the mutated nucleotides were confirmed. The plasmid was introduced into the *E. coli* Rosetta-gami (DE3) strain (Novagen). Macromolecule-production information is summarized in Table 1.

The transformant was grown in 200 ml LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ ampicillin and 15 $\mu\text{g ml}^{-1}$ kanamycin for 12 h at 310 K; this culture was then inoculated into 2.4 l Terrific Broth medium ($\text{OD}_{600} = 0.01$) similarly supplemented with antibiotics. Expression of recombinant StCGS was induced with 0.02 mM isopropyl β -D-1-thiogalactopyranoside at 303 K for 12 h when the culture reached mid-log growth phase ($\text{OD}_{600} = 0.5$). After harvesting the cells

Table 1

Macromolecule-production information.

Source organism	<i>S. tokodaii</i> strain 7
DNA source	Genomic DNA
Cloning primers	N/A†
Cloning vector	N/A†
Expression vector	pET-11a
Expression host	<i>E. coli</i> Rosetta-gami (DE3)
Complete amino-acid sequence of the construct produced	MHGLREGTKVTTEGYDEETGAITPIYQTTSYIY-PIGEKYRSREVNPTVLKLAEKISELEEAEMG-VAFSSGMGAISSTLLTLAKPGSKILHRDMFG-RTYRFFTFDMRNLGVEVDVANPGEILEMVKVK-KYDIVVETISNPLLRVIDIPALSKICKENGSL-LLITDATFSTPINQKPLVQGADIVLHSASKFI-AGHNDVIAGLGAGSKELMTKVDLMRRTLTGSL-DPHAAVLVIRGIKTLKIRMDVINSNAQKIAEY-LQEHNKIKSVYYPGLKSHPDYETARRILKGYG-GVVTFEIKGSMNDALNLITRFKVLPAQTLGG-VNSTISHPATMTHRTLTPEEKKIIGISDSMLR-LSVGIEDVNDLIEDLDKALTSLN

† The gene cloned into pET-11a vector was a kind gift from Professor Seiki Kuramitsu (Osaka University).

by centrifugation for 15 min at 7000g and 277 K, the pellet was rinsed with 20 mM potassium phosphate buffer (KPB) pH 8.0 containing 0.05% (v/v) β -mercaptoethanol and 0.02 mM PLP, and was stored at 193 K until use.

2.1.2. Purification of StCGS. The cell pellet (20 g) was resuspended in 40 ml KPB and disrupted by sonication at 277 K; it was then centrifuged at 7000g for 15 min at 277 K to remove the cell debris. The supernatant was incubated at 343 K for 15 min and heat-denatured host proteins were removed by centrifugation at 14 400 g for 20 min at 277 K. The supernatant was dialyzed twice against 5.0 l of 5 mM KPB pH 8.0 containing 0.05% β -mercaptoethanol and 0.01 mM PLP at 277 K for 2 h, and was then centrifuged to remove flocculated proteins. The supernatant containing StCGS was applied onto a hydroxyapatite column (1.6 cm internal diameter $\times$ 36 cm; Bio-Rad) pre-equilibrated with 5 mM KPB pH 7.0. After washing the column with 750 ml of the buffer, the bound protein was eluted with a linear gradient of KPB (5–500 mM in 300 ml) at a flow rate of 2.5 ml min⁻¹. The eluted StCGS was detected by measuring the A_{280} and A_{415} . The fractions containing StCGS were collected and concentrated using an Amicon Ultra centrifugal concentrator (30 kDa molecular-weight cutoff; Millipore). The concentrated protein solution was then subjected to gel-filtration chromatography using a Sephacryl S-300 HR column (1.6 cm internal diameter $\times$ 60 cm; GE Healthcare) equilibrated with 20 mM KPB pH 8.0 containing 0.01 mM PLP. The purified protein was concentrated to about 15–20 mg ml⁻¹ using an Amicon Ultra centrifugal concentrator and finally stored at 193 K. The protein concentration was estimated by measuring the A_{280} and using the calculated molar extinction coefficient for StCGS (Pace *et al.*, 1995).

2.1.3. Estimation of the apparent molecular mass. The apparent molecular mass was estimated by gel-filtration chromatography. The purified protein (0.5 mg, 10 mg ml⁻¹) was applied onto a Superdex 200 column (0.5 cm internal diameter $\times$ 15 cm; GE Healthcare) equilibrated with 50 mM HEPES pH 7.5 containing 0.02 mM PLP and eluted at a flow

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well VDX plates (Hampton Research)
Temperature (K)	Forms I and II, 293; form II', 313
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM potassium phosphate buffer pH 8.0 containing 0.01 mM PLP
Composition of reservoir solution	Form I, 100 mM acetate pH 3.6, 54% (w/v) 2-methyl-2,4-pentanediol, 200 mM magnesium chloride, 10 mM ammonium sulfate; forms II and II', 100 mM Tris-HCl pH 7.0, 12–18% (w/v) polyethylene glycol 3350, 200 mM sodium citrate, nickel(II) chloride hexahydrate
Volume and ratio of drop	1.0 µl total, 1:1 ratio
Volume of reservoir (µl)	400

rate of 0.3 ml min⁻¹. Molecular mass was calibrated using commercially available standards (Bio-Rad).

2.1.4. Enzymatic activity assay. The enzymatic activity of StCGS was measured using the α,β -elimination side reaction because this measurement is more straightforward than measurement of the γ -replacement reaction. The pH dependence of the activity was measured using 3 or 0.3 µg purified enzyme at 353 K in 60 µl 100 mM buffer [acetate (pH 4.7), MES (pH 5.6), phosphate (pH 6.8), Bicine (pH 7.5) or CAPS (pH 9.1); estimated pH values at 353 K are shown in parentheses (Fukada & Takahashi, 1998)] containing 10 mM *O*-phospho-L-serine, 0.01 mM PLP, 1 mM EDTA and 1 mM DTT. The temperature dependence of the activity between 293 and 353 K was measured using 100 mM Bicine buffer pH 8.0 prepared at 298 K (pH 7.3 at 353 K). The reaction was terminated after 10 min by the addition of 10 µl 50% trichloroacetic acid, and denatured protein was removed by centrifugation at 15 000g for 10 min at 277 K. The supernatant (48 µl) was then incubated for 1 h at 323 K with 96 µl 1 M acetate buffer pH 5.2 containing 0.1% 3-methyl-2-benzothiazolinone hydrazine hydrochloride hydrate. The amount of azine generated was estimated by measuring the A_{320} using sodium pyruvate as a standard (Soda, 1968).

2.2. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method using the reservoir solutions supplied in commercially available screening kits (Crystal Screen, Crystal Screen 2 and PEG/Ion from Hampton Research, and Wizard Screen 1 and 2 from Molecular Dimensions). A droplet made by mixing 0.5 µl purified StCGS (10 mg ml⁻¹) with an equal volume of reservoir solution was equilibrated against 100 µl reservoir solution at 293 K. Conditions providing crystals were subsequently optimized by varying the pH values and concentrations of the precipitants at 293 and 313 K. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

X-ray diffraction experiments were performed on the BL-17A beamline ($\lambda = 0.9800$ Å; ADSC Q315 CCD detector)

at the KEK Photon Factory, Tsukuba, Japan and the BL44XU beamline ($\lambda = 0.9000$ Å; MX300HE CCD detector) at SPring-8, Harima, Japan. A crystal mounted in a nylon loop was transferred briefly to reservoir solution containing 15% (v/v) ethylene glycol and then flash-cooled at 100 K in a nitrogen-gas stream. A total of 180 images were recorded with an oscillation angle of 1.0° and an exposure time of 1 s per image (Table 3). The diffraction data were processed and scaled with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

The initial phase was obtained by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) as implemented in *CCP4* (Winn *et al.*, 2011). The structure of *E. coli* CGS (PDB entry 1cs1; 35.6% amino-acid identity to StCGS; Clausen *et al.*, 1998) was modified for use as a search model. The structure was refined using iterative cycles of refinement with *REFMAC5* (Murshudov *et al.*, 2011) followed by manual rebuilding of the structure using *Coot* (Emsley *et al.*, 2010).

3. Results and discussion

Recombinant wild-type StCGS was overexpressed in *E. coli* Rosetta-gami (DE3) cells and heat treatment effectively removed contaminating proteins in the lysate (Fig. 2). The enzyme was purified by two successive column-chromatography steps: hydroxyapatite and gel filtration. Approximately 50 mg of purified enzyme with >95% purity (Fig. 2) was obtained from 2.4 l bacterial culture. The molecular weights estimated by SDS-PAGE (42.0 kDa) and by gel-filtration chromatography (150 kDa) indicate that the enzyme exists as a homotetramer in solution. The F97Y mutant enzyme was purified in the same manner as the wild-type enzyme. This enzyme exhibited high enzymatic activity at 353 K at neutral to basic pH, rather than at 293 K as for the wild type, and the wild type exhibited higher activity than the F97Y mutant enzyme at all pH values and temperatures tested (Figs. 3*a* and 3*b*).

Figure 2
SDS-PAGE gel (12%) stained with Coomassie Brilliant Blue. Lane 1, molecular-weight markers (labelled in kDa); lane 2, supernatant from the cell lysate; lane 3, supernatant after heat treatment; lane 4, pooled fractions after hydroxyapatite chromatography; lane 5, purified protein after gel filtration.

Table 3
Data collection and processing.
Values in parentheses are for the outer shell.

	Form I	Form II	Form II'
Diffraction source	BL-17A, KEK-PF	BL44XU, SPring-8	BL44XU, SPring-8
Wavelength (Å)	0.98000	0.90000	0.90000
Temperature (K)	100	100	100
Detector	ADSC Q315	MX300HE	MX300HE
Crystal-to-detector distance (mm)	256.0	350.0	340.0
Rotation range per image (°)	1	1	1
Total rotation range (°)	180	180	180
Exposure time per image (s)	5	1	1
Space group	$P2_1$	$C222_1$	$C222_1$
a, b, c (Å)	58.4, 149.3, 90.2	117.7, 117.8, 251.3	107.5, 127.7, 251.1
α, β, γ (°)	90.0, 108.9, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Mosaicity (°)	0.52	0.25	0.56
Resolution range (Å)	50–2.2 (2.24–2.20)	50–2.85 (2.90–2.85)	50–2.7 (2.75–2.70)
Total No. of reflections	262163	300377	342869
No. of unique reflections	73782	40802	47677
Completeness (%)	99.5 (99.4)	99.9 (100.0)	99.5 (100.0)
Multiplicity	3.6 (3.4)	7.4 (7.2)	7.2 (7.1)
$\langle I/\sigma(I) \rangle$	9.2 (2.2)	12.1 (2.6)	11.2 (2.5)
$R_{\text{r.i.m.}}$	0.037 (0.339)	0.034 (0.314)	0.024 (0.302)
R_{meas}	0.071 (0.637)	0.093 (0.849)	0.066 (0.812)
Overall B factor from Wilson plot (Å ²)	24.7	60.9	40.4

Crystals of forms I, II and II' (Fig. 4) were obtained for the wild-type enzyme under different crystallization conditions: form I from 100 mM acetate pH 3.6, 54%(w/v) 2-methyl-2,4-

pentanediol, 200 mM magnesium chloride, 10 mM ammonium sulfate at 293 K, form II from 100 mM Tris–HCl pH 7.0, 12–18%(w/v) polyethylene glycol 3350, 200 mM sodium citrate

Figure 3
Dependence of StCGS enzymatic activity on (a) pH and (b) temperature observed for the wild-type enzyme (black bars) and the F97Y mutant enzyme (white bars). The estimated pH values are given in parentheses in (b). Each bar represents the mean $\pm$ standard deviation of triplicate measurements.

Figure 4
(a) Form I, (b) form II and (c) form II' crystals of StCGS prepared by the hanging-drop vapour-diffusion method.

Figure 5
The X-ray diffraction patterns of StCGS crystals in (a) form I, (b) form II and (c) form II' to resolutions of 2.20, 2.85 and 2.70 Å, respectively.

Figure 6
Current structural models of the active centres observed in (a) form I, (b) form II and (c) form II' crystals. Asterisks denote residues from an adjacent protomer in the asymmetric unit. Hydrogen bonds are shown by dashed lines. The images were drawn using *PyMOL* (v.1.8; Schrödinger).

and nickel(II) chloride hexahydrate at 293 K, and form II' using the same crystallization condition as for form II but at 313 K. Form I crystals diffracted X-rays to 2.2 Å resolution (Fig. 5a) and belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 58.4$, $b = 149.3$, $c = 90.2$ Å, whereas X-ray diffraction data to 2.9 and 2.7 Å resolution were collected for form II (space group $C222_1$; unit-cell parameters $a = 117.7$, $b = 117.8$, $c = 251.3$ Å) and form II' (space group $C222_1$; unit-cell parameters $a = 107.5$, $b = 127.7$, $c = 251.1$ Å) crystals, respectively. The statistics for the X-ray diffraction data collected for these crystal forms are summarized in Table 3.

Assuming that there are four protomers ($41.6 \text{ kDa} \times 4$) in the asymmetric unit, the Matthews probability coefficients and estimated solvent contents were $2.24 \text{ Å}^3 \text{ Da}^{-1}$ and 45.0%, $2.61 \text{ Å}^3 \text{ Da}^{-1}$ and 52.9%, and $2.59 \text{ Å}^3 \text{ Da}^{-1}$ and 52.5% for forms I, II and II', respectively. The structure of StCGS was solved by molecular replacement using *E. coli* CGS (PDB entry 1cs1; 35.1% amino-acid identity to StCGS; Clausen *et al.*, 1998) as the search model. The structures are currently refined to R_{cryst} and R_{free} values of 0.205 and 0.260 for form I, 0.217 and 0.288 for form II and 0.187 and 0.252 for form II',

respectively. The obtained structures show that in form I and II' crystals the PLP cofactor binds to the active site of StCGS in a similar manner, whereas there are differences in the orientation of Phe97 and other residues that interact with the cofactor (Figs. 6a and 6c). On the other hand, the orientation of the PLP cofactor in form II (Fig. 6b) is obviously different from that in forms I and II', suggesting that the high activity of StCGS observed at basic pH and elevated temperature is caused by changes in the protein structure. In order to reveal how these structural changes activate and deactivate StCGS, we are currently preparing crystals under different pH and temperature conditions.

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