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Overexpression, purification, crystallization and preliminary crystallographic studies of a hyperthermophilic adenylosuccinate synthetase from *Pyrococcus horikoshii* OT3

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Adenylosuccinate synthetase (AdSS) is a ubiquitous enzyme that catalyzes the first committed step in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) in the purine-biosynthetic pathway. Although AdSS from the vast majority of organisms is 430–457 amino acids in length, AdSS sequences isolated from thermophilic archaea are 90–120 amino acids shorter. In this study, crystallographic studies of a short AdSS sequence from *Pyrococcus horikoshii* OT3 (PhAdSS) were performed in order to reveal the unusual structure of AdSS from thermophilic archaea. Crystals of PhAdSS were obtained by the microbatch-under-oil method and X-ray diffraction data were collected to 2.50 Å resolution. The crystal belonged to the trigonal space group $P3_212$, with unit-cell parameters $a = b = 57.2$, $c = 107.9$ Å. There was one molecule per asymmetric unit, giving a Matthews coefficient of 2.17 Å^3 Da^{-1} and an approximate solvent content of 43%. In contrast, the results of native polyacrylamide gel electrophoresis and analytical ultracentrifugation showed that the recombinant PhAdSS formed a dimer in solution.

1. Introduction

Adenylosuccinate synthetase (AdSS; EC 6.3.4.4) is a ubiquitous enzyme that catalyzes the first committed step in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) in the purine-biosynthetic pathway (Lieberman, 1956; Fromm, 1958; Bass *et al.*, 1984; Poland *et al.*, 1997). AdSS sequences from viruses, archaea and bacteria, as well as from several eukaryotes from *Dictyostelium discoideum* to humans, have been determined. Although the sequences of AdSS from the vast majority of organisms are 430–457 amino acids in length, AdSS sequences that are 90–120 amino acids shorter have been isolated (Honzatko *et al.*, 1999) from thermotolerant archaea (Mehrotra & Balaram, 2007; Bouyoub *et al.*, 1996), *Vibrio* phage VP5 (NCBI reference sequence YP_024984.1), *Rhodospirillum rubrum* (NCBI reference sequence YP_427051.1) and *Streptomyces rimosus* (McCarty & Bandarian, 2008). Based on sequence length, these short AdSSs may be categorized into a subfamily.

Genomic studies have shown that AdSS from *Pyrococcus horikoshii* OT3 (PhAdSS) contains 339 residues (Cann *et al.*, 1998; Kawarabayasi *et al.*, 1998). The sequence of PhAdSS is 21% shorter than those of homologous enzymes isolated from organisms ranging from bacteria to humans. The crystal structures of AdSS from *Escherichia coli* (EcAdSS; Poland *et al.*, 1993; Silva *et al.*, 1995), *Plasmodium falciparum* (Eaazhisai *et al.*, 2004), *Arabidopsis thaliana* (Eaazhisai *et al.*, 2004), *Triticum aestivum* (Prade *et al.*, 2000) and mouse (MiAdSS; Iancu *et al.*, 2001) exhibit common structural features: these AdSSs contain a polypeptide chain of 430–457 residues that folds to form a central β-sheet that is composed of nine parallel strands and a tenth antiparallel β-strand. The crossover connections between the first nine strands of the central β-sheet are all right-handed, typical of the parallel β-sheet structure described previously by Richardson (1981). The shortening of PhAdSS corresponds to the loss of two α-helices and three β-strands that are

present in all other homologous enzymes from bacteria to mammals (Fig. 1). The sequence of PhAdSS also contains a significant number of substitutions in residues that are conserved in all other homologous AdSS sequences. The conserved residues have been described as essential for catalytic activity and/or for maintaining the folded structure of the homodimer (Bouyoub *et al.*, 1996).

Mehrotra and Balaram have reported the temperature-dependent catalytic activity, structural stability and unusual kinetic behaviour of a short AdSS (345 residues) isolated from *Methanocaldococcus jannaschii* (MjAdSS; Mehrotra & Balaram, 2007, 2008). MjAdSS shares 69% sequence identity with PhAdSS and more than 50% with other known short AdSS sequences (Fig. 1). The temperature-dependence of MjAdSS catalysis exhibited a biphasic Arrhenius plot with a transition at 313 K. Pre-steady-state kinetics as a function of temperature indicated a change in the rate-determining step of the reaction at the inflection point. The slow release of products from the active site of MjAdSS probably accounts for its thermophilicity. Thermal unfolding of MjAdSS occurred at T_m = 358 K, with the process being only partially reversible. Even at a urea concentration of 8 *M*, the secondary and tertiary structure of MjAdSS remained intact. Denaturation of MjAdSS induced by guanidinium chloride allowed the estimation of thermodynamic parameters. The unfolding profiles could be described as a composite of at least two distinct transitions, with a stable intermediate in the unfolding pathway. The results from initial velocity and product-inhibition studies of MjAdSS suggested a rapid equilibrium random AB steady-state ordered C kinetic mechanism for the MjAdSS-catalyzed reaction. At equilibrium, MjAdSS exists as a mixture of dimers and tetramers, with the tetramer being the catalytically active form. The tetramer dissociates into dimers when the ionic strength of the buffer is increased slightly, while the dimer is extremely stable and does not dissociate even in 1.2 *M* NaCl. This is in contrast to the homologue EcAdSS, which is

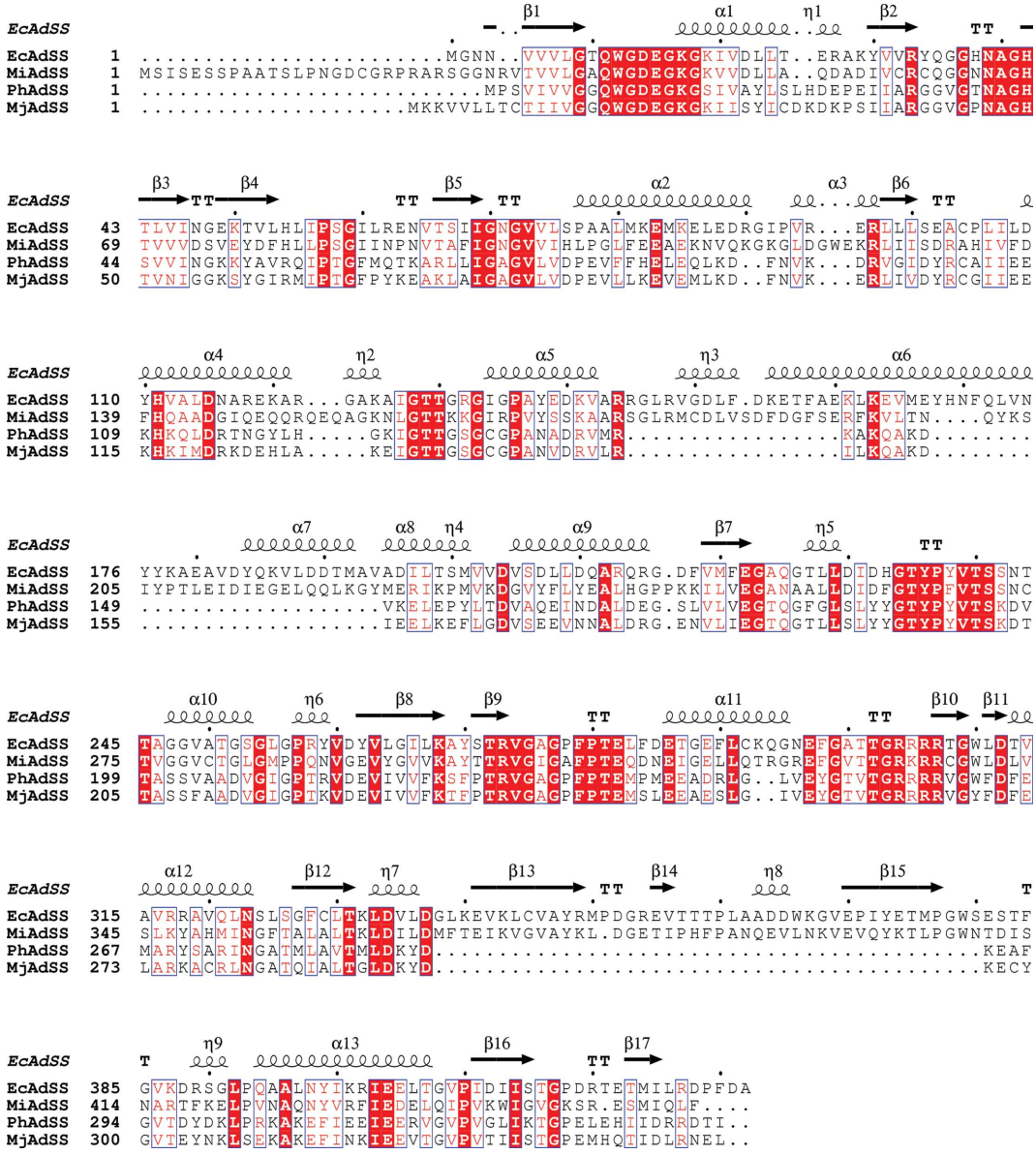

Figure 1
Alignment of the amino-acid sequences of EcAdSS, MiAdSS, PhAdSS and MjAdSS. Identical residues are shown as white letters on a red background, while similar residues are shown as red letters. The secondary-structural elements determined on the basis of the crystal structure of EcAdSS are indicated with arrows for β-strands and coils for α-helices at the top of the alignment. The alignment was made using the program *ClustalW* (Larkin *et al.*, 2007) and was coloured using the program *ESpript* (Gouet *et al.*, 1999).

known to exist in a monomer–dimer equilibrium with the dimer being implicated in catalysis (Wang *et al.*, 1997). Therefore, PhAdSS might show MjAdSS-like or other unusual functions. Determination of the crystal structure of PhAdSS will provide information towards understanding the relationship between the structure and function of hyperthermophilic AdSSs. In this paper, the results of crystallographic studies of PhAdSS are reported.

2. Experimental

2.1. Protein expression and purification

The gene encoding PhAdSS from *P. horikoshii* OT3 genomic DNA (NC_000961) was amplified by polymerase chain reaction. The forward-primer sequence 5′-GGAATTCCATA**TG**CCGAGCGTTA-**TAGTTGTAGG**-3′ contains an *Nde*I restriction site (underlined) and the initial N-terminal amino-acid codons (in bold). The reverse-primer sequence 5′-CGGGATCC*CTA***GATGGTATCCCTTCTATC-TATTATG** -3′ contains a *Bam*HI restriction site (underlined), a stop codon (in italics) and the last C-terminal amino-acid codons (in bold). The cDNA of PhAdSS was inserted into pET19b expression vector between the *Nde*I and *Bam*HI restriction sites (Novagen, USA). Recombinant PhAdSS with an N-terminal tag MG*HHHHHHHHHH-H*SSGHI**DDDDK**H containing a His tag (in italics) and an enterokinase cleavage site (bold) was produced in *E. coli* strain BL21 (DE3) Codon Plus RIL (Stratagene, USA). The *E. coli* cells were cultivated in LeMaster medium (LeMaster & Richards, 1985) at 310 K and 175 rev min^{-1}. When the optical density at 600 nm of the LeMaster medium reached 1.0, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 m*M* followed by growth at 289 K for 12 h to induce recombinant protein expression. The *E. coli* cells were harvested by centrifugation at 4000*g* for 20 min and the cell pellet was resuspended using 50 ml lysis buffer consisting of 20 m*M* Tris–HCl pH 8.0, 20 m*M* imidazole, 500 m*M* NaCl. After disruption of the cells by sonication, the lysate was heat-treated at 343 K for 10 min and then centrifuged to remove denatured proteins and cell debris. Purification of the His-tagged PhAdSS in the supernatant was performed at 277 K using an ÄKTAxpress automatic column chromatography system (GE Healthcare, USA). The supernatant was loaded onto a HisTrap column (5 ml) and the column was washed using 25 ml lysis buffer and 25 ml wash buffer consisting of 20 m*M* Tris–HCl pH 8.0, 100 m*M* imidazole, 500 m*M* NaCl to remove unbound proteins. The recombinant PhAdSS was eluted from the HisTrap column using 15 ml elution buffer consisting of 20 m*M* Tris–HCl pH 8.0, 200 m*M* imidazole, 500 m*M* NaCl. The solvent was exchanged to 50 m*M* Tris–HCl pH 8.0, 1 m*M* $CaCl_2$ using a HiPrep 26/10 desalting column. To cleave the N-terminal tag, 20 units of enterokinase were added and incubated at 310 K overnight. The product was purified using a HiLoad 16/60 Superdex 75 pg column equilibrated with a solution consisting of 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 2 m*M* dithiothreitol (DTT).

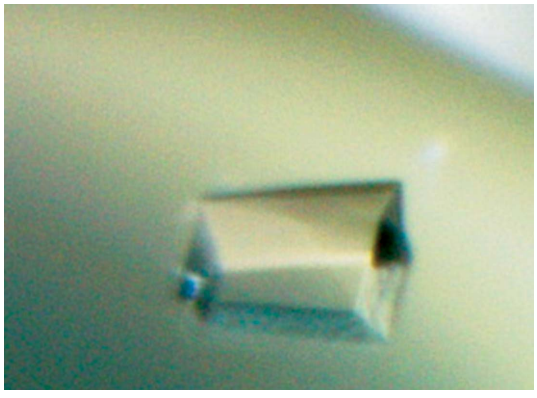

Figure 2
Typical crystal of *Ph*AdSS with approximate dimensions of 0.15 × 0.1 × 0.1 mm grown by the microbatch-under-oil method.

2.2. Crystallization

Purified PhAdSS was concentrated to approximately 9.3 mg ml^{-1} using an Amicon Ultra-15 filter unit (molecular-weight cutoff 10 000; Millipore, USA). The conditions required to crystallize PhAdSS were initially screened using the sitting-drop vapour-diffusion method with Crystal Screen, Crystal Screen 2 and Index screens (Hampton Research, USA) at 293 K. Each sitting drop consisted of 1.0 µl PhAdSS solution and 1.0 µl reservoir solution. Crystallites were obtained using condition Nos. 27 [100 m*M* MES pH 6.5, 20%(*v*/*v*) PEG 20 000] and 48 (10% PEG 20 000, 0.1 *M* Bicine pH 9.0, 2% dioxane) of Crystal Screen 2 within 3 d. Crystal-growth optimization was performed using a TERA automatic crystallization system with the microbatch-under-oil method (Sugahara & Miyano, 2002). Diffraction-quality crystals were obtained at 291 K from a drop consisting of 0.5 µl protein solution and 0.5 µl reservoir solution (13.5% PEG 20 000, 100 m*M* Tris pH 8.7) within 7 d (Fig. 2).

2.3. X-ray diffraction data collection

X-ray diffraction data were collected from the crystal on Riken beamline BL26B2 at SPring-8, Harima, Hyogo, Japan. Prior to data collection, the crystal was treated with a cryoprotectant solution consisting of 66.6%(*v*/*v*) Paratone-N, 28.6%(*v*/*v*) paraffin and 4.8%(*v*/*v*) glycerol to remove the reservoir solution. The crystal of PhAdSS was mounted in a nylon loop under a stream of nitrogen gas at 100 K. Diffraction patterns were recorded on a Jupiter CCD. The wavelength, camera distance, exposure time, oscillation range and temperature used were 1.000 Å, 250 mm, 14 s, 0.5° and 100 K, respectively. One data set was collected consisting of 440 images covering 220° in total. X-ray diffraction data were processed and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

2.4. Estimation of molecular weight by native PAGE and analytical ultracentrifugation

Polyacrylamide gel electrophoresis under native conditions (native PAGE) was performed according to the protocol of McLellan (1982) with some modifications. The gel solution consisted of 0.5 *M* Tris–HCl pH 8.8, 10% acrylamide (Bio-Rad). The running buffer was composed of 0.192 *M* glycine and 0.025 *M* Tris–HCl pH 8.3. 5 µl recombinant PhAdSS (with the N-terminal tag) solution (0.4 mg ml^{-1}) was mixed with 5 µl native dye solution consisting of Tris–HCl pH 6.8, 10% glycerol and BPB, and electrophoresis was performed at 40 mA constant current for 1.5 h at 277 K.

Analytical ultracentrifugation was performed in 50 m*M* Tris–HCl pH 7.8, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol at 293 K using a Beckman Optima XL-1 analytical ultracentrifuge. The sedimentation-velocity experiments were carried out with loading concentrations of 1.86 and 1.02 mg ml^{-1} PhAdSS at 2 000 000*g*. The data were analyzed with the program *SEDFIT*. The sedimentation-equilibrium experiments were carried out with loading concentrations of 0.98, 0.45 and 0.23 mg ml^{-1} PhAdSS and the sedimentation reached equilibrium in 14 h at 6000, 7000 and 8000 rev min^{-1}. The

Table 1
Crystal parameters and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P3_212$
Unit-cell parameters (Å)	$a = b = 57.2$, $c = 107.9$
Resolution range (Å)	47.58–2.50 (2.66–2.50)
No. of measurements	21852
No. of unique reflections	11262 (1750)
Completeness (%)	98.7 (97.5)
V_M (Å^3 Da^{-1})	2.17
Solvent content (%)	43
$\langle I/\sigma(I)\rangle$	38.3 (6.2)
R_{merge} (%)†	6.4 (18.4)
Average multiplicity	10.3 (7.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the value of the ith measurement of the intensity of reflection hkl, $\langle I(hkl)\rangle$ is the average integrated intensity obtained from multiple measurements and the summation is over all observed reflections.

equilibrium data were analyzed using *Origin* v.6 (Beckman Coulter Co.). The partial specific volume was 0.7337, which was calculated from the amino-acid composition using the *SEDNTERP* program (http://www.jphilo.mailway.com/).

3. Results

The crystal of PhAdSS belonged to the trigonal space group $P3_112$ (or $P3_212$), with unit-cell parameters $a = b = 57.2$, $c = 107.9$ Å. There is a single molecule in the asymmetric unit, giving a Matthews coefficient of 2.17 Å^3 Da^{-1} and a solvent content of 43%. The structure has been solved by molecular replacement using the crystal structure of the EcAdSS–hydantocidin 5′-monophosphate complex (PDB entry 1soo; Fonné-Pfister *et al.*, 1996) as the search model and the coordinates have been deposited in the PDB (PDB entry 2d7u). Molecular replacement allowed us to identify the space group as $P3_212$. Diffraction data statistics are presented in Table 1.

As previously reported, *E. coli*, *P. falciparum* and yeast AdSSs exhibit a monomer–dimer equilibrium in solution (Borza *et al.*, 2003; Jayalakshmi *et al.*, 2002; Lipps & Krauss, 1999), while MiAdSS predominantly forms dimers (Iancu *et al.*, 2001). The calculated molecular weight of PhAdSS with an N-terminal tag is 39 998.9 Da. The main band shown by native PAGE is estimated to correspond to approximately 80 kDa and is predicted to be a dimer (Fig. 3). The other very weak band corresponds to about 160 kDa, which would correspond to a tetramer. This result suggests that PhAdSS mainly exists as a dimer in solution. For further confirmation, sedimentation-velocity and sedimentation-equilibrium experiments were carried out by analytical ultracentrifugation. The sedimentation-velocity analysis gave a single sedimentation coefficient of 5.1 S corresponding to the dimer. The sedimentation-equilibrium experiments revealed that the molecular weight was 85.2 kDa. Presumptive dimer–tetramer equilibrium gave an equilibrium constant of $2.1 \times 10^3\ M^{-1}$ and a dimer molecular weight of 80.4 kDa, suggesting that PhAdSS forms a dimer in solution. These results are in good agreement with the native PAGE data (Fig. 3). The crystallographic dimer present in our crystal is structurally equivalent to the dimer observed for MiAdSS and is likely to correspond to the physiological dimer.

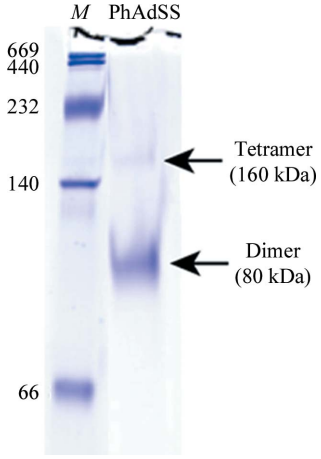

Figure 3
Estimation of molecular weight in solution by native PAGE. Lane *M* contains molecular-weight standards (High Molecular Weight Calibration Kit for native electrophoresis from GE Healthcare). The molecular weight of each band is labelled in kDa on the left. The arrows indicate the two forms of recombinant PhAdSS with an N-terminal tag.

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