Overproduction, crystallization and preliminary X-ray diffraction analysis of probable ATP sulfurylase from *Thermus thermophilus* HB8

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ATP sulfurylase catalyzes the reaction of inorganic sulfate with ATP, producing adenosine-5′-phosphosulfate (APS) and pyrophosphate. A probable ATP sulfurylase (MW = 38.8 kDa) from *Thermus thermophilus* HB8 was overproduced in *Escherichia coli* and purified. It was crystallized in the presence of 5 mM APS by the batch method. The crystal was monoclinic, space group $P2_1$, with unit-cell parameters $a = 68.8$, $b = 61.2$, $c = 128.6$ Å, $\beta = 95.4°$. Diffraction to better than 2.5 Å resolution was obtained using synchrotron radiation at SPring-8. The asymmetric unit most probably contains two subunits ($V_M = 3.48$ Å^3 Da^{-1}).

1. Introduction

ATP sulfurylases (ATPSs) are ubiquitous enzymes that catalyze the transfer of the adenylyl group from ATP to inorganic sulfate, yielding adenosine-5′-phosphosulfate (APS) and pyrophosphate (PP$_i$),

$$MgATP + SO_4^{2-} \rightarrow MgPP_i + APS.$$
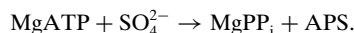
The APS produced has a high-energy mixed phosphoric–sulfuric acid anhydride bond that is used for sulfate activation and reduction in the cell. ATPS is important in the biosynthesis of methionine and cysteine and in the activation and inactivation of biological materials (Marzluf, 1997; Coughtrie *et al.*, 1998).

Crystal structures of the ATPSs from the yeast *Saccharomyces cerevisiae* (Ullrich *et al.*, 2001; Ullrich & Huber, 2001), *Penicillium chrysogenum* (MacRae *et al.*, 2001, 2002) and *Riftia pachyptila* symbiont (Beynon *et al.*, 2001) have been reported. *S. cerevisiae* and *P. chrysogenum* ATPSs form hexamers and each subunit has N-terminal, catalytic and C-terminal domains. In contrast, *R. pachyprila* symbiont ATPS, which lacks the C-terminal domain present in the yeast and fungal ATPSs, forms a dimer. The crystal structures of these ATPSs and their complexes with various substrate analogues and inhibitors have provided a wealth of information about the stereospecificity and regulation mechanisms of the enzyme reactions. The C-terminal domain of *P. chrysogenum* ATPS may function in the allosteric reaction of the enzyme (MacRae *et al.*, 2001, 2002).

In the genome of the Gram-negative eubacterium *Thermus thermophilus* HB8 (Yokoyama *et al.*, 2000), an ORF (DDBJ/EMBL/GenBank accession No. AB090277-1; project code 0343) was annotated as coding ATPS because its amino-acid sequence has identity with the sequences of *S. cerevisiae* ATPS (35%), *P. chrysogenum* ATPS (35%) and *R. pachyprila* symbiont ATPS (30%), as well as having conserved residues in the catalytic domain that are involved in catalysis. The probable ATPS from *T. thermophilus* HB8 (ttATPS) has 348 amino-acid residues and its sequence shows that it lacks the C-terminal domain as does the *R. pachyprila* symbiont ATPS. To clarify the mechanisms of the substrate-binding and enzyme action of ttATPS, we performed crystallographic analysis of the enzyme in the presence of APS. We report the crystallization and preliminary X-ray analysis of the ttATPS–APS complex.

2. Materials and methods

2.1. Cloning, sequencing and overproduction

PCR primers were synthesized based on the DNA sequence of the ttATPS gene. The primer sequences were: 5′-primer, 5′-ATAT-catatgGTAGAGACCCTTCCCGCTTTGGA-GATCG-3′ (*Nde*I site in lower case) and 3′-primer, 5′-ATATagatctTTATTAGACCCCCC-GCCGGAGGATAGGCAAA-3′ (*Bgl*II site in lower case). The ttATPS gene amplified by PCR was subcloned into the plasmid pT7Blue. After confirmation of the nucleotide sequence, the ttATPS gene was ligated into the *Nde*I and *Bam*HI sites of pET-11a. *Escherichia coli* BL21(DE3) cells carrying the resulting expression plasmid were grown at 310 K overnight in medium containing 1.0% polypeptone, 0.5% yeast extract and 0.5% NaCl (adjusted to pH 7.0 with NaOH) supplemented with 50 µg ml^{-1} ampicillin. Cells were harvested by centrifugation and stored at 193 K.

2.2. Purification

Frozen cells were thawed, suspended in 20 mM Tris–HCl buffer pH 8.0 containing 50 mM NaCl and 5 mM 2-mercaptoethanol and disrupted by sonication. The lysate was incubated at 343 K for 10 min, kept on ice for 12 min and then ultracentrifuged (200 000g) for 60 min at 277 K. The following procedures, in which Amersham Bioscience columns were used, were performed at room temperature. Ammonium sulfate (AS) was added to the resulting supernatant to 35% saturation. The solution was applied to a Resource ISO column equilibrated with 50 mM sodium phosphate buffer pH 7.0 containing 35% saturated AS. Proteins were eluted with a linear AS gradient of 35–0% saturation in 50 mM sodium phosphate buffer pH 7.0. Fractions containing ttATPS were desalted by running them through a HiPrep 26/10 desalting column equilibrated with 20 mM MES buffer pH 6.0, after which they were run through a Resource S column equilibrated with 20 mM MES buffer pH 6.0, to which ttATPS was not bound. The flowthrough fractions were adjusted to pH 8.0 with 1 M Tris and run through a Resource Q column equilibrated with 20 mM Tris–HCl buffer pH 8.0, to which ttATPS was not absorbed. The flowthrough fractions were concentrated by ultrafiltration then run through a HiLoad 16/60 Superdex 75pg column equilibrated with 20 mM Tris–HCl pH 8.0 containing 150 mM NaCl. The ttATPS fractions obtained were stored at 277 K. The N-terminal sequence of the purified protein was determined using a HP G1000A sequencer.

2.3. Circular-dichroism (CD) measurements

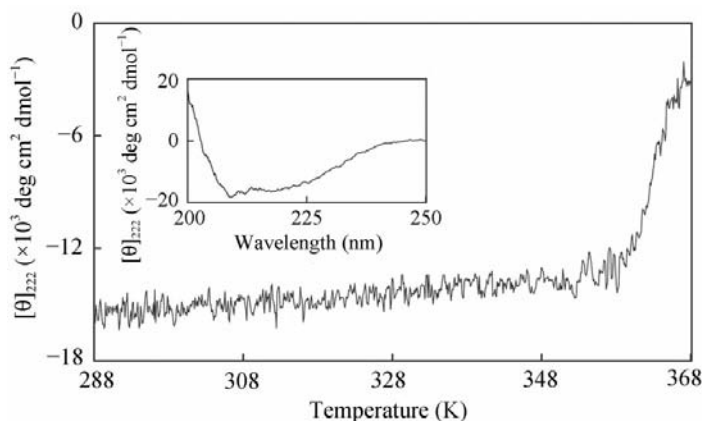CD spectra of ttATPS were measured in a 1 mm cell with a Jasco spectropolarimeter, model J-720W. The solution contained 1.8 µM ttATPS and 20 mM Tris–HCl pH 8.0.

2.4. Size-exclusion chromatography

Size-exclusion chromatography was performed using a Superdex 75pg column (Amersham Bioscience). The sample was dissolved in 20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl and then loaded onto a column equilibrated with the same buffer. The molecular size of ttATPS was estimated using a Molecular-Weight Marker Kit (Sigma).

2.5. Crystallization

Crystallization conditions were surveyed using the hanging-drop vapour-diffusion method with Crystal Screen Kits (Hampton Research) at 293 K. The protein concentration was 5 mg ml^{-1} in a solution containing 20 mM Tris–HCl pH 8.0 and 5 mM APS. Drops prepared by mixing 1 µl of the protein solution with 1 µl of the reservoir solution were equilibrated against the reservoir solution. Small crystals of ttATPS were obtained in the initial screening. Improvement of crystallization was attempted by the batch method. Final conditions were 5% PEG 6000, 0.1 M MES pH 6.0 and 5 mM APS. Microseeding was needed to grow crystals to a size suitable for X-ray diffraction measurement.

2.6. X-ray diffraction analysis

Crystals were soaked for a few minutes in solution containing 0.1 M MES and 20 mM Tris pH 6.0, 15% PEG 6000, 5 mM APS and 25% ethylene glycol, which served as a cryoprotectant. The crystal mounted on a cryoloop was flash-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected using a MAR CCD system and

Table 1
Crystal data and results of diffraction measurements for ttATPS.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 68.8$, $b = 61.2$, $c = 128.6$, $\beta = 95.4$
Resolution range (Å)	20.0–2.5
No. measured reflections	207915
No. unique reflections	37661
Redundancy	5.5
R_{sym}† (%)	6.1 (20.5)
Completeness (%)	99.7 (97.1)
Mean $I/\sigma(I)$	24.9 (8.0)

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the intensity of the ith observation and $\langle I(h) \rangle$ is the mean intensity of equivalent reflections.

synchrotron radiation ($\lambda = 1.295$ Å) at beamline BL44B2, SPring-8. The oscillation angle was 1.5° and the exposure time was 40 s per frame. A total of 180 diffraction images were recorded at a camera distance of 140 mm and were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993).

Figure 2
Crystal of ttATPS. Scale bar: 0.2 mm.

Figure 1
Temperature dependence of the molar ellipticity at 222 nm ($[\theta]_{222}$). The heating rate was 1 K min^{-1}. Inset: the CD spectrum of ttATPS.
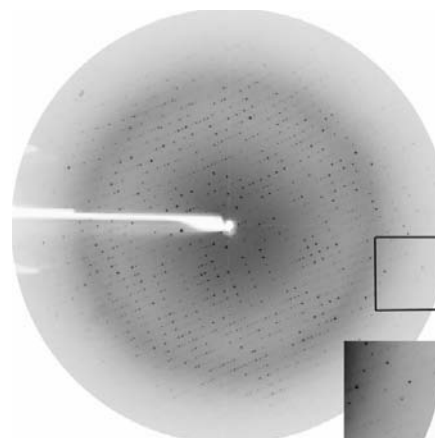
Figure 3
Diffraction pattern of the ttATPS crystal. The frame edge is at 2.5 Å resolution.

3. Results and discussion

ttATPS was purified to homogeneity as judged by SDS–PAGE. Ten amino-acid residues from its N-terminus were sequenced as VETLPALEIG. This confirms that the protein purified is indeed ttATPS and that the first methionine is missing. Its thermostability was examined by the residue molar ellipticity at 222 nm ($[\theta]_{222}$) (Fig. 1). ttATPS was stable up to 353 K, indicating high thermostability.

The molecular weight of ttATPS in solution was estimated by size-exclusion chromatography. The elution profile of ttATPS had a peak corresponding to 55.5 kDa, which is between the values for a monomer and a dimer. Therefore, it does not give a clear indication of the oligomeric state of ttATPS in solution.

ttATPS produced plate-shaped crystals with typical dimensions of about $0.7 \times 0.7 \times 0.03$ mm (Fig. 2). These crystals belong to the monoclinic system and space group $P2_1$ and have unit-cell parameters a = 68.8, b = 61.2, c = 128.6 Å, β = 95.4°. The crystal diffracts X-rays to at least 2.5 Å resolution (Fig. 3). When the asymmetric unit contains two ttATPS subunits, the V_M value is 3.48 Å^3 Da^{-1} (Matthews, 1968). Results of the X-ray intensity measurements are given in Table 1. The present crystals are suitable for X-ray analysis. We now are determining the structure by the MAD method with selenium as the anomalous scattering atom using synchrotron radiation.

References

Beynon, J. D., MacRae, I. J., Huston, S. L., Nelson, D. C., Segel, I. H. & Fisher, A. J. (2001). *Biochemistry*, **40**, 14509–14517.

Coughtrie, M. W., Sharp, S., Maxwell, K. & Innes, N. P. (1998). *Chem. Biol. Interact.* **109**, 3–27.

MacRae, I. J., Segel, I. H. & Fisher, A. J. (2001). *Biochemistry*, **40**, 6795–6804.

MacRae, I. J., Segel, I. H. & Fisher, A. J. (2002). *Nature Struct. Biol.* **9**, 945–949.

Marzluf, G. A. (1997). *Annu. Rev. Microbiol.* **51**, 73–96.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 55–62. Warrington: Darsbury Laboratory.

Ullrich, T. C., Blaesse, M. & Huber, R. (2001). *EMBO J.* **20**, 316–329.

Ullrich, T. C. & Huber, R. (2001). *J. Mol. Biol.* **313**, 1117–1125.

Yokoyama, S., Hirota, H., Kigawa, T., Yabuki, Y., Shirouzu, M., Tereda, T., Ito, Y., Matsuo, Y., Kuroda, Y., Nishimura, Y., Kyogoku, Y., Miki, K., Masui, R. & Kuramitsu, S. (2000). *Nature Struct. Biol.* **7**(*Suppl.*), 943–945.