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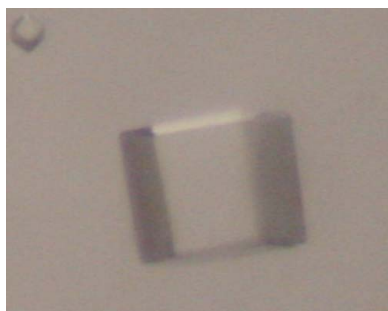
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Purification, crystallization and preliminary X-ray analysis of the regulatory subunit of aspartate kinase from *Thermus thermophilus*

Aspartate kinase (AK) from *Thermus thermophilus*, which catalyzes the first step of threonine and methionine biosynthesis, is regulated *via* feedback inhibition by the end product threonine. To elucidate the mechanism of regulation of AK, the regulatory subunit (the β subunit of *T. thermophilus* AK) was crystallized in the presence of the inhibitor threonine. Diffraction data were collected to 2.15 Å at a synchrotron source. The crystal belongs to the cubic space group $P4_332$ or $P4_132$, with unit-cell parameters $a = b = c = 141.8$ Å.

1. Introduction

Aspartate kinase (AK) catalyzes the phosphorylation of aspartic acid, the first step in the biosynthesis of the aspartic amino-acid family lysine, threonine and methionine. Similar to other enzymes involved in the first steps of amino-acid biosynthesis, AK is regulated through feedback inhibition by the end products. AK from *Thermus thermophilus* AT-62 (formerly named *T. flavus* AT-62) is inhibited by threonine (Nishiyama *et al.*, 1995), while AK from *Corynebacterium glutamicum* is inhibited by lysine and threonine in a concerted manner (Shiio & Miyajima, 1969). AK is also important for industrial production of the amino acids belonging to the aspartic amino-acid family (Tosaka *et al.*, 1983; Jetten & Sinskey, 1995), which are essential for mammals. In addition to these essential amino acids, diaminopimelic acid, an intermediate in lysine biosynthesis, is a key compound that is necessary for cell-wall synthesis in most bacteria. Therefore, an enzyme that is involved in diaminopimelic acid biosynthesis may serve as a target for the design of antimicrobial agents, especially for pathogenic bacteria (Patte, 1996; Girodeau *et al.*, 1986). Thus, AK is an attractive target for both scientific understanding and industrial applications. AK from *T. thermophilus* is composed of two subunits, α and β , which are encoded by an in-frame overlapping gene (Nishiyama *et al.*, 1995) as in *C. glutamicum* AK and *Bacillus subtilis* AK II to form an $\alpha_2\beta_2$ tetramer (Kalinowski *et al.*, 1991; Chen *et al.*, 1987). The β subunit is identical to about 160 amino acids of the C-terminus of the α subunit. In this $\alpha_2\beta_2$ -type AK, the N-terminal regions of the α subunit serve as catalytic domains and the C-terminal region of the α subunit and the β subunit function as regulatory domains (Kobashi *et al.*, 1999; Marco-Marín *et al.*, 2003; Kato *et al.*, 2004). Aravind and Koonin discovered a motif that is conserved in many allosteric enzymes involved in amino-acid and purine biosynthesis and named the motif the 'ACT domain' as an acronym for aspartate kinase, chorismate mutase and TyrA (prephenate dehydrogenase; Aravind & Koonin, 1999). The motif was expected to serve as a small-molecule-binding domain for catalytic regulation, forming a β - α - β - α - β fold (Schuller *et al.*, 1995). The crystal structures of several homo-oligomeric AKs have recently been reported (Mas-Droux *et al.*, 2006; Kotaka *et al.*, 2006; Faehnle *et al.*, 2006). In contrast to these AKs, which have dimeric quaternary structures in which two ACT domains from each subunit interact with each other to form a dimer, AK from *T. thermophilus* has a different $\alpha_2\beta_2$ subunit organization in which a single β subunit of $\alpha_2\beta_2$ -type AK contains two ACT-domain motifs. The amino-acid sequence identity of the β subunit of *T. thermophilus* AK is 20%, 17% and 27% to the ACT domain-containing portions of aspartate kinase I from *Arabi-*



dopsis thaliana, aspartate kinase III from *Escherichia coli* and aspartate kinase from *Methanococcus jannashii*, respectively. This suggests that it has a different mechanism for subunit assembly and regulation. To elucidate the regulatory mechanism, we crystallized the regulatory subunit (the β subunit) of *T. thermophilus* AK.

2. Experimental

2.1. Protein expression and purification

The *askB* gene (accession No. D37928) encoding the β subunit of *T. thermophilus* AK was amplified by polymerase chain reaction using the oligonucleotides 3'-GGGAATTCTCAAGGAGGTGTCATATGGAGATGGACAAGGCG-5' and 3'-CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGCCTTGCCAGCTC-5'. The amplified DNA fragment designed to direct the production of the full-length β subunit (Met1-Ala161) with a His₆-tag extension at the C-terminal end was cloned into the *EcoRI/HindIII* site of pBlue-scriptII SK(+). The calculated theoretical molecular weight of the protein is 17 717 Da. After verifying the nucleotide sequence, the DNA fragment was cloned into the *NdeI/HindIII* site of pET26b(+) and introduced into *E. coli* BL21-CodonPlus(DE3)-RIL cells. The cells were grown in 2×YT broth in the presence of kanamycin (50 $\mu\text{g ml}^{-1}$) and chloramphenicol (30 $\mu\text{g ml}^{-1}$) at 303 K. When the optical density at 600 nm of the culture reached about 0.6, gene expression was induced by adding 0.1 mM isopropyl β -D-thiogalactopyranoside and the culture was continued for an additional 12–14 h. The cells were harvested and washed in buffer A (20 mM Tris-HCl pH 7.5) and suspended in buffer B (20 mM Tris-HCl pH 7.5, 150 mM NaCl). Suspended cells were disrupted by sonication and centrifuged at 40 000g for 25 min. The supernatant was applied onto a Ni-resin column (Clontech) equilibrated with buffer B supplemented with 20 mM imidazole. After washing with buffer B containing 20 mM imidazole and successive washing with buffer B containing 50 mM imidazole, the proteins bound to the resin were eluted with buffer B containing 200 mM imidazole and then with buffer B containing 500 mM imidazole. The flow rate and fraction volume for the nickel-affinity chromatography were 1 ml min⁻¹ and 1 ml, respectively. Fractions containing the His₆-tagged β subunit were mixed and concentrated to about 40 mg ml⁻¹ using Vivaspinn-20 centrifugal filtration with a 10 kDa cutoff (Vivascience). The concentrated sample was applied onto a HiLoad 26/60 Superdex 75 gel-filtration FPLC column (Amersham Bioscience) equilibrated

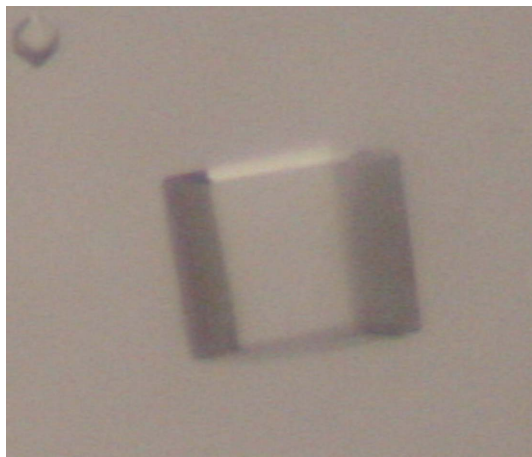


Figure 1
Cubic crystal of the β subunit of *T. thermophilus* AK.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	NW12A
Space group	<i>P</i> ₄ ₃ ₂ or <i>P</i> ₄ ₃ ₂
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = <i>c</i> = 141.8
Resolution (Å)	2.15 (2.15–2.19)
Total reflections	578149
Unique reflections	27145
<i>R</i> _{merge} † (%)	6.7
<i>I</i> /σ(<i>I</i>)	59.9 (11.1)
Completeness	100.0 (100.0)

$$\dagger R_{\text{merge}} = \frac{\sum |I_i - \langle I \rangle|}{\sum \langle I \rangle}$$

with buffer B and fractions were collected every 2 min using a flow rate of 2.5 ml min⁻¹. Based on the elution volume from the size-exclusion column, the oligomeric state of the protein was estimated to be a monomer. The >95% homogeneity of the purified β subunit was verified by SDS-PAGE. Over 60 mg of the β subunit of *T. thermophilus* AK with a His₆ tag was purified from 1 l culture and was used for crystallization without removal of the tag.

2.2. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method using Crystal Screen kits (Hampton Research). 2 μl drops consisting of 1 μl reservoir solution and 1 μl 10 mg ml⁻¹ β -subunit solution with or without 5 mM threonine were equilibrated against 500 μl reservoir solution at 293 K. A few crystals were obtained from threonine-containing droplets using solution No. 9 (0.1 M sodium acetate trihydrate pH 4.6, 2.0 M sodium chloride) from Crystal Screen II. Crystals with dimensions of 0.30 × 0.30 × 0.30 mm formed in 0.1 M sodium acetate trihydrate pH 4.6 and 1.2–2.0 M sodium chloride (Fig. 1) were used for X-ray diffraction.

2.3. Data collection and processing

Prior to data collection, crystals were briefly soaked in a cryo-protectant solution containing the same concentrations of sodium acetate and sodium chloride as in the crystallization condition and 25% (v/v) glycerol, flash-cooled in a nitrogen-gas stream at 95 K and stored in liquid nitrogen. Diffraction data ($\lambda = 1.000 \text{ \AA}$) were collected using a charge-coupled device (CCD) camera (ADSC Quantum 210) with a crystal-to-detector distance of 189.60 mm, an oscillation angle of 0.5°, a total of 180 images and an exposure time of 2.5 s at the NW12A station of the Photon Factory AR, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan. Diffraction images were indexed, integrated and scaled using the *HKL-2000* program suite (Otwinowski & Minor, 1997). The crystal belongs to the cubic space group *P*₄₃₂ or *P*₄₃₂, with unit-cell parameters *a* = *b* = *c* = 141.8 Å. Assuming the presence of two monomers of 18 kDa protein in the asymmetric unit, the calculated Matthews coefficient (*V*_M) is 3.3 Å³ Da⁻¹, with a solvent content of 63.0%. A complete data set has been obtained to 2.15 Å, corresponding to an *R*_{merge} of 6.7%. Details of the data-collection statistics are summarized in Table 1. We are now in the process of attempting to use the multiwavelength anomalous diffraction method to solve the three-dimensional structure of the β subunit of *T. thermophilus* AK. Solving this structure will provide the first structure of the regulatory subunit of an $\alpha_2\beta_2$ -type aspartate kinase.

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