

Crystallographic analysis of Aspartate kinase from *Thermus flavus*

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Introduction

Aspartate kinase (AK) catalyses the phosphorylation of aspartic acid, which is the first step of the biosynthesis of aspartic amino acid family, lysine, threonine, and methionine. AK is regulated via feedback inhibition by those end products. AK from *Thermus flavus* is inhibited by threonine [1], while AK from *Corynebacterium glutamicum* is concertedly inhibited by lysine and threonine [2]. However, the mechanism of regulation of AKs is unclear because structural information is unavailable. On the other hand, AK is an important target for the industrial biosynthesis of amino acids, because the amino acids produced from these pathways following the catalytic reaction of AK are essential for mammals. Therefore, these AKs are significant subjects for both scientific and application, however, regulatory and catalytic mechanisms have not been elucidated because no structural information is available.

AK from *T. flavus* is composed of two subunits, a and b, which are encoded by an in-frame overlapping gene [1] as in *C. glutamicum* AK and *B. subtilis* AK II and has $\alpha_2\beta_2$ type structure. β subunit is identical to around 160 amino acids of C-terminus of a subunit. In our previous studies, in this $\alpha_2\beta_2$ type AK, C-terminal region of α -subunit and b subunit were found to be regulatory domain, and N-terminus region of a subunit is catalytic domain from chimeric AK experiment [2]. To elucidate the mechanism of regulation of AK including threonine binding site and the actions for catalytic domain, we conducted crystallographic analysis of aspartate kinase from *T. flavus*.

Results and Discussion

The gene of *T. flavus* AK b subunit was obtained by PCR amplification and it has (His)₆-tag at the C-terminal end. Amplified DNA fragment was once cloned into pT7Blue-T-vector to confirm the nucleotide sequence by using DSQ-2000L automatic sequencer (Shimadzu Co., Kyoto, Japan). Checking the nucleotide sequence, the DNA fragment was introduced into the expression vector pET-26b(+). The construct was transformed into BL21-Codon-Plus (DE3)-RIL competent cells. These cells were grown in 2x YT broth in the presence of kanamycin (50 mg/ml) and chloramphenicol (30 mg/ml) at 303 K. When the O.D. 600 reached about 0.6, protein expression was induced by adding 0.1 mM isopropyl β -D-thiogalactopyranoside and the culture was continued for

additional 12-14 h. The cells were harvested by the centrifugation and suspended in buffer A (20 mM Tris-HCl (pH7.5)) and washed with buffer B (20 mM Tris-HCl (pH7.5), 150 mM NaCl). Suspended cells were disrupted by sonication and the crude extract was clarified by centrifugation (17000 rpm, 25 min). The supernatant was mixed with Ni-resin equilibrated with buffer B containing 20 mM Imidazole. The resin was washed with the buffer B containing 20 mM and 50 mM Imidazole in sequence, and proteins bound to the resin were eluted with the buffer B containing 200 mM and 500 mM Imidazole. After concentration with VIVASPIN20, the sample was loaded to gel filtration FPLC column, HiLoad 26/60 Superdex 75 (Amersham Pharmacia Bioscience). The homogeneity of the purified protein was assessed by SDS-PAGE. Protein concentration was determined by the method of Bradford using a protein assay kit (Nippon Bio-Rad). Final recovery of purified b subunit of *T. flavus* AK was over 60 mg protein per litre of starting culture. Screening of crystallization conditions are now underway.

References

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