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Crystallization of *Escherichia coli* AHAS Ⅰ Regulatory Subunit IlvN and Co-crystallization IlvN With a Valine Effector*

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Abstract Acetohydroxyacid synthase (AHAS) as the first common enzyme in the brunched-chain amino acids biosynthesis is the target of several classes of commercial herbicide. AHAS is normally composed of a larger catalytic subunit with FAD (flavin adenine dinucleotide), ThDP (thiamine diphosphate) and Mg^{2+} as cofactors and a smaller regulatory subunit bound to the end-product feedback signals such as valine, leucine or isoleucine to down regulate the holoenzyme activities. The *Escherichia coli ilv*N gene that encodes the regulatory subunit of AHAS Ⅰ was cloned into pET28a and expressed in soluble form at high levels in *E. coli* strain BL21 (DE3). The protein was purified using Ni^{2+}-chelating chromatography followed by size-exclusion chromatography. Crystals of IlvN protein were obtained and diffracted to 2.6Å. To further study the regulatory mechanism, crystals of IlvN co-crystallized with its effector valine were also obtained and diffracted to 3.0Å.

Key words acetohydroxyacid synthase (AHAS), ACT domain, crystallization
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Branched-chain amino acids (BCAAs; valine, leucine and isoleucine) biosynthetic pathway exists in bacteria, archaea, fungi, algae and plants, but not in animals, therefore, the BCAAs are all essential amino acids for human nutrition. Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) is the first common enzyme in this pathway. AHAS catalyzes the synthesis of 2-acetolactate from two molecules of pyruvate, and also the synthesis of 2-aceto-2-hydroxybutyrate from one molecule of pyruvate and one molecule of 2-ketobutyrate. Almost all the AHASes from different species (except archaea) contain two subunits, a larger catalytic and a smaller regulatory subunits. The catalytic subunit binds to FAD (flavin adenine dinucleotide), ThDP (thiamine diphosphate) and Mg^{2+} as essential cofactors; whereas the regulatory subunit interacts with the end-product feedback signals (valine, leucine or isoleucine) and down regulates the activity of the holoenzyme. The characteristics of different AHASes have been intensively studied [1] and the crystal structures of the catalytic subunits of *Saccharomyces cerevisiae* and *Arabidopsis thaliana* in complex with cofactors and different inhibitors have been published[2-6]. Furthermore, the regulatory subunit of AHAS Ⅲ without ligand bound in *Escherichia coli* (IlvH) became the first AHAS regulatory subunit with structure solved[7].

The structure of the N terminal of IlvH displayed a typical ACT domain fold[7]. The ACT domain was first defined by Arvind and Koonin who proposed this domain based on PSI-BLAST using the regulatory subunit of AHAS Ⅰ (IlvN) to search protein sequence databases[8], and the ACT domain function was defined as to control metabolism by binding small signal molecules, particularly in amino acids metabolic

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pathways[9]. IlvN has about half the molecular mass of IlvH, but with full function. Most bacteria contain only one AHAS enzyme most similar to *E. coli* AHAS Ⅲ. The sequence alignment of different regulatory subunits from several bacteria was presented in Figure 1, with the structural mask from IlvH crystal structure overlaid. The ACT domain is a widespread protein domain to sense signals from small molecules in general, as a minimal stand-alone ACT domain, the structure of IlvN with and without the bound effector molecule is of great interests to look into, and to understand the detailed mechanism of the AHAS holoenzyme has been an ultimate goal in this field, therefore, it is important to understand the structural changes of the regulatory subunit caused by the binding of effector ligands.

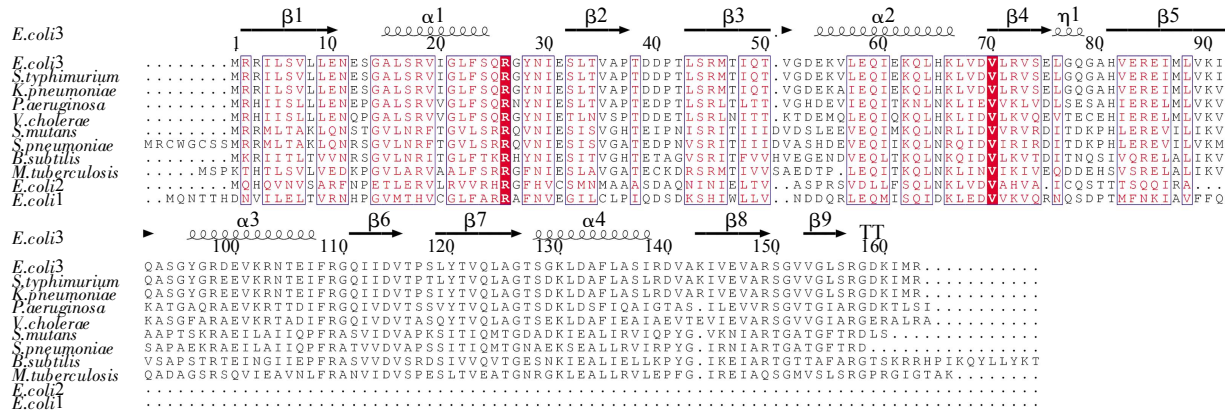

Fig. 1　Sequence alignment of regulatory subunits from some bacteria

*E. coli*3, *E. coli* AHAS Ⅲ (IlvH); *S. typhimurium*, *Salmonella typhimurium*; *K. pneumoniae*, *Klebsiella pneumoniae*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *V. cholerae*, *Vibrio cholerae*; *S. mutans*, *Streptococcus mutans*; *S. pneumoniae*, *Streptococcus pneumoniae*; *B. subtilis*, *Bacillus subtilis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *E. coli*2, *E. coli* AHAS Ⅱ (IlvM); *E. coli*1, *E. coli* AHAS Ⅰ (IlvN). The structure mask was from IlvH structure. Conserved sequences were labeled with red color and the highlight residues were the most conserved residues. Sequence alignment performed with ClustalW2 on EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and optimized manually. ESPript[10] was used to generate the figure.

On the other hand, AHAS, as many classes of herbicides' target, was used to develop new inhibitors and protein structure information could make this procedure more rational. Traditional inhibitors bound to the substrate channel in the catalytic subunit of AHAS to block the entrance of substrates to the active site of the enzyme[1]. The structural study of AHAS regulatory subunit will provide important insights into new pathway to develop new types of inhibitors.

In this project, we have aimed and obtained the crystal of IlvN, also co-crystallized IlvN with one of the effector molecules, valine; the valine bound and unbound crystals were with completely different crystal growth habit and space group, and we have collected complete data sets for both type of crystals.

1　Materials and methods

1.1　Cloning, expression and purification of *E. coli* IlvN

The *ilv*N gene of *E. coli* was amplified by the PCR (polymerase chain reaction) from the plasmid pQE-BNwt which contained *ilv*BN gene (encoding the holoenzyme of AHAS Ⅰ) using the primers 5′ggaattcc-atatgcaaaacacaactc 3′ and 5′ ccgctcgagttactgaaaaaaca-ccg 3′. The amplified fragment was inserted into the expression vector pET-28a (+) (Novagen) between *Nde* Ⅰ and *Xho* Ⅰ restriction sites to yield plasmid pNXH103a. This construct expressed IlvN protein fused with a 20-residue (2.18 ku) vector-coded N-terminal tag containing the hexahistidine for further purification. This construct was verified by DNA sequencing.

The expression plasmid pNXH103a was transformed into *E. coli* strain BL21 (DE3) cell. A single colony was inoculated into 20 ml of lysogeny broth (LB) medium containing 50 mg/L kanamycin. After 200 r/min shaking overnight at 310 K, the culture was scaled up to 1 L LB medium containing 50 mg/L kanamycin and until A_{600} reached about 0.6. After cooling the culture to 291 K, 0.5 ml of 1 mol/L

isopropyl β-D-thiogalactoside was added to induce the expression of the target protein (final concentration was 0.5 mmol/L) overnight at 291 K and 200 r/min. The cells were harvested by centrifugation at 3 000 *g* at 277 K, and the pellet was resuspended in buffer A (20 mmol/L Tris-HCl, 500 mmol/L NaCl, pH 7.5) and frozen at 253 K.

Two-step purification was used to obtain the target protein fused with hexahistidine-tag: nickel ion chelate affinity chromatography followed by gel filtration. PMSF (phenylmethylsulfonyl fluoride), a serine protease inhibitor, was added into fresh thawed bacterial suspension to a final concentration of 0.5 mmol/L. The bacterial cells were disrupted by sonication in ice water bath. The insoluble particles were removed by centrifugation at 58 000 *g* for 30 min at 277 K. The supernatant was loaded onto a 1 ml Ni^{2+}-chelating affinity column (HiTrap Chelating HP 1 ml, GE Healthcare, USA) by a peristaltic pump after passing through a 0.22 μm filter. The nickel column was previously charged with 100 mmol/L Ni_2SO_4 and equilibrated with 5 column volume (CV) buffer A. All subsequent chromatography steps were performed with an AKTA Purifier 100 (GE Healthcare, USA) at room temperature.

The unbound cell debris was eliminated with 5 CV buffer A and the non-specifically bound proteins were washed out with 5 CV 20% buffer B (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 500 mmol/L imidazole，pH 7.5) in buffer A. Then the hexahistidine-tagged protein was eluted with 3 CV buffer B. Fractions with the target protein were pooled together, and dithiothreitol (DTT) was added to a final concentration of 5 mmol/L. The total volume of the protein solution was about 1 ml. Then the denatured proteins were eliminated by centrifugation at 16 000 *g* for 10 min at 277 K and the supernatant containing the desired protein was purified further using a Hiload Superdex 75 column (GE Healthcare, USA) with buffer C (20 mmol/L Tris-HCl, 200 mmol/L NaCl, 2 mmol/L DTT, pH 7.5). Fractions containing IlvN were collected and concentrated to approximately 20 g/L (Bio-Red Protein Assay, Bio-Red Laboratories Inc., USA; bovine serum album as standard) by ultrafiltration. The purified IlvN was flash frozen with liquid nitrogen and stored at 193 K in small aliquots for further use. The purity of the protein was checked by SDS-PAGE during each operation step. Only one main band with an expected molecular mass (～13 ku) was visible on the gel after size-exclusion purification.

1.2 Crystallization and data collection

Crystallization was carried out at 293 K using the sitting-drop vapor-diffusion method on a 48-well-plate (XtalQuest, Beijing, China). The initial screen was carried out using Crystal Screen, Crystal Screen 2, Index Screen and Natrix (Hampton Research, CA, USA). Each droplet contained 1 μl protein solution and 1 μl reservoir solution and was equilibrated against 0.1 ml reservoir solution.

After screening and optimization, IlvN protein crystallized in the condition of 100 mmol/L KCl, 10 mmol/L $MgCl_2$, 50 mmol/L Tris-HCl pH 7.8, 20% PEG MME 550; IlvN and valine co-crystallized in the condition of 2 mol/L ammonium acetate, 10 mmol/L $CaCl_2$, 10 mmol/L $MgCl_2$, 100 mmol/L sodium cacodylate pH 6.3, 13% PEG 4000. Protein concentration could be 10～20 g/L. For co-crystallizing IlvN with valine, valine was added to IlvN solution to a final concentration of 5 mmol/L right before the crystal screening or optimization. Crystals were first observed the next day and took about 5 days to reach maximum size of about 0.1 mm × 0.1 mm × 0.2 mm.

Complete diffraction data sets were collected on a MAR 165 CCD detector at beamline I911-3 at MAX-lab, Lund, Sweden. The crystals were flash-frozen in liquid nitrogen and maintained at 100 K using nitrogen gas(Oxford Instruments Inc., UK) during data collection. For cryo-protection, the crystals were flash soaked in a reservoir solution containing 20% glycerol, and the time of soaking in the cryo-protection solution should be less than 5 s. The data were processed using the program suite XDS[11].

2 Results and discussion

Although the holoenzyme structure has been the final goal for the field worldwide and many groups including our laboratory have tried to work a lot on the holoenzyme structure without success, in the absence of the holoenzyme structure, we will need to crystallize the subunits with bound effectors one by one. The regulatory (small) subunit of *E. coli* AHAS Ⅰ has been crystallized successfully for the first time in this work (Figure 2a). The crystals were grown in 100 mmol/L KCl, 10 mmol/L $MgCl_2$, 50 mmol/L Tris-HCl pH 7.8, 20% PEG MME 550. They diffracted to a resolution of 2.6Å and belonged to the space group $P2_12_12$, with unit-cell parameters a = 87.2Å, b = 133.5Å, c = 60.9Å. For 6 molecules per asymmetric unit, the Matthews

coefficient was 2.66Å^3 u^{-1} and the solvent content was 53.81%[12].

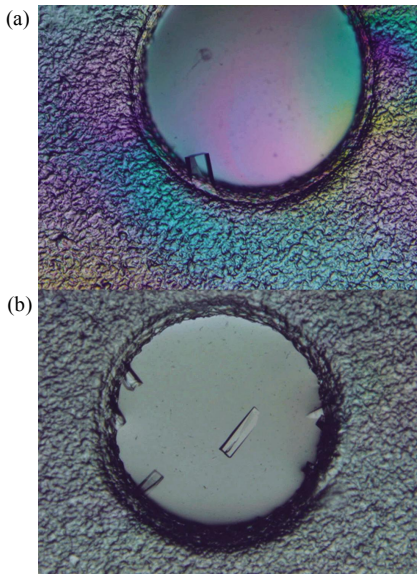

Fig. 2　Photos of crystals

(a) The crystals of IlvN protein. (b) The crystals of IlvN co-crystallized with valine.

To obtain the valine-IlvN complex crystals, we tried to soak IlvN crystals with valine at first. But no mater how gentle we treated, the IlvN crystals just collapsed. Then, we screened IlvN crystals with addition of different concentration of valine. Finally, IlvN co-crystallized with 5 mmol/L valine in the condition of 2 mol/L ammonium acetate, 10 mmol/L $CaCl_2$, 10 mmol/L $MgCl_2$, 100 mmol/L sodium cacodylate pH 6.3, 13% PEG 4000 (Figure 3b). These crystals were fragile and very difficult to handle, after many struggles we have finally managed to collect one set of data. This crystal form only diffracted to a maximal resolution of 3.0Å with the space group $P222_1$, and unit-cell parameters a = 74.5Å, b = 89.2Å, c = 178.7Å. Assuming six molecules per asymmetric unit, the Matthews coefficient[12] was 2.20Å^3•u^{-1} and the solvent content was 44.10%, falling into the reasonable amount of solvent. The data-collection statistics are listed in Table 1.

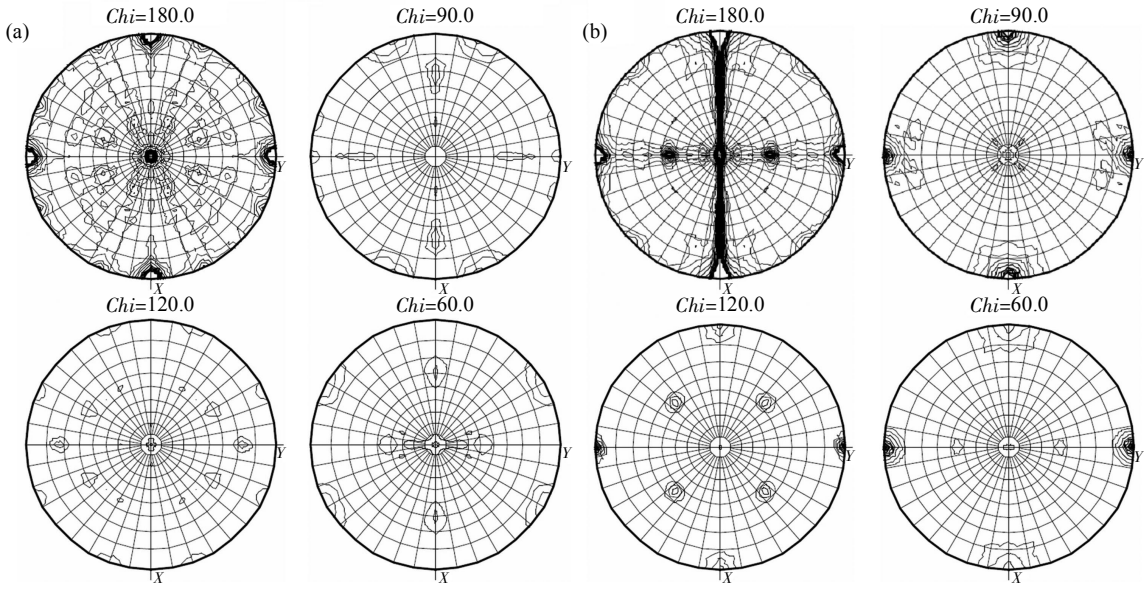

Fig. 3　The self-rotation plots of IlvN crystal (a) and valine-IlvN co-crystallized crystal (b)

These plots were generated using MOLREP[13] from X-ray data.

During the procedure of purification, IlvN protein tended to aggregation in solution and could not concentrated to higher concentration than 20 g/L. When valine added into the IlvN protein solution, the protein became more stable without changing any other conditions. Therefore, binding with valine can change the performance of IlvN protein in solution and affect the crystallization.

During crystal screening and optimization trials, IlvN protein both with and without valine were tried in the same conditions and at the same time. Two types of crystals grew in very different conditions and neither of them could grow in the other's conditions. The different space groups and cell unit parameters

showed two types of crystals(Table 1). The self-rotation functions calculated by the program MOLREP[13] were plotted in Figure 3, these plots showed the two types of crystals had apparently very different patterns.

Evidences above made us to assume that IlvN had co-crystallized with the valine bound to the regulatory site and to further confirm this assumption, we need to solve these two structures. The phases of these two structures have been determined and the refinement of the structures is in progress.

Table 1 Data collection statistics of IlvN crystals

	IlvN	IlvN + valine
Wavelength/Å	0.978 7	0.978 7
Resolution range/Å	50.00～2.59 (2.74～2.59)	50.00～3.00 (3.10～3.00)
Space group	$P2_12_12$	$P222_1$
Unit-cell parameters/Å	a=87.2, b=133.5, c=60.9	a=74.5, b=89.2, c=178.7
No. of observed reflections	159 605	165 249
No. of unique reflections	22 639	21 691
Completeness/%	97.4 (94.0)	89.4(82.2)
R_{sym}† /%	6.5 (49.5)	9.5 (65.6)
Average I/σ (I)	25.44 (4.05)	21.31 (2.85)
Molecules per ASU	6	6
V_M /(Å^3•u^{-1})	2.66	2.20
Solvent content/%	53.81	44.10

† $R_{sym} = \sum |I_{obs} - Iavg| / \sum I_{obs}$. Values in parentheses are for the highest resolution shell.

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大肠杆菌乙酰羟基酸合酶 I 调控亚基 IlvN 的结晶及其与配体缬氨酸的共结晶 *

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摘要　乙酰羟基酸合酶(acetohydroxyacid synthase，AHAS)是生物体内支链氨基酸合成通路中的第一个通用酶，它是目前市售多种除草剂的靶标．AHAS 通常由分子质量较大的催化亚基和分子质量较小的调控亚基组成．催化亚基结合催化必需的辅基(FAD、ThDP 和 Mg^{2+})；调控亚基可以结合终产物(缬氨酸、亮氨酸或异亮氨酸)作为负反馈信号调节全酶的活性．大肠杆菌中 AHAS 有 3 个同工酶，每种同工酶都由催化亚基和调控亚基组成．大肠杆菌 *ilv*N 基因编码了 AHAS 同工酶 I 的调控亚基．*ilv*N 基因克隆到 pET28a 表达载体中，在大肠杆菌 BL21(DE3)菌株中得到可溶性的大量表达．表达的蛋白质通过镍离子亲和层析和分子筛层析得到纯化．为了对调控亚基的调节机理有深入了解，对 IlvN 蛋白进行结晶并对蛋白质与其配体缬氨酸进行共结晶．IlvN 蛋白晶体衍射能力为 2.6Å，IlvN 与缬氨酸共结晶的晶体衍射能力为 3.0Å．

关键词　乙酰羟基酸合酶，ACT 结构域，结晶

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