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Crystal structure of the single-stranded RNA binding protein HutP from *Geobacillus thermodenitrificans*

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ABSTRACT

RNA binding proteins control gene expression by the attenuation/antitermination mechanism. HutP is an RNA binding antitermination protein. It regulates the expression of *hut* operon when it binds with RNA by modulating the secondary structure of single-stranded *hut* mRNA. HutP necessitates the presence of L-histidine and divalent metal ion to bind with RNA. Herein, we report the crystal structures of ternary complex (HutP–L-histidine–Mg^{2+}) and EDTA (0.5 M) treated ternary complex (HutP–L-histidine–Mg^{2+}), solved at 1.9 Å and 2.5 Å resolutions, respectively, from *Geobacillus thermodenitrificans*. The addition of 0.5 M EDTA does not affect the overall metal-ion mediated ternary complex structure and however, the metal ions at the non-specific binding sites are chelated, as evidenced from the results of structural features.

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1. Introduction

Expression of bacterial operons is controlled at the transcriptional level. RNA binding proteins regulate this kind of mechanism, which either act as activator/repressor or by modulating the structure of the corresponding RNA. The site responsible for the transcription mechanism of operon is located within or at the end of the operons [1]. Gene expression through transcription regulation in operons is referred as transcriptional attenuation and antitermination [2]. Protein mediated antitermination mechanism is described in nine operons, out of which eight from the catabolic system. Basically six operons are found in *Bacillus subtillis* [3].

Antitermination proteins from *B. subtilis* are classified as (i) bgl-Sac family (ii) glP and (iii) *hutP* types of operons based on the mode of regulation. Antitermination/attenuation mechanism of gene regulation in many systems, such as TRAP, hutP, pgR, bglP, sacT, glpP, licT, SacY, amir and nasR are reported earlier [3]. The antitermination that leads to full-length transcription of genes by (i) modification in RNA polymerase and (ii) destabilizing the terminator structure. The TRAP attenuation process is carried out in *trp* operon. In the presence of tryptophan, 11 subunits of TRAP bind with trp leader mRNA and induce the terminator [4] whereas in the absence of tryptophan, TRAP is unable to bind with mRNA due to the formation of an alternative conformation. This kind of regulation is named as negative regulation [5–7].

In hutP, histidine is an important and essential amino acid, synthesized in cells involving different steps of biochemical mechanism. When nitrogen and carbohydrates are lacking in their sources, its *hut* operon gets activated and utilize the freely available histidines as nitrogen source. The *hutP* gene is located near the promoter in *hut* operon with five additional genes *hutH*, *hutU*, *hutI*, *hutG* and *hutM*, which are located downstream from *hutP* and the promoter [8–11].

Hexameric form of HutP binds with specific sequence of *hut* mRNA in the presence of L-histidine [10]. HutP not only requires L-histidine for RNA binding but also requires divalent metal ions like Mg^{2+}, Mn^{2+} [12]. In the apo-form, HutP crystallized as a dimer and three dimers were related by 3-fold symmetry to form the hexamer. In binary complex, HutP recognizes the L-histidine at the dimer interface by forming an open hydrophobic pocket with its surrounding residues. This hydrophobic pocket is essential to clarify whether the incoming residues possesses the imidazole group or not. HutP–L-histidine binding with mRNA confirms that the L-histidine analogs with imidazole ring possess higher affinity

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for binding [13]. Once the ternary complex (HutP–L-histidine–Mg^{2+}) was achieved, the metal ion makes the structural rearrangement in L3 and L5 loop regions and L-histidine binding sites in the structure. These structural rearrangements are responsible for recognition of RNA. Upon binding to the HutP, RNA terminator structure is destabilized and changed its conformation in a novel triangular fashion; but the protein will not go for any further conformational changes. Thus, divalent metal ions play crucial role in HutP–RNA interactions and structural rearrangement of HutP–RNA complex formation [14].

The literature survey reveals that *hutP* gene also exists in other *Bacillus* species including *Bacillus anthracis, Bacillus cereus* [15], *Bacillus halodurans* [16], *Geobacillus kautophilus* [17] and *Bacillus amyloliquefaciens* [18].

Recent study indicates the availability of *hutP* gene in *Geobacillus hermodenitrificans* that it is a facultative aerobic thermophilic bacterium. It was isolated from oilfield in Dagang (Northern China) at a depth of 2000 m and a temperature of 73 °C. The complete genome sequence consists of 3,550,319-bp chromosome and 57,693-bp plasmid and the *hutP* gene consists of 149 amino acids [19]. About 60% sequence similarity is observed between *G. thermodenitrificans* and *B. subtilis*. Thermophilic proteins differ from mesophilic in structure and function and remain stable around 73 °C. In view of the above reasons, we plan to determine the crystal structures of HutP–L-histidine–Mg^{2+} and HutP–L-histidine–Mg^{2+}–RNA to understand the RNA binding mechanism of HutP from *G. thermodenitrificans.*

In this present study, we show the ternary complex structure (HutP–L-histidine–Mg^{2+}) of antitermination protein HutP. The importance of L-histidine and Mg^{2+} ions for RNA binding is also demonstrated by biochemical studies. To understand the role of metal ion, we solved the EDTA treated ternary complex structure.

2. Materials and methods

2.1. Cloning, expression and purification

The *hutP* gene from *G. thermodenitrificans* was chemically synthesized with His-tag and cloned into PCR 2.1vector. The cloned gene was sub-cloned by digesting with *Nde*I and *BamH*I enzymes, and the resultant fragment was inserted into pHCE constitutive expression vector containing a His6-tag and thrombin cleavage site at the N-terminus, which was digested earlier with the same enzyme. The positive clones was confirmed by DNA sequencing and transformed into BL21(DE3) cells. The recombinant strain was expressed overnight in LB medium containing 50 μg/ml ampicillin without any inducer such as IPTG [20]. Cells were harvested by centrifugation at 8000 rpm for 15 min and the pellet was resuspended in a 50 ml of purification buffer containing 50 mM Tris–HCl, 500 mM NaCl, 5% glycerol and 2 mM β-mercaptoethanol. The cells were lysed by sonication for 10 min with 1 min interval in ice-cold condition. Protein containing the supernatant was separated from lysate through centrifugation for about 15,000 rpm for 10 min.

The his-tagged protein was purified using Ni–NTA column. The column is pre equilibrated with buffer containing 50 mM Tris–HCl, 500 mM NaCl, 5% glycerol and 2 mM β-mercaptoethanol. The protein sample was applied to the column and elution was carried out by imidazole gradient method. HutP protein eluted at 300 mM of imidazole gradient and was confirmed by SDS–PAGE. The imidazole has been removed from protein through dialysis with the same equilibration buffer.

Later, the engineered his-tag was removed by thrombin digestion. The his-tag containing protein sample digested by adding thrombin (1.5 U/mg) for 2 h, digestion was confirmed by SDS–PAGE. The digested sample was applied into HiTtrap QFF column and the bound sample was eluted by salt gradient method. The purity of protein was confirmed in SDS–PAGE (Fig. 1A).

2.2. Crystallization

Purified protein sample was concentrated (14 mg/ml) and used for crystallization. Initial screening of HutP crystallization was performed by hanging drop vapour-diffusion method using the following kits, Wizard screen I & II (Emerald Bio Systems), Hampton crystallization kits crystal screen and crystal screen 2 and MPD suite I & II. All the crystallization experiments were performed at 293 K. For HutP–L-histidine–Mg^{2+} complex, we have mixed 1 μL of protein solution, 1 μL each of 50 mM L-histidine and 10 mM $MgCl_2$, alongwith 3 μL of reservoir solution (1:1) and equilibrated against 500 μL reservoir solution. The preliminary crystals were appeared in Wizard I condition No. 26. The good quality single crystals suitable for diffraction were obtained within 3 days with the following condition; 10% W/V PEG 300; 0.1 M CHES pH 9.5 (Fig. 1B).

2.3. Data collection and processing

The crystals were cryo-protected with the mother liquor solution containing 15% glycerol and incubated for 3 min before data collection. Complete data set was collected at 1.9 Å resolution. For the EDTA-treated data, the crystal was soaked in the previous cryo condition (mother liquor plus15% glycerol) along with 0.5 M EDTA for about 1 min and the data set was collected up to 2.5 Å at 100 K using structural genomics beam line I, BL26B1, (SPring-8, Hyogo, Japan).

The soaking of the crystal in 0.5 M EDTA for longer time, for example, 1.5 and 2 min was also carried out, but unfortunately the crystal degradation had occurred and could not able to mount the crystal for data collection. Plausibly, crystal damage was caused by the chelation of metal ions.

The HutP protein crystal belonged to $P6_3$ space group with unit cell parameters $a = 90.811$; $b = 90.811$; $c = 76.672$. The Matthews co-efficient indicates the presence of two monomers in asymmetric unit with 56.98% solvent contents. The diffraction data sets were processed, integrated and scaled with HKL2000 (Table 1).

2.4. Gel mobility shift assay

The gel mobility shift assay was performed to clarify RNA binding with HutP protein. 21-mer RNA was purchased (Fasmac, Japan) and used for protein–RNA complex preparation. To analyze and understand the importance of L-histidine and $MgCl_2$, different complexes were prepared with varying concentrations as follows; HutP–RNA, HutP–RNA–L-histidine, HutP–RNA–$MgCl_2$ and HutP–RNA–L-histidine–$MgCl_2$. The shift of the band in gel was visualized using native gel electrophoresis. The conditions were optimized using different running buffer, binding buffer, pH, protein concentration, RNA, L-histidine and $MgCl_2$.

The prepared complexes were incubated in binding buffer containing 10 mM HEPES for about 15 min. Initially pre-run was allowed for 10 min/200 V at 4 °C and then the incubated sample was centrifuged and loaded after mixing with the dye. Protein–RNA complex was resolved through 6% non-denaturing polyacrylamide gel running 200 V at 4 °C. The gel was stained in SYBR green solution and visualized using Fuji film gel document instrument (Fig. 1C).

3. Results

3.1. Sequence and structure alignment

The protein sequence alignment was done using BLASTp [21]. A total of 93 hits observed in search against non-redundant database.

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