Crystallization and Preliminary X-ray Crystallographic Studies of HslU Mutant in *Escherichia coli*

Ji-Joon Song, Cheol Soon Lee,† Yun Sik Kim, Jung Jin Kim, Young-Jun Im, Hyun Hee Kim,† Soon Ji Yoo,† Ihn Sik Seong,† Chin Ha Chung,† and Soo Hyun Eom*

Department of Life Science, Kwangju Institute of Science and Technology, Kwangju 500-712, Korea
†School of Biological Sciences, Seoul National University, Seoul 151-742, Korea
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HslVU is an ATP dependent protease in *E. coli* like proteases La (Lon) and Ti (ClpAP/ClpXP).[1] It is composed of two multimeric components, 19 kDa HslV and 50 kDa HslU proteins.[2] While HslU itself has an ATPase activity, HslV has a weak peptidase activity so that it slowly degrades certain hydrophobic peptides, such as *N*-carbobenzoxy-Gly-Gly-Leu-7-amino-4-methyl-coumarin and polypeptides such as insulin B-chain and casein.[3,4] But the proteolytic activity of HslV is increased significantly (up to 150 fold) by associating with HslU in the presence of ATP.[3] The primary amino acid sequence of HslV is similar to certain β-type subunits of the 20S proteasomes of archaebacterium *Thermoplasma acidophilum* with 18% identity.[5] While β-type subunits of the 20S proteasomes show 72-point symmetry, HslV is a dimer of hexamers with 62 point symmetry. The crystal structure of HslV solved at 3.8 Å resolution shows that in spite of the different symmetry, the folds and the contacts between subunits are conserved, compared with β-type subunits of the 20S proteasomes.[6] In the case of HslU, it is 50% identical to the ClpX protein of *E. coli* in amino acid sequence. According to the analysis of HslU using electron microscopy, HslUs make ring-shaped forms in the presence of ATP or AMP-PNP (ATP analogue). This ring is composed of 6 or 7 HslU molecules to form hexameric or heptameric rings.[7] HslU contains two Cys residues, Cys261 and Cys287. It has been suggested that Cys261 is involved in oligomerization and that Cys287 is related to the ATPase function.[8] In order to reveal the three-dimensional structure, and the mechanism of oligomerization between HslUs, and between HslU and HslV, the $HslU_{C261V}$ was crystallized and studied with X-ray crystallographic method.

Experimental Section

The pGEM-T vector (Promega) carrying the *hslVU* operon (named pGEM-T/HslVU) was constructed as described previously.[6,9] Site-directed mutations were created by the PCR method, which consists of two sequential PCRs, using pGEM-T/HslVU for HslU mutant as the templates. The primary PCR was carried out using mutagenic primers, which were designed to replace Cys^{261} of HslU with Val, and then the second PCR was performed. After the secondary PCR, the mutated fragments were ligated into pGEM-T vector. The resulting plasmids were digested with *Nru*I and *Bgl*II, followed by ligation of the restriction fragments into the pGEM-T/$HslVU_{C261V}$ plasmids. The resulting plasmids were transformed into *E. coli* strain XL2 Blue. Substitution of the nucleotide by mutagenesis was confirmed by DNA sequencing.

E. coli cells harboring pGEM-T/$HslVU_{C261V}$ were grown overnight, lysed in 2% (w/v) SDS, and electrophoresed on 13% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol. Two major bands representing 19 kDa (HslV) and 50 kDa ($HslU_{C261V}$) polypeptides were detected in cells carrying the recombinant plasmids, but not in cells containing only the vector. To purify $HslU_{C261V}$, the crude extracts of the *E. coli* cells harboring pGEM-T/$HslVU_{C261V}$ were loaded onto a phosphocellulose column equilibrated with 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.5) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (referred to as buffer A). After collecting the flow-through fraction, the column was washed extensively with the same buffer, and the proteins bound to the column were eluted with the same buffer containing 0.4 M phosphate. $HslU_{C261V}$ was recovered in the flow-through and HslV in the 0.4 M phosphate eluate. For purification of $HslU_{C261V}$, the flow-through fraction from the initial phosphocellulose column was dialyzed against buffer containing 20 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol (referred to as buffer B) and loaded onto a DEAE-cellulose column equilibrated with the same buffer. After washing the column with buffer B containing 0.15 M NaCl, proteins were eluted with a linear gradient of 0.15-0.3 M NaCl. The fractions containing $HslU_{C261V}$ were pooled, diluted 1 : 1 with buffer B, and applied to a heparin-agarose column equilibrated with the same buffer. Proteins bound to the column were eluted with a linear gradient of 0.2-0.5 M NaCl. The $HslU_{C261V}$ containing fractions were pooled, concentrated by ultrafiltration using a YM30 membrane (Amicon), and chromatographed on a Sephacryl S-300 column equilibrated with buffer B containing 0.1 M NaCl. Then $HslU_{C261V}$ was concentrated.

Two crystal forms that belong to different space groups were grown with hanging drop-vapour diffusion method at room temperature (293 ± 1 K). One form of $HslU_{C261V}$ crystals (Figure 1A) was grown on a siliconized cover slip by equilibrating a mixture containg 1 μL of protein solution (10.6 mg mL^{-1}) containing 2 mM ATP in buffer A and 1 μL reservoir solution (100 mM Tris-HCl pH 8.0, 6-8% PEG 4K,

*Corresponding author. Tel: +82-62-970-2493; Fax: +82-62-970-2484; E-mail: eom@kjist.ac.kr

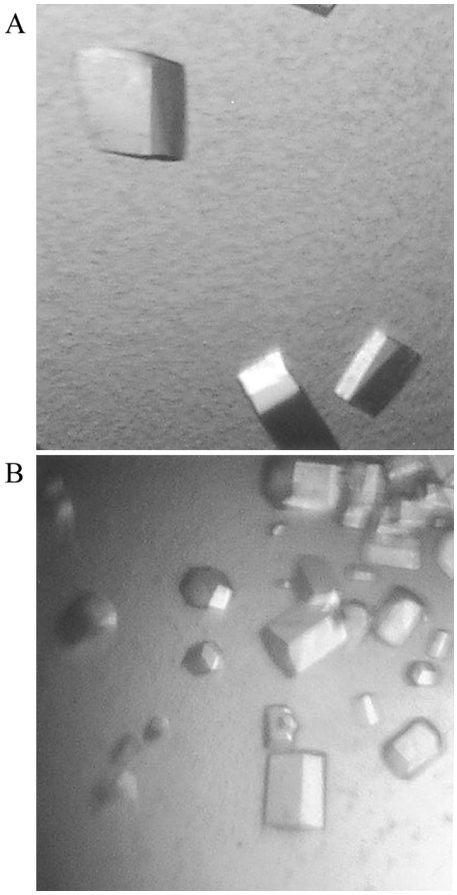

Figure 1. Orthorhombic (A) and hexagonal (B) crystals of HslU mutant (C261V) from *Escherichia coli*. The maximun dimensions are 0.1 × 0.1 × 0.1 mm.

100-300 mM NaCl, 20 mM $MgCl_2$, 2 mM DTT, and 1 mM EDTA) against 1 ml of resorvoir solution. The crystals with maximum dimensions of 0.1 × 0.1 × 0.1 mm grew in one week. X-ray diffaction data were collected on an R-AXIS IV image-plate system attached to a Rigaku rotating-anode generator (RU-300) providing Cu$K\alpha$ radiation and running at 50 kV and 90 mA with a 0.3 mm focus cup at room temperature (293 ± 1 K).

Preliminary intensity data were collected where the diffraction beyond 5.5 Å resolution was recorded, and the crystals belong to the primitive orthorhombic space group ($P2_12_12$ or $P2_12_12_1$). The unit cell parameters of the crystal were determined to be a=80.0, b=138.1, and c=175.0 Å with 1933400 $Å^3$ unit cell volume, using DENZO.[10] Assuming that asymmetric unit contains three molecules with a molecular mass of 49.6 kDa, V_M value is calculated as 3.25 $Å^3D^{-1}$, resulting in a solvent content of 62.2%. The other form of crystals (Figure 1B) was grown under the same crystallization condition, and experiment was performed in the same manner. The crystal diffracted beyond 6.5 Å. The unit cell parameters were determined as a=b=81.0 and c=174.6 Å, and this crystal belongs to primitive hexagonal space group ($P6_1$ or $P6_4$). Assuming that asymmetric unit contains one molecule, V_M value is calculated as 3.33 $Å^3D^{-1}$, resulting in a solvent content of 63.1%.

This work is the initial step toward revealing not only the structure of HslU mutant and its complex with HslV complex but also the mechanism of oligomerization. A search for better-diffracting crystals is continuing by varying the crystallizing conditions.

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References

1. Goldberg, A. L. *Eur. J. Biochem.* **1992**, *203*, 9.
2. Chuang, S. E.; Burland, V.; Plunkett, G.; Daniels, D.; Blattner, F. *Gene* **1993**, *134*, 1.
3. Yoo, S. J.; Seol, J. H.; Shin, D. H.; Rohrwild, M.; Kang, M. S.; Tanaka, K.; Goldberg, A. L.; Chung, C. H. *J. Biol. Chem.* **1996**, *274*, 14035.
4. Seol, S. J.; Yoo, S. J.; Shin, D. H.; Shim, Y. K.; Kang, M. S.; Goldberg, A. L.; Chung, C. H. *Eur. J. Biochem.* **1997**, *247*, 1143.
5. Seemüller, E.; Lupas, A.; Stock, D.; Löwe, J.; Huber, R.; Baumeister, W. *Science* **1995**, *268*, 579.
6. Bochtler, M.; Ditzel, L.; Groll, M.; Huber, R. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6070.
7. Rohrwild, M.; Coux, O.; Pfeifer, G.; Santarius, U.; Müller, S. A.; Huang, H.-C.; Engel, A.; Baumeister, W.; Goldberg, A. L. *Nat. Struct. Biol.* **1997**, *4*, 133.
8. Yoo, S. J.; Shim, Y. K.; Seong, I. S.; Seol, J. H.; Kang, M. S.; Chung, C. H. *FEBS Lett.* **1997**, *412*, 57.
9. Yoo, S. J.; Kim, H. H.; Shin, D. H.; Lee, C. S.; Seong, I. S.; Seol, J. H.; Shimbara, N.; Tanaka, K.; Chung, C. H. *J. Biol. Chem.* **1998**, *273*, 22929.
10. Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1996**, *276*, 307.