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STRUCTURE OF MACROMOLECULAR COMPOUNDS

Isolation, Purification, Crystallization, and Preliminary X-ray Diffraction Study of the Crystals of HU Protein from *M. gallisepticum*

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Abstract—HU proteins are involved in bacterial DNA and RNA repair. Since these proteins are absent in cells of higher organisms, inhibitors of HU proteins can be used as effective and safe antibiotics. The crystallization conditions for the *M. gallisepticum* HU protein were found and optimized by the vapor-diffusion method. The X-ray diffraction data set was collected to 2.91 Å resolution from the crystals grown by the vapor-diffusion method on a synchrotron source. The crystals of the HU protein belong to sp. gr. $P4_12_12$ and have the following unit-cell parameters: $a = b = 97.94$ Å, $c = 77.92$ Å, $\alpha = \beta = \gamma = 90°$.

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INTRODUCTION

The DNA-binding HU proteins ("H" for histone-like and "U" for U93—an *E. coli* strain used at that time to isolate the protein [1]) are present in all bacteria and belong to the family of *nucleoid-associated proteins* (NAPs). These proteins can be considered precursors to eukaryotic histones [2]. The main function of these proteins is to maintain genomic DNA supercoiling and compaction in prokaryotic cells [3]. These proteins are involved in replication [4], recombination [5], repair [6], transcription [7], and adaptation [8]. The HU protein from *E. coli* was the first to be characterized [9]. In particular, it was found that, although the genetic knockout of genes encoding two subunits of the HU protein does not kill this bacterium, it substantially impairs bacterial growth and adaptation [10]. Meanwhile, the absence of the HU protein is lethal for organisms, in which it is the only representative of NAPs [11, 12]. The HU proteins are absent in eukaryotic cells and, consequently, they are potential pharmacological targets for the design of antibacterial agents to use in human and veterinary medicine. In 2014 it was shown that the low-molecular-weight inhibitor predicted by molecular docking based on the three-dimensional structure of the *M. tuberculesis* HU protein can disrupt nucleoid architecture and reduce bacterial growth [13]. From this point of view, HU proteins from pathogenic parasitic microorganisms of the genus *Mycoplasma* (the class *Mollicutes*) [14] are of particular interest. The reduced genome of these microorganisms lacking HU protein is lethal for bacteria [15]. Histone-like HU proteins from the following two classes of mycoplasmas were functionally characterized: *Acholeplasma laidlawii* [16] and *Mycoplasma gallisepticum* [17]. In the latter study, evidence was obtained that it is the HU protein that replaces components of the mismatch repair (MMR) system lacking in mycoplasmas [18, 19]. The first three-dimensional structure of the HU protein from the mycoplasma *Spiroplasma melliferum* KC3 was published in 2015 [20]. In the present work, we report the preparation of the recombinant HU protein from *Mycoplasma gallisepticum* (HUMgal), its crystallization, and preliminary X-ray diffraction study.

MATERIALS AND METHODS

Isolation and Purification

Two genes encoding proteins homologous to the *E. coli* HU protein (HimA/Hup_1 and HimA/Hup_2) were annotated in the genome of *M. gallisepticum* [21]. However, only one of these proteins (HimA/Hup_2) exhibits DNA-binding ability and restores the growth of the *E. coli* knockout strain with deleted genes of its own HU proteins [17]. The cloning of this gene was performed as described in [20] by the conventional procedure—the polymerase chain reaction (PCR) on a genomic DNA as a template. The oligonucleotides used in the PCR were synthesized based on the sequences of 5' and 3' ends of the encoding gene region (Hu-M.gal.F 5'-TATTTCCCATGGCAAAAATCAAATC-3' and Hu-M.gal.R 5'-ATCTTGAATTCCTATTTGTGCGA-3') containing the Nco1 and EcoR1 restriction sites (underlined) at the 5' ends. The reaction was performed using the Encyclo polymerase (Evrogen, Russia) according to recommendations of the manufacturer. The amplification product was subjected to 2% agarose gel electrophoresis, followed by the isolation from agarose and the treatment with the Nco1 and EcoRI restriction enzymes. The restriction product was again purified by electrophoresis. The resulting restriction fragment was ligated into the pHisTEV plasmid vector, which is a derivative of the pET22b expression vector (Novagen, Germany) with the Nde1–Nco1 region encoding the pelB leader peptide replaced by the fragment encoding the sequence of an N-terminal hexahistidine tag, fused with the tobacco etch virus (TEV) protease recognition/cleavage site: MSYYHHHHHHDYDIPTTENLYFQGA. The vector was pretreated with the Nco1 and EcoRI restriction enzymes. Then *E. coli* cells of the Match1 strain were transformed with the constructed HisTEV-mgHU plasmid. The grown colonies were tested for the presence of the inserted gene by PCR, and the plasmid DNA, which was isolated from positive clones, was checked for the absence of mutations occurred during the cloning procedure using automated sequencing. In order to obtain the producing strain, the expression construct was transformed into the *E. coli* K12 cells of the BL21(DE3) RIPL strain (Stratagene, USA), which maintained the expression of bacteriophage T7 tRNA and RNA polymerases rare in *E. coli*, thus providing a high level and satisfactory regulation of the expression of recombinant proteins in these cells.

Cells of the producing strain were cultured in the LB medium containing ampicillin at 100 μg/mL concentration. When the culture reached the optical density of 0.8 OU, the expression was induced by the addition of IPTG to a concentration of 0.4 mM. The induction was performed for 16 h at 24°C. Then the cells were precipitated by centrifugation at 6000 rpm for 20 min. The cell sediments were stored at –70°C. The recombinant protein was isolated as described in [20]. The isolation involves the following three steps of chromatographic purification: two high-performance metal-chelate chromatography steps separated by the treatment with TEV protease and the final gel-filtration chromatography. The cell sediment, which was obtained from 1 L of the culture, was lysated with 25 mL of the cold buffer solution A (50 mM TrisHCl, 500 mM NaCl, pH 8.0, with the addition of 5% glycerol, 0.2% Triton X100, 1 mM PMSF) and treated with ultrasound using an Ultrasonic Processor (Cole Parmer) for 5 × 30 s, with cooling in ice. The insoluble fraction was precipitated by centrifugation at 20 000 rpm for 20 min at 4°C. The clarified lysate was loaded onto a column containing the metal-affinity Ni-NTA Superflow resin (Quigen). After the removal of proteins, which have not bound or have been nonspecifically bound to the resin (the buffer A containing imidazole and NaCl at concentrations increased to 30 mM and 1 M, respectively), the target product was eluted with the buffer A containing 300 mM imidazole. The eluate was treated with TEV protease fused with an N-terminal hexahistidine tag (2–3 h at room temperature). Then, in order to decrease the imidazole concentration, the sample was dialyzed against the buffer A. The second metal-chelate chromatography step was performed to separate the target protein from the N-terminal hexahistidine tag and TEV protease. In this case, the target protein was eluted in the breakthrough fraction, while polypeptides containing hexahistidine clusters were bound to the resin. The resulting sample was concentrated using centrifugal concentration tubes (Amicon Ultra 3kD cutoff, Millipore ltd, Ireland) and finally purified on a gel-filtration column (GE Superdex G75 10/300 GL column, E LifeSciences, United States) equilibrated with the buffer (20 mM TrisHCl, 200 mM NaCl, 10% glycerol, pH 8.0). All protein fractions obtained during the protein isolation were analyzed by denaturing gel electrophoresis in 15% polyacrylamide, followed by staining with Coomassie G-250. The electrophoretic mobility of the protein HUMgal corresponds to the calculated molecular weight of 12.6 kDa. The yield of the recombinant protein was 6 mg per liter of the bacterial culture. The purity was no lower than 96%.

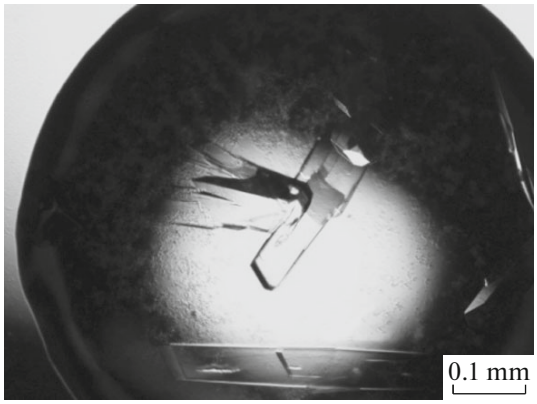
Crystal Growth

The crystallization conditions for the histone-like protein HUMgal were screened by the sitting-drop vapor-diffusion method on a Rigaku robotic crystallization system (Japan) using the following Hampton Research crystallization screen kits for globular proteins: Crystal Screen HT, Crystal Screen Cryo HT, Index HT, PEG/Ion HT, PEGRx HT, and SaltRx HT. The protein concentration was 12 mg/mL. The crystallization was performed in 96-well plastic plates (ArtRobbins, Hampton Research) at 20°C. The crystals appeared within 19 days in the conditions Index HT B11(2.1 M DL-Malic Acid, pH 7.0).

Crystallographic data and the X-ray-data-collection statistics for the crystal of HUMgal

sp. gr.	$P4_12_12$
$a = b$, c, Å; $\alpha = \beta = \gamma$, deg	97.94, 77.92; 90
T, K	100
λ, Å	0.984
Resolution, Å	34.63–2.91 (3.07–2.91)*
Number of unique reflections	8699 (1590)
Redundancy	12.75 (6.70)
Completeness, %	99.20 (95.87)
$I/\sigma(I)$	4.02 (2.01)
Rmrgd-F, %	13.7 (35)

*The data for the last resolution shell are given in parentheses.



Crystal of HUMgal grown by the vapor-diffusion method. The crystal size is about 0.3 mm.

X-ray Diffraction Data Collection and Processing

Before the collection of the X-ray diffraction data set, the crystals were withdrawn from a capillary and transferred into a harvesting solution composed of equal volumes of the protein solution and the reservoir solution. For the X-ray diffraction study, a crystal was picked out with a loop and transferred into a cryoprotectant solution, which contained the same components as the harvesting solution with the addition of 15% of glycerol. Then the crystal in the loop was frozen in a stream of nitrogen vapor. The X-ray diffraction data set was collected from one crystal at 100 K on the Belok station equipped with a MARCCD detector at the Kurchatov synchrotron radiation source. The X-ray data were obtained by the rotation method at a wavelength of 0.984 Å. The rotation angle was 247°, the oscillation angle was 1.0°, and the crystal-to-detector distance was 180 mm. The X-ray data set was processed using the imosflm program [22]. The X-ray-data-collection statistics are given in the table.

RESULTS AND DISCUSSION

The crystallization conditions for HUMgal were found by the hanging-drop vapor-diffusion method using the Rigaku robotic crystallization system (National Research Centre "Kurchatov Institute"). In the course of the optimization, the precipitant and protein concentrations were varied. The photo of the crystal is shown in the figure.

The crystals were characterized by X-ray diffraction using synchrotron radiation. The crystals grown by the vapor-diffusion method are suitable for the collection of X-ray diffraction data to 2.91 Å resolution. The crystals belong to sp. gr. $P4_12_12$ and have the following unit-cell parameters: $a = b = 97.94$ Å, $c = 77.92$ Å, $\alpha = \beta = \gamma = 90°$. There are four protein molecules per asymmetric unit. The Matthews coefficient is 2.32 Å^3/Da. The solvent content of the unit cell estimated by Matthews's method [23] using the CCP4 suite [24] is 47.00% of the unit-cell volume.

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