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Novel hexamerization motif is discovered in a conserved cytoplasmic protein from *Salmonella typhimurium*

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Abstract

The cytoplasmic protein Stm3548 of unknown function obtained from a strain of *Salmonella typhimurium* was determined by X-ray crystallography at a resolution of 2.25 Å. The asymmetric unit contains a hexamer of structurally identical monomers. The monomer is a globular domain with a long β -hairpin protrusion that distinguishes this structure. This β -hairpin occupies a central position in the hexamer, and its residues participate in the majority of interactions between subunits of the hexamer. We suggest that the structure of Stm3548 presents a new hexamerization motif. Because the residues participating in interdomain interactions are highly conserved among close members of protein family DUF1355 and buried solvent accessible area for the hexamer is significant, the hexamer is most likely conserved as well. A light scattering experiment confirmed the presence of hexamer in solution.

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Keywords

β -hairpin; Protein family DUF1355; *Salmonella typhimurium*

Introduction

The three-dimensional structure of the protein Stm3548 obtained from strain LT2 of bacteria *Salmonella typhimurium* was solved as a part of the Protein Structure Initiative. *S. typhimurium* is a serovar of *S. enterica*, a rod-shaped gram-negative bacteria that is a leading cause of human gastroenteritis and serves as a mouse model of human typhoid fever. The number of cases of salmonellosis, mostly from contaminated food, is increasing worldwide causing millions of infections in the human population each year. A structural genomics survey of proteins from this organism may lead to new therapeutic or vaccine targets. The *S. typhimurium* strain LT2 was isolated in 1940s, and sequencing of the complete genome was completed in 2001 [1].

Gene locus Stm3548 encodes a 253 amino acid protein with a calculated molecular weight of 28.6 kDa. This conserved protein belongs to Pfam07090 which contains several bacterial proteins of a similar length and unknown function (DUF1355). This domain was classified with the A4 beta-galactosidase middle domain family according to the Structural Classification of Proteins (SCOP) [2]. A type 1 glutamine amidotransferase (GATase1)-like domain, this group includes proteins similar to β -galactosidase from *Thermus thermophilus*. However, the catalytic Cys–His–Glu triad of typical GATase1 domains has not been conserved for the members of this family. The proteins of the DUF1355 family belong to the $\alpha/\beta/\alpha$ protein class and have a flavodoxin-like fold.

In this paper, we present the crystal structure of Stm3548 determined at a resolution of 2.25 Å and analyze its three dimensional structure and subunit interactions. We propose that this protein has a novel structural feature that facilitates formation of a hexameric state.

Materials and methods

Protein cloning, expression and purification

The gene was cloned, then a recombinant protein was expressed, purified, and the initial crystal trials were performed following procedures described previously [3,4]. The ORF of Stm3548 was amplified by PCR from *S. typhimurium* genomic DNA. The recombinant construct in pMCSG7 provides for an N-terminal hexahistidine tag separated from the fusion protein by a TEV protease recognition site (ENLYFQ↓G). The cells were grown in LB at 37°C to an OD₆₀₀ of 0.6–0.8 followed by 1 mM IPTG induction overnight at 20°C. The cultures were harvested and flash-frozen in a –80°C freezer. The purification procedure including sample loading, washing, eluting and desalting was automatically performed on an AKTA Explorer 3D (General Electric Health Systems) using buffers containing 50 mM HEPES, pH 8.0, 500 mM NaCl, 5% glycerol, and 10, 20, 250 and 0 mM imidazole for the binding, wash and elution as well as desalting buffers, respectively. The His₆-tag was removed using TEV protease and the cleaved protein was resolved through a second Ni²⁺-column. The sample buffer was exchanged to 200 mM NaCl, 20 mM Tris–HCl, pH 7.6, and 2 mM DDT for crystallization. The SeMet-labeled protein was purified by using the same procedure.

Protein crystallization

Crystals of a SeMet-substituted Stm3548 were initially grown in sitting drops and further modified with the hanging-drop vapor diffusion technique at room temperature. A protein

solution of ~25 mg/ml in 20 mM HEPES at pH 8.0, 50 mM NaCl, and 1 mM DTT was mixed with an equal volume of reservoir solution and left to equilibrate at 18°C. The best crystals were obtained against a reservoir of 0.1 M Tris-HCl pH 8.5, 1.2 M tri-sodium citrate dihydrate. A 32% sucrose solution was added as a cryoprotector to the crystallization solution. Crystals were mounted on cryo loops and flash-frozen in liquid nitrogen.

Light scattering analysis

The molecular mass of SeMet-labeled Stm3548 in solution was estimated by light scattering analysis. A DAWN HELEOS instrument with ASTRA 5 analytical software (Wyatt Technology) was used as described earlier [5]. The peak fraction from a size exclusion column was diluted to 0.3, 0.5, and 1.0 mg/ml in crystallization buffer and injected into cell. The dilutions produced average molecular weights from 161 to 168 kDa, 5.6 to 5.9 times the predicted molecular weight of monomer.

Data collection

A two-wavelength anomalous diffraction (MAD) data set was collected at the Advanced Photon Source (Argonne, USA) 19ID beamline [6] at 0.97941 Å (peak) and 0.97952 Å (inflection). The temperature of data collection was 100 K. The data were integrated and scaled with the HKL2000 suite [7]. Some data collection statistics are given in Table 1. Data analysis was carried out using the programs of the CCP4 suit [8].

Structure determination, refinement and deposition

Selenium sites were located by the SHELXD program [9] as incorporated in the structure solution package HKL3000 [10]. A total of 40 sites were optimized and expanded to a total of 42 by iterative residual analysis and used for phasing, density modification, and structure solution with the autoSHARP program suite [11]. Manual rebuilding with the program Coot [12] was required to identify the six monomers in the asymmetric unit which form the cohesive hexamer. Upon tracing one complete polypeptide, it was docked onto partial builds of the other protomers to complete the model. The model was then subject to iterations of refinement, manual correction, and solvent addition. TLS (each monomer defined as a group) and restrained refinement was performed with REFMAC5 [13] against peak data at 2.25 Å resolution and Hendrickson-Lattmann coefficients that were calculated by autoSHARP prior to density modification. No non-crystallographic symmetry restraints were applied. The final model contained six Stm3548 monomers, 558 water molecules and six glycerol molecules. There was no interpretable electron density for the N-terminal cloning tag residues 1–7 in chains A, B, C, D, and F and for residues 1–5 of chain E. The geometry of the final model was inspected by the program PROCHECK [14]. A summary of refinement statistics and model quality is given in Table 2. Atomic coordinates and experimental structure factors were deposited into the Protein Data Bank and are accessible under the code 2GK3.

Results and discussion

The tertiary structure description

The *S. typhimurium* Stm3548 protein expressed well in *E. coli* and was soluble and well behaving. In the Stm3548 crystal, the asymmetric unit contains a striking hexamer of structurally identical monomers: A, B, C, D, E and F (Fig. 1). A ribbon diagram of the Stm3548 monomer structure is shown in Fig. 2. The monomer has a globular domain and a hairpin protrusion. The globular domain contains eight α -helixes and thirteen β -strands and has a three-layer $\alpha/\beta/\alpha$ sandwich flavodoxin-like fold. There are 3 α -helixes ($\alpha 1$, $\alpha 7$ and $\alpha 8$) at the first level, five parallel β -strands ($\beta 4$, $\beta 1$, $\beta 5$, $\beta 6$, $\beta 13$) and two antiparallel β -strands ($\beta 11$ and $\beta 12$) at the second level, and five α -helixes ($\alpha 2$ – $\alpha 6$) at the third level (Fig. 2). An extended 23 residue

β -hairpin containing strands $\beta 2$ and $\beta 3$ extrudes from the core β -sheets and forms the very distinctive feature of this structure. For the globular part of the monomer several structural homologs were found. However, no proteins containing a similar β -hairpin were revealed among homologs. Note that this β -hairpin occupies a central position in the hexamer, and the residues of the hairpin participate in most interactions between different subunits of the hexamer (see above).

The quaternary structure description

The monomers are located around a three-fold noncrystallographic rotation axis. In Fig. 3a, the three-fold NCS axis is parallel to the y axis, while in Fig. 3b this axis is perpendicular to the plane of the figure. For example, monomers A and B are related by a 120.1° rotation angle with RMSD of overlap 0.4° (calculated for $C\alpha$ atoms). The structure of Stm3548 can be described as two shamrock-shaped layers connected by a narrow triangular column formed by anti-parallel β -sheets of extended $\beta 2$ – $\beta 3$ hairpins.

There are several distinct interactions within the hexamer. Two diagonally positioned monomers that interact with their long β -hairpins forming an antiparallel β -sheet (A and D, B and F, C and E) are related by a two-fold noncrystallographic rotation axis and can be considered dimers (Fig. 3a). For example, monomers A and D are related by 180.0° rotation angle with RMSD of overlap equal to 0.6° (calculated for $C\alpha$ atoms). Therefore, the structure can be also described as a trimer of dimers.

In the central part of the hexamer, the antiparallel β -sheets of crossing β -hairpins of all monomers form a narrow channel (see Fig. 3b). The inner part of this channel surface in the center is composed of the side chains of six His20 and six Ile22 each pair contributed by a different subunit. The distance between the closest CD1 atoms of Ile22 residues of different monomers is equal to $5.2 \text{ \AA}$. Therefore, a channel diameter in the center of the hexamer can be estimated to be approximately $6 \text{ \AA}$. The loops consisting of residues 130–133 and 150–153 of monomers of the same level form the narrowest part and the bottom of the channel, correspondingly. The diameter is about $4 \text{ \AA}$ at its narrowest and $7.5 \text{ \AA}$ at the widest. The channel walls are composed mostly of polar residues (except Ile22). The entrance to the channel on both sites is quite acidic and central part rather basic. A number of ordered water molecules are found hydrogen bonded to protein in this channel. It is likely that no molecule larger than water or a dehydrated ion could pass through narrowest orifice of this channel.

An electrostatic potential map for the molecular surface of the protein has been calculated by GRASP [15] and is shown in Fig. 4. The acidic nature of the monomer ($pI = 4.6$) surface is apparent. Note, that a deep equatorial cleft has a relatively large negative charge.

Subunit interaction

An analysis of the interfaces between subunits was done using the PISA server http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html [16]. The largest contact area is observed between the monomers that are positioned diagonally and form dimers A and D, B and F, C and E (Fig. 1). These monomers interact mainly using the long β -hairpins. The interface area, e.g., for subunits A and D is $1270 \text{ \AA}^2$, which is about 13% of the monomer surface. There are 17 hydrogen bonds and four possible salt bridges (Lys25–Asp82) between monomers A and D. The distance between NZ of Lys25 of D monomer and OD1 and OD2 of Asp82 of A monomer is $3.3 \text{ \AA}$.

The three monomers in the same layer, for example, monomers A, B and C interact with each other by their neighboring globular domains and also via the β -hairpins in the center of the hexamer. The size of the interface area, e.g., between subunits A and B is about 820 and 840

Å² respectively, which is approximately equal to 8% of the monomer surface. Ten hydrogen bonds (six bonds between the residues of neighboring globular parts and four between the residues of the β -hairpins in the center) and two possible salt bridges (between NZ of Lys133 and OE1 and OE2 of Glu131) were revealed.

Some of the interactions between different monomers are mediated by ordered water molecules. For example, the atoms OE2 of residues Glu131 of the monomers A, B and C interact with the same water molecule 352, which is placed at a distance of 2.6, 2.4 and 2.4 Å from the atoms OE2 of Glu131 of the monomers A, B and C, correspondingly. Another example of interdomain interactions through water molecules is a network of water molecules between His20 of different monomers.

Note that residue Asn86 participates in two interdomain interactions. The distance between the atom OD1 of Asn86 (A) and the atom NH1 of Arg155 (B) is 2.9 Å, and the distance between the atom ND2 of Asn86 (A) and the atom OD1 of Asp28 (D) is 2.8 Å.

Far fewer interactions are observed between the monomers of different layers not arranged diagonally. For example, the only contact areas between monomer A and F are between the β -hairpin of monomer A and helices α_2 and α_5 of monomer F and, accordingly, between the β -hairpin of monomer F and helices α_2 and α_5 of monomer A. Eight hydrogen bonds were found between these monomers. No hydrogen bond was found between A and E monomers.

Searching for structural homologs

A search for structural homologs in the Protein Data Bank was performed with the DALI server (<http://www.ebi.ac.uk/dali>). Several homologs with relatively high Z-score values were found. The best match with a Z-score and RMSD of 18.1 and 2.7, respectively, was found for a monomer of the thua-like protein of unknown function from *Bacillus stearothermophilus* (PDB ID 1t0b). The superposition of monomers of Stm3548 and the thua-like protein reveals structural similarity for seven β -strands (β_4 , β_1 , β_5 , β_6 , β_{13} , β_{11} , β_{12}) and three helices (α_1 , α_6 , α_8) (Fig. 5). However, the sequence identity between Stm3548 and the thua-like protein was very low (only 16% over equivalent positions) and the quaternary structures of these proteins were completely different.

A rather high degree of homology was found with the anthranilate synthase from *Sulfolobus solfataricus* (Z-score = 11.2, RMSD = 3.3). In this case, as in several other cases, the similarity was to the β -strands and three α -helices. Among all structural homologs that were found, no monomer had a similar extended β -hairpin and no structure had a similar packing of the monomers in the asymmetric unit. This is why we suggest that Stm3548 presents a unique protein structure and a new protein hexameric state.

Analysis of conserved residues

In an attempt to reveal residues that can be important for the function of Stm3548, a primary sequence alignment of close members of the protein family, DUF1355, was performed. The sequences were aligned using the program Clustal X [17]. Figure 6 shows an alignment of 10 members of the DUF1355 family. All these sequences belong to proteins of unknown function. Approximately 20% of the residues are fully conserved in this family. The structure of Stm3548 is the first in this family. Structural analysis maps sequence conservation to few areas of the protein. Most of the conserved residues are observed in the regions of interdomain interactions. For example, the largest number of conserved residues are revealed at the twist of the β -hairpin (residues 23–30), in the region between the β_5 strand and the α_4 helix (residues 81–90), on the β_6 strand and on the loop that follows the β_6 strand (residues 112–130), at the β_8 strand (residues 153–161) and on the loop between the β_{13} strand and the α_7 helix (residues 222–228). This

suggests that the oligomeric nature of this protein must be strongly conserved. All these residues, except residues 112–120, are on the surface of the monomer in the regions that facilitate interactions with other domains.

In addition, residues Asp28, Asn86 and Arg155 are involved in interdomain interactions, and are also conserved. The salt bridge between Asn86 and Arg155 is most likely bifurcated; therefore, this interaction is very tight.

Different criteria can be used for predicting the oligomerization state of a protein. For example, the PQS server (<http://pqs.ebi.ac.uk>) uses among other criteria a value of 400 Å² for a lower limit of lost solvent accessible area and the presence of interchain salt bridges. Another criterion is based on the idea that residues of true oligomeric contacts have to be conserved across a series of homologous proteins [18]. There are also criteria based on a theoretical evaluation of chemical stability of possible oligomers [16].

As evaluated by the PQS server, the hexamer shows a loss of 4016 Å² solvent accessible surface area upon complex formation and a gain of −134.6 kcal/mol in solvation free energy of folding. Based on the findings that the residues that participate in interdomain interaction are highly conserved, including interdomain salt bridges, and the buried solvent accessible area for the hexamer is significant, we propose that the hexamer is most likely the biologically relevant unit for the whole protein family. Furthermore, studies of Stm3548 in solution using static light scattering at various Stm3548 concentrations in crystallization buffer showed the average molecular weights 5.6–5.9 times the predicted molecular weight of monomer confirming the presence of the hexamer in solution.

Putative functional site

The function of the protein Stm3548 is unknown. Nevertheless, based on analysis of the structure, we hypothesize about the possible functional/catalytic site. On the surface of the monomer, in the area of interdomain interactions, there is a region that contains a high concentration of residues with a potential for catalysis. These are Glu15, Ser81, Cys224, Asp82, Tyr124, Arg155, Glu157, Ser222, Asp223, Cys224, Ser225, His227, and Trp228. Catalytic sites often occur at domain or subunit interfaces. All these residues are conserved. In weighted difference map, all six subunits had additional positive density in front of residues Asp82, Asp223 and His227, which was modeled as a glycerol molecule. Perhaps the glycerol, used as a cryo-protectant, locates an empty ligand binding site. Further investigations are needed to confirm or reject this hypothesis.

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Fig. 1. Overall structure of Stm3548 hexamer. The subunits are colored blue (A), green (B), magenta (C), pale blue (D), pale pink (E) and pale green (F)

Fig. 2.
The ribbon diagram of the monomer Stm3548. The monomer is colored blue to red, from the amino- to carboxy-terminus. The most prominent feature is the hairpin containing $\beta 2$ and $\beta 3$ strands

Fig. 3.

Two orthogonal views of Stm3548 structure. The hexamer presented in (a) is related to the hexamer presented in (b) by a 90 degree rotation toward the reader. (a) Subunits A and D (blue and pale blue) are shown down the two-fold rotation axis that relates them. (b) A view down the three-fold noncrystallographic rotation axis with subunits from one layer colored

Fig. 4.
Electrostatic potential mapped on the surface of the Stm3548 protein. The surface is colored red for negative charge, and blue for positive charge

Fig. 5. Superposition of the monomers of Stm3548 (rainbow N to C) and the thua-like protein (grey) that is the closest structural homolog found by DALI server

Fig. 6.

Multiple sequence alignment of close members of the protein family DUF1355 [17]. Secondary structure elements of Stm3548 are plotted above the sequence and consensus residues for the DUF1355 family are shown below sequence alignment. A list of proteins and organisms which sequences were used in the sequence alignment: >gi|16766834 Stm3548 putative cytoplasmic protein [*S. typhimurium* LT2]; >gi|91211258 hypothetical protein UTI89_C2245 [*E. coli* UTI89]; >gi|16502824 Orf 245 protein; putative pathogenicity island protein; [*S. enterica* subsp. *enterica* serovar *Typhi*]; >gi|56127903 hypothetical protein SPA1472 [*S. enterica* subsp. *enterica*; serovar *Paratyphi A* str. ATCC 9150]; >gi|90576406 hypothetical protein CdifQ_02000344 [*Clostridium difficile* QCD-32g58]; >gi|88856977 putative cytoplasmic protein [*Marine actinobacterium* PHSC20C1], >gi|14027126 [*Mesorhizobium loti*

MAFF303099]; >gi|77390117 putative cytoplasmic protein [*Rhodobacter sphaeroides* 2.4.1]; >gi| 17741430 conserved hypothetical protein [*Agrobacterium tumefaciens* str. C58]; >gi| 66044027 Protein of unknown function DUF1355 [*Pseudomonas syringae* pv.; *syringae* B728a]

Table 1

Data collection statistics

Space group	P2 ₁ 2 ₁ 2	
Unit cell parameters	a = 182.89 Å, b = 79.37 Å, c = 115.66 Å, α = β = γ = 90.0	
Wavelength (Å)	0.9794	0.9795
Resolution (Å)	50–2.25	50–2.25
Number of unique reflections	75833	70652
Completeness (%)	94.1 (68.4)	87.1 (63.6)
all range (last shell)		
R-merge (%)	10.1 (56.5)	8.0 (53.2)
all range (last shell)		
I/σ all range (last shell)	24.6 (2.1)	25.5 (1.7)

Table 2

Refinement statistics

Resolution range (Å)	50–2.25
No. reflections in the working set	72006
R _{work} /R _{free} (%)	17.7/22.6
No. residues	1478
No. water molecules	548
No. glycerol molecules	6
No. Se atoms	42
Average atomic B-factor (Å ²)	38.5
% residues in the favored region of the Ramachandran plot	97.1
RMS deviation	
Bond lengths (Å)	0.012
Bond angles (deg.)	1.417