PROTEIN STRUCTURE REPORT

Crystal structure of secretory abundant heat soluble protein 4 from one of the toughest “water bears” micro-animals Ramazzottius Varieornatus

Yohta Fukuda and Tsuyoshi Inoue*

Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstract: Though anhydrobiotic tardigrades (micro-animals also known as water bears) possess many genes of secretory abundant heat soluble (SAHS) proteins unique to Tardigrada, their functions are unknown. A previous crystallographic study revealed that a SAHS protein (RvSAHS1) from one of the toughest tardigrades, Ramazzottius varieornatus, has a β-barrel architecture similar to fatty acid binding proteins (FABPs) and two putative ligand binding sites (LBS1 and LBS2) where fatty acids can bind. However, some SAHS proteins such as RvSAHS4 have different sets of amino acid residues at LBS1 and LBS2, implying that they prefer other ligands and have different functions. Here RvSAHS4 was crystallized and analyzed under a condition similar to that for RvSAHS1. There was no electron density corresponding to a fatty acid at LBS1 of RvSAHS4, where a putative fatty acid was observed in RvSAHS1. Instead, LBS2 of RvSAHS4, which was composed of uncharged residues, captured a putative polyethylene glycol molecule. These results suggest that RvSAHS4 mainly uses LBS2 for the binding of uncharged molecules.

Keywords: tardigrades; X-ray crystallography; secretory abundant heat soluble protein; anhydrobiosis

Introduction
Water is indispensable for all living things. Therefore, severe loss of water results in death for almost all organisms. However, some species of tardigrades, or water bears, can survive extremely desiccated conditions by stopping metabolic processes.1,2 This state is called anhydrobiosis. In association with loss of water, anhydrobiotic tardigrades form structures called “tun”, which show a high tolerance to desiccation. They can restart metabolic processes once their tuns are given water.3,4 The tun is also tolerant to very high or low temperature,5,6 exposure to radiation,7–9 vacuum,10,11 high pressure,12,13 and toxic compounds.14,15 This resistance of tardigrades is
further highlighted by an experiment in which two tardigrades in dehydrated states could survive in the vacuum of outer space for 10 days.\(^{16}\) Several research groups have recently launched genomics\(^{17–22}\) and molecular biological studies\(^{23–26}\) to reveal the molecular basis for anhydrobiosis of tardigrades. Some proteins discovered in these studies are thought to be keys to anhydrobiosis because they have not been found in phyla other than Tardigrada.\(^{23,25}\) Secretory abundant heat soluble (SAHS) protein is one of them and is constantly expressed at high levels in Ramazzottius varieornatus.\(^{21}\) This tardigrade can enter an anhydrobiotic state in a shorter time than other species such as Hypsibius dujardini.\(^{24,27}\) Although the expression levels of SAHS proteins are low in active H. dujardini, they are significantly increased when the tardigrade undergoes anhydrobiosis.\(^{21}\) These findings suggest that SAHS proteins play an important role in stress tolerances; however, their functions are unknown. Moreover, it has been recently reported that an anhydrobiotic tardigrade, Milnesium tardigradum, does not have SAHS proteins,\(^{22}\) making the role of SAHS proteins more enigmatic. One of SAHS proteins from R. varieornatus (RvSAHS1) is secreted into the culture medium when it is expressed in human cells; therefore, SAHS proteins are thought to protect extracellular components and/or secretory organelles on anhydrobiosis.\(^{23}\) A crystal structure of RvSAHS1 revealed that it has a \(\beta\)-barrel structure resembling fatty acid binding proteins (FABPs).\(^{28}\) Residues found in the \(\beta\)-barrel of RvSAHS1 were bulky and hydrophilic, while smaller and/or hydrophobic residues are assembled in FABPs. Some FABPs conserve tyrosine and arginine at the binding site for fatty acids (FAs) and RvSAHS1 also has Arg161 and Tyr163, which are superimposed on conserved tyrosine and arginine in FABPs. Moreover, electron density that can be interpreted as an FA molecule is observed near these residues and this site is designated as ligand binding site 1 (LBS1). The analysis of RvSAHS1 also showed electron density that can be interpreted as acetate at the putative ligand binding site 2 (LBS2). The sequence alignment indicates that residues located around LBS1 and LBS2 are conserved among many SAHS proteins [Fig. 1(A)]. However, several SAHS proteins such as RvSAHS4 have different sets of residues around LBS1 and LBS2, which implies differences in functions. Here we analyzed a crystal structure of RvSAHS4 to compare it with RvSAHS1.

**Results and Discussion**

The unit cell of the RvSAHS4 crystal contained two RvSAHS4 molecules (MolA and MolB) [Fig. 1(B)]. The RvSAHS4 structure was refined at 1.5 Å resolution. The overall structure of RvSAHS4 shared a typical FABP fold having an antiparallel \(\beta\)-barrel composed of 10 \(\beta\)-strands, and a helix-turn-helix lid between \(\beta\)A and \(\beta\)B. Because main chain atoms could not form hydrogen bonds between \(\beta\)A and \(\beta\)E, there was a gap as is found in RvSAHS1 and FABPs.\(^{28}\) The N-terminal region of the adjacent molecule was inserted into this gap in the crystal structure [Fig. 1(B)]. The structure of RvSAHS4 was well superimposed on that of RvSAHS1. Helix \(\alpha\)II in the RvSAHS4 structure had only a single turn and shorter than those in FABPs as is observed in RvSAHS1 structures. However, MolB in the RvSAHS4 structure had a 3\text{,}10 helix (\(\eta\)1) following \(\alpha\)II and MolA also had a helix-like structure in the same region [Fig. 1(C)]. Consequently, RvSAHS4 had a longer helical region than that in RvSAHS1. The \(\alpha\)II-\(\eta\)1 and following loop region showed higher diversity in amino acid sequences as compared to other regions [Fig. 1(A)], and tended to have proline, glycine, and tyrosine residues, which often inhibit the formation of a helix.\(^{28}\) B-factor values showed that the lid region of RvSAHS4 was highly flexible (Fig. S1, Supporting Information), as is observed in RvSAHS1.\(^{28}\) The variety of residues and the flexibility in the lid structure may be related to ligand binding.

As was predicted by alignment of amino acid sequences, the interior of RvSAHS4 included residues distinct from those found in RvSAHS1 [Fig. 2(A, B)]. Superimposition of the RvSAHS4 structure on the RvSAHS1 structure revealed that although Arg and Tyr, key residues for the binding of FAs at LBS1 in RvSAHS1, were conserved in RvSAHS4 (Arg164 and Tyr166), Tyr65 positioning on \(\eta\)1 could inhibit the binding of FAs at LBS1 [Fig. 2(B)]. Moreover, residues giving positive charges to LBS1 in RvSAHS1 (His72 and Lys150), which can stabilize negative charges of FA molecules, were replaced by Gln153 and Leu173 in RvSAHS4. While LBS1 in RvSAHS1 possesses an endogenous FA molecule originating from _Escherichia coli_ in the crystal structure, no electron density corresponding to an FA molecule was observed around LBS1 in RvSAHS4. Because RvSAHS4 was expressed and purified by the same methods as those for RvSAHS1 and crystallized under a condition (Materials and Methods section) similar to that for RvSAHS1 [100 mM HEPES pH 7.8, 150 mM MgCl\(_2\), 1 mM ZnSO\(_4\), 21% v/v polyethylene glycol (PEG) 600, and 5% v/v 1-butanol or 3% v/v 2-propanol at 20\textdegreeC],\(^{28}\) the absence of the FA molecule indicates that LBS1 of RvSAHS4 has lower affinity to FAs than that of RvSAHS1. To use LBS1 in RvSAHS4, a dramatic structural change in the helix-turn-helix lid may be needed, by which Tyr65 is moved away from LBS1. Residues located at LBS2 in RvSAHS4 were also distinct from those in RvSAHS1. LBS2 in RvSAHS4 was constituted by Cys120, Val142, Tyr144, and Gln153, which respectively correspond to Tyr117, Asp139, Tyr141, and Lys150 in RvSAHS1 [Fig. 2(B)]. Moreover, Tyr75 and Thr129 were located
around LBS2 where Leu74 and Ala126 are, respectively, located in Rv\textsuperscript{SAHS1}. Cys120 was specific to Rv\textsuperscript{SAHS4} [Fig. 1(A)] and could form a hydrogen bond with Thr129 [Fig. 2(C)] and Tyr75. Slender electron density was observed near Thr129 and Tyr75. Hydrophobic compounds such as FAs, bile acids, and retinoid, which are ligands for FABP family proteins\textsuperscript{30} could not fit well into the electron density. Therefore, we modeled short PEG molecules contained in the crystallization solution (triethylene glycol in MolA and tetraethylene glycol in MolB) [Fig. 2(C), S2, Supporting Information]. Although PEG molecules seemed to be the best assignment, we do not exclude the possibility that there was another compound such as a disordered FA molecule. The terminal oxygen atom of the PEG molecules could form hydrogen bonds with Thr129, Tyr75, and a nearby water molecule. Other oxygen atoms in the PEG molecules could form hydrogen bonds with water molecules in the \( \beta \)-barrel. Our observation that LBS1 in Rv\textsuperscript{SAHS4} is blocked by Tyr65 and that PEG molecules were found at LBS2 [Fig. 2(C), S2, Supporting Information].
suggests that RvSAHS4 mainly uses LBS2 to capture ligands. Because neither positively nor negatively charged residues were positioned at LBS2, it may be used to capture uncharged ligands such as alcohols and aldehydes. RvSAHS4 contained several water molecules stabilized by hydrogen bonds around the bottom of the β-barrel [Fig. 2(C), S2, Supporting Information]. In contrast, there are only one or two water molecules around the β-barrel bottom of RvSAHS1.28 This is because hydrophobic residues in RvSAHS4 (Leu73, Val86, and Phe155) were substituted by residues forming hydrogen bonds with each other in RvSAHS1 (His72, His83, and Tyr152). A hypothesis suggests that RvSAHS1 having the hydrogen bond network being independent of water is stable even under dehydrated conditions.28 In line with this thinking, RvSAHS4 containing more water molecules might be more vulnerable to desiccation than RvSAHS1, implying that roles of SAHS proteins may not be limited to responses to dehydration stresses.

Materials and Methods

Protein expression and purification

A synthetic gene of RvSAHS4 (RvSAHS428–171) was purchased from GenScript and subcloned into the pET-28a(+) vector (Novagen). A 6× His tag and a TEV protease site (H6-ENLYFQS) was fused at the N-terminus of the RvSAHS4 sequence. Therefore, the purified protein contained an extra serine residue at its N-terminus (The full DNA and amino acid sequences are shown in the text of Supporting Information). The resulting plasmid was used to transform E. coli strain BL21 Star(DE3). The cells were cultivated in Luria-Bertani medium supplemented
Table I. Data Collection and Refinement Statistics

|                      | RvSAHS4 |
|----------------------|---------|
| Data collection      |         |
| Wavelength (Å)       | 0.90000 |
| Space group          | P1      |
| a, b, c (Å)          | 32.8, 41.9, 53.5 |
| α, β, γ (°)          | 89.97, 80.75, 78.76 |
| Resolution range (Å) | 50.0–1.50 (1.53–1.50) |
| No. of unique reflections | 42,396 (2,099) |
| Completeness (%)     | 95.9 (95.5) |
| Redundancy           | 2.0 (2.0) |
| Rmerge (%)           | 3.6 (2.9) |
| Rfree (%)            | 5.1 (36.8) |
| CC1/2                 | (0.818)/(0.949) |
| R-work/R-free (%)    | 15.9/20.3 |
| No. of non-H atoms   | 2543 |
| Bonds (Å)            | 0.007 |
| Angles (°)           | 1.224 |
| Average B factors (Å²) |         |
| Overall              | 23.8 |
| Protein              | 23.4 |
| Water                | 34.7 |
| Other                | 30.6 |
| Ramachandran plot    |         |
| Favored (%)          | 100 |
| Disallowed (%)       | 0 |
| PDB code ID          | 5Z4G |

*a Data in the parenthesis was calculated based on the highest resolution shell. 
*b CC1/2 and CC* are Pearson correlation coefficient of two half data sets and an estimate of the true CC value, respectively. CC* is calculated from the following equation: CC* = [2CC1/2/(1 + CC1/2)]1/2.

with 100 µg/mL kanamycin at 37°C to an optical density at 600 nm of 0.6. Expression of RvSAHS4 was induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside, and culturing continued for further 19 h at 20°C. Collected cells were sonicated in a lysis buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, and 5 mM imidazole). After the lysate was centrifuged, the supernatant was purified by a HisTrap TALON column (GE Healthcare). Fractions containing RvSAHS4 were collected and dialyzed with TEV protease against a buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl). The dialysate was further purified by a HisTrap Excel column (GE Healthcare) and a HiLoad 16/60 superdex 75 column (GE Healthcare).

Crystalization
Crystallization was performed by the hanging drop vapor-diffusion method. RvSAHS4 crystals appeared within 1 week in the presence of 17 mg/mL protein, 50 mM HEPES pH 7.8, 1 mM ZnSO4, and 24% v/v PEG 600 at 20°C. The reservoir solution (400 µL) was poured into 0.5 mL sample cups (Sanplatec), and 1 µL protein solution was mixed with 1 µL reservoir solution on a siliconized cover glass plates. A single plate crystal was peeled off from a petal-like cluster. Before the crystal was frozen by liquid nitrogen, it was soaked in the crystallization solution supplemented by 20% v/v ethylene glycol.

Data collection, processing, structure solution, and refinement
X-ray diffraction experiments were performed on the BL44XU beamline of SPring-8, Hyogo, Japan, at 100 K using a MX300-HE detector (Rayonix). The dataset was processed using HKL2000. The phases were determined by the molecular replacement method with a monomer of RvSAHS1 (PDB code ID: 5XNA) as a search model using Phaser. Manual model building was performed using Coot. The program Refmac5 implemented in the CCP4 suite and phenix.refine were used for refinement. The final model was checked by MolProbity. Data collection and processing statistics are summarized in Table I. The diffraction dataset has been deposited in Integrated Resource for Reproducibility in Macromolecular Crystallography (https://proteindiffraction.org/). The atomic coordinate and structure factor have been deposited in the Protein Data Bank (PDB code ID: 5Z4G).

Sequence alignment
Amino acid sequence alignment was performed by Clustal Omega. The figure of sequence alignment was generated by ESPript.

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