SUPPLEMENTARY MATERIALS

Materials and methods

Cloning, expression, and purification of afGspE

The AfGspE gene was amplified from *A. fulgidus* genomic DNA by standard PCR method, and cloned into the pET-11a expression vector (Novagen). Native afGspE was expressed in *E. coli* BL21 (DE3) RIL (Stratage). Proteins expression was induced by addition of 0.4 mM isopropyl-β-galactopyranoside when cultures reached an O.D. of ~0.6 at 600 nm. The cells were thawed in the lysis buffer containing 20 mM Tris-HCl (pH7.5), 250 mM NaCl, 0.1 mM EDTA. After the disruption by sonication, sample was heat treated at 70 °C for 10 min. Supernatant was separated by centrifuge, and final 20% saturated ammonium sulfate was added. Sample was then loaded onto HiTrap Phenyl sepharose column equilibrated with the buffer A, containing 20 mM Tris-HCl (pH7.5), 20% saturated ammonium sulfate, and 0.1 mM EDTA. Proteins were eluted in linear gradient from 20 to 0% saturated ammonium sulfate. Pooled fractions were dialyzed against the buffer B, containing 20 mM Tris-HCl (pH7.5), 100 mM NaCl, 0.1 mM EDTA, and applied onto Q sepharose HP column. AfGspE was eluted at 0.25 M NaCl by the linear gradient from 0.1 to 0.3 M NaCl. AfGspE was further purified by gel filtration using Sephacryl S-300 equilibrated with buffer B. The Se-Met substituted afGspE was expressed in *E. coli* B834 (DE3) in the presence of 50 mg/l of Se-Met. Se-Met afGspE was purified by the same procedure as native afGspE, except for the adding of 2 mM DTT to lysis buffer. AfGspE mutant K273A was generated using QuickChange site-directed mutagnesis kit (Stratagene), and proteins were purified with the same procedure as the wild-type protein.

Crystallization of Se-Met afGspE

All afGspE crystallizations were done with the Se-Met substitute proteins, because the native protein did not form single crystals. The Se-Met afGspE (10 mg/ml in 20 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 7.5) with 10 mM AMP-PNP and 10 mM MgCl₂ was crystallized by hanging drop vapor diffusion by mixing 1 µl of protein solution and 1 µl of reservoir solution (10% PEG 8k, 5% PEG 400, 1.5 M NaCl, 0.1 M Na/K phosphate, pH 8.9). Adding 10 mM L-cysteine improved crystal quality. Crystals were transferred into stabilization buffer (10% PEG 8k, 10% PEG 400, 1.5 M NaCl, 0.1 M Na/K phosphate, pH 8.9), and then flash cooled at liquid nitrogen temperature. A 3.10 Å resolution MAD data set (Supplementary table 1) were collected at beamline 9-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). Peak data set to 2.95 Å resolution was further processed as the reference data set (non-anomalous) used for refinement. Crystals were in space group R32 with cell
Hexameric structures of archaeal secretion ATPase

Yamagata and Tainer, 2006

dimensions \(a = b = 132.864, c = 442.261\) with two afGspE in the asymmetric unit. In addition, afGspE was co-crystallized in the presence of 0.2, 0.5, 1 mM AMP-PNP and 10 mM Mg\(^{2+}\). These data were collected at beamline 5.0.2 and 12.3.1 at advanced light source (ALS). The data was processed to 3.10 Å resolution, except for that the data in the presence of 0.2 mM AMP-PNP reached 3.25 Å resolution. The data for apo-afGspE to 3.15 Å resolution was collected at beamline 5.0.2 at ALS. All data were processed with the HKL2000 suite (Otwinowski and Minor, 1997).

**Structure determination and Refinement**

The AMP-PNP-afGspE structure was determined by multi-wavelength anomalous dispersion (MAD) method. As afGspE has 17 methionine residues in monomer, there are 34 methionine residues in one asymmetric unit. The dual space direct method of the program Shake-n-Bake (Weeks and Miller, 1999) successfully identified 28 sites in 34 sites. These sites were then used for MAD phasing by SHARP (de La Fortelle and Bricogne, 1997). The electron density map was improved by solvent flipping with SOLOMON (Abrahams and Leslie, 1996). We manually built the initial model by the program XFIT (McRee, 1999) including partial models with RESOLVE (Terwilliger, 2002). The model was refined by simulated-annealing and energy-minimization with CNS (Brunger et al., 1998). Stereochemistry was assessed by PROCHECK(Laskowski et al., 1993), with 84.2 % in most favored region and no residues in the disallowed region in the Ramachandran plot for molecule B, and with 81.5 % in most favored region and one residue in the disallowed region for molecule A (Laskowski et al., 1993). The models for the other afGspE structures were first obtained by rigid body refinement using AMP-PNP-afGspE structure without AMP-PNP and Mg\(^{2+}\) and then by model building to the \(2F_O - F_c\) and \(F_O - F_c\) difference Fourier electron density maps.

In Apo afGspE structure, there are positive electron densities within the P-loop residues in both \(2F_O - F_c\) and \(F_O - F_c\) difference Fourier maps. A single phosphate ion was fitted to this density peak in molecule A, whereas three phosphates ions in that in the closed form (molecule B). As the B-factors for two of the three phosphates fitted in the closed form were significantly higher (~90 Å\(^2\)) than the other phosphate (~60 Å\(^2\)), they have lower occupancy (~60%). Yet, replacement of either phosphate with a water molecule yielded B-factors in the 1~3 Å\(^2\) range, excluding water molecules. The formation of hexameric rings for the apo-state under the crystallization conditions (0.1 M Na/K phosphate, 1.5 M NaCl, but no precipitants) was confirmed by EM analysis (not shown), while the apo-state in Tris buffer without phosphate (i.e. 20mM Tris-HCl, 100 mM or 1.5 M NaCl), did not form ring structures.
Electron Microscopy

For electron microscopy, we basically used two different buffers; Tris buffer (20 mM Tris, 100 mM NaCl, at pH 7.5), and phosphate based buffer, such as 1× PBS buffer. In the case of Tris buffer, the excess amount of Mg\(^{2+}\) ion against nucleotide was required for ring formation of afGspE. For example, a 0.1 mg/ml afGspE incubated with 10 mM AMP-PNP and 25 mM MgCl\(_2\) in Tris buffer was shown in Figure 2B. Similarly, ring structures in the presence of ADP or ATP plus 4-5 fold excess of Mg\(^{2+}\) were observed in Tris buffer. In phosphate-based buffer, such as 1× PBS buffer, afGspE forms ring structure even in the same Mg\(^{2+}\) concentration as nucleotide, such as 10 mM AMP-PNP and 10 mM Mg\(^{2+}\). In both buffer conditions, we observed no ring structures without nucleotide, except for the crystallization condition buffer, containing 0.1 M Na/K phosphate and 1.5 M NaCl.

Solution small angle x-ray scattering

SAXS data for afGspE hexamer were collected at the SIBYLS beamline at ALS using MAR 165 CCD area detector (165 mm diameter). A 10 mg/ml afGspE was incubated in the presence of 2 mM nucleotide and 10 mM MgCl\(_2\) in 20 mM Tris-HCl, 100 mM NaCl at pH 7.5 for 10 min at 37 °C, and then centrifuged to remove the aggregation. A 15 µl of samples was exposed for 6 s and 60 s, with an energy of 10 kV (\(\lambda = 1.239\) Å), in the momentum transfer range 0.008 < s < 0.30 Å\(^{-1}\) (s = 4\(\pi\)sin\(\theta\)/\(\lambda\), where \(\theta\) is the scattering angle). For ATP state, afGspE was incubated with 2 mM ATP and 10 mM MgCl\(_2\) for 5 min, and after the centrifugation, SAXS data was immediately measured. The data with different exposure times were merged together using PRIMUS(Kovnarev, 2003). The pair distance distribution function, \(P(r)\), was calculated by GNOM(Svergun, 1992). The maximum dimension of the particle (\(D_{max}\)), was calculated by estimating the \(P(r)\) function by changing \(r_{max}\) in 1 Å increment, with GNOM. The scattering curves were calculated from the crystal structure and the models, and compared with the experimental scattering curves using CRYSOL(Svergun, 1995). In addition, the fitting with multiple models were carried out by OLIGOMER(Kovnarev, 2003).

Fluorescence nucleotide binding assays

The fluorescence nucleotide analogs 2′, 3′-O-trinitrophenyl (TNP)-ATP and TNP-ADP were purchased from Molecular Probes, Inc. The concentrations of TNP-nucleotides were determined based on an extension coefficient of 26,400 M\(^{-1}\)cm\(^{-1}\) at 408 nm. A 0.187 or 0.177 µM of TNP-ATP or TNP-ADP, respectively, was consecutively added to and mixed with micro stirrer in a 3 ml of cuvette, in the presence or absence of 0.94 µM afGspE in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM
MgCl₂. After 2 min incubation at 37 °C, fluorescence emission at 540 nm was measured upon excitation at 410 nm. The fluorescence difference (ΔF) between in the presence and absence of afGspE was titrated by the concentration of TNP-nucleotide. The hydrolysis activity for TNP-ATP by afGspE was measured by detecting the release of inorganic phosphate using EnzCheck phosphate assay kit (Invitrogen). A 0.2 mM TNP-ATP was mixed with 40 µM of afGspE in 20 mM Tris, 100 mM NaCl, with 200 µM of 2-amino-6-mercapto-7-methylpurine riboside, and purine nucleoside phosphorylase, according to manufacturer’s instruction, and then monitored for absorbance at 360 nm. No hydrolysis more than the background level was observed.

**Figure preparation**

All figures are prepared using PyMol (DeLano, 2002).
### Supplementary Table 1. Crystallographic data and Refinement statistics

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<th>AMP-PNP-afGspE</th>
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<td>23.6/5.3</td>
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</table>

Refinement statistics

| No. of reflections (working/test) | 29697/1542 | 24657/1200 |
| Total amino acids              | 1001       | 1001       |
| $R/R_{free} (%)^{c}$           | 22.6/23.3  | 22.0/23.5  |
| Rms deviation                  |            |            |
| bond length (Å)                | 0.007      | 0.009      |
| bond angle (°)                 | 1.4        | 1.5        |


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### Supplementary Figure legend

**Supplementary Figure 1.** The phylogenetic tree of the secretion ATPase superfamily. The figures are made according to Planet, et al (Planet et al., 2001).

**Supplementary Figure 2.** Bound AMP-PNP occupancies in the distinct open and closed subunits of the hexamer. The electron density shows the bound AMP-PNP in afGspE structures co-crystallized with 1 mM, 0.5 mM, and 0.2 mM AMP-PNP concentrations with 10 mM Mg$^{2+}$. The $Fo-Fc$ difference Fourier map (contoured at 3σ) was calculated using protein model and shown superimposed with the final model. The nucleotide binding site of the open form structure (left column) and shows similar occupancies of AMP-PNP to that of the closed form structure (right column) except at the lowest concentration, but only the closed form shows density for the bound Mg$^{2+}$ ion.

**Supplementary Figure 3.** (A) AMP-PNP type SAXS curves with various nucleotide analogs. To prove that the presence of γ-phosphate of nucleotide analogs induces afGspE all closed hexamer, SAXS...
data in the presence of ATPγS, and ADP-Vi, were collected. In addition, to investigate the effect of L-cysteine, used in crystallization, for the hexmer stabilization, SAXS data with L-cysteine was also analyzed. From top to bottom, green; AMP-PNP, dark green; AMP-PNP and 10 mM L-cysteine, magenta; ATPγS, black; ADP-Vi. These curves are all well fitted with all closed hexamer models, of which fitting $\chi^2$ values are as follows; 3.8 for AMP-PNP + L-cysteine, 3.7 for ATPγS, 5.0 for ADP-Vi. (B) The almost identical curves between ADP and ADP plus L-cysteine. SAXS data with ADP is shown in deep blue, and that with ADP plus 10 mM L-cysteine in light blue. In addition, the calculated scattering curve of all open hexamer is shown in orange. (C) SAXS curve in the presence of ATP. SAXS curve in the presence of ATP (magenta) shows similar profile with SAXS curve in the presence of ADP (blue) in 0.05 < s< 0.3 Å, rather than that with AMP-PNP (green). However, in the very low angle range (s<0.05), SAXS curve with ATP shows the steep rise, which might be due to the aggregation. Ring structures in the presence of ATP were confirmed by negative-staining EM analysis (not shown).

References


