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Archaeal flagellar ATPase motor shows ATP-dependent hexameric assembly and activity stimulation by specific lipid binding

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Ghosh *et.al.***Abstract**

Microbial motility frequently depends on flagella or type IV pili. Using recently developed archaeal genetic tools, archaeal flagella and its assembly machinery have been identified. Archaeal flagella are functionally similar to bacterial flagella and their assembly systems are homologous to type IV pili assembly systems of Gram negative bacteria. Therefore, elucidating their biochemistry may result in insights in both archaea and bacteria. Flal, a critical cytoplasmic component of the archaeal flagella assembly system in *Sulfolobus acidocaldarius*, is a member of the type II/IV secretion system ATPase superfamily, and is proposed to be bi-functional in driving flagella assembly and movement. Here we show that purified Flal is a Mn^{2+} -dependent ATPase that binds MANT-ATP with a high affinity and hydrolyses ATP in a cooperative manner. Flal has an optimum pH and temperature of 6.5 and 75°C for ATP hydrolysis. Remarkably, archaeal, but not bacterial lipids, stimulated the ATPase activity of Flal 3-4 fold. Analytical gel filtration indicated that Flal undergoes nucleotide-dependent oligomerization. Furthermore, small angle X-ray scattering (SAXS) analysis revealed an ATP-dependent hexamerization of Flal in solution. These results report the first detailed biochemical analyses of the motor protein of an archaeal flagellum.

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INTRODUCTION

Motility is vitally important for most prokaryotes, and various mechanisms utilized by bacteria and archaea for motility have been described in recent years [1]. In bacteria, the two most commonly used mechanisms, movement via flagellar rotation as well as twitching using the extension and retraction of type IV pili (T4P) have been studied extensively [2-3]. This has led to the identification and characterization of many components required for the assembly and function of these systems. Very little however is known about the assembly of archaeal surface appendages like flagella and pili, although a diverse variety has been identified [4-5]. In *Halobacterium salinarum* flagella have been reported to be involved in swimming motility [6-8] and shown to be important for swarming on solid surfaces in methanogens and *Sulfolobus solfataricus* [7-8]. Recent evidence further revealed that flagella in *S. solfataricus* are involved not only in surface attachment, but also in late phases of bio-film maturation [9-11].

Remarkably, available structural and genetic information indicated that the archaeal flagella are more similar to bacterial T4P than to the bacterial flagella [4, 12-14]. Genetic analysis in different flagellated archaea recently has been used to identify a genetic locus that encodes an archaeal flagella assembly system (AFAS) [15]. Within different archaea, this genetic locus contains seven to thirteen genes. Usually it contains several flagellin genes (*flaA* and/or *flaB* genes) followed by a conserved set of flagella-related protein genes (*flaC-flaJ*) [16]. The genetic locus in *Sulfolobales*, however, contains apart from the conserved set of flagella-related protein genes only one gene encoding a flagellin. Several components of the archaeal flagella assembly machinery show homology to proteins involved in type IV pilus (T4P) assembly, type II secretion (T2S), and type IV secretion (T4S) systems of Gram negative bacteria [4, 7, 17-21]. T2S systems in bacteria play a role in the secretion of toxins and enzymes, e.g. the cholera toxin of *Vibrio cholerae* [22], whereas T4S systems are used for the transport of DNA to either bacterial or eukaryal host cells as exemplified by *Agrobacterium tumefaciens* [23]. T4P are involved in a variety of functions such as twitching motility and cell adhesion which are essential for the pathogenicity of several harmful bacteria [14]. All three systems have significant structural and functional similarities pointing to a common origin [18]. Interestingly, the subunits that are found to be similar in those systems are also present in the AFAS [24]. These components include pre-flagellin peptidases (e.g. PibD of *S. solfataricus*) [4], FlaJ, that has homology to the polytopic membrane protein found in T4P systems (e.g. PilG of *Neisseria*) [17-18, 25] and FlaI, that has homology to type II secretion ATPases and T4P assembly ATPases (e.g. PilB, GspE, EpsE, HP0525) [18-19, 26-28]. Recently, three-dimensional reconstructions of electron microscopic images of archaeal flagella and of T4P revealed a considerable structural homology with type IV pili [13, 20-21, 29-30]. These collective results showed that the archaeal flagella and T4P systems show strong similarities and might be evolutionary related.

A central component found in both systems is the cytoplasmic ATPase that provides the energy. These ATPases belong to the "secretion superfamily ATPase" or "type II/IV secretion ATPase" family [18]. Proteins of this superfamily function in multiple macromolecular transport systems in bacteria and archaea, like T2S, T4S, T4P assembly, and in archaeal flagella assembly

systems [14, 18, 26-27, 31]. Members of this superfamily contain characteristic conserved motifs such as Walker A, Walker B and P-loop motifs (GX4GK(S/T)), which are involved in nucleotide binding. They also contain the Asp box motif involved in divalent cation binding and the His box motif possibly is involved in stabilization of the nucleotide binding [32].

Based on their sequence similarities, secretion ATPases were grouped into different clusters, including clusters for ATPases from T2S systems, T4P systems, and a cluster for archaeal ATPases [18]. Although both T2S and T4S are powered by secretion ATPases, the basic mechanism is different for these secretion systems. In T2S system, the ATPase, GspE is involved in assembly of the 'pseudopilin' filament called pseudopilus. In comparison, T4S system ATPases may function as protein translocators by interacting with the secreted protein already folded in the cytoplasm. In T4P assembly systems, two ATPases, PilB and PilT are present and function in assembly and disassembly of the pilus, respectively [33-35]. Interestingly, the amino acid sequence of Flal resembles bacterial T2S and T4P type ATPases, suggesting a common evolutionary root for all these secretion ATPases.

The archaeal flagellum is able to move clockwise and counterclockwise, which results in a switch in swimming direction [36]. The question arises how a type IV pilus would be able to move in this fashion, as normally twitching motility is conferred by filament assembly and disassembly and not by any rotational movement. Because Flal is the only ATPase identified in archaeal flagella operons, it is most likely involved in two functions: first in the assembly process and secondly in the movement leading to motility. Therefore, it is important to understand how this protein can perform both functions.

The specific role of the secretion ATPase in archaeal flagella assembly systems has not been addressed before. It has been shown for *Methanococcus maripaludis* and *Halobacterium salinarum* that Flal deletion mutants are impaired in assembly of their flagella [16, 19, 37], but neither have been biochemically characterized. The relation between ATP binding and hydrolysis and flagellum assembly and rotation remains unknown. In the present work, the secretion ATPase Flal from *Sulfolobus acidocaldarius*, a thermoacidophilic crenarchaeon that grows optimally at 70-80°C and pH 2-3, is biochemically and structurally characterized.

MATERIALS AND METHODS

Construction of a Flal overexpression vector. To enable expression of non tagged Flal or Flal with an N-terminal His-tag, the *flal* gene (*saci_1173*) was amplified by PCR from genomic DNA of *Sulfolobus acidocaldarius* DSM639 using primer-pairs Flal₇₁₃/Flal₇₁₄ or Flal₇₂₈/Flal₇₂₉ and cloned as NdeI/BamHI or EcoRI/HindIII fragment into plasmid pETDuet-1(Novagen), yielding plasmids pSVA251 and pSVA263 respectively. For expression analysis, the respective vectors were transformed to *Escherichia coli* C43 cells containing the RIL^{Chl}-plasmid (Stratagene) [38].

Site-directed mutagenesis of flal. Mutagenesis of *flal* was performed by overlap PCR on vector pSVA263. Mutants were created by changing K²⁶⁸ within the predicted Walker A motif, D²⁹⁰ within the Asp-box and E³³⁶ within the Walker B motif to alanines. The resultant plasmids were named pSVA265 (K^{268A}), pSVA307 (D^{290A}), pSVA308 (E^{336A}) and pSVA314 (K^{268A}, E^{336A}). Sequences of the oligonucleotides used for mutagenesis are listed Table 1.

Expression of recombinant proteins in Escherichia coli. Ten milliliters of an overnight culture of *E. coli* C43 cells containing the RIL plasmid [38] and one of the respective over-expression plasmids was used to inoculate 1 liter of Luria-Bertani medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). Cells were grown at 37°C to an optical density at 600 nm of 0.6 was reached, at which moment 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The temperature was reduced to 16°C, and growth was continued over-night to reduce inclusion body formation. The cells were collected by centrifugation, resuspended in lysis buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol) containing the complete EDTA-free protease inhibitor cocktail (1 tablet/ 50 ml of lysate; Roche, Mannheim, Germany), frozen in liquid nitrogen and stored at -80°C.

Purification of recombinant proteins. Before purification, frozen resuspended cell pellets were thawed on ice. Cells were lysed by sonication with a Sonoplus HD3100 sonicator (Bandelin Sonorex Biotechnique, Germany) using probe HD3100. Cell debris was removed by centrifugation at 6000 x g for 15 min followed by centrifugation at 150,000 x g for 30 min at 4°C in a OptimaTM MAX-XP ultracentrifuge (rotor MLA 55) (Beckman Coulter, USA) to remove the membranes. The supernatant was incubated at 70°C for 20 min in a water bath. The sample was cooled down on ice and precipitated proteins were removed by centrifugation at 150,000 x g for 30 min at 4°C in a OptimaTM MAX-XP ultracentrifuge (rotor MLA 55) (Beckman Coulter, USA). For purification of His-tagged proteins, the supernatant was applied to a Ni²⁺-affinity column (Native IMAC) on the ProfiniaTM protein purification system (BioRad Laboratories, Inc, USA). Bound protein was eluted in lysis buffer containing 500 mM imidazole. The eluted fraction was dialyzed overnight at 4°C against 50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol. The dialyzed fraction was loaded on a Superdex 200 10/300 gel filtration column on an AKTA-Purifier system (GE Health Care, Germany), equilibrated with 50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol (flow rate 0.5 ml/min). The untagged Flal was expressed, sonicated and heat treated, as described above. The protein was then precipitated at room temperature with

20% ammonium sulfate (w/v) for 30 min. The precipitated protein was re-suspended in buffer Blue A (50mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA) and loaded on a 5 ml HiTrap BLUE column in the AKTA-Purifier system (GE Health Care, Germany). The column was washed with buffer Blue A and the bound protein was eluted with buffer Blue B (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM EDTA). The eluted protein was further purified on a 5 ml HiTrap PHENYL column. The protein was eluted using a gradient from buffer Blue B to buffer PHENYL (50 mM Tris-HCl, pH 8.0, 2 mM EDTA). The eluted protein was then concentrated (<2 ml) using an amicon concentrator system (Milipore, USA), and loaded on to a Superose 6 10/300 size-exclusion column pre-equilibrated with PHENYL buffer and eluted with 50 mM HEPES-NaOH buffer, pH 7.5 containing 150 mM NaCl.

Protein purity was monitored by reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Biosafe (BioRad) protein stain. Protein concentration was determined with the Bio Rad protein assay dye-dependent reagent (Bio Rad).

ATPase assay. ATPase activities were measured using the malachite green assay, as described previously [39]. Briefly, purified protein (2 µg) was pre-heated for 2 min in 100 µl assay buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 5 mM MgCl₂). Reactions were initiated by addition of 1 mM ATP, and incubated for 5 min at 70°C. The reaction was stopped by freezing in liquid nitrogen. The amount of released inorganic phosphate was determined using the malachite green assay. The data were corrected for non-enzymatic ATP hydrolysis. To determine the activity of Flal as a function of temperature, pH, nucleotides and divalent cations, the assay was performed at different temperatures, in different buffers (50 mM Citrate, pH 3.0-5.5; MES, pH 6.0-6.5; HEPES, pH 7.0-7.5; Tris-Cl, pH 8.0-9.5), with different NTPs (5 mM of ATP, GTP, CTP, and UTP), and in the absence of, or with different divalent cations (5 mM Na-EDTA or 5 mM MnCl₂ or 5 mM CaCl₂,) respectively. The ATPase activity was determined as a function of the ATP concentration, and the resulting data were fitted to the Hill equation $y = (ax^n)/(b^n + x^n)$, to obtain the V_{max} (a), the dissociation constant, $K_{0.5}$ (b) and the hill coefficient (n). To analyze the effect of tetraether lipids on ATP-hydrolysis by Flal, total lipids of *S. acidocaldarius* were extracted, as described previously [40]. The ATPase activity of Flal (1 µM purified protein) was measured as described above in the presence of different concentrations of isolated lipids (40, 80, 100, 200, 400, and 800 µg).

MANT-ATP binding assay. Binding of MANT-ATP (2'-/3'-O-(N'-Methylantraniloyl)adenosine-5'-O-triphosphate) was followed by FRET measurements using a temperature-controlled ISS PC1 spectrofluorometer (ISS Inc., USA). The excitation wavelength was set to 285 and the emission spectrum was recorded from 400 to 500 nm. The slit widths for the excitation and the emission beam were set to 1 nm. MANT-ATP binding affinities were determined by titrating 0.1 µM of protein with increasing concentration of MANT-ATP (0.125 µM – 25 µM) at 25°C in binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MnCl₂). Fluorescence spectra were corrected for the fluorescence of unbound MANT-ATP, and the resulting fluorescence at 450

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nm was fitted to the Hill equation $y = (ax^n/(b^n + x^n))$, to obtain the $\Delta F_{\max}$ (a), the dissociation constant, $K_{0.5}$ (b) and the hill coefficient (n).

Liposome pull-down assay. The liposome pull-down assay was performed as described previously [41]. Briefly, isolated and dried tetraether lipids from *S. acidocaldarius* were hydrated with liposome-binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$) and vigorously vortexed to ensure dispersion of the various lipids in a Sonoplus HD3100 sonicator (Bandelin Sonorex Biotechnique, Germany) using probe HD3100. Liposomes were collected from the supernatant after centrifugation (2,500 xg for 5 min) and used in subsequent assays. To perform experiments with *E. coli* polar lipids, a liquid form of polar lipid mixture was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). The polar lipids were dried in a vacuum desiccator for approximately 5 h. Dried lipids were hydrated with liposome-binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$) and treated in a similar manner as tetraether lipids to generate differentially sized liposomes for pull-down assay.

The pull-down assay was performed separately with 1 μ M of 6xHis-tagged Flal, Flal^{K268A} or Flal^{E336A} purified protein. The proteins were pre-incubated with 1 mM ATP at 25°C for 30 min in the nucleotide binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$) in a total volume of 100 μ L. Liposomes were added to a final lipid concentration of 80 μ g/ml and reactions were incubated at room temperature for 1 h. By centrifugation at 100,000 x g in a Beckman TLA100.3 rotor in a Optima™ MAX-XP ultracentrifuge (Beckman Coulter, USA) at 4°C for 30 min, the lipid vesicles were separated from the soluble fraction. The supernatant and pellet were analyzed on a 11% polyacrylamide gel and visualized with Coomassie blue.

Analytical gel-filtration. Purified Flal was concentrated to 1.0 mg/ml in 50 mM HEPES-NaOH buffer, pH 7.5, 150 mM NaCl by centrifugation in an amicon filter (10 kDa cutoff; Millipore). The sample was then pre-incubated at 50°C for 10 min followed by incubation at 4°C for 6 h with either 1 mM AMP-PNP (Adenylyl-imidodiphosphate) or ADP in the presence of 10 mM $MgCl_2$. The sample was loaded on a Superdex 200 10/300 column on the AKTA purifier system as described above. All the fractions were analyzed on SDS-PAGE and immunoblotted using anti-His antibodies (Abcam).

Small angle X-ray scattering (SAXS) analyses. SAXS data were collected at the ALS beamline 12.3.1 (SIBYLS, Lawrence Berkeley National Laboratory, Berkeley, CA) [42]. The scattering vector is defined as $q = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle. Prior to SAXS data collection Flal wild-type and Flal^{E336A} were applied to a superose 6 size exclusion column in 20 mM Tris, pH 7.0, 200 mM NaCl and 20 mM Tris pH 7.0, 200 mM NaCl, 1 mM ATP, 2.5 mM $MgCl_2$, respectively. Fractions from the column were analyzed directly and after 2-fold and 4-fold concentration of the sample using Corning Spin-X concentrators. All experiments were performed at 20°C, and data were processed as described [43]. The data acquired at short and long time exposure (1 and 10 s) were merged for calculations using the entire scattering spectrum. The experimental SAXS data for different protein concentrations were investigated for aggregation using Guinier plots [44]. The program

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GNOM [45] was used to compute the pair distance distribution functions, $P(r)$. This approach also provided the maximum dimension of the macromolecule, $D_{\max}$. The overall shapes were restored from the experimental data using the program GASBOR and averaging of 15 independent structures using the program DAMAVER [46]. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081, Pettersen et al, 2004). Theoretical scattering curves from crystal structures were calculated using FoXS [47].

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RESULTS

Expression, purification and biochemical characterization of Flal

Both His-tagged and wt Flal were expressed in *E. coli* and purified to homogeneity. Purified Flal was analyzed by SDS-PAGE, and found to be over 99% pure. Flal was tested for the ability to hydrolyze ATP under different conditions. The optimal conditions for ATP hydrolysis by Flal were determined to be pH 6.5 and 75°C (Figure 1A and 1B), which are close to the cytoplasmic pH and the optimal growth temperature of *Sulfolobus acidocaldarius*. Mn^{2+} was the preferred divalent cation for ATP hydrolysis (Figure 1C), as was observed for other secretion ATPases [26]. Considerable activity was however also detected in the presence of Ca^{2+} and Mg^{2+} , respectively (Figure 1C). Flal hydrolyzed ATP with the highest rate, but it was also able to hydrolyze GTP, CTP and UTP (Figure 1D), as seen for other ATPases. No conversion of ADP to AMP was detected (Figure 1D).

To determine the concentration dependency of ATP hydrolysis, the ATP hydrolysis rate was determined as a function of the ATP concentration (Figure 1E and 1F). The fit of the data with the Hill equation resulted in a V_{max} of 32 nmol Pi/mg protein/min, a $K_{0.5}$ of 10 mM and a Hill coefficient of 2.0. The Hill coefficient demonstrates that ATP hydrolysis is cooperative, suggesting that Flal oligomerizes during ATP hydrolysis. Flal had a very low turn-over rate (1 ATP/min), consistent with observations on other secretion ATPases [26]. Most of these ATPases are part of large macromolecular assemblies and other protein components of these complexes, for example, the membrane protein EpsL in type II secretion system, stimulate the nucleotide hydrolyzing activity of the ATPases [48-49]. Furthermore, lipids can enhance the ATPase activity of these proteins [48].

To assess the influence of archaeal tetraether lipids on the Flal ATPase activity, the specific activity of the enzyme was measured in the presence of increasing concentrations of *S. acidocaldarius* tetraether lipids (Figure 2). Remarkably, the tetraether lipids increased the ATPase activity 3-4 fold, whereas the addition of an *E. coli* total lipid extract only led to a 1.5 fold stimulation of the ATPase activity. This is in contrast to EpsE, which activity was only activated in the presence of both lipids and the cytoplasmic loops of the corresponding inner membrane protein and not by lipids alone [48].

Active site mutants of Flal

Secretion ATPases contain key conserved motifs for the Walker A and B motifs and the Asp-box. Alignment of *S. acidocaldarius* Flal with GspE, i.e. a secretion ATPase of *Archaeoglobus fulgidus* [27], led to the identification of these motifs in Flal (Figure S1). To determine the effects of mutations in these motifs, the lysine of the Walker A (K^{268A}), the glutamate of the Walker B (E^{336A}), and the aspartate of the Asp-box (D^{290A}) were mutated. A double mutant of the Walker A and B mutations Flal ^{K^{268A}/E^{336A}} was also constructed. These mutants were expressed and purified, and the expression levels and final purity of these proteins were equal to wt Flal (data not shown). The different mutants were tested for their ability to hydrolyze ATP (Figure 3A), and for their ability to bind the fluorescent ATP analog MANT-ATP (Figure 3B). Remarkably, mutation of the lysine of the Walker A motif to an alanine only resulted in a reduction of approximately 50% of ATP-hydrolysis

compared to wild type Flal, while mutagenesis of the aspartate of the Asp-box had no effect on ATP hydrolysis. By substituting E³³⁶ of the Walker B with an alanine, ATPase activity was reduced upto 90%. Analysis of the crystal structure of GspE from *A. fulgidus* (pdb: 2OAP) revealed that the glutamate residue (E³³⁶ in Flal) in the Walker B motif is involved in the interaction with Mg-ATP and also is highly conserved among secretion ATPases superfamily members from various archaeal species (Figure S1) [27]. Also for the *Myxococcus xanthus* PilT, which is the disassembly ATPase of the T4P assembly system, mutagenesis of the conserved glutamate residue in the Walker B box to alanine resulted in loss of ATPase activity [34]. Almost no ATP hydrolysis (2-3%) was detected for the Flal^{K268A/E336A} double mutant.

The MANT-ATP binding assay demonstrated that wt Flal bound MANT-ATP with a K_d of 0.46 μ M, a fit of the data with the Hill equation resulted in a Hill coefficient of 0.9, demonstrating that ATP binding, contrary to ATP hydrolysis was not co-operative. Remarkably, MANT-ATP binding to both the Walker B motif (K_d = 0.58 μ M) and Asp-box mutant (K_d = 0.43 μ M) was comparable to wt Flal, whereas binding to the Walker A mutant was strongly reduced (K_d = 9.6 μ M). Thus our data showed that mutagenesis of the Asp-box residue of Flal does not have any effect on ATP binding and hydrolysis, while mutation of the Walker B motif does not affect ATP binding, but heavily abolishes ATP hydrolysis. Notably, mutagenesis of the Walker A motif results in a 20-fold lower binding affinity but an only 2-fold reduction of ATPase hydrolysis activity. Presumably, the Walker A box substitution, i.e. K^{268A} in Flal, indirectly reduces ATPase activity by interfering with ATP binding. The Walker B box substitution on the other hand, i.e. E^{336A} in Flal, abolishes ATPase activity by directly interfering with the ATP hydrolysis, although ATP binding seems normal in this mutant (E^{336A}). Furthermore, the data suggest no role of the conserved D²⁹⁰ in the Asp-box motif in ATP binding and hydrolysis.

Oligomerization of Flal

Members of the secretion ATPases superfamily can oligomerize into higher oligomeric structures [27, 50-51]. To assess the oligomeric state of purified Flal (monomer molecular weight 55 kDa), his-tagged Flal was analyzed by size exclusion chromatography. Due to the presence of a small shoulder in the chromatogram, small amounts of a dimeric Flal seem to be present (Figure 4, fraction 13). To determine whether Flal could form higher oligomeric structures, the effect of nucleotide binding on Flal oligomerization was studied using the non-hydrolyzable ATP analog AMP-PNP. After preincubation with or without AMP-PNP and Mg²⁺, purified Flal was analyzed by size exclusion chromatography. Remarkably, after incubation with AMP-PNP, Flal was eluted at estimated molecular weights of 330 kDa and 55 kDa, respectively. This shows that Flal assembles in an oligomeric structure in a nucleotide-dependent manner in the presence of non-hydrolyzable ATP analogs. To ascertain that the same phenomenon occurs in Flal mutants, Flal with a mutation in either the Walker A motif (Flal^{K268A}) or the Walker B motif (Flal^{E336A}), which showed a reduced (~50%) and very low (~10%) ATPase activity, respectively, were also analyzed. After incubation with ATP, a higher oligomeric form was observed only for the Flal protein containing a mutation in the Walker B (Flal^{E336A}), but not for the Flal with a mutation in the Walker A motif (Flal^{K268A}) (Figure S2). This demonstrated that Flal with a mutation in the

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Walker B motif (FlaI^{E336A}) forms a stable oligomer after ATP binding. Therefore, wt FlaI and FlaI with a mutation in the Walker B motif (FlaI^{E336A}) oligomerize upon ATP binding. However, the oligomerization potential of Walker B motif mutant (FlaI^{E336A}) seems higher compared to wt FlaI, suggesting a more stable complex formation with ATP by the Walker B mutant (FlaI^{E336A}) (Figure S2). As expected, incubation of either the wt FlaI, or FlaI with a mutation in either the Walker A motif (FlaI^{K268A}) or the Walker B motif (FlaI^{E336A}) with ADP did not result in oligomerization of FlaI (data not shown).

SAXS analysis of oligomerization states of FlaI

To further analyze the oligomeric structure of FlaI, Small Angle X-ray Scattering (SAXS) was performed. SAXS is a powerful method to structurally analyze proteins in solution under physiological conditions [43]. In Figure 5, the SAXS analysis of wild-type FlaI is visualized in comparison to the Walker B mutant (FlaI^{E336A}) in the presence of ATP and Mg²⁺. The experimental data was compared with theoretical SAXS curves from the crystal structures of monomeric and hexameric GspE. In order to obtain a reasonable fit of the monomeric GspE to the SAXS data from monomeric FlaI, the N-terminal domain (in orange) had to be extended from the ATPase domain (green), which is an indication that there is a flexible conformation of these domains relative to one another. FlaI^{E336A} with ATP clearly forms a hexameric ring similar to the one determined for GspE.

Ab initio structures for both complexes agree well with the GspE models. For calculating the structure of the hexameric protein, we used both no symmetry constraint and 6-fold symmetry. Even without symmetry constraints, we obtained the closed ring structure, but the conformation of the N-terminal domains (orange) seems to be different for the single subunits. The SAXS data from FlaI in monomeric and hexameric form can be analyzed by the Kratky plot (Figure S3) to determine amounts of intrinsic flexibility. In the monomeric form of FlaI that is present in the cytoplasm, the amount of flexibility within the protein is much higher compared to the hexameric, membrane associated form. From this data, we propose that the N-terminal domains are flexible when FlaI has a cytoplasmic localization but may adopt a more rigid conformation when the proteins attach to the membrane or membrane proteins.

Liposome interaction studies

The structure of GspE showed an accumulation of hydrophobic patches at the interface which probably interacts with membrane-integral components. The same hydrophobicity was observed in FlaI models based on the GspE structures. Based on the 3-4 fold increase in FlaI ATPase activity upon addition of archaeal tetraether lipids, it was essential to ascertain that a direct interaction between lipids and FlaI occurs.

To this end, a liposome pull-down assay was performed in which FlaI, FlaI^{K268A} and FlaI^{E336A} were incubated either with *S. acidocaldarius* tetraether lipid liposomes (Figure 6A) or *E. coli* lipid liposomes (Figure 6B). In the case of the tetraether lipids, the FlaI association with the liposomes was ATP-dependent. When ATP was present, FlaI^{E336A} associated most strongly with

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the tetraether liposomes whereas Flal^{K268A} bound the least. The same results were observed when the same experiment was performed in the presence of AMP-PNP (Figure S4). These data imply that ATP binding by Flal is essential for its interaction with tetraether lipids. In contrast, the interaction of Flal with the *E. coli* lipids was independent of the presence or absence of ATP, showing that this is an unspecific interaction that is caused by the hydrophobic nature of the upper surface of the hexameric protein.

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DISCUSSION

Although archaeal flagella systems were identified some time ago, the role that the various flagellar subunits play during assembly and rotation and how they interact with each other during this process has not been elucidated [14, 18]. The secretion ATPase Flal is a central component of the flagellum assembly system. Previously, it was shown that Flal could hydrolyze ATP under *in vitro* condition [26]. Genetic analysis revealed its importance in the assembly process. *flal* deletion mutants of *M. maripaludis* and *H. salinarum* were impaired in flagella assembly [16, 19, 37]. Furthermore, in *H. salinarum* it has been shown that flagellar rotation is dependent on the intercellular ATP concentration and not on the proton motive force, as is the case in bacteria [52]. These findings implicate Flal as an essential ATPase for flagellar assembly and/or rotation. However, to provide further insights into its roles, it is necessary to understand the molecular mechanism that Flal employs. Here, we demonstrated that Flal hydrolyzes ATP and undergoes an ATP-dependent oligomerization.

Computational analysis has identified the conserved ATP binding (Walker A), metal binding (Asp box), and ATP hydrolysis (Walker B) motifs in Flal. We demonstrated the importance of each of these predicted domains in ATP binding and hydrolysis by site-directed mutagenesis. ATP-hydrolyzing activity of Flal is more efficient using Mn^{2+} as the co-factor instead of Mg^{2+} or Ca^{2+} . This preference for Mn^{2+} as co-factor is congruent with secretion ATPases and kinases characterized from *S. solfataricus* [26]. Our data also revealed that the ATPase activity of Flal displays positive cooperativity, indicating that interactions between subunits occur in an ATP-dependent manner. However, we found that MANT-ATP binding is non-cooperative, probably because ATP analogs bind the monomeric or dimeric state of Flal under *in vitro* conditions. Analytical gel filtration demonstrated that in the presence of a non-hydrolyzable ATP analogue the monomer shifted to a higher oligomer and SAXS confirmed that the higher molecular species was a hexamer.

Importantly, archaeal tetraether lipids were found to enhance the ATPase activity of Flal, indicating that the Flal transition state intermediate is possibly stabilized in an ATP-bound state. The interactions of the ATPase motor with membrane lipids may therefore help regulate its actions.

Our data thus provide the first substantial insights into archaeal Flal function and probably preface the understanding of a common mechanism of Flal homologues in the Archaea. Generally, these data suggest a hexameric state of Flal in its ATP-bound state, when it is involved in flagellum assembly. Collectively, these data provide the basis for a model for the mode of function of Flal (Figure 7). Under *in vivo* conditions, Flal hydrolyses ATP and provides energy to the AFAS. It is still a matter of contention, whether the energy gained in this process is utilized for flagellar assembly by directing the translocation of structural components like flagellins or for the rotational movement of flagella or even for both.

Crystal structures of the archaeal secretion ATPase GspE, the *H. pylori* and *E. coli* hexameric T4S ATPases, as well as the hexameric PilT ATPase in the T4P assembly system of *P. aeruginosa* have been shown to use N-terminal domain movement to facilitate closed ATP-bound and open ADP-bound hexameric conformations [27-28, 51, 53-55]. Furthermore, it has been

postulated that these ATPases act as inner membrane gated transporters for structural proteins and may thus mediate the whole cycle of assembly by executing a conformational change from closed to open upon ATP hydrolysis. It is possible that ATP hydrolysis is closely coupled to flagellin export into the growing flagellar filament. In contrast to this idea, ATP hydrolysis of Flal might generate energy for the rotation of the flagellum as it was shown that in *H. salinarum* flagellar rotation is ATP dependent [52].

Understanding how components in AFAS function to effect proper assembly on the archaeal surface and also how they communicate to execute these functions are still at an early stage. The secretion ATPase Flal and polytopic membrane protein FlaJ are potentially involved in formation of the core of the assembly complex, and it has been postulated that on this Flal-J core is the main platform for flagellar assembly [12, 56]. However, another incomplete ATPase, FlaH has also been postulated to function in association with the FlaJ core to facilitate the process of assembly, disassembly or rotation. Understanding the biochemical and structural features of the Flal protein provides a basis to understand the function that this protein fulfills in AFAS which can be transferred to homologous ATPases from other T4P systems of bacteria.

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Table 1 Primers and plasmids used for assays

Bacterial strains, primers and plasmids	Relevant characteristics	Source or reference
Strains		
<i>E.coli</i>		
Dh5α	12 f80d/ <i>lacZDM15</i> D(<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (rK2 mK1) <i>supE44 thi-1 gyrA relA1</i>	GIBCO BRL Stratagene [38]
BI21(DE3)- RIL	B F- <i>ompT</i> hsdS(rB- mB-) <i>dcm</i> + Tetr <i>E. coli</i> gal λ (DE3) <i>endA</i> Hte [<i>argU ileY leuW</i> Camr]	
Walker cells- RIL (C43)	F- <i>ompT</i> gal <i>hsdSB</i> (rB-mB-) <i>dcm lon</i> _DE3 <i>endA</i> Hte [<i>argU ileY leuW</i> Camr]	
<i>Sulfolobus acidocaldarius</i>		
DSM639	Hyperthermo-acidophilic crenarchaeon which grows optimally at 80°C and pH 2-3	DSMZ
Primers		
FlaI713	5'-GGGGGCATATGAGCTTCGTGGAAGAC-3'; Forward primer for FlaI containing NdeI restriction site	This study
FlaI714	5'-GGGGGCTCGAGTCAAATATTACTGACATATTTTATC-3'; Reverse primer for FlaI containing XhoI restriction site	This study
FlaI728	5'- CCCCCGAATTCGATGAGCTTCGTGGAAGAC-3'; Forward primer for FlaI containing EcoRI restriction site	This study
FlaI729	5'- CCCCCAAGCTTCAAATATTACTGACATATTTTATC-3'; Reverse primer for FlaI containing HindIII restriction site	This study
FlaI742	5'- ATCTGGAGCGACAACACTAC-3'; Overlapping internal forward primer for K ₂₆₈ A mutation in FlaI	This study
FlaI743	5'- AATGTAGTTGTCGCTCCAG-3'; Overlapping internal reverse primer for K ₂₆₈ A mutation in FlaI	This study
FlaI782	5'- TATTGAGGCGACTCCAG-3'; Overlapping internal forward primer for D ₂₉₀ A mutation in FlaI	This study
FlaI783	5'- TCTGGAGTCGCCTCAATA-3'; Overlapping internal reverse primer for D ₂₉₀ A mutation in FlaI	This study
FlaI784	5'-ACTTGTGGGAGCAATTAGAG-3'; Overlapping internal forward primer for E ₃₃₆ A mutation in FlaI	This study
FlaI785	5'- CTTTGTCTCTAATTGCTCCCACAAG-3'; Overlapping internal reverse primer for E ₃₃₆ A mutation in FlaI	This study
Plasmids		
pETDuet-1	Amp ^r , Car ^r , expression plasmid containing replicon Cole1 (pBR322) and two MCS (MCS1 and MCS2)	Novagen
pSVA251	Amp ^r , Car ^r , pETDuet-1 carrying untagged FlaI in MCS2 using restriction sites NdeI-XhoI	This study
pSVA263	Amp ^r , Car ^r , pETDuet-1 carrying N-terminal His-tagged FlaI in MCS1 using restriction sites EcoRI-HindIII	This study
pSVA265	Amp ^r , Car ^r , pETDuet-1 carrying N-terminal His-tagged FlaI(K ₂₆₈ A) in MCS1 using restriction sites EcoRI-HindIII	This study
pSVA307	Amp ^r , Car ^r , pETDuet-1 carrying N-terminal His-tagged FlaI(D ₂₉₀ A) in MCS1 using restriction sites EcoRI-HindIII	This study
pSVA308	Amp ^r , Car ^r , pETDuet-1 carrying N-terminal His-tagged FlaI(E ₃₃₆ A) in MCS1 using restriction sites EcoRI-HindIII	This study
pSVA314	Amp ^r , Car ^r , pETDuet-1 carrying N-terminal His-tagged FlaI(K ₂₆₈ A, E ₃₃₆ A) in MCS1 using restriction sites EcoRI-HindIII	This study

Figures and figure legends

Figure 1 ATPase activity of Flal under various conditions

Total Pi released upon ATP (NTP) hydrolysis was measured by the malachite green assay. (A) Influence of pH on Flal ATPase activity. (B) Influence of temperature on Flal ATPase activity. (C) Preference for optimal divalent metal ion as cofactor in Flal ATPase activity. The final concentration of protein used in the assays was 1 μM and the final concentration of metal ions was 5 mM. (D) Analysis of optimal substrate for hydrolysis. NTPs were added to a final concentration of 5 mM. (E) Flal ATPase activity as a function of the ATP concentration measured at 70°C with 1 μM of Flal. (F) The same Flal ATPase

activity plotted according to Hill equation (Hill equation = 2.0). All experiments were performed in triplicate and standard deviations are indicated.

Figure 2 Effect of tetraether lipids from *S. acidocaldarius* and total lipids from *E. coli* on Flal ATPase activity

ATP hydrolysis was measured at 70°C with 1 μM of purified Flal in presence of different concentrations (40 μg- 800 μg) of isolated tetraether-linked lipids from *Sulfolobus acidocaldarius* and total lipids from *E. coli* against respective controls in which an equivalent amount of respective lipids was present in the assay mixture without any protein and treated in a similar manner as for the enzymatic reaction described in materials and methods. All experiments were performed in triplicate and standard deviations are indicated.

Figure 3 Characterization of ATP-binding and hydrolysis motifs in Flal

(A) Comparative ATP-hydrolyzing activity of Walker A (Flal^{K268A}), Asp- box (Flal^{D290A}), Walker B (Flal^{E336A}), and Walker A & B double (Flal^{K268A, E336A}) mutants against the wild type Flal protein (black). (B) Analysis of the binding of MANT-ATP by wild type Flal and the mutants as monitored by FRET. The change in fluorescence (ΔF) upon MANT-ATP binding is shown as a function of the concentration of MANT-ATP. Fluorescence spectra were corrected for the fluorescence of unbound MANT-ATP. The K_d values were calculated for each of the mutants and wild type Flal. Closed circles, Flal; closed triangle, Flal^{K268A, E336A}; open rectangle, Flal^{K268A}; vertical stripe, Flal^{D290A}; closed diamonds, Flal^{E336A}.

Figure 4 Size exclusion chromatography of Flal, Flal with ADP and Flal with AMP-PNP

Flal, Flal in the presence of ADP or AMP-PNP were analyzed on an analytical size exclusion column (Superdex 200). The elution fractions were analyzed on a SDS-PAGE and stained with Coomassie. Arrows indicate the position of the marker proteins used to calibrate the column. Prior to analytical gel filtration the purified Flal (1 μ M) was pre-incubated at 50°C for 10 min followed by an incubation at 4°C for 6 h with either 1 mM AMP-PNP or ADP, and 10 mM $MgCl_2$. (Molecular weight standards used are: Ribonuclease A M_r 13,700; Carboxy anhydrase M_r 29,000; Ovalbumin M_r 43,000; Conalbumin M_r 75,000; Aldolase M_r 158,000; Ferritin M_r 440,000).

A

B

Figure 5 Solution structures of *S. acidocaldarius* Flal with and without ATP

(A) Experimental scattering profiles are shown for wild-type Flal (lower curve) and for the Walker B mutant Flal^{E336A} in the presence of ATP and MgCl₂ (upper curve). The theoretical scattering from *A. fulgidus* GspE is shown in green for a hexamer and in orange for a monomer. In the presence of ATP and MgCl₂ Flal forms a hexameric structure similar to the hexameric ring of GspE, without nucleotide this ring falls apart in what seems to be similar to the GspE monomer. **Inset.** Distance distribution functions $P(r)$ of the Flal hexamer and monomer computed from experimental SAXS data are shown in green and orange, respectively. The $P(r)$ functions are normalized to unity at their maxima.

(B) Average of 15 *ab initio* models for Flal with and without ATP overlaid with the crystal structures of hexameric and monomeric GspE, respectively. The model for hexameric Flal was calculated without and with applied 6-fold symmetry in the modeling.

A *S. acidocaldarius* lipids

B *E. coli* lipids

Figure 6 Liposome pull-down assay of Flal in the presence of *S. acidocaldarius* tetraether lipids and *E.coli* lipid extract

The interaction of either Flal, Flal^{K268A} or Flal^{E336A} protein was tested either with *S. acidocaldarius* tetraether liposomes (A) or lipids from *E. coli* (B) in presence or absence of ATP. After centrifugation, proteins remaining in the supernatant (s) or those associated with liposomes in the pellet (p) were separated on an SDS-PAGE.

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Figure 7 Model describing the sequence of events that Flal undergoes in AFAS as inferred from this study

Flal undergoes ATP-dependent oligomerization and attains a hexameric conformation. This ATP bound hexameric Flal is possibly stabilized via interactions with other membrane components (like FlaJ) in AFAS and also with tetraether lipids in the membrane. ATPase activity of Flal, already associated to the macromolecular archaeal flagella assembly complex, might provide energy to drive the flagellum assembly and/or rotation.