Characterization of the ATPase Flal of the motor complex of the *Pyrococcus furiosus* archaellum and its interactions between the ATP-binding protein FlaH

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The archaellum, the rotating motility structure of archaea, is best studied in the crenarchaeon *Sulfolobus acidocaldarius*. To better understand how assembly and rotation of this structure is driven, two ATP-binding proteins, Flal and FlaH of the motor complex of the archaellum of the euryarchaeon *Pyrococcus furiosus*, were overexpressed, purified and studied. Contrary to the Flal ATPase of *S. acidocaldarius*, which only forms a hexamer after binding of nucleotides, Flal of *P. furiosus* formed a hexamer in a nucleotide independent manner. In this hexamer only 2 of the ATP binding sites were available for binding of the fluorescent ATP-analog MANT-ATP, suggesting a 2-fold symmetry in the hexamer.

*P.furiosus* Flal showed a 250-fold higher ATPase activity than *S. acidocaldarius* Flal.

Interaction studies between the isolated N- and C-terminal domains of Flal showed interactions between the N- and C-terminal domains and strong interactions between the N-terminal domains not previously observed for ATPases involved in archaellum assembly. These interactions played a role in oligomerization and activity, suggesting a conformational state of the hexamer not observed before. Further interaction studies show that the C-terminal domain of *Pf* Flal interacts with the nucleotide binding protein FlaH. This interaction stimulates the ATPase activity of Flal optimally at a 1:1 stoichiometry, suggesting that hexameric *Pf* Flal interacts with hexameric *Pf* FlaH. These data help to further understand the complex interactions that are required to energize the archaellar motor.
Characterization of the ATPase FlaI of the motor complex of the Pyrococcus furiosus archaellum and its interactions between the ATP-binding protein FlaH

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Abstract
The archaellum, the rotating motility structure of archaea, is best studied in the crenarchaeon *Sulfolobus acidocaldarius*. To better understand how assembly and rotation of this structure is driven, two ATP-binding proteins, FlaI and FlaH of the motor complex of the archaellum of the euryarchaeon *Pyrococcus furiosus*, were overexpressed, purified and studied. Contrary to the FlaI ATPase of *S. acidocaldarius*, which only forms a hexamer after binding of nucleotides, FlaI of *P. furiosus* formed a hexamer in a nucleotide independent manner. In this hexamer only 2 of the ATP binding sites were available for binding of the fluorescent ATP-analog MANT-ATP, suggesting a 2-fold symmetry in the hexamer. *P. furiosus* FlaI showed a 250-fold higher ATPase activity than *S. acidocaldarius* FlaI. Interaction studies between the isolated N- and C-terminal domains of FlaI showed interactions between the N- and C-terminal domains and strong interactions between the N-terminal domains not previously observed for ATPases involved in archaellum assembly. These interactions played a role in oligomerization and activity, suggesting a conformational state of the hexamer not observed before. Further interaction studies show that the C-terminal domain of *Pf*FlaI interacts with the nucleotide binding protein FlaH. This interaction stimulates the ATPase activity of FlaI optimally at a 1:1 stoichiometry, suggesting that hexameric *Pf*FlaI interacts with hexameric *Pf*FlaH. These data help to further understand the complex interactions that are required to energize the archaellar motor.
Motility in archaea is driven by a rotating cell surface appendage, called the archaellum (Jarrell and Albers, 2012; Albers and Jarrell, 2015). The archaellum is widely spread in archaea, and was identified in many of the archaeal phyla, e.g. in crenarchaeota, euryarchaeota, thaumarchaeota and nanoarchaeota (Makarova et al., 2016). Although in function it resembles the bacterial flagellum, it is structurally different as it is evolutionarily related to archaeal and bacterial type IV pilus assembly systems (T4PSs) and Type II secretion systems (T2SS) (Jarrell and Albers, 2012; Berry and Pelicic, 2015; Albers and Jarrell, 2015). The archaellum consists of 7-13 proteins, which are all essential for the assembly and function (Patenge et al., 2001; Thomas et al., 2001; Chaban et al., 2007; Lassak et al., 2012). Similar to the pilins of T4PSs, archaellins also possess N-terminal class III signal peptides, which are processed by a dedicated membrane bound aspartic acid peptidase (Albers et al., 2003; Bardy and Jarrell, 2003). After N-terminal cleavage, the mature archaellins are inserted into the growing archaellum filament. Recently, cryo EM structures of the euryarchaeotes Methanospirillum hungatei and Pyrococcus furiosus archaellum revealed that the archaellin monomer has two domains: an N-terminal domain, which forms a long hydrophobic α-helix, and a C-terminal domain with an eight-stranded anti-parallel β-barrel (Poweleit et al., 2016; Daum et al., 2017). The assembled archaellins showed different inter-subunit interactions than the assembled pilins of T4PSs (Craig et al., 2006; Wang et al., 2017) and the Lho670 adhesion filament of non-motile Ignicoccus hospitalis (Braun et al., 2016). Similar to the assembly of the pilus in T4PS and T2SSs (Jakovljevic et al., 2008; Chiang et al., 2008; Yamagata and Tainer, 2007), assembly of the archaellum is energized by ATP hydrolysis (Thomas et al., 2001; Reindl et al., 2013). Operons encoding components of the archaellum contain two ATP binding proteins, Flai and Flah. Flai of the crenarchaeon Sulfolobus acidocaldarius (SaFlai) forms an ATP-dependent hexamer and the nucleotide bound crystal structure showed a conserved C-terminal ATPase domain (CTD) which is connected via a flexible linker to the variable N-terminal domain (NTD) (Fig.1A) (Ghosh et al., 2011; Reindl et al., 2013). In the hexameric crystal structure, an intrinsic 2-fold symmetry results in three unique subunit conformations, and superimposition of unique NTDs and CTDs shows that the individual NTD and CTD structures are similar, with only small changes in the NTD. The intra-subunit interface is largest between the CTDs. Indeed, SaFlai lacking the NTD still exhibits 75% of the ATPase activity compared to the full-length Flai (Reindl et al., 2013). In the crystal structures,
the interaction between the NTD and CTD from one subunit is small compared to the interaction
between one NTD and the neighboring subunit CTD (Reindl et al., 2013). Together with the
hexameric structures of Type II secretion and Type IV pili systems (Yamagata and Tainer, 2007;
Kenneth et al., 2007; Misic et al., 2010; Lu et al., 2013; McCallum et al., 2017) a model evolved
in which successive rounds of ATP binding, ATP hydrolysis and ADP release in the three unique
subunits result in conformational changes of the subunits. Based on the homology of FlaI and
FlaJ with the T4PS proteins, it seems likely that FlaI interacts with FlaJ (Chiang et al., 2005;
Takhar et al., 2013; Bischof et al., 2016). Indeed, the flexible crown groove (residues 61-128) of
the structure of SaFlaI contains negatively charged amino acid patches which were proposed to
interact with the positively charged cytoplasmic loops of SaFlaJ (Reindl et al., 2013; Banerjee et
al., 2013). A similar interaction was recently proposed between the PilB and PilC of Geobacter
metallireducens (McCallum et al., 2017). Conformational changes in the membrane platform
protein then might result in insertion or extrusion of the pilin or archaellin into the
pilus/archaellum (Chang et al., 2016; McCallum et al., 2017). Indeed, the ATPase activity of
Myxococcus xanthus PilB is stimulated by interaction with PilC (Bischof et al., 2016).
Additional to its assembly, the archaellum also needs to rotate. How the switch between
assembly and rotation occurs is currently unknown, but possibly FlaH, a second ATPase only
identified in operons encoding the archaellum but not in operons encoding archaeal Type IV pili
systems, is involved in this switch (Chaudhury et al., 2016). FlaH belongs to the RecA
superfamily of ATPases. S. acidocaldarius FlaH (SaFlaH) can bind ATP, but is unable to
hydrolyze it, most likely due to the presence of a non-canonical walker B motif. The crystal
structures of SaFlaH and Methanocaldococcus jannaschii FlaH were solved (Meshcheryakov
and Wolf, 2016; Chaudhury et al., 2016). SaFlaI and SaFlaH interact with each other in an ATP
dependent manner (Chaudhury et al., 2016). SaFlaI and SaFlaH were also shown to interact with
S. acidocaldarius FlaX (SaFlaX) (Banerjee et al., 2013). FlaX, which was only identified in
crenarchaea, contains an N-terminal transmembrane domain and a C-terminal cytoplasmic
domain which, for SaFlaX, forms a ring-like oligomeric structure with a diameter of 30 nm
(Banerjee et al., 2012). Deletion of 57 amino acids which correspond to three helices from the C-
terminus of SaFlaX abolished formation of the ring and interaction with Flal in vitro (Banerjee et
al., 2012; Banerjee et al., 2013). Electron microscopy revealed that, in vitro, SaFlaH could
assemble as a second ring inside the SaFlaX ring (Chaudhury et al., 2016). Thus, it was proposed
that the central core of the crenarchaeal archaellum is formed by FlaI together with FlaH, FlaX and FlaJ (Banerjee et al., 2013). In addition to these proteins, a minimal functional archaellum requires the FlaF and FlaG proteins. FlaF and FlaG are monotopic membrane proteins where SaFlaF has a β-sandwich fold and interacts with S-layer proteins suggesting that it might act as a stator that anchors the rotating archaellum (Banerjee et al., 2015).

Although the archaellum is widely spread among archaea, the archaellum has mainly been biochemically characterized in the crenarchaeote *S. acidocaldarius*. Several differences have been observed between the archaellum systems of crenarchaea and euryarchaea. For example, whereas most species of crenarchaeota contain only one archaellin, euryarchaeota may possess up to five different archaellins (Jarrell and Albers, 2012). In *Methanococcus maripaludis*, the archaellum showed a hook-like structure which was not observed in a deletion mutant of the minor archaellin *flaB3* (Chaban et al., 2007). Furthermore, FlaX was only identified in crenarchaea (Ghosh and Albers, 2011), whereas euryarchaeota contain the *flaC, flaD* and *flaE* genes which conversely are not found in crenarchaea (Jarrell and Albers, 2012; Albers and Jarrell, 2015). Finally, many euryarchaea exhibit chemotaxis systems, which have not been identified in crenarchaea till date (Wuichet et al., 2010). In *Halobacterium salinarum* and *Halofex volcanii*, it has been demonstrated that the FlaC, FlaD and FlaE proteins link rotation of the archaellum to the CheY signal transduction cascade and thus to the chemotaxis system (Schlesner et al., 2009; Quax et al., 2018).

Recently, a low resolution image of the archaellar basal body of the euryarchaeote *Thermococcus kodakaraensis* was obtained by cryo-tomography (Briegel et al., 2017). This structure shows similarities to the structures of bacterial T4P (Chang et al., 2016), but also shows several unique features. For example, a large conical frustum of up to 500 nm in diameter was observed at the cytosolic base of the structure. The resolution of this structure is however not high enough to distinguish or identify individual components (Briegel et al., 2017). The cryo-EM structure of the *P. furiosus* archaellum allowed a more detailed view of the archaellum motor complex (Daum et al., 2017). Like in *T. kodakaraensis* a cone structure is present below the archaellum motor complexes. After modelling the structures of SaFlaI and SaFlaH in the densities close to the membrane, several remaining densities were observed. These probably contain the FlaCDE proteins.
In this study, we set out to characterize the motor subunits of the archaellum of *Pyrococcus furiosus*, an anaerobic, heterotrophic hyperthermophilic euryarchaeote that can grow at temperatures between 70°C and 103°C, and a pH between 5 and 9 (Fiala and Stetter, 1986). *P. furiosus* contains monopolar polytrichous archaella (Fiala and Stetter, 1986), which it does not only use to swim, but also to form cable-like cell-cell connections to adhere to solid surfaces (Näther *et al.*, 2006). Here, we continue our previous studies on the biochemical characterization of *P. furiosus* FlaI (*PfFlaI*) and FlaH (*PfFlaH*).

**Materials & Methods**

**Strains and plasmids.** *Escherichia coli* strains NEB 10-beta (New England BioLabs) and Rosetta (DE3) (Novagen) were used for cloning purposes and overexpression respectively. Genomic DNA of *Pyrococcus furiosus* DSM 3638 (Robb *et al.*, 2001) was used as a template for PCR reactions. Plasmids and their construction are described in Supplementary Table S1. Primers used are described in Supplementary Table S2. All plasmids sequences were confirmed by PCR and sequencing.

**Overproduction and purification.** Overproduction and purification of His-tagged *P. furiosus* FlaI (*PfFlaI*), *PfFlaI*-NTD, *PfFlaI*-CTD, and *P. furiosus* FlaH (*PfFlaH*) and the *PfFlaH* (K39A) and *PfFlaH* (D126N) mutants were performed as described previously (Chaudhury *et al.*, 2016). Overproduction and purification of StrepII-tagged *PfFlaI* (E336A) were essentially performed the same except that Streptactin column material (IBA GmbH, Göttingen, Germany) was used, and that the protein was eluted with 2.5 mM d-desthiobiotin. Samples were stored at -80°C until use.

**Analytical gel filtration.** *PfFlaI*, *PfFlaI*-NTD and *PfFlaI*-CTD were concentrated to 1 mg/ml in buffer containing 20 mM Tris HCl pH 8.0, 150 mM NaCl (buffer A) using Amicon concentrators (Millipore) with a 10 kDa cut-off. 500 µl of the concentrated samples or 250 µl *PfFlaI*-NTD mixed with 250 µl *PfFlaI*-CTD and applied to Superdex 200 10/300 GL or Superdex 75 10/300 GL size exclusion columns equilibrated with buffer A. Fractions were analyzed on SDS-PAGE. Thyroglobulin (669 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa) were used as size standards.
**MANT-ATP binding.** Binding of the fluorescent ATP analog 2'-/3'-O-(N-methylantraniloyl)adenosine 5'-triphosphate (MANT-ATP, JENA biosciences) was detected by titrating PfFlaI (20 nM, 100 nM and 5 µM in buffer A containing 5 mM MgCl$_2$) with increasing concentrations of MANT-ATP in a 150 µl cuvette at 20°C in a Fluoromax-4 fluorimeter (Horiba Scientific). Excitation and emission wavelengths were set to 285 and 450 nm respectively with slit widths of 10 nm. Fluorescence was corrected for MANT-ATP fluorescence in the absence of protein. To determine the binding affinity of ATP, competition assays between MANT-ATP and ATP were performed. Total fluorescence was determined under the conditions described above after addition of increasing amounts of ATP to a solution containing 20 nM PfFlaI and 10 nM of MANT-ATP. The data were fitted with the Hill equation: 

$$ F = \frac{F_{\text{max}} + (F_{\text{min}} - F_{\text{max}}) \times [\text{ATP}]^n}{IC_{50}^n + [\text{ATP}]^n}, $$

where $F$= Fluorescence, $F_{\text{min}}$= minimal fluorescence, $F_{\text{max}}$= maximal fluorescence, $[\text{ATP}]$ is the ATP concentration, $IC_{50}$ is the ATP concentration where the fluorescence is reduced by half, and $n$= Hill coefficient.

**ATPase Assay.** Release of inorganic phosphate after ATP hydrolysis was determined using the Malachite green assay (Lanzetta et al., 1979) by determining the colorimetric change at 620 nm using a Clariostar plate reader (BMG labtech). ATP hydrolysis at different temperatures was determined by incubating 12.5 µg/ml PfFlaI in buffer A containing 5 mM MgCl$_2$ and 1 mM ATP for 5 minutes at different temperatures. ATP hydrolysis at different pHs was determined by incubating 12.5 µg/ml PfFlaI in different buffers (20 mM citrate (pH 3.0), 20 mM 2-(N-morpholino)ethane sulfonic acid (MES, pH 6.0), 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES, pH 7.2), 20 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS, pH 8.0/ 9.5), 20 mM sodium carbonate-bicarbonate (pH 10.0)) containing 5 mM MgCl$_2$ and 1 mM ATP for 5 minutes at 70°C. To determine the ATPase activity of PfFlaI at different ATP concentrations, 12.5 µg/ml PfFlaI was incubated for 5 minutes at 70°C in buffer A containing 5 mM MgCl$_2$ and different ATP concentrations. The curve was fitted to the Michaelis-Menten equation ($v = \frac{v_{\text{max}} \times [\text{ATP}]}{K_m + [\text{ATP}]}$). The Hill coefficient was determined from the slope of a plot of log ([ATP]) vs log ($v/(v_{\text{max}} - v)$). ATPase activity of PfFlaI, PfFlaI-NTD, PfFlaI-CTD and the stoichiometric mixture of PfFlaI-NTD and PfFlaI-CTD was determined by incubating the proteins at a concentration of 12.5 µg/ml in buffer A containing 5 mM MgCl$_2$ at 70°C.
Microscale thermophoresis. Microscale thermophoresis was performed as described previously (Chaudhury et al., 2016). To determine the binding affinity of CTD and NTD of PfFlaI, 28 nM labeled PfFlaH in buffer A containing 0.05 % (v/v) Tween-20 was titrated with increasing concentrations (1.3 nM-22.5 µM) of Pf-CTD and (5.6 nM-11.6 µM) Pf-NTD of PfFlaI on a Nano Temper Monolith NT.115 Pico instrument. The data were fitted as described previously (Chaudhury et al., 2016).

RESULTS

Overproduction, purification and characterization of P. furiosus FlaI

To compare the crenarchaeal and euryarchaeal archaellum, we set out to biochemically characterize the archaellum of Pyrococcus furiosus. The genetic region that encodes the components of the archaellum of P. furiosus encodes the major archaellin (flaB0), followed by two minor archaellins (flaB1, flaB2) and the flaCDFGHJI genes (Robb et al., 2001; Näther et al., 2006; Näther-Schindler et al., 2014). Here, we focus on the FlaI and FlaH proteins. Similar to SaFlaI, Pyrococcus furiosus FlaI (PfFlaI) contains both an N-terminal and a C-terminal domain, which show respectively 36 % and 57 % identity with SaFlaI (See Fig. 1A and 1B). PfFlaI was previously overexpressed and purified (Chaudhury et al., 2016), and is here characterized further. Analysis of the oligomeric state of PfFlaI using size exclusion chromatography showed that PfFlaI eluted at a position corresponding to a hexamer (Fig. 2A and 2B). Thus, in this respect, PfFlaI differs from SaFlaI, which after purification elutes as a monomer and formed a hexamer in the presence of the non-hydrolysable ATP analog adenylyl-imidodiphosphate (AMP-PNP) (Ghosh et al., 2011) (Fig. 2A and 2B). The Abs260/Abs280 ratio of 0.3 suggested that PfFlaI was isolated in the nucleotide free form. Indeed, further purification steps, dialysis or ammonium sulphate precipitation did not result in a change in the Abs260/Abs280 ratio. To test whether PfFlaI could bind ATP, titrations with the fluorescent ATP analog MANT-ATP were performed. Similar experiments were performed to determine the nucleotide binding affinity of SaFlaI (Ghosh et al., 2011). For PfFlaI, the fluorescence increased linearly upon addition of MANT-ATP, until a maximum was reached after which no further increase was observed. This saturation was dependent on the protein concentration, and when 20 nM, 100 nM or 5 µM protein PfFlaI (Fig. 3A) was used, the maximum was reached for all three concentrations at 1/3 of the PfFlaI concentration used, demonstrating that only 2 of the ATP binding sites in the
hexamer are available for binding of MANT-ATP. Indeed, SaFlaI crystallized as a hexameric ring with an intrinsic 2-fold symmetry resulting in three different conformations of the monomers (Reindl et al., 2013), suggesting that also for PfFlaI such a 2-fold symmetry with three unique subunits occurs. To determine the affinity for ATP, bound MANT-ATP was competed with ATP resulting in an IC$_{50}$ of 260 nM at 20°C (Fig. 3B). The hydrolysis of ATP by PfFlaI was tested at different temperatures and at different pHs (Fig. 3C and 3D) and the highest activity was found at 70°C and pH 8.0. Even though *P. furiosus* can live up to 103°C, the ATPase activity in vitro decreased above 70°C at 70°C and pH 8.0, ATP was hydrolyzed with a $v_{\text{max}}$ of 8 µmoles mg$^{-1}$ min$^{-1}$ (Chaudhury et al., 2016) and a K$_{m}$ of 580 µM (Fig. 3E). ATP hydrolysis did not show cooperativity with increasing ATP concentrations (Hill coefficient = 0.9) (Fig. 3E, inset). The maximum activity observed for PfFlaI was 250-fold higher than the maximum activity (at pH 6.5 and 75 °C) observed for SaFlaI (Chaudhury et al., 2016), and equals a turn-over of ~500 ATP min$^{-1}$.

The N-terminal domains of PfFlaI interact with each other and with the C-terminal domains to stimulate ATP hydrolysis

The crystal structures of SaFlaI revealed that, similar to other described T2SS and T4PS ATPases (Robien et al., 2003; Yamagata and Tainer, 2007; Satyshur et al., 2007; Misic et al., 2010; Lu et al., 2013; McCallum et al., 2017), SaFlaI consists of a variable NTD and a CTD that binds and hydrolyses ATP (Reindl et al., 2013). These domains are connected via a short flexible linker (Reindl et al., 2013). The hexameric structure of SaFlaI was only observed in the crystal structure after incubation with AMP-PNP. Since PfFlaI forms a much more stable hexamer, we set out to study the interactions between the NTDs and CTDs of PfFlaI.

Both the NTD (PfFlaI-NTD; 29 kDa) and the CTD (PfFlaI-CTD; 31 kDa) of PfFlaI were overexpressed in *E. coli*, purified, and were then analyzed on analytical size exclusion chromatography (Fig. 4A and 4B). The PfFlaI-CTD eluted as a monomer whereas, contrary to what was expected, the PfFlaI-NTD eluted not only as monomer but also as dimer and possibly a higher order oligomer. This demonstrates that the NTDs of PfFlaI interact with each other and might play a role in the formation of the hexameric ring. Since in the crystal structure of the SaFlaI hexamer the NTDs interact with the CTDs of the neighboring subunit, it was tested...
whether also the PfFlaI-NTD and the PfFlaI-CTD interacted. Equimolar concentrations of PfFlaI-NTD and PfFlaI-CTD were mixed, analyzed on analytical size exclusion chromatography. This resulted in a co-elution of the PfFlaI-NTD and PfFlaI-CTD and a shift of especially the elution position of the PfFlaI-CTD, demonstrating an interaction between the PfFlaI-NTD and the PfFlaI-CTD. To test whether interaction between the PfFlaI-NTD and the PfFlaI-CTD influenced the ATPase activity, ATP hydrolysis of the single domains and of the mixed domains was determined (Fig. 4C). In these experiments, ATP hydrolysis was only observed when both the PfFlaI-NTD and PfFlaI-CTD were present, demonstrating that the PfFlaI-NTD can stimulate ATP hydrolysis by the PfFlaI-CTD.

**PfFlaH stimulates the ATPase activity of PfFlaI**

We have overexpressed and purified *P. furiosus* FlaH (*PfFlaH*) and used microscale thermophoresis (MST) to show that nucleotide-bound *PfFlaH* bound to *PfFlaI* with a $K_D$ of 1 μM (*Chaudhury et al.*, 2016). *PfFlaH* containing mutations in the Walker A (*PfFlaH K39A*) and Walker B motifs (*PfFlaH D126N*) had a strongly reduced affinity for nucleotides, and bound *PfFlaI* with a strongly reduced affinity, demonstrating that nucleotide binding by *PfFlaH* is important for its interaction with *PfFlaI* (*Chaudhury et al.*, 2016). Here, we further investigated the influence of this interaction on the ATPase activity of *PfFlaI*. As observed, *PfFlaI* hydrolyzed ATP, whereas for *PfFlaH*, no ATPase activity could be observed (Fig. 5A, *Chaudhury et al.*, 2016). Also no ATP hydrolysis was observed for *PfFlaI* with an E336A mutation in the Walker B motif, and for the *PfFlaHK39A* and *PfFlaHD126N* proteins. Addition of *PfFlaH* to *PfFlaI* stimulated the total ATPase activity. To test whether the stimulation of the ATPase is derived from an increase of the activity of *PfFlaH* or of *PfFlaI*, different combinations of mutants in *PfFlaH* and *PfFlaI* with WT proteins were tested (Fig. 5A). This demonstrated unequivocally that binding of nucleotide bound *PfFlaH* stimulates the ATPase activity of *PfFlaI*. To test the stoichiometry of this interaction, 1 μM *PfFlaI* was incubated with increasing concentrations of *PfFlaH*. A maximal stimulation was observed when *PfFlaH* was present in stoichiometric amounts to *PfFlaI* (Fig. 5B). The stimulation decreased when further increasing amounts of *PfFlaH* were added. Thus *PfFlaI* and *PfFlaH* interact in a 1:1 stoichiometry.

**FlaH interacts at the C-terminal domain of FlaI**
Nucleotide-bound PfFlaH can interact with PfFlaI with an affinity of 1 µM (Chaudhury et al., 2016). To test whether FlaH interacts with the NTD or CTD of FlaI, interaction assays using microscale thermophoresis were performed using PfFlaH and the P. furiosus NTD and CTD of FlaI (Fig. 6). The PfFlaI-CTD and the PfFlaI-NTD interacted with PfFlaH with affinities of 100 nM and > 8 µM, respectively. Thus, it was concluded that PfFlaH interacts specifically with the PfFlaI-CTD. This interaction occurs with a 10-fold higher affinity than the interaction between full length hexameric PfFlaI and PfFlaH, showing that PfFlaH can interact in different manners with the hexameric PfFlaI and the monomeric PfFlaI-CTD.

Discussion

Many archaeal cell surface structures are homologous to bacterial T4PSs, which function in cell attachment to surfaces, DNA transport, biofilm formation and motility (Jarrell et al., 2013; Makarova et al., 2016; Chaudhury et al., 2018). Similar to the T2SSs and T4PSs, most of these systems possess a pilin protein, a prepilin peptidase which cleaves at the N-terminus of the prepilin, an ATPase of the superfamily of traffic ATPases, and a membrane platform protein (Peabody, 2003; Nishida and Chen, 2004). The traffic ATPase was suggested to interact with the membrane platform protein for several systems (Reindl et al., 2013; Banerjee et al., 2013; Bischof et al., 2016; Takhar et al., 2013), and it has been proposed that interactions between the processed pilin, the traffic ATPase protein and the platform protein drive the assembly of the pilus (Chang et al., 2016; McCallum et al., 2017). FlaI, the traffic ATPase of the archaellum differs from the other traffic ATPases in the fact that it not only energizes the assembly of the archaella, but also should drive its rotation. Comparison of the different crystal structures of traffic ATPases showed many different conformations of the NTD relative to the CTD, but in general, binding of ATP results in large domain movements bringing the NTD and CTD closer together (Reindl et al., 2013; Lu et al., 2013; Satyshur et al., 2007). Comparison of the conformations in SaFlaI and VcGspE, the traffic ATPase of the T2SS of Vibrio cholerae, showed that in VcGspE, ATP hydrolysis results in an up and down movement of the domains, whereas, SaFlaI shows a more rotating movement of the domains, possibly explaining the differences between SaFlaI and other traffic ATPases (Reindl et al., 2013). The rotating movement of the SaFlaI hexamer is the result of three different alternating conformations which mostly differ in the position of the NTD relative to the CTD.
Here the euryarchaeal PfFlaI was characterized, and compared to the well characterized crenarchaeal SaFlaI. Firstly, it was observed that PfFlaI forms a stable hexamer whereas SaFlaI was a monomer in solution. The SaFlaI hexamer was observed only after incubation with a non-hydrolyzable ATP analogue and at high protein concentrations in the crystal structure. In the ADP-bound SaFlaI crystal structure, 3 different alternating conformations were observed, which all contained a nucleotide. ATP binding assays with PfFlaI showed that only 2 of the 6 positions in the PfFlaI hexamer were accessible for MANT-ATP, suggesting that PfFlaI also contains alternating conformations, and that 4 of the 6 subunits of the hexamer are not accessible to fluorescently labelled nucleotides. ATP hydrolysis by SaFlaI was highly co-operative, whereas ATP hydrolysis by PfFlaI did not show any cooperativity, suggesting that cooperativity occurs during the assembly of the SaFlaI hexamer, while no cooperativity occurs in the assembled PfFlaI hexamer. Indeed, the PfFlaI hexamer is, contrary to SaFlaI, formed in an ATP independent manner. This suggest that PfFlaI and SaFlaI might differ structurally. Another suggestion that PfFlaI and SaFlaI differ in their structures comes from the experiments with the isolated NTD and CTD. Whereas in the different SaFlaI crystal structures obtained, interactions are found between the CTDs and between the CTDs and the NTD, no strong interactions were observed between the NTDs. Indeed, SaFlaI lacking the NTD still exhibits 75% of the ATPase activity compared to the full-length FlaI (Reindl et al., 2013), suggesting that the NTD plays no important role in oligomerization. In PfFlaI however, deletion of the NTD strongly reduces or abolishes oligomerization and ATPase activity. The activity and oligomerization can be partly recovered by the addition of the isolated NTD, suggesting that the NTD for PfFlaI also plays an intrinsic role in oligomerization. Based on the crystal structure of SaFlaI, it could be expected that this is caused by the stabilization of the interaction between 2 CTDs by an NTD, but our observation that the isolated NTDs of PfFlaI also oligomerize suggests that interactions between NTDs might also play a role in the oligomerization and activity of PfFlaI. Strong interactions between NTDs of traffic ATPases, have currently only been observed for the HP0525 traffic ATPase of the Helicobacter pylori type IV secretion system (Yeo et al., 2000).

Next to the characterization PfFlaI, the interaction of PfFlaI with PfFlaH was further analyzed. Binding of nucleotide bound PfFlaH to PfFlaI stimulated its ATPase activity 2-fold, further demonstrating the importance of this interaction. Maximum stimulation was found at a 1:1 stoichiometry, suggesting that hexameric PfFlaH interacts with hexameric PfFlaI. Previously, it
was demonstrated that both *SaFlaI* and *SaFlaH* interact with *SaFlaX* (Banerjee *et al.*, 2013). *In vitro* assembly of the C-terminal domain of *SaFlaX* resulted in ring-like structures with 15- to 23- fold symmetry with widely different diameters (Banerjee *et al.*, 2012). After incubation with *SaFlaH*, monomeric *SaFlaH* particles were observed inside these *SaFlaX* rings (Chaudhury *et al.*, 2016). The amount of *FlaH* bound inside the rings varied with the size of the ring, but in the most occurring rings with a 20- fold symmetry, 9–10 *SaFlaH* monomers could be observed (Chaudhury *et al.*, 2016). The size of the *in vivo* *FlaX* ring in the *S. acidocaldarius* archaellum complex is currently still unknown. However, since *SaFlaX* also interacts with *SaFlaI*, and a *FlaI* hexamer interacts with hexameric *FlaH*, it seems likely that the motor complex in *S. acidocaldarius* consists of hexameric *SaFlaI*, bound to hexameric *SaFlaH*, surrounded by a *SaFlaX* ring. Like all euryarchaeal, *P. furiosus* does not encode a *FlaX* homolog, but encodes the *FlaC* and *FlaD* proteins. However, it is currently not known whether they also form a ring-like structure and whether possible interactions with *FlaI* or *FlaH* exist. Remarkably, *P. furiosus* does not encode a chemotaxis system (Maeder *et al.*, 1999) and thus the *FlaCDE* proteins should not be related to the CheY signal transduction cascade.

It was proposed that the NTD of *FlaI* interacts with *FlaJ* (Reindl *et al.*, 2013; Banerjee *et al.*, 2013), making it likely that the CTD domain of *FlaI* would interact with *FlaH*, and we found that *PfFlaH* interacts with the *PfFlaI-CTD*. This interaction occurs with a 10-fold higher affinity than the interaction between full length *PfFlaI* and *PfFlaH*. This suggest that the affinity of the interaction between *FlaH* and *FlaI* might be modulated by factors that increase or decrease the accessibility of the CTD of *FlaI* to *FlaH*, and this might facilitate a switch between assembly and rotation of the archaellum.

**Conclusions**

Our results showed that *FlaI* of *P. furiosus* differs significantly from the extensively studied *FlaI* of *S. acidocaldarius*. Contrary to *FlaI* of *S. acidocaldarius*, which only forms a hexamer after binding of nucleotides, *FlaI* of *P. furiosus* forms a stable hexamer in a nucleotide independent manner. The presence of the stable hexamer allowed us to study nucleotide binding to the hexamer. This showed that only 2 of the 6 ATP binding sites were available for binding of the fluorescent ATP-analog MANT-ATP, suggesting a 2-fold symmetry in the hexamer and further
suggesting that individual proteins in the hexamer alternate between the empty, ATP and ADP bound states. We also identified strong interactions between the N-terminal domains *S. acidocaldarius* FlaI not identified before for *S. acidocaldarius* FlaI. These interactions played a role in oligomerization and activity, suggesting a conformational state of the hexamer not observed previously. We further showed that interaction between FlaI and FlaH stimulates the ATPase activity of FlaI. This occurs optimally at a 1:1 stoichiometry, suggesting that a FlaI hexamer can interact with six FlaH proteins or with a FlaH hexamer. Further interaction studies showed that FlaH interacts with the C-terminal domain of *Pf*FlaI.
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References

Albers, S.-V., and Jarrell, K.F. (2015) The archaellum: how Archaea swim. *Front Microbiol* **6**: 23.

Albers, S.V., Szabó, Z., and Driessen, A.J.M. (2003) Archaeal homolog of bacterial type IV prepilin signal peptidases with broad substrate specificity. *J Bacteriol* **185**: 3918–3925.

Banerjee, A., Ghosh, A., Mills, D.J., Kahnt, J., Vonck, J., and Albers, S.-V.V. (2012) FlaX, a unique component of the crenarchaeal archaellum, forms oligomeric ring-shaped structures and interacts with the motor ATPase FlaI. *J Biol Chem* **287**: 43322–30.

Banerjee, A., Neiner, T., Tripp, P., and Albers, S.-V. (2013) Insights into subunit interactions in the *Sulfolobus acidocaldarius* archaellum cytoplasmic complex. *FEBS J* **280**: 6141–9.

Banerjee, A., Tsai, C.L., Chaudhury, P., Tripp, P., Arvai, A.S., Ishida, J.P., Tainer, J.A, and Albers, S.V. (2015) FlaF is a β-sandwich protein that anchors the archaellum in the archaeal cell envelope by binding the S-layer protein. *Structure* **23**: 863–872.

Bardy, S.L., and Jarrell, K.F. (2003) Cleavage of preflagellins by an aspartic acid signal peptidase is essential for flagellation in the archaeon *Methanococcus voltae*. *Mol Microbiol* **50**: 1339–1347.

Berry, J.-L.J.L., and Pelicic, V. (2015) Exceptionally widespread nanomachines composed of type IV pilins: the prokaryotic Swiss Army knives. *FEMS Microbiol Rev* **39**: 1–21.

Bischof, L.F., Friedrich, C., Harms, A., Søgaard-Andersen, L., and Does, C. van der (2016) The Type IV Pilus Assembly ATPase PilB of Myxococcus xanthus Interacts with the Inner Membrane Platform Protein PilC and the Nucleotide-binding Protein PilM. *J Biol Chem* **291**: 6946–6957.

Braun, T., Vos, M.R., Kalisman, N., Sherman, N.E., Rachel, R., Wirth, R., Schröder, G.F., and Egelman, E.H. (2016) Archaeal flagellin combines a bacterial type IV pilin domain with an Ig-like domain. *Proc Natl Acad Sci* **113**: 10352–10357.

Brriegel, A., Oikonomou, C.M., Chang, Y., Kjær, A., Huang, A.N., Kim, K.W., Ghosal, D., Nguyen, H.H., Kenny, D., Orgorzaæek Loo, R.R., Gunsalus, R.P., and Jensen, G.J. (2017) Morphology of the archaellar motor and associated cytoplasmic cone in *Thermococcus kodakaraensis*. *EMBO Rep* e201744070.

Chaban, B., Ng, S.Y.M., Kanbe, M., Saltzman, I., Nimmo, G., Aizawa, S.I., and Jarrell, K.F. (2007) Systematic deletion analyses of the fla genes in the flagella operon identify several genes essential for proper assembly and function of flagella in the archaeon, *Methanococcus maripaludis*. *Mol Microbiol* **66**: 596–609.
Chang, Y.W., Rettberg, L.A., Treuner-Lange, A., Iwasa, J., Søgaard-Andersen, L., and Jensen, G.J. (2016) Architecture of the type IVa pilus machine. Science (80-) 351: aad2001.

Chaudhury, P., Neiner, T., D’Imprima, E., Banerjee, A., Reindl, S., Ghosh, A., Arvai, A.S., Mills, D.J., van der Does, C., Tainer, J.A., Vonck, J., and Albers, S.V. (2016) The nucleotide-dependent interaction of FlaH and FlaI is essential for assembly and function of the archaellum motor. Mol Microbiol, 99: 674-85

Chaudhury, P., Quax, T.E.F., and Albers, S.-V. (2018) Versatile cell surface structures of archaea. Mol Microbiol 107: 298–311.

Chiang, P., Habash, M., and Burrows, L.L. (2005) Disparate subcellular localization patterns of Pseudomonas aeruginosa Type IV pilus ATPases involved in twitching motility. J Bacteriol 187: 829–39.

Chiang, P., Sampaleanu, L.M., Ayers, M., Pahuta, M., Howell, P.L., and Burrows, L.L. (2008) Functional role of conserved residues in the characteristics secretion NTPase motifs of the Pseudomonas aeruginosa type IV pilus motor proteins PilB, PilT and PilU. Microbiology 154: 114–126.

Craig, L., Volkmann, N., Arvai, A.S., Pique, M.E., Yeager, M., Egelman, E.H., and Tainer, J.A. (2006) Type IV Pilus Structure by Cryo-Electron Microscopy and Crystallography: Implications for Pilus Assembly and Functions. Mol Cell 23: 651–662.

Daum, B., Vonck, J., Bellack, A., Chaudhury, P., Reichelt, R., Albers, S.-V., Rachel, R., and Kühlbrandt, W. (2017) Structure and in situ organisation of the Pyrococcus furiosus archaellum machinery. Elife 6.

Fiala, G., and Stetter, K.O. (1986) Pyrococcus furiosus sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch Microbiol 145: 56–61.

Ghosh, A., and Albers, S.-V. (2011) Assembly and function of the archaeal flagellum. Biochem Soc Trans 39: 64–9.

Ghosh, A., Hartung, S., Does, C. van der, Tainer, J.A., and Albers, S.-V. (2011) Archaeal flagellar ATPase motor shows ATP-dependent hexameric assembly and activity stimulation by specific lipid binding. Biochem J 437: 43–52.

Jakovljevic, V., Leonardy, S., Hoppert, M., and Søgaard-Andersen, L. (2008) PilB and PilT are ATPases acting antagonistically in type IV pilus function in Myxococcus xanthus. J Bacteriol 190: 2411–21.

Jarrell, K., Ding, Y., Nair, D., and Siu, S. (2013) Surface Appendages of Archaea: Structure, Function, Genetics and Assembly. Life 3: 86–117.

Jarrell, K.F., and Albers, S.V. (2012) The archaellum: An old motility structure with a new name. Trends Microbiol 20: 307–312.

Satyshur, K.A., Worzalla, G.A., Meyer, L.S., Heiniger, E.K., Aukema, K.G., Misić, A.M., and Forest, K.T. (2007) Crystal Structures of the Pilus Retraction Motor PilT Suggest Large Domain Movements and Subunit Cooperation Drive Motility. Structure 15: 363–376.
Lanzetta, P.A., Alvarez, L.J., Reinach, P.S., and Candia, O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem* **100**: 95–97.

Lassak, K., Neiner, T., Ghosh, A., Klingl, A., Wirth, R., and Albers, S.V. (2012) Molecular analysis of the crenarchaeal flagellum. *Mol Microbiol* **83**: 110–124.

Lu, C., Turley, S., Marionni, S., Park, Y.-J., Lee, K.K., Patrick, M., Shah, R., Sandkvist, M., Bush, M.F., and Hol, W.G. (2013) Hexamers of the Type II secretion ATPase GspE from *Vibrio cholerae* with Increased ATPase Activity. *Structure* **21**: 1707–1717.

Maeder, D.L, Weiss, R.B., Dunn, D.M., Cherry, J.L., González, J.M., DiRuggiero, J. and Robb, F.T. (1999) Divergence of the hyperthermophilic archaea *Pyrococcus furiosus* and *P. horikoshii* inferred from complete genomic sequences. *Genetics*. 152(4):1299-305.

Makarova, K.S., Koonin, E. V., and Albers, S.-V. (2016) Diversity and Evolution of Type IV pili Systems in Archaea. *Front Microbiol* **7**: 667.

McCallum, M., Tamمام, S., Khan, A., Burrows, L.L., and Lynne Howell, P. (2017) The molecular mechanism of the type IVa pilus motors. *Nat Commun* **8**: 15091.

Meshcheryakov, V.A., and Wolf, M. (2016) Crystal structure of the flagellar accessory protein FlaH of *Methanocaldococcus jannaschii* suggests a regulatory role in archaean flagellum assembly. *Protein Sci* **25**: 1147–1155.

Misic, A.M., Satyshur, K.A., and Forest, K.T. (2010) *P. aeruginosa* PilT structures with and without nucleotide reveal a dynamic Type Iv pilus retraction motor. *J Biol Chem* **400**: 1011–1021.

Näther-Schindler, D.J., Schopf, S., Bellack, A., Rachel, R., and Wirth, R. (2014) *Pyrococcus furiosus* flagella: Biochemical and transcriptional analyses identify the newly detected flaB0 gene to encode the major flagellin. *Front Microbiol* **5**: 695.

Näther, D.J., Rachel, R., Wanner, G., and Wirth, R. (2006) Flagella of *Pyrococcus furiosus*: Multifunctional organelles, made for swimming, adhesion to various surfaces, and cell-cell contacts. *J Bacteriol* **188**: 6915–6923.

Nishida, T., and Chen, D.G. (2004) Incorporating spatial autocorrelation into the general linear model with an application to the yellowfin tuna (*Thunnus albacares*) longline CPUE data. *Fish Res* **70**: 265–274.

Patenge, N., Berendes, A., Engelhardt, H., Schuster, S.C., and Oesterhelt, D. (2001) The fla gene cluster is involved in the biogenesis of flagella in *Halobacterium salinarum*. *Mol Microbiol* **41**: 653–663.

Peabody, C.R. (2003) Type II protein secretion and its relationship to bacterial type IV pili and archaean flagella. *Microbiology* **149**: 3051–3072.

Poweleit, N., Ge, P., Nguyen, H.H., Loo, R.R.O., Gunsalus, R.P., and Zhou, Z.H. (2016) CryoEM structure of the *Methanospirillum hungatei* archaellum reveals structural features distinct from the bacterial flagellum and type IV pilus. *Nat Microbiol* **2**: 16222.

Quax, T.E.F., Altegoer, F., Rossi, F., Li, Z., Rodriguez-Franco, M., Kraus, F., Bange, G., and
Albers, S.V. (2018) Structure and function of the archaeal response regulator CheY. Proc Natl Acad Sci 115: 201716661.

Reindl, S., Ghosh, A., Williams, G.J., Lassak, K., Neiner, T., Henche, A.-L., Albers, S.V., and Tainer, J.A. (2013) Insights on Flal functions in Archaeal Motor Assembly and Motilify from Structures, Conformations and Genetics. Mol Cell 49 (6): 1069–82.

Robb, F.T., Maeder, D.L., Brown, J.R., DiRuggiero, J., Stump, M.D., Yeh, R.K., Weiss, R.B., and Dunn, D.M. (2001) Genomic sequence of hyperthermophile, Pyrococcus furiosus: Implications for physiology and enzymology. Methods Enzymol 330: 134–157.

Robien, M. a., Krumm, B.E., Sandkvist, M., and Hol, W.G.J. (2003) Crystal Structure of the Extracellular Protein Secretion NTPase EpsE of Vibrio cholerae. J Mol Biol 333: 657–674.

Schlesner, M., Miller, A., Streif, S., Staudinger, W.F., Müller, J., Scheffer, B., Siedler, F., and Oesterhelt, D. (2009) Identification of Archaea-specific chemotaxis proteins which interact with the flagellar apparatus. BMC Microbiol 9: 56.

Takhar, H.K., Kemp, K., Kim, M., Howell, P.L., and Burrows, L.L. (2013) The platform protein is essential for type IV pilus biogenesis. J Biol Chem 288: 9721–8.

Thomas, N.A., Pawson, C.T., and Jarrell, K.F. (2001) Insertional inactivation of the flaH gene in the archaeon Methanococcus voltae results in non-flagellated cells. Mol Genet Genomics 265: 596–603.

Wang, F., Coureuil, M., Osinski, T., Orlova, A., Altindal, T., Gesbert, G., Nassif, X., Egelman, E.H., and Craig, L. (2017) Cryoelectron Microscopy Reconstructions of the Pseudomonas aeruginosa and Neisseria gonorrhoeae Type IV Pili at Sub-nanometer Resolution. Structure 25: 1423–1435.e4.

Wuichet, K., Cantwell, B.J., and Zhulin, I.B. (2010) Evolution and phyletic distribution of two-component signal transduction systems. Curr Opin Microbiol 13: 219–225.

Yamagata, A., and Tainer, J.A. (2007) Hexameric structures of the archaeal secretion ATPase GspE and implications for a universal secretion mechanism. EMBO J 26: 878–890.

Yeo, H.J., Savvides, S.N., Herr, A.B., Lanka, E., and Waksman, G. (2000) Crystal structure of the hexameric traffic ATPase of the Helicobacter pylori type IV secretion system. Mol Cell 6: 1461–1472.
Figure 1 (on next page)

Current models of the euryarchaeal and crenarchaeal archaellum motor complex

In both shown archaellum motor complexes, FlaH, Flal and Flaj are conserved. FlaB, the archaellin, builds the filament in the archaeal cell envelope (S-layer). The euryarchaeal archaellum motor complex contains FlaC/D/E which are thought to interact with the chemotaxis system. Additionally, a polar cap structure was identified very recently. However, its function is so far unknown. In the crenarchaeal archaellum motor complex FlaX forms a ring-like structure and is thought to act as a scaffold protein for motor protein assembly.
Figure 2 (on next page)

PfFlal forms a stable hexamer

(A) Relative absorbance at 280 nm of size exclusion chromatography of PfFlal (64 kDa) shown in black line and SaFlal (59 kDa) is shown in grey line using Superdex 200 10/300GL column. Elution positions of molecular mass standards [kDa] were indicated with arrows. (B) Coomassie stained SDS-PAGE analysis of the different elution fractions.
A. Relative absorbance versus elution volume [ml]

B. SDS-PAGE gel images showing bands at different molecular weights for PfFlal and SaFlal.
Figure 3 (on next page)

ATP binding and hydrolysis of PfFlaI

(A) Fluorescence increase at increasing concentrations of MANT-ATP upon addition of 20 nM, 100 nM and 5 µM PfFlaI. Lines depict the linear fits of the two observed phases. The lines cross at a MANT-ATP concentration of 1.7 µM. (B) Total fluorescence after addition of increasing amounts of ATP to a solution containing 20 nM PfFlaI and 10 nM of MANT-ATP. The data were fitted with the Hill equation: $F = \frac{(F_{\text{max}} + (F_{\text{min}} - F_{\text{max}}) \cdot [\text{ATP}])^n}{(IC_{50}^n + [\text{ATP}])^n}$ resulting in a best fit ($R^2 = 0.98$) with $IC_{50} = 260$ nM and $n = 0.67$. (C,D) ATP hydrolysis by 12.5 µg/ml PfFlaI at different temperatures and at different pHs respectively. (E) ATPase activity of PfFlaI at different ATP concentrations. The curve was fitted to the Michaelis-Menten equation ($V = V_{\text{max}} \cdot [\text{ATP}] / (K_m + [\text{ATP}])$), resulting in a $K_m$ of 580 nM. The inset shows the same data plotted according to the Hill equation (Hill coefficient = 0.9). Experiments were performed with at least 2 biological and 3 technical replicates. Error bars depict the standard error obtained from the technical replicates.
Analysis of the interaction between the N- and C-terminal domains of PfFlaI

(A) Relative absorbance at 280 nm of size exclusion chromatography at 280 nm of size exclusion chromatography of PfFlaI-NTD (black line), PfFlaI-CTD (grey line) or a stoichiometric mixture of PfFlaI-NTD and PfFlaI-CTD (dashed line) using Superdex 75 10/300GL column. Elution positions of molecular mass standards [kDa] are indicated with arrows. (B) SDS-PAGE analysis of the elution fractions described in A. (C) ATPase activity at 70°C of the main elution fraction after size exclusion chromatography of PfFlaI, PfFlaI-NTD, PfFlaI-CTD and the stoichiometric mixture of PfFlaI-NTD and PfFlaI-CTD at a concentration of 12.5 µg/ml. For A and B, a representative experiment is shown. For C, graph shows the average of 2 biological replicates with 2 technical replicates.
Figure 5 (on next page)

Nucleotide bound PfFlaH stimulates the ATPase activity of PfFlaI

(A) The ATPase activity at 70°C was determined for PfFlaI, PfFlaH and these proteins with mutations in their respective Walker A PfFlaH(K39A) and walker B motifs PfFlaI(E336A), PfFlaH(D126N) and of different combinations of these proteins. Proteins were added to a final concentration of 1 µM. (B) The ATPase activity at 70°C was determined for 1 µM PfFlaI and increasing amounts of PfFlaH, PfFlaH(K39A) and PfFlaH(D126N) shown in solid line, dashed line and dotted line respectively. The graphs show the average of two biological replicates with two technical replicates.
**Figure 6** (on next page)

*PfFlaH interacts with higher affinity with PfFlaI-CTD than PfFlaI-NTD*

The binding of 28 nM fluorescently labeled Pf FlaH to increasing concentrations of 1.4 nM-22.5 µM for PfFlaI-CTD (black) and 5.7nM- 11.7 µM for PfFlaI-NTD (grey) was studied by microscale thermophoresis. Binding is depicted as the fraction bound and binding curves were fitted to the Hill equation. Curves were obtained from at least two independent experiments.
