Unique ATPase Site Architecture Triggers cis-Mediated Synchronized ATP Binding in Heptameric AAA⁺-ATPase Domain of Flagellar Regulatory Protein FlrC*

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Background: ATP binding, hydrolysis, and σ²⁺ interactions by oligomeric bEBPs are poorly understood.

Results: First structure of the flagellar regulatory protein FlrC revealed a heptameric AAA⁺ domain with unique cis-mediated synchronized ATP binding.

Conclusion: ATPase site architecture of bEBPs influences oligomerization, ATP binding, and σ²⁺ interaction.

Significance: Novel cis-mediated synchronized ATP binding in FlrC without nucleotide-dependent subunit remodeling is delineated.

Bacterial enhancer-binding proteins (bEBPs) oligomerize through AAA⁺ domains and use ATP hydrolysis-driven energy to isomerize the RNA polymerase-σ²⁺ complex during transcriptional initiation. Here, we describe the first structure of the central AAA⁺ domain of the flagellar regulatory protein FlrC (FlrC⁺), a bEBP that controls flagellar synthesis in Vibrio cholerae. Our results showed that FlrC⁺ forms heptamer both in nucleotide (Nt)-free and -bound states without ATP-dependent subunit remodeling. Unlike the bEBPs such as NtrC1 or PspF, a novel cis-mediated “all or none” ATP binding occurs in the heptameric FlrC⁺, because constriction at the ATPase site, caused by loop L3 and helix α7, restricts the proximity of the cis-acting “Glu switch” Glu-286, facilitates ATP binding and hydrolysis. Fluorescence quenching and ATPase assays on FlrC⁺ mutants revealed that although Arg-349 of sensor II, positioned by trans-acting Glu-286 and Tyr-290, acts as a key residue to bind and hydrolyze ATP, Arg-319 of α7 anchors ribose and controls the rate of ATP hydrolysis by retarding the expulsion of ADP. Heptameric state of FlrC⁺ is restored in solution even with the transition state mimicking ADP-AlF₃. Structural results and pull-down assays indicated that L3 renders an in-built geometry to L1 and L2 causing σ²⁺ FlrC⁺ interaction independent of Nt binding. Collectively, our results underscore a novel mechanism of ATP binding and σ²⁺ interaction that strives to understand the transcriptional mechanism of the bEBPs, which probably interact directly with the RNA polymerase-σ²⁺ complex without DNA looping.

The bacterial enhancer-binding proteins (bEBPs)² are molecular machines belonging to the AAA⁺ (ATPases associated with various cellular activities) superfamily (1). The conserved AAA⁺ domain of the bEBPs, which is made of the signature motifs like Walker A, Walker B, sensor I and sensor II (Fig. 1), controls the oligomeric states, nucleotide (Nt) binding, hydrolysis, and/or conformational changes in the loops, implicated in RNAS-σ²⁺ binding to initiate transcription (2). Despite the conserved nature of the AAA⁺ domain, variation in the functional state of oligomerization and mode of Nt-binding of bEBPs emerged as matter of interest. Although Salmonella enterica NtrC and Aquifex aeolicus NtrC1 and NtrC4 are homologs made of regulatory (R), AAA⁺, and DNA binding domains, structural studies showed that their regulatory mechanisms are significantly different (3–6). Upon phosphorylation, the R domain of NtrC gains interactions with neighboring AAA⁺ domain stabilizing the oligomeric form (3, 6). In the case of NtrC1 and NtrC4, phosphorylation or BeF₃ + Mg²⁺ activation at the R domain (7) converts the inactive dimer of the AAA⁺ domain to the active hexa/heptamers (4, 5). The ATP-bound AAA⁺ domain of A. aeolicus NtrCl²⁺AlF₃, revealed a heptameric assembly with asymmetry in the central channel (8). Recently, the AAA⁺ domain of NtrC1 is found to form a split ring hexamer in the presence of ADP + BeF₃ that argues for flexibility in packing stoichiometry and interface angles of the constituting monomers (9). ESI-MS results of A. aeolicus NtrC4 showed that, although the full-length and activated R-AAA⁺ proteins form hexamers, the isolated AAA⁺, unactivated R-AAA⁺, and AAA⁺-DNA binding domains form heptamers (10). Interestingly, despite the variation in the functional oligomeric states, the structural results of the aforesaid bEBPs underscores Nt-dependent subunit remodeling and participation of a trans-acting Arg (named as “R-finger”) in ATP binding and hydrolysis (11–13).

In this study, we have investigated for the first time the state of oligomerization, molecular mechanism of ATP binding, and hydrolysis of a bEBP, FlrC, which is involved in flagellar synthesis of Vibrio cholerae. Transcriptional regulation of the flagellar

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²The abbreviations used are: bEBP, bacterial enhancer binding protein; Nt, nucleotide; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate; PDB, Protein Data Bank; Ni-NTA, nickel-nitrilotriacetic acid; DLS, dynamic light scattering; RNAS, RNA polymerase.

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system of *V. cholerae* acts as an important signaling component of the pathogenic process positively regulating the factors that assist in arrival at the colonization site (14). The transcription of the flagellar genes is organized in a hierarchy of four classes (15). The class I gene product FlrA activates /H9268-dependent transcription of the class II genes *flrBC*, which encode FlrC and its cognate kinase FlrB (16). The expression of class III genes, which encode the basal body hook and the flagellin FlaA, is controlled by modulation of the activity of FlrC (16). Although deletion of *flrC* produces a nonmotile *V. cholerae* strain with a modest colonization defect, a strain producing hyperactive FlrC shows altered cell morphology (16–18). Homologs of FlrC that regulate a similar class of flagellar genes are found in all the *Vibrio* spp. studied (19), along with *Pseudomonas aeruginosa* (20) and *Campylobacter jejuni* (21, 22), suggesting that similar mechanisms underlie the regulation of class III genes in polar flagellates. The /H9268-dependent activators typically bind to the sites located upstream of the /H9268 holoenzyme-binding site and contact RNAP-/H9268 complex by a DNA looping mechanism (23). In contrast, FlrC binds the elements located downstream of the /H9268 binding and transcriptional start sites of the *flaA* and *flgK* promoters (17). Although the downstream location of FlrC-binding sites is unusual, similar downstream binding is also observed in FleQ of *P. aeruginosa* for *flhA*, *fliE*, and *fliL* genes. Ramphal and co-workers (24) suggested that the close proximity of FleQ-binding sites to the /H9268-dependent transcriptional start sites is incompatible with a DNA looping mechanism and argues for a direct interaction between activator and RNAP without looping, which may occur in the case of FlrC as well.

FlrC includes the N-terminal R domain, central AAA+/[^1] domain of *V. cholerae* strain containing an inactive (D54A) or constitutively active (M114I) FlrC mutant showed more severe colonization defects than strain lacking FlrC entirely, which implies that both unphosphorylated and phosphorylated forms of FlrC are required for the colonization, and locking FlrC into either an active or an inactive state would send incorrect stimuli into this stepwise colonization process (18).

Here, we describe the crystal structure of the central AAA+/[^1] domain of FlrC (FlrC[^1]) in Nt-free and AMP-PNP (nonhydrolysable ATP analog)-bound states. Our results provide the first structural evidence for an AAA+/[^1]-ATPase implicated in flagellar synthesis that forms heptamer both in Nt-free and -bound states without any Nt-dependent subunit remodeling. The major presence of the heptameric species is established in solution by the size exclusion chromatography and dynamic light scattering, in the ground and ADP[^2]/[^3]AlF3-mediated transition states of FlrC[^1]. Strikingly, in contrast to the other bEBPs, FlrC[^1] does not use any trans-acting residue for Nt binding, and the reason lies in the ATPase site architecture of the individual monomer. A unique “closed to open” conformational change occurs in Walker A of FlrC[^1] to facilitate ATP binding. Structural observations coupled with fluorescence quenching and ATPase assay identified a novel trans-acting “Glu switch” that promotes the displacement of Walker A for ATP binding and hydrolysis. With a relatively wider central channel and an in-built architecture of the L1 loop, heptameric FlrC[^1] interacts with /H9268 both in Nt-free and -bound states. These intriguing observations open up a new avenue to further explore σ[^4]'-dependent transcriptional mechanism of such activators.
cis-Mediated ATP Binding in Heptameric AAA⁺ Domain of FlrC

EXPERIMENTAL PROCEDURES

Cloning, Overexpression, and Purification—The genes of FlrC⁺ (amino acids 132–381) and σ⁵⁴ (amino acids 1–487) from V. cholerae were cloned in pET28a⁺ within NdeI and BamHI restriction sites. The recombinant proteins with N-terminal His₆ tag were overexpressed in BL21(DE3) and purified by Ni-NTA affinity chromatography as per protocol described in Dey and Dasgupta (25). Mutants E286A, R319A and R349A were prepared by two-step PCR. All the mutants were verified by commercial sequencing, and purifications were done following the same protocol as WT protein.

Crystallization and Diffraction Data Collection—Crystals of Nt-free and AMP-PNP-bound FlrC⁺ were grown at 20°C using the hanging drop vapor diffusion method. Optimal crystals of Nt-free FlrC⁺ were obtained when 4 μl of the protein solution (12 mg/ml) in a buffer consisting of 50 mm Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM MgCl₂, and 10% glycerol was mixed with 2 μl of precipitant solution consisting of 10% (w/v) PEG 6000, 0.1 M MES (pH 6.0) and was incubated over a reservoir solution of same composition. Initially, Nt-free FlrC⁺ crystals diffracted up to 2.8 Å, but after further standardization, the resolution improved to 2.3 Å.

AMP-PNP-bound FlrC⁺ was prepared by mixing 20 μl of the protein solution (20 mg/ml) (in the same buffer as for Nt-free FlrC⁺) with 1 mM AMP-PNP and incubated for 30 min at room temperature. AMP-PNP-bound crystals of FlrC⁺ were grown when 4 μl of the above mixture and 1.5 μl of the precipitant solution (consisting of 10% (w/v) PEG 6000, 0.1 M MES (pH 6.0)) were mixed and equilibrated for 4 days against 12% (w/v) PEG 6000, 0.1 M MES (pH 6.0), 0.1 M NaCl. Crystals were transferred to a cryo-protectant solution consisting of 40% (v/v) ethylene glycol, 2.5% (w/v) PEG 6000, and 50 mM Tris-HCl (pH 8.0). Crystals were then flash-frozen in liquid nitrogen, and diffraction data were collected to 2.6Å.

Diffraction data were collected at BM14 beamline of the European Synchrotron Radiation Facility (ESRF) at Grenoble, France. Data were processed and scaled using HKL2000 (26). Data collection and processing statistics are given in Table 1.

Structure Determination and Refinement—The initial phases for both Nt-free and AMP-PNP-bound FlrC⁺ were obtained by molecular replacement using PHASER (27). Packing considerations indicated the presence of seven molecules in the asymmetric unit for both the structures. Seven molecules were organized in the form of a closed heptamer with a central channel.

Truncated coordinates of one subunit of the inactive dimeric σ⁵⁴ activator NtrC4 of A. aeolicus (PDB code 3DZD) (4) having residues 133–369 (where the coordinates of the N-terminal regulatory domain were truncated) were retrieved, which produced acceptable solution in molecular replacement calculations for Nt-free FlrC⁺. The model of Nt-free FlrC⁺ was then used to solve the structure of AMP-PNP-bound FlrC⁺. Few cycles of simulated annealing, positional refinement, individual B-factor, and TLS refinement were accomplished by PHENIX (28), and model building was done by WinCoot (29). The structures were refined well with Rcryst of 19.4% (Rfree = 25.47%) and with Rcryst of 18.63% (Rfree = 24.66%) for Nt-free and AMP-PNP-bound FlrC⁺, respectively. Data collection and refinement statistics are given in Table 1.

Fluorescence Quenching Study—Fluorescence measurement was carried out using a spectrophotometer, Hitachi F-7000. Changes in tryptophan (Trp-299) fluorescence were measured at an excitation wavelength of 295 nm, and the emission spectra were recorded between 300 and 400 nm with slit widths of 2.5 nm for both excitation and emission. All reactions were carried out at 25°C. Equilibrium titrations of FlrC⁺, R319A, R349A, and E286A were carried out with AMP-PNP. The reactions were performed in a buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 5 mM MgCl₂. For all proteins, the final concentrations were 5 μM and AMP-PNP concentration varied from 0 to 0.39 mM. The binding stoichiometry was determined using the protocol described in Mani et al. (30). The plot of log(F₀ – F)/F against log [AMP-PNP], where F₀ is the fluorescence intensities of FlrC⁺ alone, FlrC⁺ in the presence of various concentrations of AMP-PNP, and FlrC⁺ saturated with AMP-PNP, respectively, yielded a straight line whose slope was a measure of the binding stoichiometry.

The dissociation constant, Kd was determined using nonlinear curve fitting analysis as per Equations 1 and 2 (31). All experimental points for the binding isotherms were fitted by the least squares methods.

\[ K_d = \frac{(C_o - \Delta F/\Delta F_{max})C_o'(C_o + \Delta F/\Delta F_{max})}{(\Delta F/\Delta F_{max})C_o} \]  
\[ (Eq. 1) \]

\[ C_o'(\Delta F/\Delta F_{max})^2 - (C_o' + C_p + K_d)(\Delta F/\Delta F_{max}) + C_p = 0 \]  
\[ (Eq. 2) \]

Although C₀ denotes the input concentrations of the ligand AMP-PNP, C₀' denotes the same for FlrC⁺ and its mutants. ΔF is the change in fluorescence intensity at 340 nm (λex = 295 nm) for each point of titration curve, and ΔFmax is the same parameter when ligand is totally bound to the protein. A double-reciprocal plot of 1/ΔF against 1/(C_p – C₀) as shown in Equation 3 was used to determine the ΔFmax.

\[ 1/\Delta F = 1/\Delta F_{max} + K_d/(\Delta F_{max}(C_p - C_o)) \]  
\[ (Eq. 3) \]

ΔFmax was calculated from the slope of the best fit line corresponding to the above plot. All experimental data points of the binding isotherms were fitted by linear fit analysis using Microsoft EXCEL and Origin 8.0. The equilibrium titrations of FlrC⁺ and the mutant R319A were also carried out in the presence of ADP following the same process as of AMP-PNP.

ATPase Assay—ATPase activity was determined with a procedure from the malachite green assay (32, 33) to monitor the release of inorganic phosphate (P_i). For ATPase assay, reaction mixtures containing FlrC⁺ and the mutants E286A, R319A, and R349A (final concentration of 2.5 μM) were individually incubated with 0.1 mM ATP at 25°C. The reaction buffer was made of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 5 mM MgCl₂. After 25 min of incubation, the reaction mixture was assayed for P_i. FlrC⁺ in the absence of MgCl₂ served as a negative control. Colored reagent containing 10 ml of 0.44 g of Malachite green dissolved in 0.5 ml H₂SO₄, 2.5 ml of 7.5% ammonium
cis-Mediated ATP Binding in Heptameric AAA+ Domain of FrlC

RESULTS

Structural Determination of FrlC<sup>c</sup>—Molecular replacement calculations for Nt-free FrlC<sup>c</sup> were systematically performed using different search models of AAA<sup>+</sup> domain prepared from the coordinates of inactive dimeric A. aeolicus NtrC1 (PDB code 1NY5), ADP-bound heptameric NtrC1 of A. aeolicus (PDB code 1NY6), ATP-bound heptameric variant of NtrC1 (PDB code 3MOE), inactive dimeric NtrC4 of A. aeolicus (PDB code 3DZD), ZraR of Salmonella typhimurium (PDB code 1OJL), and PspF of E. coli (PDB code 2BJW). In each case, the coordinates of AAA<sup>+</sup> domain were retrieved from one subunit, and mismatched residues were mutated to alanine. Although molecular replacement calculations with the models of NtrC1 and PspF produced no solution, the coordinates of NtrC4 having residues 133–369 of chain A yielded a clear-cut solution. PHASER (27) identified seven monomers (with RFZ = 5.1, TF2 = 27.9, and LLG = 1771) organized in a heptameric ring, in the space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, using data between 49 and 2.3 Å resolutions. A search model of ZraR also produced a heptameric solution, but the statistics were better for the previous one. After a few cycles of refinement and model building, Nt-free FrlC<sup>c</sup> produced a final R<sub>crys</sub> of 19.4% (R<sub>free</sub> = 25.47%) (Table 1). The structure of AMP-PNP-bound FrlC<sup>c</sup> was solved using a monomer of Nt-free FrlC<sup>c</sup> structure as search model and the resulting heptameric structure refined up to the R<sub>crys</sub> of 18.63% (R<sub>free</sub> = 24.66%) (Table 1). Noncrystallographic symmetry was not used during the refinement of these two structures.

Heptameric Structure of FrlC<sup>c</sup> in Nt-free State—Nt-free FrlC<sup>c</sup> forms a closed heptameric ring with a central channel (Fig. 2A). The diameter and height of the ring are ~126 and ~55 Å, respectively, whereas that of the central channel is ~26 Å (Fig. 2A). Contact surface areas between the subunits (calculated by PISA) (34) are consistently similar with an average value of ~780 Å<sup>2</sup>. Adjacent protomers of Nt-free FrlC<sup>c</sup> are related roughly by a rotation angle of 50–53° indicating that the heptamer is symmetrical.

Each protomer of FrlC<sup>c</sup> is kidney-shaped consisting of a α/β subdomain that is common for P-loop NTPases and a κ-helical subdomain that carries a signature of AAA<sup>+</sup>-ATPases (Fig. 2, B and C). A monomer of FrlC<sup>c</sup> in the Nt-free state superposes on that of the inactive NtrC4 (4), NtrC1 (13), and PspF (35) with root mean square deviations of 1.1 Å (for 205 Ca), 1.2 Å (for 216 Ca), and 1.7 Å (for 187 Ca), respectively. Structural superposition shows that although the central part of the α/β subdomain of FrlC<sup>c</sup>, containing the helices α1, α2, and the central β-sheet, matches well with aforesaid eEBPs, the loop regions of the α/β subdomain and a portion of the κ-helical subdomain differs significantly (Fig. 2C). Interestingly, a protrusion of the L3 loop and an inclination of helix α7 constric the ATP binding pocket of FrlC<sup>c</sup> by ~7 Å compared with the eEBPs like NtrC1, NtrC4, and PspF (Fig. 2C).

Heptameric State of FrlC<sup>c</sup> Is Retained upon AMP-PNP Binding—AMP-PNP-bound FrlC<sup>c</sup> is also a heptamer (Fig. 2D) whose average contact surface area between subunits (calculated by PISA) (33) is ~750 Å<sup>2</sup>, which is comparable with that of Nt-free FrlC<sup>c</sup>. Angular rotation values are similar to the Nt-free state indicating that the symmetry of the heptamer is retained.
upon AMP-PNP binding (Fig. 2D). Heptamer of AMP-PNP bound FlrC

superposed on the apo-structure with an r.m.s.d. of 0.85 Å, further indicating that no major change in the oligomeric state occurs here. Inter-protomeric interactions were largely retained upon AMP-PNP binding (Table 2). However, significant local conformational changes occurred to accommodate AMP-PNP, which will be discussed subsequently.

Determination of Binding Stoichiometry through Fluorescence Spectroscopy— Stoichiometry of binding between AMP-PNP and FlrC

has been determined using fluorescence spectroscopy. Because Trp-299 is within the Forster distance of the ATP binding pocket, fluorescence quenching of this Trp was monitored in the presence of AMP-PNP. The effect of AMP-PNP binding to each FlrC monomer was determined by measuring the change in fluorescence intensity with increasing concentrations of the AMP-PNP. The plot of fluorescence intensities against ligand concentration (Fig. 2A) yielded a slope of 1.13 indicating 1:1 interaction between AMP-PNP and FlrCC.

Table 1

| Data collection statistics | AMP-PNP-bound FlrC<sup>C</sup> | Nt-free FlrC<sup>C</sup> |
|---------------------------|-----------------------------|------------------------|
| Beamline                  | BM14                        | BM14                   |
| Wavelength (Å)            | 0.980                       | 0.980                  |
| Detector                  | MarCCD                      | MarCCD                 |
| Oscillation (°)           | 1                           | 1                      |
| Space group               | P2<sub>1</sub> 2<sub>1</sub> | P2<sub>1</sub> 2<sub>1</sub> |
| Unit cell parameters (Å)  | a = 81.29, b = 153.28, c = 193.75 | a = 80.96, b = 152.56, c = 193.14 |
| Resolution (Å)            | 49.38-2.3 (2.34-2.30)       | 49.19-2.56 (2.60-2.56) |
| No. of heptamers per asymmetric unit | 1 | 1 |
| R<sub>merge</sub> (%)     | 11.5 (51.4)                 | 5.2 (42.4)             |
| Completeness (%)          | 89.01 (86.2)                | 99.45 (99.5)           |
| Redundancy                | 5.9 (4.1)                   | 4.3 (4.1)              |

Refinement statistics

| Resolution (Å)            | 2.3-4.94                     | 2.6-49.2               |
| No. of reflections        | 96,306                       | 77,494                 |
| R<sub>work</sub>/<R<sub>free</sub> (%) | 19.4/25.47                  | 18.63/24.66             |
| No. of atoms              | 14,061                       | 14,114                 |
| All                       | 14,061                       | 14,114                 |
| Proteins                  | 13,297                       | 13,692                 |
| Ligand                    | 7                           | 26                     |
| Water                     | 757                          | 396                    |
| B-factor (Å<sup>2</sup>)  | 55.23                        | 54.54                  |
| Average                   | Protein                      | 54.23                  |
|                            | Ligand                       | 55.08                  |
|                            | Water                        | 54.70                  |
| Root mean square deviations | Bond lengths/bond angles (Å<sup>2</sup>) | 0.008/1.2 |
| Ramachandran statistics (%) | Most favored                 | 92.36                  |
|                            | Additionally allowed         | 5.27                   |
|                            | Disallowed                   | 2.37                   |

Conserved Arg-349 of sensor II interacts canonically with the γ-phosphate of AMP-PNP (Fig. 3A and C). Mg<sup>2+</sup> that binds the γ-phosphate of AMP-PNP is stabilized by Asp-230, the first conserved acidic residue of Walker B (Fig. 3A and C). Conserved Asn-187 of β2 and Thr-270 (Ala in case of NtrC1, NtrC4, and PsPf) of β4 stabilize the conformation of Asp-230 (Figs. 1 and 3E). Lys-165 of α2 and Asn-272 of sensor I also participate in positioning the γ-phosphate of AMP-PNP (Fig. 3C). Superposition of all seven chains of AMP-PNP-bound FlrC shows conserved water Wat1 that coordinates consistently with Mg<sup>2+</sup> and the γ-phosphate (Fig. 3E). Few more water molecules are also identified in “near apical” position, as observed in case of LTag structure of SV40 (36), to act as potential nucleophile (Fig. 3E).
Figure 2. Overall structure of monomer and heptamer of FlrC\(^{\text{C}}\) in Nt-free and AMP-PNP-bound states. A, at left is a top view illustrating how the protomers pack to form the heptamer of FlrC\(^{\text{C}}\); at right is a side view of the same. B, kidney-shaped monomer of FlrC\(^{\text{C}}\). GAFTGA motif is colored in green. Walker A (yellow), Walker B (pink), sensor I (cyan), and sensor II (blue) residues are shown as spheres. C, superposition of the Nt-free AAA\(^{\text{C}}\) structures of NtrC1 (blue), NtrC4 (yellow), and Pspf (pink) on Nt-free FlrC\(^{\text{C}}\) (red) showing constriction at the ATP binding pocket of FlrC\(^{\text{C}}\), caused by the inclination of \(\alpha 7\) and protrusion of L3 loop. D, electrostatic surface of AMP-PNP bound FlrC\(^{\text{C}}\) showing synchronized binding of AMP-PNP in the ATP binding pockets that are marked by yellow boxes. E, sample plot of fluorescence data from titration of FlrC\(^{\text{C}}\) with AMP-PNP. \(F_0, F, F_{\text{sat}}\) are the relative fluorescence intensities at 338 nm of FlrC\(^{\text{C}}\) alone, FlrC\(^{\text{C}}\) in the presence of a given concentration of AMP-PNP, and FlrC\(^{\text{C}}\) saturated with AMP-PNP, respectively. Slope of the straight line indicates binding stoichiometry. F, at left is \(2F_{\text{Fo}} - F_{\text{Fc}}\) electron density map (contoured at 1\(\sigma\)) around AMP-PNP molecules bound to FlrC\(^{\text{C}}\) heptamer, and at right is the zoomed view of the electron density around an AMP-PNP molecule. G, conformation of Walker A in AMP-PNP-bound FlrC\(^{\text{C}}\) resembles those of ADP-bound NtrC1\(^{\text{C}}\) (accession code 1NY6) and ATP-bound NtrC1\(^{\text{C}}\) (accession code 3M0E). AMP-PNP molecule bound to FlrC\(^{\text{C}}\) also superposes on ADP and ATP bound to NtrC1\(^{\text{C}}\), reflecting canonical binding.

Table 2
Polar and hydrophobic interactions at the subunit interface of Nt-free and AMP-PNP-bound FlrC\(^{\text{C}}\)

| Polar interactions | Nt-free FlrC\(^{\text{C}}\) | AMP-PNP bound FlrC\(^{\text{C}}\) |
|--------------------|--------------------------|-------------------------------|
| Lys-147 NZ        | Leu-361 O                 | Lys-147 NZ                   |
| Val-294 O         | Arg-357 NH1               | Val-294 O                    |
| Tyr-290 O         | Asn-353 OD1               | Tyr-290 O                    |
| Glu-286           | Asp-349                   | Glu-286 OE1, OE2             |
| Arg-285 NH1, NH2  | Ala-189 O                 | Arg-285 NH1                  |
| Asn-238 OD1       | Asp-193 OD2               | Asn-238 OD1                  |
| Lys-242 NZ        | Tyr-203 OH                | Lys-242 NZ                   |
| Arg-258 NH1       | Arg-258 NH2, Arg-258 NE   | Arg-258 NH2, Arg-258 NE      |
| Hydrophobic interactions | Leu-144 | Leu-144 |
| Phe-295, Val-148  | Ile-360, Leu-361          | Ile-360, Leu-361             |

cis-Mediated ATP Binding in Heptameric AAA\(^{\text{C}}\) Domain of FlrC
Conformational Rearrangement of Walker A Is Essential to Accommodate AMP-PNP—Comparison of the AMP-PNP-bound FlrC with the Nt-free structure demonstrates that Walker A motif undergoes a unique conformational rearrangement to facilitate AMP-PNP binding (Fig. 3). In the native (closed) conformation Walker A would exert steric hindrance to the incoming AMP-PNP. A displacement of ~2.5 Å of Walker A relieves this hindrance to facilitate AMP-PNP binding where the side chain of Ser-161 experiences a maximum shift of ~5.3 Å (Fig. 3, F and G). In the Nt-free state, Glu-231 of Walker B and Asn-272 of sensor I bind Ser-161 to stabilize the closed conformation of Walker A (Fig. 3G). Hydrogen bond between Ser-163 and Trp-299 also stabilizes that closed conformation (Fig. 3G). Both of these interactions are abrogated upon AMP-PNP binding, and the open conformation of Walker A is stabilized by trans-acting Arg-285 with cis-acting Ala-189 of L3 loop is also shown here. D, superposition of AMP-PNP-bound FlrC (violet) on the Nt-free structure (green) showing structural changes occurred due to AMP-PNP binding. E, interaction of Mg2+ to γ-phosphate and Asp-230. Conserved water Wat1 (W1) and some the other water molecules located close to the γ-phosphate are also shown here. F, superposition of AMP-PNP bound FlrC (violet) on the Nt-free structure (green) to show the conformational rearrangement of Walker A upon AMP-PNP binding. G, interactions of Walker A before and after AMP-PNP binding.
that aforesaid conformational rearrangement of Walker A, which is unique in FlrC, starts from Pro-160 (Fig. 3).

In the Nt-free state, cis-acting Arg-349 is positioned by an inter-protomeric salt bridge with trans-acting Glu-286 and a hydrophobic barrier made of trans-acting Tyr-290 (Fig. 3G). Upon AMP-PNP binding, Arg-349 slightly alters its conformation, breaks the salt bridge with trans-acting Glu-286, and binds γ-phosphate (Fig. 3G). trans-Acting Glu-286, on the other hand, participates in stabilizing the open conformation of Walker A. Similarly, cis-acting Asn-272 is relieved from anchoring Ser-161 and is recruited to stabilize the γ-phosphate (Fig. 3G). The movement of Walker A causes a lateral shift of Val-167 with adenine base and salt bridge interactions of Lys-165 with β- and γ-phosphates (Fig. 3D).

Contribution of the cis-Acting Arginines and trans-Acting Glu-286 to Nt Binding, Fluorescence Quenching Studies—Based on the structural results, we investigated the potential contribution of Arg-319, Arg-349, and Glu-286 toward AMP-PNP binding through fluorescence quenching studies. Because Trp-299, which is unique in FlrC (Fig. 1), experiences conformational change upon AMP-PNP binding (Fig. 3D), fluorescence quenching of Trp-299 was monitored for FlrCC and the mutants R319A, R349A, and E286A with the addition of AMP-PNP. As expected, FlrCC showed maximum quenching by AMP-PNP with a $K_d$ value of 11.5 ± 0.575 μM (Fig. 4 and Table 3). The minimum quenching was observed for R349A with a $K_d$ of 309 ± 15.45 μM (Fig. 4A and Table 3). Substitution of Arg-
cis-Mediated ATP Binding in Heptameric AAA$^+$ Domain of FlrC

319 by Ala showed almost \( -7 \) fold higher \( K_d \) value compared with FlrC$^C$, although the impact of this substitution was much less compared with that of Arg-349 (Fig. 4A). These observations imply that although Arg-319 renders significant contribution in the Nt binding through its interaction with ribose, stabilization of \( \gamma \)-phosphate is more important in terms of ATP binding, which is severely affected upon mutation at Arg-349. Binding of ADP with FlrC$^C$ has also been tested in a similar fashion. The result showed that the binding efficiency of ADP to FlrC$^C$ is only \( -4 \) fold weaker than AMP-PNP, which might be attributed to the contribution of Arg-319 in stabilizing ribose sugar that may restrict the expulsion of ADP upon hydrolysis. Although Glu-286 shows no direct interaction with AMP-PNP, quenching of E286A was lesser compared with FlrC$^C$ with an \( -5 \) fold higher \( K_d \) value (Fig. 4A) suggesting that in the absence of Glu-286 stabilization of the open conformation of Walker A would be compromised.

**ATPase Activity of FlrC$^C$ and Its Mutants**—We further investigated the ability of FlrC$^C$ and the aforesaid mutants to hydrolyze ATP through Malachite green assay (32, 33). The reactions were carried out in a buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 5 mM MgCl$_2$. Each protein was tested with Malachite green without ATP to measure the contaminant inorganic phosphate if any, and the negligible absorbance thus obtained at 630 nm, ranging between 0.001 and 0.003, was subtracted from the absorbance produced by that protein upon hydrolysis of the added ATP. The highest rate of ATP hydrolysis was observed for FlrC$^C$ (Fig. 4B). We also tested the effect of Mg$^{2+}$ in ATP hydrolysis by measuring generation of inorganic phosphate from ATP by FlrC$^C$ in the absence of Mg$^{2+}$. About 90% reduction in the rate of ATP hydrolysis was observed for FlrC$^C$ without Mg$^{2+}$ (Fig. 4B). Interestingly, the ATP hydrolysis rate of R349A was as low as FlrC$^C$ without Mg$^{2+}$, whereas R319A showed about 30% reduction compared with that of FlrC$^C$ + Mg$^{2+}$ (Fig. 4B). These observations further suggest that although Arg-319 contributes to ATP binding through its interactions with ribose, Arg-349 that stabilizes \( \gamma \)-phosphate is more effective in terms of ATP binding and hydrolysis. Interestingly, similar high thermal vibration was also observed in L1 loops of ATP bound NtrC$^C$ structure (Fig. 5G).

**FlrC$^C$ Interacts with \( \sigma^{54} \), Pulldown Assay**—In-built architecture of L1, L2, and L3 loops prompted us to qualitatively assess the binding ability of FlrC$^C$ with \( \sigma^{54} \) through in vitro pulldown assays. \( \sigma^{54} \) having N-terminal His$_6$ tag was immobilized on Ni$^{2+}$-NTA resin. The resin was then washed thoroughly and incubated with Nt-free FlrC$^C$ as well as FlrC$^C$ treated with ATP and AMP-PNP individually. Our results consistently showed interaction between \( \sigma^{54} \) and Nt-free, AMP-PNP and ATP-bound FlrC$^C$ (Fig. 5H). Only FlrC$^C$, FlrC$^C$ + ATP, and FlrC$^C$ + AMP-PNP without \( \sigma^{54} \) were used as controls to see the basal level of adherence of FlrC$^C$ (if any) with Ni-NTA in free and Nt-bound states. Only \( \sigma^{54} \) nullifies the possibility of any contaminating band. Variation in ATP or AMP-PNP concentration did not show any measurable variation in binding (data not shown).

**Size Exclusion Chromatography and DLS Showed Retention of the Oligomeric State in Solution**—We have investigated the oligomeric states of Nt-free as well as AMP-PNP and ADP + AlF$_3$ (that mimics transition state)-treated FlrC$^C$ in solution through gel filtration experiments in Superdex-200 column. All three profiles consistently show a major presence of the heptamers with a minor trailing at the lower molecular weight region (Fig. 6A). The observations indicate that the state of oligomerization of FlrC$^C$ remains intact upon Nt binding.

To further investigate, we performed DLS experiments with the eluted fractions corresponding to the peak region and trailing region of gel filtration. The peak regions of Nt-free FlrC$^C$, AMP-PNP-treated FlrC$^C$, and ADP + AlF$_3$-treated FlrC$^C$ showed a monomodal population with a hydrodynamic radius \( R_h \) of 6.6 ± 0.3 nm (Fig. 6B) that corresponds to the molecular mass of 277 ± 30 kDa. An oligomeric assembly with a central pore is expected to have a larger hydrodynamic radius than the compact globular proteins of similar molecular weight. The trailing region identified species with \( R_h \) of 4.5 ± 0.1 and 5.1 ± 0.1 nm that correspond to molecular masses 115 ± 10 and 150 ± 10 kDa, which probably appear due to gradual dilution. Nonetheless, DLS results further established that the major oligomeric structure of FlrC$^C$ in solution is not influenced by Nt binding (Fig. 6B).
DISCUSSION

Two models were proposed to explain how hydrolysis is coordinated in the AAA+ family of proteins (38). Homogeneous Nt occupancy was observed for a number of AAA+/H11001 protein crystal structures, such as SV40 helicase LTag (2, 39–41), where Nt binds simultaneously to all the pockets of the oligomer with full occupancy, supporting a model of concerted/synchronized hydrolysis. Other AAA+ structures showed mixed occupancy with ATP/ADP, which supports a model of sequential hydrolysis (42, 43). The 1:1 binding stoichiometry between FlrCC monomer and AMP-PNP indicates that all seven pockets of the heptamer are able to act simultaneously as potential ATP-binding sites (Fig. 2E). Our structural results also show that AMP-PNP binds to all seven pockets of FlrCC.

FIGURE 5. Structural details of L1, L2, and L3 loops and interaction of FlrCC with σ54. A, stereo view of 2Fo-Fc electron density map (contoured at 1σ) around L1 loops (in yellow) and L3 loops (in blue) in AMP-PNP-bound FlrCC. B, overall comparison of the structures of Nt-free (gray) and AMP-PNP-bound FlrCC. C, comparisons of the intra-subunit interactions at L1, L2, and L3 loops between Nt-free (gray) and AMP-PNP-bound (magenta) FlrCC. D, inter-protomeric interactions between L1 (magenta) with trans-acting L2 (yellow) and L3 (magenta) with trans-acting α4 (yellow). Nt-free FlrCC is shown in gray. E, comparison of the main chain B-factors for Nt-free (black) and AMP-PNP-bound (red) FlrCC showing an increase of thermal vibration of L1 and L3 loops and a decrease of thermal vibration in σ7 and sensor II upon AMP-PNP binding. F, clustering of the high thermal vibration regions (L1 and L3) in AMP-PNP-bound FlrCC (from low, blue, to high, red). G, clustering of the high thermal vibration regions (L1 loops) in ATP-bound NtrC1C (accession code 3M0E) (from low, blue, to high, red). H, pulldown assay showing interactions between FlrCC and σ54 of V. cholerae in the presence and absence of ATP/AMP-PNP. The interaction between FlrCC and σ54 was determined by Coomassie staining. Left gel, lane 1 shows His6-tagged full-length σ54 bound to Ni-NTA resin; lane 2 is the molecular weight marker; lane 3 shows the interaction between FlrCC and His6-tagged σ54 in the absence of Nt; lanes 5 and 7 show the interactions between σ54 and FlrCC in the presence of ATP and AMP-PNP, respectively. Lanes 4, 6, and 8 show background binding of FlrCC with Ni-NTA free and in the presence of ATP and AMP-PNP, respectively. Right gel, purified σ54 (lane 1) and FlrCC (lane 3).
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![Graphs and diagrams](https://example.com)

The closed conformation of Walker A in the presence or absence of Nt show exclusive formation of the heptamers. The developed chromatograms are shown for FlrC alone (black), FlrC + AMP-PNP (blue), and FlrC + ADP-AlF\(_3\) (red). The molecular weight of the peaks was determined from the calibration curve prepared using molecular weight standards. A, from left to right, the DLS profiles of Nt-free FlrC, FlrC + AMP-PNP, and FlrC + ADP-AlF\(_3\), C, monomer and heptamer of NtrC1 in ATP-bound state. D, monomer and heptamer of FlrC in AMP-PNP-bound state. ATP binding pocket of the monomers is shown by black bar in C and D.

Heptamer with full occupancy (Fig. 2, D and F) which resembles the “all or none” Nt-binding model proposed in support of synchronized hydrolysis. Unlike PspF or NtrC1, where inter-subunit interactions confer cooperativity in Nt binding (8, 44), FlrC\(^+\) in its heptameric state, renders an intriguing cis-mediated all or none ATP binding without any Nt-dependent subunit remodeling.

A unique closed to open type conformational change of Walker A facilitates ATP binding and subsequent hydrolysis in FlrC (Figs. 3 and 4). Interestingly, the conformation of Walker A in the “native” (ADP-bound) NtrC1 (PDB code 1NY6) and ATP-bound NtrC1\(^\text{C}\) (PDB code 3MOE) (8, 13) closely resemble the open conformation of Walker A of FlrC\(^+\) implying that no such rearrangement of Walker A is required in NtrC1 (Fig. 2G). In FlrC, the closed conformation of Walker A exerts steric hindrance to the \(\beta\)- and \(\gamma\)-phosphates of the incoming AMP-PNP (Fig. 3F). A closed to open movement, assisted by the trans-acting sensor I residue Glu-286, is thus required for efficient binding (Fig. 3, F and G). Upon AMP-PNP binding, Lys-165, along with the helix \(\alpha2\), moves toward the ATP binding pocket and stabilizes \(\beta\)- and \(\gamma\)-phosphates. Consequent repositioning of the neighboring residues like Val-167 of \(\alpha2\), Leu-312 and His-315 of \(\alpha7\), and N-terminal Val-132 constitutes a hydrophobic pocket capped by Trp-299 to house the adenine base (Fig. 3D).

Further stabilization to AMP-PNP binding is incurred by the novel cis-acting Arg-319 of \(\alpha7\) that binds the ribose of AMP-PNP (Fig. 3C). The corresponding residue Lys-327 of NtrC1 resides about 6 Å away from the ribose (8). Likewise, Lys-230 of PspF, instead of interacting with ribose, forms a salt bridge with neighboring Glu-234 (45). In FlrC, the inclination of \(\alpha7\) toward the ATP binding pocket makes the interaction of Arg-319 with ribose feasible, which was not the case for NtrC1 or PspF.

Asn-272 of sensor I and Arg-349 of sensor II contribute significantly in positioning the \(\gamma\)-phosphate. Comparison of the \(K_d\) values and release of \(P_i\) showed that substitution of Arg-349 by Ala drastically reduces ATP binding and hydrolysis, although the effect of R319A is not so damaging (Table 3 and Fig. 4). ATP binding and hydrolysis are thus influenced more by the stabilization of \(\gamma\)-phosphate than that of the ribose. However, the contribution of Arg-319 cannot be under-rated. Only a 4-fold increase in \(K_d\) upon ADP binding (Table 3 and Fig. 4A) suggests that Arg-319, because of its interaction with ribose, acts as a deciding factor in the rate of ATP hydrolysis by retarding the release of ADP.

Walker B and sensor I also play regulatory roles in ATP binding and hydrolysis. Asp-230 of Walker B is positioned by the neighboring Asn-187 and Thr-270 for efficient Mg\(^{2+}\) binding. Conversely, the next residue Glu-231 stabilizes the closed conformation of Walker A in the Nt-free state and that of Asn-272 in the AMP-PNP-bound state, features which point toward a regulatory role of Glu-231. Additionally, Asn-272 of sensor I has emerged as a new “cis-acting Asn switch” in FlrC because of its dual role in stabilizing the closed conformation of Walker A and in sensing \(\gamma\)-phosphate upon AMP-PNP binding (Fig. 3G).

A trans-acting Arg, defined as R-finger, is observed in quite a few AAA\(^+\)-ATPases that participate in ATP binding and hydrolysis, although the mechanistic conclusions on the role(s) of such an R-finger have yet to be drawn (2). In NtrC1 or PspF, trans-acting arginine(s) belonging to the “RXDXXR” motif of sensor I stabilize the \(\gamma\)-phosphate of ATP. Strikingly, despite the conservation of 2MMDREDXXXR in FlrC (Fig. 1), neither of...
these two arginines participates in AMP-PNP binding. Rather, trans-acting Arg-285 stabilizes the cis-acting L3 loop beneath the γ-phosphate (Fig. 5D), and the Arg-291 side chain stays about 10 Å away from the phosphates of AMP-PNP (Fig. 3C). Despite that, the role of the 285REDXXR291 motif in ATP binding and hydrolysis of FlrC cannot be ignored. Stabilization of γ-phosphate is the most essential step in ATP hydrolysis, and in FlrC, the cis-acting Arg-349 of sensor II is one of the major contributors in this direction (Figs. 3G and 4). Even in the Nt-free state, Arg-349 remains oriented toward the ATP binding pocket and by the hydrophobic packing with trans-acting Tyr-290, both of which belong to the 285REDXXR291 motif. Additionally, trans-acting Glu-286 stabilizes the open conformation of Walker A and thus serves as a novel trans-acting “Glu switch” that facilitates ATP binding and hydrolysis.

The precise organization of the interface between two adjacent subunits is the key element for oligomerization of bEBPs. Starting from very similar monomeric structures, when PsPF(1–275) and NtrC1cis-organize into hexamers and heptamers, respectively, their inter-protonic interfaces adopt different configurations (11). Interestingly, the unique architecture of the FlrCcis-monomer leads to a heptamer with a much wider central channel (diameter ~26 Å) compared with NtrC1trans (diameter ~17 Å) (Figs. 2C, and 6, C and D). The cis-acting mode of AMP-PNP binding in FlrCcis is also guided by the characteristic architecture of the individual monomer. The inclination of α7, coupled with the protrusion of L3 loop, constricts the ATP binding pocket of FlrCcis-protomer by about 7 Å compared with that of NtrC1trans (Figs. 2C, and 6, C and D). Because of this constriction, the adjacent protomers in FlrCcis stay relatively away at the ATP-binding site (Fig. 6, C and D), eventually occluding the trans-acting residues from ATP binding. However, the binding and hydrolysis of ATP are not compromised in FlrCcis. Packing of the adenine base in a hydrophobic pocket, stabilization of the ribose, and most importantly the locking of the γ-phosphate make an efficient cis-mediated productive ATP binding without any direct contribution from the trans-acting residues.

The necessary amount of ATP hydrolyzed by an AAA+ATPase might be different for different functions (11). An all or none binding of AMP-PNP + Mg2+ complex (with waters molecules in near apical positions) in FlrCcis points toward a synchronized mechanism of ATP hydrolysis. Local conformational changes occur here in a subtle manner to accommodate ATP without destroying the inter-protonic interactions (Table 2). ATP binding and hydrolysis are, however, regulated by the conformational restriction of Walker A and the retarded expulsion of ADP. Upon requirement of ATP hydrolysis, Walker A moves to the open conformation, stabilized by the trans-acting Glu switch as a result of which the hydrophobic pocket for the adenine base is created. Hydrolysis of ATP and release of P, then allow Walker A to return to its closed conformation that eventually destroys the hydrophobic pocket for adenine base causing expulsion of ADP.

The bEBPs typically bind to the enhancer elements far upstream of the σ54-binding site, and upon DNA looping interact with RNAP-σ54 at the promoter (2). FlrC, together with the flagellar regulators FlrA of V. cholerae and FleQ of P. aeruginosa, forms a new set of bEBPs that bind to the enhancer elements located downstream of the σ54-binding and transcriptional start sites (17, 24, 46). Interestingly, a sequence comparison of these flagellar regulators with NtrC1, NtrC4, and PsPF showed that they consistently possess a 40–50-residue longer linker region between ATPase and the DNA binding domain. Although the process of DNA looping seems to be incompatible with such downstream enhancer binding (17, 24, 46), their mechanism of RNAP-σ54 binding at the promoter to initiate DNA melting has yet to be investigated.

Cross-linking and EM reconstruction studies have recently shown that only one oligomeric assembly of bEBP is sufficient enough to simultaneously bind RNAP-σ54 and the upstream promoter region using varying numbers of participating L1 loops (2, 9, 47, 48). Notably, σ54 binds bEBP and RNAP through its highly conserved regions I and III. Considering the very high degree of sequence conservation at these two regions of σ54 and the conserved nature of the GAFTGA loop of bEBPs, a similar binding stoichiometry may be expected between FlrC heptamer and RNAP-σ54.

Extensive studies on PsPF or NtrC1, however, showed that participation of L1 loops in binding σ54 at the promoter is actually guided by Nt binding, hydrolysis-induced subunit remodeling, and a spatio-chemical environment offered by the asymmetric arrangement of the L1 loops (8, 44, 45). Although our results on FlrCcis exclude the possibility of any such Nt-dependent subunit remodeling, even in the presence of ADP, the probability of local structural changes are not ruled out. In FlrCcis, loop L1 and its neighboring regions have in-built architecture that is qualitatively supported by in vitro pulldown assay with σ54 (Fig. 5, C, D, and H). It seems that FlrCcis has a potential to recognize RNAP-σ54 even without Nt binding, although the exact scenario in the presence of promoter has yet to be investigated. This is not a very rare observation because Lee and Huber (49) reported that Rhizobium meliloti C4-dicarboxylic acid transport protein D cross-links with σ54 even without Nt binding. In FlrCcis, the small asymmetry created in the central pore and their elevated thermal vibration upon AMP-PNP binding probably point toward the generation of local asymmetry upon ATP binding, which may enhance during ATP hydrolysis, as observed previously for the other bEBPs (8, 9), although the extent of asymmetry may differ in this case. Dimension of the central pore and disposition of the L1 loops around the central pore would seemingly play a crucial role in causing asymmetry (8, 9). The central pore of FlrCcis heptamer is strikingly wider having a diameter of ~26 Å compared with the other bEBP structures determined so far (~17 Å for NtrC1trans heptamer) (Fig. 6, C and D). In spiral or split ring hexamers, asymmetric movement of the L1 loops makes them separate enough to simultaneously interact with RNAP-σ54 and the upstream promoter region (9, 48). However, the extent of asymmetry to make a productive complex with σ54 at promoter may not be that dramatic with an ATPase ring having much wider central pore where adjacent GAFTGA loops already stay substantially away (~12 Å in FlrCcis) from each other.

Collectively, the structure of FlrCcis with a wide central pore in the ATPase ring guided by unique ATPase site architecture,
cis-mediated synchronized ATP binding, and hydrolysis without subunit remodeling and a long linker that connects the ATPase ring with the DNA binding domain are indicative of a novel transcriptional initiation mechanism for this bEBP, involved in downstream enhancer binding. In the future, additional structures in the presence of ADP-AlF₄⁻ combined with systematic biochemical and structural analysis of σ²⁸ binding in the presence of cognate promoters should facilitate progress in defining the mechanochemical action of such unusual bEBPs.

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