Chapter 4

Measurement of relative contribution of the key residues to ATP binding and hydrolysis: fluorescence quenching and ATP hydrolysis studies on FlrC and its mutants
Summary

Structural data of FlrC-AMP.PNP complex suggest that the cis-acting Arginines, R319 and R349 and the trans-acting Glutamate, E286 play crucial role in sensing and stabilizing AMP.PNP binding. Mutation of these important residues, followed by fluorescence quenching studies and ATP hydrolysis assays with FlrC and the mutants allowed us to determine the relative contribution of these key amino acids toward ATP binding and hydrolysis. This chapter contains methods and results of fluorescence quenching studies and malachite green assays with FlrC and its mutants. The binding affinity and ATP hydrolysis data were analyzed to understand the relative contribution of these key residues.

4.1 Fluorescence spectroscopic studies of ATP binding to FlrC

4.1.1 Preparation of the mutants

Mutants R319A, R349A and E286A were prepared by two steps PCR and the mutant clones were verified by commercial sequencing. All the mutant proteins were overexpressed and purified using the same protocol described for the wild type protein in chapter 2.

4.1.2 Fluorescence Studies

Steady-state fluorescence spectra were measured at 25°C on a Hitachi F-7000 spectrofluorometer for the wild type and the mutant proteins, R319A, R349A and E286A. The method of experiment has been described in the section 3.5 of Chapter 3. Briefly, changes in tryptophan (W299) fluorescence were measured at an excitation wavelength of 295 nm and the emission spectra were recorded between 300 and 400 nm. All the reactions were carried out at 25 °C and equilibrium titrations of FlrC, R319A, R349A and E286A were carried out with AMP.PNP. The effect of AMP.PNP on protein fluorescence intensities were monitored by adding
AMP.PNP to protein samples from the stock solutions. The fluorescence intensities were corrected for dilution factors. Background quenching was eliminated by subtracting the signal obtained from a buffer solution that contained the appropriate quantity of AMP.PNP. The total AMP.PNP concentration varied from 0 to 0.39 mM.

4.1.3 Analysis of Fluorescence Quenching Data

The dissociation constant, $K_d$, was determined using nonlinear curve fitting analysis as per Equations 1 and 2 (90). All experimental points for the binding isotherms were fitted by the least squares methods.

\[ K_d = [C_0 - (\Delta F / \Delta F_{\text{max}}) C_0] [C_p - (\Delta F / \Delta F_{\text{max}}) C_0] / [(\Delta F / \Delta F_{\text{max}}) C_0] \] \[ 1 \]

\[ C_0 (\Delta F / \Delta F_{\text{max}})^2 - (C_0 + C_p + K_d) (\Delta F / \Delta F_{\text{max}}) + C_p = 0 \] \[ 2 \]

While $C_0$ denotes the input concentrations of the ligand AMP.PNP, $C_p$ denotes the same for FlrC and its mutants. $\Delta F$ is the change in fluorescence intensity at 338 ($\lambda_{ex}=295$ nm) nm for each point of titration curve, and $\Delta F_{\text{max}}$ is the same parameter when ligand is totally bound to the protein. A double-reciprocal plot of $1/\Delta F$ against $1/(C_p - C_0)$, as shown in equation [3] was used to determine the $\Delta F_{\text{max}}$.

\[ 1/\Delta F = 1/\Delta F_{\text{max}} + K_d/[\Delta F_{\text{max}}(C_p-C_0)] \] \[ 3 \]

$\Delta F_{\text{max}}$ was calculated from the slope of the best-fit line corresponding to the above plot. All experimental data points of the binding isotherm were fitted by linear fit analysis using Origin 8.0. The equilibrium titration of FlrC and the mutant R319A were also carried out in the presence of ADP following the same process as of AMP.PNP.
4.1.4 Relative contributions of the cis-acting Arginines and trans-acting E286 to ATP binding

Since W299 experiences conformational change upon AMP.PNP binding (Fig. 3.4F), fluorescence quenching of W299 was monitored for FlrC and the mutants R319A, R349A and E286A with the addition of AMP.PNP. As expected, FlrC showed maximum quenching by AMP.PNP with a $K_d$ value of 11.5±0.575 µM while it was minimum for R349A with a $K_d$ of 309±15.45 µM (Fig. 4.1 and Table 4). Substitution of R319 by Ala showed almost ~7-fold higher $K_d$ value compared with FlrC, although the impact of this substitution was much less compared with that of R349 (Fig. 4.1). These observations imply that although R319 renders significant contribution in 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 R349. Binding of ADP with FlrC has also been tested in a similar fashion. The result showed that the binding efficiency of ADP to FlrC is only ~4-fold weaker than AMP.PNP, which might be attributed to the contribution of R319 in stabilizing ribose sugar that may restrict the expulsion of ADP upon hydrolysis. Although E286 shows no direct interaction with AMP.PNP, quenching of E286A was lesser compared with FlrC with a ~5 fold higher $K_d$ value (Fig. 4.1) suggesting that in the absence of R286 stabilization of the open conformation of Walker A would be compromised.

![Figure 4.1](image_url)

**Figure 4.1:** Fluorescence studies. At left are the plots of $\Delta F/\Delta F_{\text{max}}$ vs AMP.PNP/ADP concentration (mM) and corresponding $K_d$ values (both in graphical and numerical modes) for FlrC and its mutants at right.
Table 4.1: $K_d$ values of FlrC$^C$ and its mutants calculated using AMP.PNP and ADP as ligands

<table>
<thead>
<tr>
<th>Protein+Ligand</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlrC$^C$+AMP.PNP</td>
<td>11.5±0.575</td>
</tr>
<tr>
<td>E286A+AMP.PNP</td>
<td>53.9±2.695</td>
</tr>
<tr>
<td>R319A+AMP.PNP</td>
<td>84.7±4.235</td>
</tr>
<tr>
<td>R349A+AMP.PNP</td>
<td>309±15.45</td>
</tr>
<tr>
<td>FlrC$^C$+ADP</td>
<td>44.9±2.245</td>
</tr>
</tbody>
</table>

4.2 ATP hydrolysis study

4.2.1 Methods of ATPase Assay

ATPase activity was determined with a procedure from the malachite green assay (91, 92) to monitor the release of inorganic phosphate ($P_i$). For ATPase assay, reaction mixtures containing FlrC$^C$ 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 MgCl$_2$. After 25 min of incubation, the reaction mixture was assayed for $P_i$. FlrC$^C$ in the absence of MgCl$_2$ served as a negative control. Colored reagent containing 10 ml of 0.44 g of Malachite green dissolved in 0.3 M H$_2$SO$_4$, 2.5 ml of 7.5% ammonium molybdate, and 0.2 ml of 11% Tween 20 was added to the reaction mixture after 25 min, and the absorbance was measured at 630 nm within 5 min of adding the coloring reagent. The total $P_i$ for each reaction was compared with a $P_i$ standard curve prepared using KH$_2$PO$_4$. All the experiments were minimally performed in triplicates.

4.2.2 ATPase activity shown by FlrC$^C$ and its mutants

The ability of FlrC$^C$ and the aforesaid mutants to hydrolyze ATP through Malachite green assay (91, 92). Each protein was tested with Malachite green without ATP to measure the contaminant inorganic phosphate if any, and the negligible O.D. thus obtained at 630 nm, ranging
between 0.001-0.003 was subtracted from the O.D. produced by that protein upon hydrolysis of the added ATP. Highest rate of ATP hydrolysis was observed for FlrC\(^{C}\) (Fig. 4.2). We also tested the effect of \(\text{Mg}^{2+}\) in ATP hydrolysis by measuring generation of inorganic phosphate from ATP by FlrC\(^{C}\) in the absence of \(\text{Mg}^{2+}\). About 90% reductions in the rate of ATP hydrolysis was observed for FlrC\(^{C}\) without \(\text{Mg}^{2+}\) (Fig. 4.2). Interestingly, the ATP hydrolysis rate of R349A was as low as FlrC\(^{C}\) without \(\text{Mg}^{2+}\) while R319A showed about 30% reduction compared to that of FlrC\(^{C}\)+\(\text{Mg}^{2+}\) (Fig. 4.2). These observations further suggest that although R319 contributes to ATP binding through its interactions with ribose, R349 that stabilizes \(\gamma\)-phosphate is more effective in terms of ATP binding and hydrolysis. Interestingly, although E286 has no direct interaction with AMP.PNP, the mutant E286A shows about 20% reduction in the rate of ATP hydrolysis (Fig. 4.2).

![Figure 4.2: ATPase Assay. ATPase activities of FlrC\(^{C}\pm\text{Mg}^{2+}\) and the mutants were measured by Malachite green assay and the release of inorganic phosphate (\(P_i\)) was estimated against the standard curve of \(\text{KH}_2\text{PO}_4\) (left).](image-url)