Chapter 6

Enhancer DNA stabilize complex formation of FlrC with $\sigma^{54}$
Summary

Although it has been established that FlrC binds to the enhancer elements located downstream of the σ^{54}-dependent transcription start site, the affinity and mechanism of binding are still unknown. This chapter presents the results of fluorescence quenching studies which has been performed to establish downstream promoter binding of a bigger construct of FlrC, FlrCΔR, that contains both AAA^{+} and DNA binding domain and lacks only the R domain and to measure the promoter binding affinity of FlrCΔR using flaA promoter. This chapter further contains the gel filtration data showing the oligomeric state of (FlrCΔR) in the presence and the absence of AMP.PNP. We have also investigated the influence of AMP.PNP and the enhancer elements on the complex formation between FlrC with σ^{54} and the results are reported here.

6.1 σ^{54} and downstream enhancer binding by FlrCΔR

FlrC^{C} not only forms heptamers both in Nt-free and -bound states without ATP dependent subunit remodeling, but also recognizes σ^{54} without Nt binding. To understand functional oligomeric state of FlrC in further detail I have prepared a bigger construct of FlrC i.e. FlrCΔR that contains both AAA^{+} and DNA binding domain and lacks only the R domain (Fig. 6.1A). The state of oligomerization of this construct in solution has been determined in the presence and absence of Nt.

Correa et al (2005) showed that FlrC binding site(s) in the flaAp lie between +24 to +85 with respect to the transcription start site (60) (Fig. 6.1B). Within this region there is a 6-bp sequence motif, CGGCAA that is repeated four times (Fig. 6.1B). They have further demonstrated that FlrC binds flaAp stronger than flgKp and suggested that the flaAp sites are likely the strongest FlrC binding sites within the genome (60). Therefore, a 62 bp DNA fragment corresponding to the proposed FlrC binding site of flaAp has been prepared. This fragment is utilized not only to check
the influence of the enhancer elements on the oligomerization of FlrCΔR and σ^{54} binding but also to measure its binding affinity toward FlrC.

![Diagram](image)

**Figure 6.1:** Domain organization of FlrC and sequence of enhancer element. (A) Domain organization of FlrC protein containing R, AAA^+ and DNA binding domain (DBD). (B) flaA promoter with σ^{54} and FlrC binding region. Grey shaded regions are the σ^{54} binding site and pink region is FlrC binding regions that includes four "CGGCA" (magenta box) motifs (60).

### 6.2 Preparation of FlrCΔR and the DNA fragment

#### 6.2.1 Cloning and purification of FlrCΔR

FlrCΔR (132-479) is a truncated construct of FlrC which contains AAA^+ and C-terminal DNA binding domain (Fig. 6.1A). FlrCΔR was cloned into kanamycin resistance pET28a^+ vector using the clone of full-length FlrC as template. The protein was overexpressed with an N-terminal 6×His-tag (Fig. 6.2A) and optimal expression level and solubility have been standardized. For overexpression, a single colony was picked, transferred into 100 ml LB broth and grown overnight. 1 litre LB broth was inoculated with 10 ml overnight culture and the culture was grown at 37°C until the OD_{600} reached 0.6. The temperature of the incubator was then reduced to 20°C and cells were induced with 0.5 mM IPTG at 20°C overnight. After induction, the cells were harvested at 4500g for 20 min and the pellet was resuspended in 10 ml ice-cold lysis buffer consisting of 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 5 mM MgCl_2, 10% (v/v) glycerol. PMSF
(final concentration of 1 mM) and 1 mg/ml lysozyme were added to the resuspended solution and it was lysed by sonication on ice. The cell lysate was then centrifuged (12 000g for 50 min) at 4°C and the supernatant was collected. Ni$^{2+}$-NTA (Qiagen) resin was equilibrated with the above mentioned lysis buffer and the supernatant containing 6×His-tagged FlrCΔR was immobilized on the resin for Ni$^{2+}$-NTA based immobilized metal-ion affinity chromatography. The resin was washed with different wash buffers containing an increasing concentration (from 5 to 50 mM) of imidazole and the 6×His-tagged FlrCΔR was eluted with the buffer containing 150 mM imidazole. The purity of the eluted fractions was checked using SDS-PAGE with 12% polyacrylamide concentration and the fractions were found to be nearly homogeneous. The eluted protein was first dialysed overnight against the buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM MgCl$_2$, 10% (v/v) glycerol and then concentrated using an Amicon ultracentrifugation unit (with molecular weight cutoff of 30 kDa). The homogeneity of the purified protein was checked by SDS-PAGE using the above mentioned polyacrylamide concentration (Fig. 6.2B)

**Figure 6.2**: Purification of FlrCΔR. (A) Overexpression of 6×His-tagged FlrCΔR: Uninduced (UI) and induced (I) proteins are shown together with the molecular-weight markers (lane M). (B) The homogeneity of the purified FlrCΔR has been checked by 12% SDS–PAGE. Lane 1 and 2 contain 2µl and 4µl of samples, lane M indicates molecular weight marker labelled in kDa.
6.2.2 Preparation of the DNA fragment

The 172 bp size fragment of flaA (-34 to +137) promoter region containing the binding sites for both FlrC and σ^{54} was amplified from *V. cholerae* genomic DNA using specific primers (Forward: 5′-TAAAAGTGGCAGGGAAGTTGC-3′ and Reverse: 5′-CATCGCTTA TTTCAATGAG-3′). The amplicon was further purified using PCR purification kit (invitrogen). The enhancer element only for FlrC binding at flaAp with four CGGCAA motif is of 62 bp ranging between +24 to +85. Therefore, a smaller DNA fragment (of 62bp) corresponding to the region of flaAp, specific for FlrC binding, was generated by hybridization of oligonucleotide strands (5′–GGGTAACCAAGCGCAAGTCAGCGGCAAAATGGATTGCCGCCTCACCAG AATCGGCAACTTT–3′) and its reverse complemented partner, synthesized by IDT service.

6.3 Fluorescence quenching study

Binding affinity (K_d) and binding stoichiometry of FlrCΔR with the downstream enhancer elements was determined by fluorescence quenching studies. Fluorescence quenching of W380 was monitored since W380 of FlrC is expected to be within the Foster distance of the C-terminal DNA (enhancer) binding domain. Negative control experiments were performed with FlrC^C (Fig. 6.3A) that contains W380 but lacks the DNA binding domain.

6.3.1 Determination of the binding affinity and stoichiometry of FlrCΔR with 62bp *flaAp* fragment

We have studied the effect of 62 bp (+24 to +85) enhancer element binding to FlrCΔR by determining the response of its fluorescence to the increasing concentrations of that DNA ligand. The binding affinity (K_d) and the stoichiometry was determined by plotting fluorescence
quenching as a function of ligand concentration. At saturating concentration of DNA fragment the maximum quenching of fluorescence intensity was observed. The DNA was bound with high affinity with $K_d$ value 0.21 pmole (Fig. 6.3A, Table 6.1). The log $(F_0 - F)/(F - F_\infty)$ is plotted against log [DNA fragment], where $F_0$, $F$, and $F_\infty$ are the fluorescence intensities of FlrCΔR alone, FlrCΔR in the presence of various concentrations of DNA fragment and FlrCΔR saturated with DNA respectively. The value of the slope for the logarithmic plot was 1.20 indicating 1:1 binding stoichiometry (Fig. 6.3B). $K_d$ cannot be calculated for the negative control study with FlrC (Fig. 6.3A).

FlrCΔR binds to AMP.PNP with $K_d$ value 19.5 µM (Table 6.1) with a binding ratio of 1:1. Therefore, a quenching experiment with DNA fragment and FlrCΔR has been conducted in the presence of 100 µM AMP.PNP since at this concentration of AMP.PNP, FlrCΔR is expected to be saturated with AMP.PNP and no further quenching will occur in W299 which responds to AMP.PNP binding. The observed fluorescence emission intensity of this complex of AMP.PNP and FlrCΔR was taken as the control value in the absence of the DNA fragment. Quenching of the fluorescence emission intensity was then monitored as a function of the concentration of added DNA fragment to determine the binding affinity of the DNA to this complex. The affinity of FlrCΔR for 62bp flaAp was not significantly affected by the presence of AMP.PNP ($K_d$=0.23 pmole) (Fig. 6.3C, Table 6.1) which indicate towards the formation of a ternary complex between FlrC, AMP.PNP and flaAp with 1:1 binding stoichiometry (Fig. 6.3D).
Figure 6.3: Fluorescence titration of FlrCΔR vs oligonucleotide. (A) Plot of ΔF/ΔF_{max} vs oligonucleotide concentration, FlrCΔR plot is of black square and control plot of FlrC^C is of red circle; (B) Sample plot of fluorescence data from titration of FlrCΔR with oligonucleotide. Slope of the *straight line* indicates binding stoichiometry; (C) FlrCΔR plot of ΔF/ΔF_{max} vs oligonucleotide concentration in presence of saturated condition of AMP.PNP; (D) Sample plot of fluorescence data from titration with oligonucleotide in presence of AMP.PNP to show binding stoichiometry.

Table 6.1: K_d values of FlrCΔR calculated using oligonucleotide (enhancer element) as ligands

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ligand</th>
<th>K_d</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlrCΔR</td>
<td>Oligo</td>
<td>0.21 pmole</td>
</tr>
<tr>
<td>FlrCΔR</td>
<td>AMP.PNP</td>
<td>19.8 μm</td>
</tr>
<tr>
<td>FlrCΔR</td>
<td>Oligo+AMP.PNP</td>
<td>0.23 pmole</td>
</tr>
</tbody>
</table>
6.4 Gel filtration study

6.4.1 Size exclusion chromatography also shows the presence of heptamer of FlrCΔR in solution

To investigate the state of oligomerization of FlrCΔR, size exclusion chromatography was performed with 6×His-tagged FlrCΔR protein alone and in complex with AMP.PNP or the 62 bp downstream enhancer element using a Superdex 200 column (16×700 mm, GE Healthcare) as described in chapter 4. The chromatographic profile of FlrCΔR also showed the presence of heptamer both in the Nt-free, AMP.PNP bound and 62 bp enhancer elements bound states. Based on the reference elution volumes obtained with different protein standards, FlrCΔR in Nt-free state, AMP.PNP bound state and in presence of the enhancer eluted as an apparent heptameric species (Fig. 6.4). These results further support that, the functional oligomeric state of FlrC is heptamer and no remodeling occurs in FlrC either by the influence of ATP or of the downstream enhancer elements.

![Graph showing size exclusion chromatography profiles](image.png)

Figure 6.4: Size-exclusion chromatography profiles of FlrCΔR in Nt-free state, AMP.PNP bound state and in presence of enhancer region show exclusive formation of the heptamers.
6.4.2 FlrCΔR interacts with $\sigma^{54}$ as observed from the gel filtration studies

The heptameric state of FlrCΔR even in the presence of enhancer element prompted us to revisit the questions that whether FlrC can interact with $\sigma^{54}$ in absence of Nt and if the enhancer element has any influence on FlrC on $\sigma^{54}$ binding. Earlier, our in-vitro pull down assay showed interaction between $\sigma^{54}$ and Nt-free, AMP.PNP/ATP bound FlrC. However, we could not identify any such complex during size exclusion chromatography. To understand this phenomenon in further detail, we performed gel filtration chromatography on FlrCΔR incubated with $\sigma^{54}$, in the presence and absence of 172bp flaAp.

FlrCΔR was incubated with $\sigma^{54}$ at 1:1 molar ratio in the presence of 172 bp flaAp that contains both $\sigma^{54}$ and FlrC binding regions. The incubation was done at 20°C for 15 min either in the presence of 2mM AMP.PNP or in the absence of any Nt. The pre-incubated samples were then gel filtered through Superdex 200 column individually. As control, the premix FlrCΔR+$\sigma^{54}$ sample without enhancer element was gel filtered.

FlrCΔR in alone and in the presence of AMP.PNP and/or enhancer element eluted with pseudo-partition co-efficient $K_{av}$ of 0.19±0.01, which corresponds to a heptamer (Fig. 6.4A). The $\sigma^{54}$ in the same condition eluted with 0.40±.02 $K_{av}$ apparently representing monomeric species. A new peak eluted with 0.075 $K_{av}$ along with 0.19/0.40 $K_{av}$ peaks, when FlrCΔR and $\sigma^{54}$ were combined in presence of the enhancer element. SDS-PAGE analysis of the aliquots of peak 1 (0.075 $K_{av}$) showed the presence of both FlrCΔR and $\sigma^{54}$. We could identify the similar complex in the presence of AMP.PNP (Fig. 6.4A-B). Quantitative SDS–PAGE analysis of the new peak reconfirmed it. Interestingly, no such complex is observed in the absence of the enhancer element. In that case $\sigma^{54}$ and FlrCΔR, have eluted separately with respective $K_{av}$, 0.40 and 0.19 (Fig. 6.4C). These observations suggest that heptameric FlrCΔR is able to form a strong and sustainable complex with $\sigma^{54}$ in the presence of flaAp.
Figure 6.5: Interaction of FlrCΔR with σ^{54} in presence of flaA promoter. (A) Elution profile of FlrCΔR with σ^{54} in presence of flaA promoter template (inset – fraction of respective peak). (B) Profile of FlrCΔR with σ^{54} in presence of flaA promoter template and AMP.PNP (inset – fraction of respective peak). (C) Profile of FlrCΔR with σ^{54} in absence of flaA promoter template.