Purification, crystallization and preliminary X-ray analysis of the AAA⁺ σ^{54} activator domain of FlrC from *Vibrio cholerae*

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Received 1 May 2013
Accepted 4 June 2013

A σ^{54} -dependent transcriptional activator FlrC containing an N-terminal regulatory domain, a central AAA⁺ domain and a C-terminal DNA-binding domain has been implicated both in flagellar synthesis and enhanced intestinal colonization. FlrC is phosphorylated by the kinase FlrB at the regulatory domain and both nonphosphorylated and phosphorylated states of FlrC seem to be important for its functions. Oligomerization plays a key role in the functions of such transcriptional activators and the AAA⁺ σ^{54} interaction domain is critical in deciding the oligomerization state. Therefore, to obtain structural insights into FlrC at the atomic level, the AAA⁺ σ^{54} interaction domain of FlrC was cloned, overexpressed and crystallized using PEG 6000 as precipitant at pH 6.0, and diffraction data were collected to 2.8 Å resolution. Molecular-replacement calculations and subsequent refinement confirmed the presence of a closed heptamer in the asymmetric unit.

1. Introduction

Intestinal colonization of *Vibrio cholerae*, which is a prerequisite for virulence, is influenced by flagellar synthesis and motility, and a σ^{54} -dependent transcriptional activator FlrC has been implicated both in flagellar synthesis and enhanced intestinal colonization (Klose & Mekalanos, 1998; Correa *et al.*, 2000; Correa & Klose, 2005). A *V. cholerae* strain containing a deletion of *flrC* was nonmotile with modest colonization defects, while a strain producing hyperactive FlrC had altered cell morphology (Klose & Mekalanos, 1998). In a transcriptome analysis, an *flrC* mutant showed decreased expression of 115 genes, of which 50 genes were annotated as flagellar/chemotaxis genes and 46 genes were annotated as hypothetical, pointing towards the as yet unknown function of FlrC (Syed *et al.*, 2009).

FlrC possesses an N-terminal response regulator (RR) domain followed by a central AAA⁺ σ^{54} interaction domain and a C-terminal DNA-binding domain (North *et al.*, 1993). Phosphorylation occurs on the conserved Asp (D54) of the RR domain by its cognate kinase FlrB (Correa *et al.*, 2000). Interestingly, a *V. cholerae* strain containing an inactive (D54A) or constitutively active (M114I) FlrC mutant showed more severe colonization defects than strains lacking FlrC entirely (Correa *et al.*, 2000), 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.

σ^{54} -dependent transcriptions often control metabolic processes such as nitrogen- and sulfate-limitation responses. Although the nitrogen-assimilatory σ^{54} activators NtrC of *Salmonella enterica* and NtrC1 and NtrC4 of *Aquifex aeolicus* are highly homologous, structural studies have shown that their regulatory mechanisms are significantly different (Lee *et al.*, 2000; Doucleff *et al.*, 2005; De Carlo *et al.*, 2006; Batchelor *et al.*, 2008). Structure analysis of the ATPase and RR-ATPase domains of NtrC and NtrC1 showed that the RR domain receives a cellular signal and converts the protein from its inactive dimeric state to its active oligomeric state (Studholme & Dixon, 2003; Lee *et al.*, 2003). In contrast to this, ESI-MS data on isolated ATPase, RR-ATPase and ATPase-DNA binding domains of *A. aeolicus* NtrC4 suggested that, while the unactivated state of NtrC4 forms a heptamer, its activated state forms a hexamer

(Batchelor *et al.*, 2009), although no direct structural evidence is available to establish this fact.

FlrC of *V. cholerae* (UniProt accession code A5F6D4) shares 56% similarity (34% identity) to NtrC of *S. enterica*. Since no structural information is yet available for any σ^{54} activator involved in flagellar synthesis, either from *V. cholerae* or any other bacterial source, the structure of FlrC is required to understand the molecular basis of its oligomerization that is essential to control its diverse functions. The central $\text{AAA}^+ \sigma^{54}$ interaction domain of FlrC hydrolyses ATP and induces the transcription by remodelling σ^{54} RNA polymerase so that it can form open complexes at promoters of class III genes leading to flagellar synthesis as well as enhanced colonization (Correa *et al.*, 2000). Here, we report the cloning, overexpression, crystallization and preliminary X-ray crystallographic analysis of the central $\text{AAA}^+ \sigma^{54}$ interaction domain of FlrC (FlrC-AAA) from *V. cholerae* O395 strain in its nucleotide-free state.

Figure 1

(a) 12% SDS-PAGE of 6 $\times$ His-tagged FlrC-AAA. Lane 1, uninduced overexpression; lane 2, induced overexpression; lane 3, molecular-weight markers (labelled in kDa); lane 4, 2 μ l purified 6 $\times$ His-tagged FlrC-AAA; lane 5, 4 μ l purified 6 $\times$ His-tagged FlrC-AAA. (b) Crystals of FlrC-AAA grown in the presence of precipitant solution consisting of 10% PEG 6000 in 0.1 M MES pH 6.0.

2. Materials and methods

2.1. Cloning, overexpression and purification of FlrC-AAA

The FlrC-AAA gene was cloned into kanamycin-resistant pET28a⁺ (Novagen) vector using specific primers (forward primer: 5'-GACTGACATATGGTGGTTCGCGGATACGAAAAGC-3'; reverse primer 5'-CATTCGGGATCCTCAATCATGCCAATCAACCCC-3'). The primers were synthesized (NeuProCell) with *Nde*I and *Bam*HI restriction-enzyme sites. Chromosomal DNA of *V. cholerae* strain O395, isolated using the protocol described in the Molecular Biology Laboratory Manual of UMBC (<http://userpages.umbc.edu/~jwolf/m1.htm#genomicDNA>), was used as template to amplify the region encoding FlrC-AAA. The 753 bp FlrC-AAA PCR amplicon and the pET28a⁺ vector were double-digested with the restriction enzymes *Nde*I and *Bam*HI and the digested products were purified from 1.0% agarose gel using a gel-extraction kit (Qiagen). These two double-digested DNA fragments were then ligated using T4 DNA ligase. The clones were selected appropriately using *Escherichia coli* XL1-Blue cells with kanamycin resistance. FlrC-AAA domain (residues 131–381; 250 amino acids) was then overexpressed (Fig. 1a) in *E. coli* BL21(DE3) cells in the presence of kanamycin as a fusion protein with a 6 $\times$ His tag at the N-terminus followed by a thrombin cleavage site.

For overexpression, 100 ml LB broth was inoculated with a single colony and was grown overnight at 310 K. 1 l LB broth was inoculated with 10 ml of the overnight culture and the culture was grown at 310 K until the OD₆₀₀ reached 0.6. The cells were then induced with 1 mM IPTG. After induction at 310 K for 3 h, 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 MgCl₂, 10% (v/v) glycerol. PMSF (final concentration of 1 mM) and 10 mg 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 277 K and the supernatant was collected. Ni²⁺-NTA (Qiagen) resin was equilibrated with the abovementioned lysis buffer and the supernatant containing 6 $\times$ His-tagged FlrC-AAA was immobilized on the resin for Ni²⁺-NTA-based immobilized metal-ion affinity chromatography. The resin was washed with the lysis buffer containing an increasing concentration (from 5 to 50 mM) of imidazole and the 6 $\times$ His-tagged FlrC-AAA was eluted with lysis buffer containing 200 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 Tris buffer consisting of 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 10% (v/v) glycerol and concentrated using an Amicon ultracentrifugation unit (molecular-weight cutoff 10 kDa). The homogeneity of the purified protein was checked by SDS-PAGE using the abovementioned polyacrylamide concentration (Fig. 1a).

2.2. Crystallization

For crystallization, FlrC-AAA was concentrated to 12 mg ml⁻¹ using an Amicon ultracentrifugation unit (molecular-weight cutoff 10 kDa) in buffer consisting of 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 10% (v/v) glycerol. The concentration of the protein was measured spectrophotometrically using a calculated extinction coefficient of 25 690 M⁻¹ cm⁻¹. Initial crystallization trials were performed by the hanging-drop vapour-diffusion method in 24-well crystallization trays (Hampton Research, Laguna Niguel, California, USA) using the precipitants of Grid Screen PEG 6000, Grid Screen Ammonium Sulfate, Crystal Screen and Crystal Screen 2

Table 1

Data-collection and data-processing parameters.

Values in parentheses are for the outermost resolution shell.

Beamline	BM14
Wavelength (Å)	0.980
Detector	MAR CCD
Oscillation (°)	1
No. of frames	360
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 81.10, b = 153.15, c = 194.17$
Resolution (Å)	51.0–2.80 (2.95–2.80)
No. of molecules in asymmetric unit	7
Mathews coefficient (V_M) (Å ³ Da ^{−1})	2.87
Solvent content (%)	57.19
No. of observations	871142 (119809)
No. of unique reflections	60446 (8671)
Multiplicity	14.4
Mosaicity (°)	0.74
Completeness (%)	100.0 (100.0)
R_{merge} † (%)	13.8 (88.6)
Average $I/\sigma(I)$	16.0 (4.1)

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the observed intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of reflection hkl calculated after scaling.

from Hampton Research (Jancarik & Kim, 1991). Typically, 2 μ l protein solution was mixed with an equal volume of the screening solution and equilibrated against 600 μ l of the latter. Initially, crystals with a largest dimension of 0.05 mm were observed in the presence of 10% PEG 6000 at pH 6.0 (a condition from Grid Screen PEG) incubated at both 293 and 277 K for 48 h. This crystallization condition was further optimized using different additives. A mixture of 4 μ l protein solution and 2 μ l precipitant solution consisting of 10% PEG 6000, 0.1 M MES pH 6.0 and an additive such as 10% (v/v) ethylene glycol, 7.5% (v/v) dioxane or 10% (v/v) PEG 400 was equilibrated against a reservoir solution consisting of 12% PEG 6000, 0.1 M MES pH 6.0, 0.1 M NaCl at 293 K for 4 d to obtain diffraction-quality crystals. The diffracting crystals (Fig. 1b) obtained both in the

presence and absence of the additives were sent to the synchrotron for data collection.

2.3. Data collection and processing

Crystals of FlrC-AAA were looped out using 0.3 mm LithoLoops (Molecular Dimensions), soaked in a cryoprotectant consisting of 40% (v/v) ethylene glycol, 2.5% PEG 6000, 50 mM Tris-HCl and immediately flash-cooled in liquid nitrogen. The qualities of the crystals were judged and the cooling conditions were optimized using a MAR Research image-plate detector (345 mm) and Cu $K\alpha$ radiation generated by a Bruker-Nonius FR591 rotating-anode generator equipped with Osmic MaxFlux confocal optics. Crystals were then sent to the ESRF, Grenoble, France, and diffraction data were collected to 2.8 Å resolution using a MAR CCD detector on beamline BM-14 of the ESRF with an oscillation range of 1° and an exposure time of 20 s. A total of 360 images were collected and the data were processed and scaled with *MOSFLM* (Powell, 1999) from the *CCP4* program suite (Winn *et al.*, 2011). Data-collection and processing parameters are given in Table 1.

3. Results

Crystals of FlrC-AAA were of good quality and diffraction data were collected to 2.8 Å resolution. Although the R_{merge} in the outermost bin was 88.6%, the average $I/\sigma(I)$ and the completeness for the outermost resolution bin were 4.1 and 100%, respectively, and therefore the data were processed to 2.8 Å resolution (Table 1). A typical diffraction image is shown in Fig. 2.

FlrC-AAA crystals belonged to the orthorhombic crystal system, with unit-cell parameters $a = 81.10, b = 153.15, c = 194.17$ Å (Table 1). Calculation of the Matthews coefficient indicated the most probable presence of eight molecules (considering the molecular weight of 31 kDa; residues 131–381) in the asymmetric unit with a V_M (Matthews, 1968) value of 2.4 Å³ Da^{−1} and a solvent content of 49%.

The initial phases were obtained by molecular replacement (MR) using *Phaser* (McCoy *et al.*, 2007). Truncated coordinates of the σ^{54} activator NtrC4 of *A. aeolicus* in its inactive state (PDB entry 3d2d; Batchelor *et al.*, 2008) consisting of residues 133–369 of chain A (the coordinates for the N-terminal response regulator domain were truncated) produced an acceptable solution in MR. Before proceeding with the MR calculations, waters were deleted from the coordinates of the search model. Additionally, mismatched residues were mutated to alanine. Seven molecules of this modified search model in the asymmetric unit produced a rotation-function Z-score (RFZ) of 4.4, a translation-function Z-score (TFZ) of 21.4 and a log-likelihood gain (LLG) of 1898 using *Phaser* in space group $P2_12_12_1$ with data between 51.05 and 2.9 Å resolution. Interestingly, the models of NtrC or NtrC1 did not produce any convincing solution. Seven molecules of FlrC-AAA were organized in the form of a closed heptamer with a central channel. The resulting model was refined by a few cycles of restrained refinement using *REFMAC* (Murshudov *et al.*, 2011) to an R value of 36.5% ($R_{\text{free}} = 40.9\%$) with the data between 50 and 2.9 Å resolution. During the refinement a sevenfold noncrystallographic symmetry (NCS) restraint was maintained and 5% reflections were used for R_{free} calculations. A sevenfold NCS-averaged $2F_o - F_c$ map, calculated with this refined model, was of good quality and overlaid properly with the model. Additionally, a careful investigation of the $F_o - F_c$ map calculated with this refined model ruled out the presence of any other molecule in the asymmetric unit. Further model building and refinements to a resolution of

Figure 2

X-ray diffraction image of the FlrC-AAA crystal. The crystal diffracted to 2.8 Å resolution. The boxed region is enlarged to show a spot at 2.8 Å resolution.

2.8 Å are in progress using the programs *WinCoot* (Emsley & Cowtan, 2004) and *REFMAC* (Murshudov *et al.*, 2011), respectively.

We are extremely grateful to Professor Udayaditya Sen of the Saha Institute of Nuclear Physics, Kolkata for his very useful suggestions during crystallization and his help in checking the quality of the crystals. We are also very grateful to Ramanuj Banerjee of Professor U. Sen's laboratory and the beamline scientists of the ESRF for helping us to collect the diffraction data. SD is very grateful to Maitree Biswas of JD's laboratory at SXC, Kolkata for her help with the experiments. We are grateful to Dr Felix Raj SJ, Principal, St Xavier's College, Kolkata for his encouragement and constant support. This work is supported by DBT (IYBA-2010) project No. BT/03/IYBA-2010 of the Government of India.

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