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Research Article

BINDING OF ppGpp AND cAMP TO TRANSCRIPTIONAL REGULATOR PROTEIN BolA

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ABSTRACT. *E. coli* BolA is a DNA-binding transcription factor that responds to various stresses during the stationary phase by regulating the transcription of stringent response-related genes. It also protects bacteria from stressful environments by changing the cell wall morphology and inducing biofilm formation. The present study examined the physical interaction between ppGpp and cAMP to BolA by fluorescence spectroscopy. Recombinant BolA from *E. coli* was cloned, overexpressed, and purified to purity. BolA shows an intrinsic fluorescence light at 339 nm when excited at 280 nm, and the maximum intensity decreases in the presence of cAMP and ppGpp. The dissociation constant for ppGpp and cAMP was determined to be 164 ± 20 and 165 ± 36 µM, respectively. The experimental evaluation suggests ppGpp competes with cAMP for the ligand-binding pocket of BolA. Under stringent conditions, ppGpp bind to BolA with a strong affinity to control gene regulation and biofilm formation. However, when the cell goes back to normal environmental conditions, cAMP occupies the binding site to repress the activity of BolA. This study showed for the first time that ppGpp and cAMP bind to BolA with strong affinity.

Keywords: *Guanosine pentaphosphate (ppGpp), cAMP, glutathione (GSH), protein-ligand interaction, morphogene bolA.*

INTRODUCTION

More than 70% of bacterial infections are related to biofilm formation. For the treatment of chronic infections, biofilm formation of the bacteria reduces the susceptibility to antibiotics. Bacteria in biofilm form can resist 10-1000 times more the effect of antibiotics [1,2]. Most bacteria have flagellum to migrate. During stress conditions, bacterial cells shift from the planktonic stage to biofilm formation by turning off the flagellum to produce extracellular polysaccharides and fimbriae [3]. BolA-like protein family can be found in a wide range from eukaryotes to bacteria. In *E. coli*, BolA downregulates flagellum biosynthesis and bacterial movement. Moreover, overexpression of the BolA regulates biofilm formation [4,5]. BolA is a DNA-binding protein, and it binds DNA with a high affinity to regulate many stringent genes. BolA is a transcriptional factor that directly binds the promoter region of many important genes to promote bacterial persistence for harsh environmental conditions [6]. mRNA of the bolA gene is highly expressed during various stresses such as acidic stress, heat shock, cold shock, osmotic and oxidative stress, and carbon starvation [7].

Lange and Henge-Aronis [8] showed that the mRNA transcription of the bolA gene is controlled by the cAMP molecule. Expression of the bolA gene is downregulated in response to the elevated levels of cAMP. Also, histone-like protein (H-NS), cAMP

binding protein (CRP), and OmpR negatively regulate the transcription of bolA under normal conditions. However, ppGpp positively regulates bolA transcription under starvation conditions [9]. Glutaredoxins (Grxs) is wildly spread between prokaryotes and eukaryotes [10]. The function of the enzymes is to reduce glutathione (GSH) to survive during oxidative stress. Grxs form a complex with BolA protein to reduce GSH [11]. Li *et al*. [12] showed biochemical characterization of the Grx-BolA complex. Glutaredoxins complex showed three iron-binding sites, GSH binding site, and BolA binding site, which is close to the active site of the enzyme.

The present study examined the interaction between ppGpp, cAMP, and BolA by the fluorescence spectroscopy method. First, BolA from *E. coli* was cloned, overexpressed, and purified to purity. Utilizing fluorescence spectroscopy, the physical binding feature was measured by calculating the K_D (dissociation constant) values of ppGpp, GSH, and cAMP. The present study showed that ppGpp, a stress response molecule, binds to BolA with high affinity. Also, cAMP, which makes a complex with CRP (cAMP binding protein) and works as a signaling molecule, binds to BolA with a similar affinity. The present study also examined the interaction between GSH and BolA and found that BolA did not bind to GSH and that BolA has no GST activity.

MATERIALS AND METHODS

Reagents

All PCR reagents were purchased from Takara, the restriction endonucleases were purchased from NEB Biolabs, the ligation reagents were purchased from Roche, Ni-NTA, DEAE, and the Superdex 75 columns were purchased from GE Healthcare. Tris, Imidazole, NaCl, potassium phosphate, Glutathione (GSH), and cAMP were purchased from Sigma-Aldrich, and the oligonucleotide DNA fragment was generated by Bioneer Korea.

Instrumentation

Purification of his_6-tagged protein was performed using AKTA FPLC (GE Healthcare). Protein-ligand binding was performed using a fluorescence spectrophotometer F-4500 (HITACHI), and activity assay measurement was performed using UV-Visible ChemStation (HP 8453).

Cloning, overexpression, and purification of recombinant protein

E. coli BL21 (DE3), DH5α strains, and pET21a(+) vector were used for cloning and over-expressing of recombinant his_6-tagged protein. The bolA gene encoding DNA sequence was amplified by PCR, using the following primers; 5'-CGCGCATATGATGATACGTGAGCGGATAG-3' (forward) and 5'-CGCGCCTCGAGTTACGCGATGCTTCCTGCTCC-3' (reverse). BolA was cloned using double restriction enzyme digest using *Nde*I and *Xho*I enzymes and inserted in pET21a(+) vector by ligation and transformed to the DH5α for screening and subsequently transferred to BL21 strain for over-expression. BL21 containing inserted plasmid were cultured in the LB medium containing 50 μg/ml ampicillin at 37 ºC when the OD_{600} reached 0.4-0.6, IPTG was added to the final volume of 0.2 mM and the culture was further induced for 4h. Cells were harvested using a centrifuge and lysed by French

Press 2 loading at 4,000 psi in the presence of 0.1 mM PMSF. The lysed was clarified by centrifugation for 30 minutes at 10,000xg. The supernatant was applied to the Ni-NTA column (5ml) equilibrated with 50 mM Tris-HCl, 20 mM Imidazole buffer by gradient pump and connected to the FPLC. Protein elution was achieved by continuous imidazole gradient with the same buffer containing 500 mM Imidazole. Elution fractions that contained target proteins were pulled, dialyzed, and applied to the DEAE column (5ml) equilibrated with 50 mM Tris-HCl for further purification. BolA was eluted with a linear gradient (1M) of NaCl in the same buffer. The concentrations of soluble proteins were determined by measuring the absorbances at 280nm. The purity of the purified BolA was assessed in Coomassie-stained 15% SDS-PAGE gels.

Synthesis and purification of ppGpp

The ppGpp molecule was synthesized from the ribosome which was isolated from *E. coli* K12. The *in vitro* synthesis of the molecule was performed as described previously [13]. ppGpp was purified by ion-exchange chromatography using the DEAE column. The purity of the molecule was examined by HPLC.

Binding kinetics studies performed on Fluorescence Spectrometer

All the protein-ligand binding studies were performed at room temperature, and protein and ligands were prepared in a filtered buffer containing 50mM Tris-HCl pH 7.5. For the measurements, the instrument was set to excitation wavelength at 280nm, both the emission and excitation slit were 5nm, and the emission spectra were recorded from 290-400nm. The BolA concentration was 5 μM. The ligands in this study were prepared in water or a buffer similar to the protein solution and were titrated 20-30 times. The fluorescence changes by titration were measured at various concentrations of ppGpp, GSH, and cAMP. K_D values were calculated using the Origin software package. The dissociation constant, K_D, was calculated by the equation, $\Delta F/\Delta F_M=(([L]+[P]+K_D)-(([L]+[P]+K_D)^2 - 4\cdot[L]\cdot[P])^{0.5})/2\cdot[P]$, where ΔF, ΔF_M, [P], and [L] were fluorescence change, the maximal fluorescence change, total protein concentration, and total ligand concentration, respectively [14]. The corrections for the inner filter effect, introduced by the ligands, were performed by the formula, $F_{cor} = F \times 10^{(P + \Delta A)/2}$, where F and F_{cor} are fluorescence intensity before and after the correction, while P and ΔA denote the initial sample absorption at the excitation wavelength and the change in absorption introduced by the ligand, respectively [15].

Assay of GST activity

Specific activities of the enzyme were determined as described by [16]. Accordingly, enzyme activity was determined by monitoring the changes in absorbance at 340nm at room temperature, scanning for 3min. The assay mixture contained 100mM KH_2PO_4 pH 6.5, 1 mM CDNB, 1 mM GSH and BolA (65 μg/ml). The assay mixture without the enzyme was used as the control.

Modeling of BolA interaction to ppGpp and cAMP

E. coli BolA (PDB code: 2DHM) crystal structure was used for docking simulation to ppGpp and cAMP. The interaction of ppGpp and cAMP to BolA was generated by using GalaxyDockWEB. The ligand-binding pockets of BolA were predicted by the GalaxySite

web server as described previously [17,18]. The interaction model was visualized and prepared using the PyMOL software [19].

RESULTS AND DISCUSSION

Cloning, overexpression, and purification of BolA from E. coli

Genomic manipulations were performed using conventional cloning techniques. The bolA gene inserted plasmid-containing cells were overexpressed as described in the methodology section, and the protein expression was analyzed by Coomassie blue staining. The soluble BolA was purified by Ni-NTA affinity chromatography using the HisTrap HP column and ion-exchange chromatography using the DEAE column. The elution fractions containing the protein of interest were collected, and the concentrations of soluble proteins were determined by measuring the absorbances at 280nm. Pure BolA was migrated as a single band in SDS-PAGE and the estimated molecular weight was approximately 17 kDa (Fig. 1A). BolA was eluted as a single peak in a size exclusion chromatography, and the molecular mass was determined to be approximately 27 kDa, suggesting that the isolated BolA, as expected, was a dimer (Fig. 1B) [20].

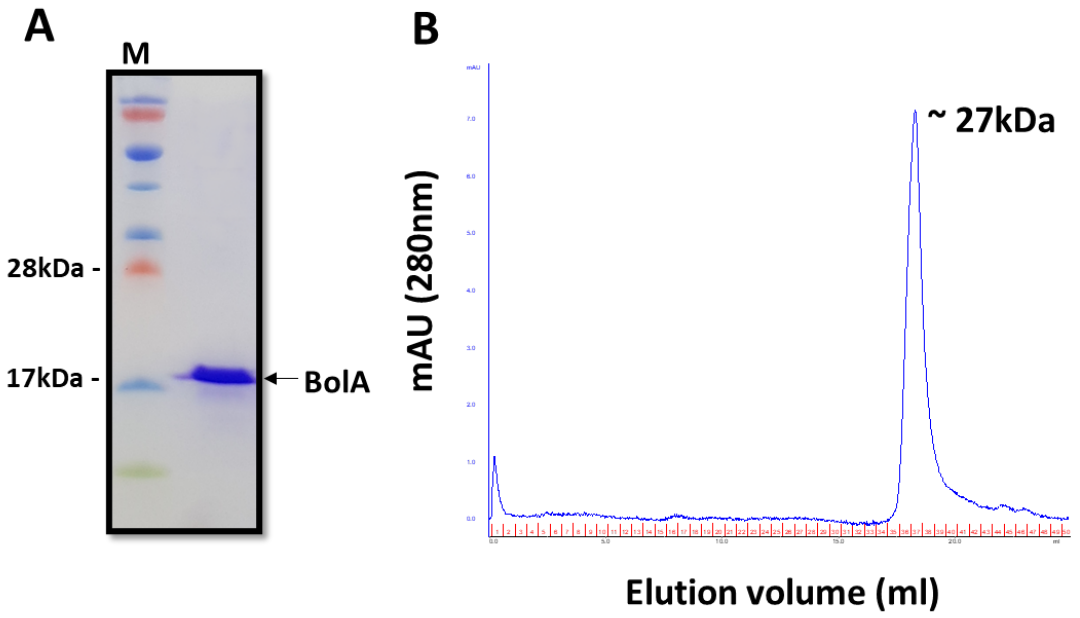

Fig. 1. *SDS-PAGE and size-exclusion chromatography of BolA. (A) SDS-PAGE of purified BolA derived from affinity chromatography and ion-exchange chromatography. The purity of DegP was analyzed by SDS-PAGE (15%) and visualized by staining (Coomassie blue). (B) Size-exclusion chromatography of purified BolA on Superdex 75 column (GE Healthcare).*

ppGpp and cAMP binding site of BolA

E. coli BolA protein consists of a helix-turn-helix structure with a DNA-binding domain that interacts with the DNA and regulates gene expression [21]. The NMR structure of *E. coli* BolA has been previously presented (PDB ID: 2DHM) [22]. According to the solved structure, two Serin residues, 26 and 45, have been shown to be

highly conserved among the different species. The binding pocket of the protein was determined by the GalayxSITE (GalaxyWEB) server. Accordingly, three amino acids, 56R, 77L, and 79T, are constituted to the binding site of the BolA. In the present study, the interaction between ppGpp and cAMP to BolA was rendered and compared using computational prediction. The software predicted that four amino acids residues, 25E, 26S, 34G, and 73H, constituted the interaction site for cAMP, and the calculated distance between the molecule were 3.1Á, 2.9Á, 3.1Á, and 3.2Á, respectively (Fig. 2A). Four amino acids residues, 1M, 26S, 27Y, and 71T, constituted the binding site for ppGpp, and the distance from ppGpp to interacting amino acids were 2.7Á, 2.1Á, 2.8Á, and 3.1Á, respectively (Fig. 2B). According to the software predictions, both ligands bind to BolA in an almost similar position, suggesting that both ligands might affect the activity of BolA by binding. Also, ppGpp might generate a more stable complex with BolA, suggesting that interaction with ppGpp might regulate BolA activity in general.

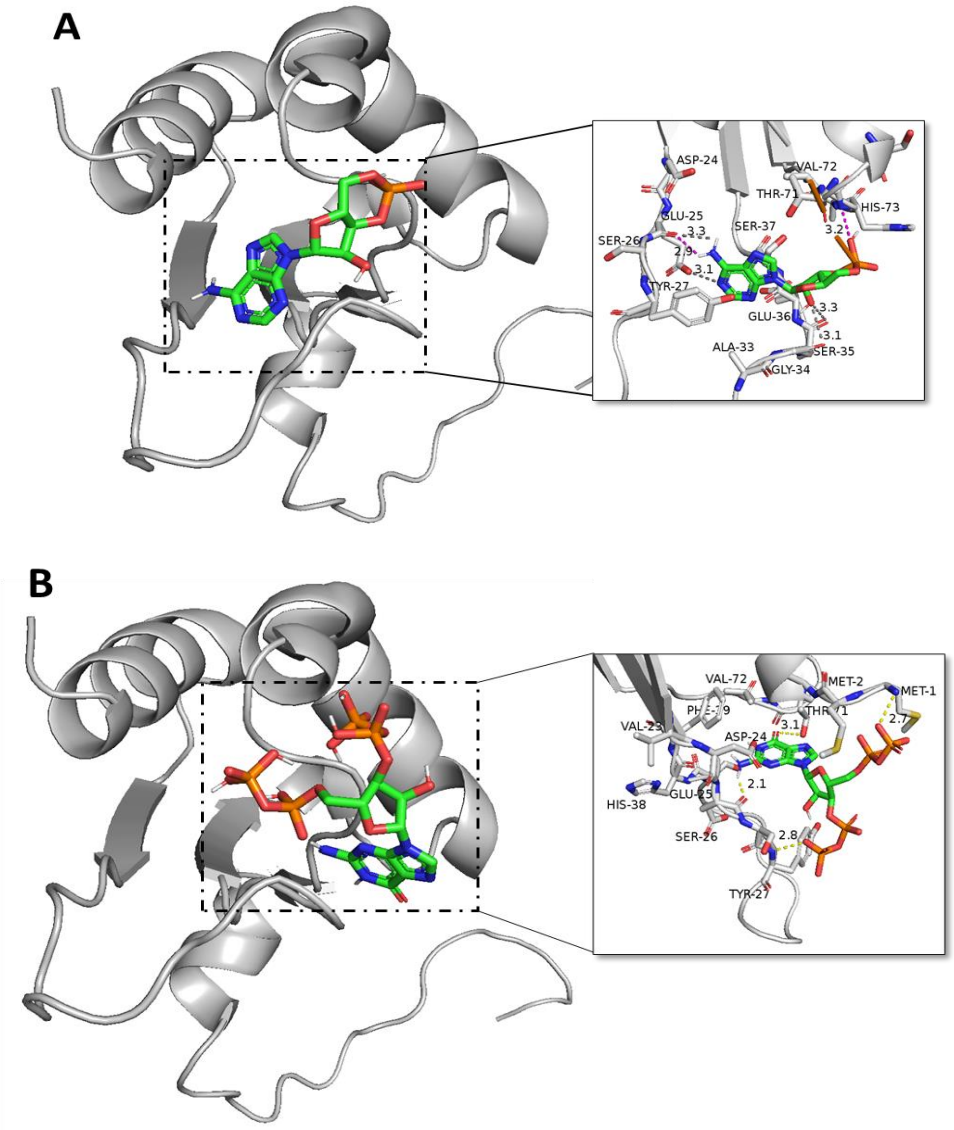

Fig. 2. *Interaction of cAMP and ppGpp to BolA. (A) cAMP interaction site determined by molecular docking. (B) ppGpp interaction site of BolA predicted by molecular docking.*

Determination of ppGpp and cAMP binding by fluorescence spectroscopy

E. coli BolA shows sequence similarities with the BolA-like protein family. The family is present in eukaryotes and bacteria and has a conserved amino acid sequence. The function of the BolA protein family is to protect the cells under stress conditions. BolA protein is not required for normal growth conditions for bacteria; synthesis is most dramatically stimulated by a stringent response [23]. Numerous studies have reported that the stringent response is characterized by the synthesis of ppGpp, where ppGpp synthesis is catalyzed by the relA gene. The synthesis of the ppGpp is required to overcome the starvation conditions [24-26]. The synthesis pattern and cellular localization of BolA protein let us assume whether protein is an accumulator of interaction between RNA polymerase and ppGpp. In the present study, BolA was identified as a new ppGpp-interacting protein. Also, BolA binds to cAMP with high physical affinity. However, BolA does not interact with GSH and does not show any GST activity. The K_D (dissociation constant) of three interactions was determined using fluorescence spectroscopy, a highly sensitive, quantitative analytical tool and it provides structural and dynamic information on interaction studies [27]. *Escherichia coli* BolA showed a strong intrinsic fluorescence at 339 nm when excited at 280 nm. Protein has one tryptophan residue whose fluorescence decreases when bound to ligands (Fig. 3 and 4). The binding affinities of BolA to ligands were determined and calculated by plotting changes in fluorescence light. The dissociation constant for cAMP to BolA was determined to be 165 ± 36 μM, and K_D for ppGpp was determined to be 164 ± 20 μM.

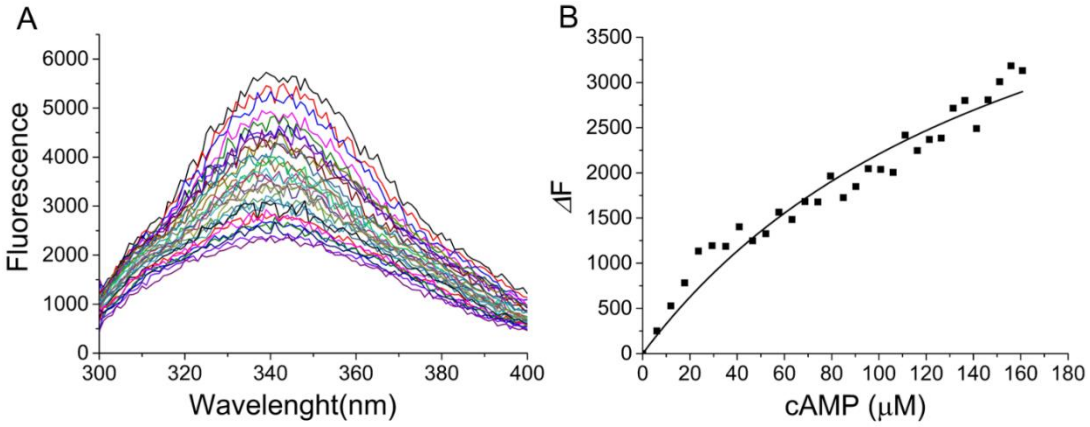

Fig. 3. *Dissociation constant K_D determination of cAMP to BolA by fluorescence spectroscopy. (A) The fluorescence emission spectra of BolA in the presence of various concentrations of cAMP. BolA (5 μM) was excited at 280 nm and emission spectra were recorded at room temperature. (B) The fluorescence changes at 339nm against ligand were calculated.*

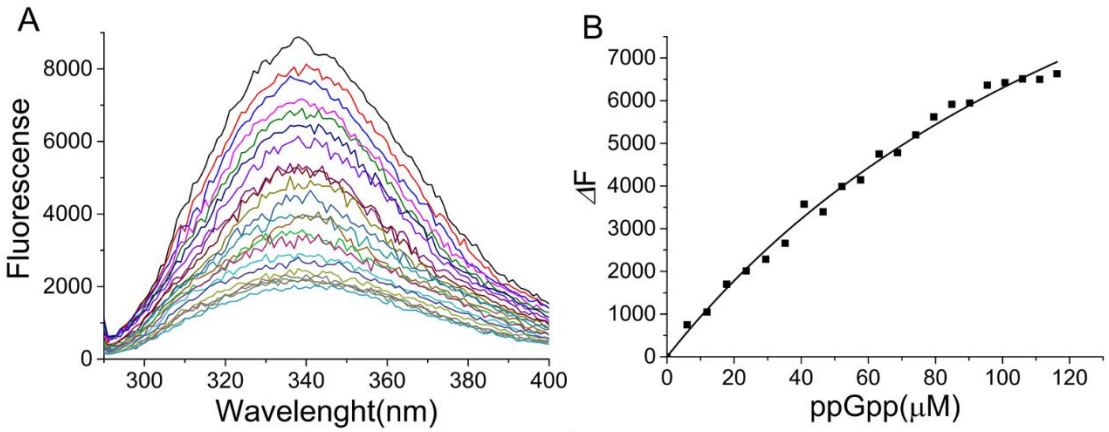

Fig. 4. *Dissociation constant K_D determination of ppGpp to BolA by fluorescence spectroscopy. (A) The fluorescence emission spectra of BolA in the presence of various concentrations of ppGpp. BolA (5 μM) was excited at 280 nm and emission spectra were recorded at room temperature. (B) The fluorescence changes at 337nm against ligand were calculated.*

CONCLUSION

E. coli BolA showed sequence homology to BolA-like proteins from different species and acts as a morphogen. BolA binds to ppGpp, which may describe the molecular basis of biofilm formation during the stringent response. The interaction may elevate the activity of the protein under stress conditions. BolA also interacts with cAMP, which is a CRP-biding and -activating molecule. This interaction suggested that the binding with cAMP to BolA may turn off the activity of BolA under normal conditions. BolA also showed oxidative stress response when working with Grx proteins, which bind and reduce GSH. However, BolA did not bind glutathione and lacks the glutathione S-transferase activity.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept, Design, Data Collection or Processing, Analysis or Interpretation, Literature Search, Writing: T.D.

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