A Study on the Binding Interactions of cAMP Receptor Protein (CRP) from *Mycobacterium Tuberculosis* with *whiB1* Gene

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Abstract

Mycobacterium tuberculosis, the causative agent of tuberculosis, remains one of the deadliest human pathogens claiming millions of lives each year. It's genes and their associated regulators are a growing interest to the scientific and medical fields because of their implications in pathogen's survival. The cAMP receptor protein from *M. tuberculosis* (CRP_{MTB}) is an essential transcription factor that regulates several important genes for host infection and antibiotic resistance in *M. tuberculosis*. Of specific interest is understanding how CRP_{MTB} binds to DNA in the absence and presence of cAMP release during host infection. Gárate *et al*. has shown a role for cAMP signaling in enhancing of CRP binding to specific DNA sequences. This study is centered around the *whiB1* gene, which encodes Wb1 proteins that control developmental processes for the pathogen. By utilizing fluorescence anisotropy for both nonstoichiometric and stoichiometric titrations of CRP_{MTB} and *whiB1*, in the absence and presence of cAMP, this study offers visual confirmation that CRP_{MTB} binds to the *whiB1* gene. Moreover, we found that CRP_{MTB} binds to to the upstream binding site of the *whiB1* gene with higher affinity than the downstream site. These two sites do not exhibit any measurable cooperativity or allosteric effects between each other which was confirmed utilizing stoichiometric titrations. It was also found that in the presence of cAMP, the creation of high-order CRP-DNA oligomers are reduced. While the computational fit of the averaged anisotropy values was not successful, it is attributed to the fact that the current fitting equation did not account for the unexpected stoichiometry of CRP:*whiB1* binding. Future work will include creating a computational model that considers the stoichiometry of *whiB1* binding sites and the proper length of the promoters. This study is vital to further understanding the ways in which *M. tuberculosis* survives in hosts via DNA transcription and gene regulation, as well as for the promotion of study with the goal of creating effective treatments against TB.

Introduction

Despite misconceptions of its decreasing relevance in American scientific study, a growing global health problem that remains prevalent today is tuberculosis (TB). *Mycobacterium tuberculosis* (MTB) is one of the most dangerous human pathogens, claiming ~2 million lives per year[2]. Tuberculosis is spread through inhalation of droplet nuclei in the atmosphere, released by other infected individuals. The disease is also able to remain dormant until favorable conditions occur, called reactivation tuberculosis, making it difficult to detect and treat. Although the Bacille Calmette-Guerin (BCG) vaccine against tuberculosis has been administered to countless individuals since the 1920s and is effective in preventing disease in infants, recent studies show it does not promise the same efficacy against pulmonary tuberculosis in adults[3]. MTB also exhibits extreme antibiotic resistance to other treatments, with rates as high as 82% multi-drug resistant TB measured in almost 600,000 cases in 2017[4]. However, by understanding the components fundamental to the survival of *Mycobacterium tuberculosis*, there is potential to develop non-antibiotic treatments to prevent the spread of disease and move towards the end of this ongoing public health crisis.

Allosteric proteins are the focus of a growing field of research due to their importance in fundamental biological signaling. The ability to have multiple sites, as referenced by the term "allosteric," allows for the protein to undergo conformational changes upon binding with other molecules, allowing for the transduction of signals within and between biological systems[1]. One such group of proteins are the cyclic AMP (cAMP) receptor proteins (CRP), transcription factors that target promoter sequences of DNA upon binding with signaling molecule cAMP and thus control gene expression during transcription into mRNA[2]. cAMP, and thus CRP, is of interest due to its connection to bacterial survival and infection. Many bacterial species survive after infecting macrophages, or white blood cells, through a cAMP burst, which both interferes with host signaling pathways and controls bacterial gene expression through CRP that promotes survival[5].

The cAMP receptor protein from *Escherichia coli* ($CRP_{E.\ coli}$) is a well-studied transcription factor that performs the aforementioned regulation responses upon hundreds of transcriptional units[6]. $CRP_{E.\ coli}$ is structured as a dimeric transcription factor with two identical subunits, with each subunit containing a cAMP binding domain that is coupled to a DNA binding domain[7] **(Figure 1A).** Previous biophysical studies have shown that cAMP binding to $CRP_{E.\ coli}$ stimulates extensive conformational changes to the coupled DNA-binding domains, allowing for enhanced binding affinity with the promoters of genes of interest[1].

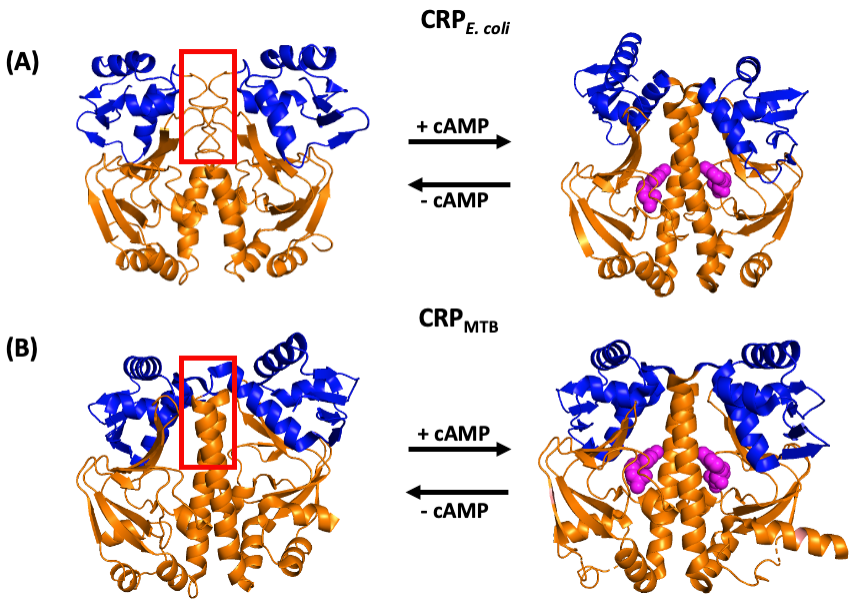

Figure 1. Crystal structures of $CRP_{E.coli}$ and CRP_{MTB}. Crystal structures of (A) apo-CRP_{Ecoli} (left) and cAMP-bound CRP_{Ecoli} (right) and (B) apo-CRP_{MTB} (left) and cAMP-bound CRP_{MTB} (right). Adapted from Sanuja Mohanaraj of the Maillard Lab.

cAMP receptor protein from *Mycobacterium tuberculosis* (CRP_{MTB}) has been previously compared to $CRP_{E.\ coli}$ and has been shown to have 53% similarity between the amino acids of the two proteins[2]. Despite this similarity, there are both structural and functional differences

between the proteins in the two bacterial species. Structurally, CRP_{MTB} is slightly larger than $CRP_{E.\ coli}$. Another difference is that unlike the heightened DNA binding affinities of cAMP-bound $CRP_{E.\ coli}$, cAMP-bound CRP_{MTB} (**Figure 1B**) has been shown to have similar binding affinities to DNA as apo- or unbound CRP_{MTB}[1].

The study of cAMP signaling and CRP transcriptional regulators is prevalent to addressing the TB crisis specifically because previous study has shown that cAMP signaling in *Mycobacterium tuberculosis* upregulates genes related to intracellular growth in host cells[8]. By analyzing the binding interactions of cAMP-bound CRP_{MTB} with such genes, possibilities for treatment avenues can be explored.

Previous *in vivo* analyses have indicated that CRP_{MTB} activates expressions of two prominent genes in the bacterial life cycle, the *rpfA* and *whiB1* genes. The *whiB1* is particularly relevant in this field of study because the gene encodes for a Wb1 protein, typically known for functioning in the control of developmental processes[2]. In this study, we focused on characterizing the binding interactions between CRP_{MTB} and *whiB1*, as well as studied the mechanisms of regulation by cAMP that affect such CRP_{MTB}-*whiB1* interactions. Studies utilizing electrophoretic mobility shift assays (EMSA) have shown that the *whiB1* promoter contains two adjacent binding sites as identified in Stapleton *et al.* and Bai *et al.*) **(Figure 2)**[2,8]. It has been shown that cAMP-bound CRP_{MTB} binds with higher affinity to the upstream binding site (CRP1), and while it still binds to the downstream binding site (CRP2), it does so with lower affinity. These interactions have been shown to be implicated in the activation of *whiB1* expression[9]. This study seeks to further understand the interactions amongst the CRP_{MTB} and these two binding sites on the *whiB1* promoter using fluorescence anisotropy.

Fluorescence anisotropy as a method allows for the determination of protein-nucleic acid interactions utilizing the principle that a fluorophore excited with light results in a somewhat polarized emission[12]. Anisotropy is measured as a function of the fluorescence intensities of vertically and horizontally polarized emissions. When struck with polarized light, a fluorophore that rotates slowly will exhibit a relatively larger anisotropy value. If the fluorophore rotates quickly, then a relatively smaller anisotropy value will be measured.

In addition, this study will further illuminate the effects of cAMP on such CRP_{MTB}-*whiB1* binding interactions utilizing stoichiometric binding assays. Previous studies have shown that cAMP promotes sequence-specific binding interactions between CRP_{MTB} and DNA and preventing the formation of high-ordered oligomers.[1] Such high-ordered oligomers are identified by non-specific complexes with multiple CRP and DNA promoter monomers in its arrangement.

Figure 2. ***whiB1 promoter region nucleotide sequence.*** Diagram of the promoter nucleotide sequence, showing the two CRP_{MTB} binding sites (boxed). Adapted from Stapleton *et al, Journal of Biological Chemistry.*

Materials and Methods

Protein Purification of CRP_{MTB} Wild Type (WT)

CRP_{MTB} WT was purified from *E. coli* strain T7 Express pLysY cells (NEB). Bacteria were grown overnight at 37ºC on LB-agar plates with ampicillin (AMP). Colonies were picked and grown overnight at 37ºC in a 100 mL volume of LB media (100 µL AMP). 1 L of LB media was initially prepared to reach an optical density (O.D.) of 0.2 using the bacteria that was previously grown overnight. The broth was then incubated at 37ºC until an O.D. of ~0.6 was reached, before inducing with 1mM IPTG for two hours. Following centrifugation (JLA 9.1 rotor, 15 minutes, 5,000 rpm, 4ºC) (Beckman-Coulter, Brea, CA), the bacterial pellets were isolated and resuspended in lysis buffer on ice (20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) supplemented with protease inhibitors (10 mM Benzamidine, 0.4 mM AEBSF, 1 µM Pepstatin, 1 µM Leupeptin, 28 µM TPCK/TLCK, 10 µM IBMX, 1 mM PMSF). The bacterial solution was initially homogenized with a glass homogenizer, followed by cell lysis using the M-110P microfluidizer at 10,000 psi (Microfluidics, Newton MA). The resulting lysate was centrifuged (JA 25.5 rotor, 45 minutes, 15,000 rpm, 4ºC) (Beckman-Coulter, Brea, CA). The supernatant was then added to a nickel resin (Clontech) supplemented with 30 mM imidazole followed by an overnight incubation at 4ºC with continuous rotation. The resin-supernatant mic was added to a column and nonspecific proteins were washed away with 30 mM imidazole. 500 mM imidazole was used to elute the protein of interest followed by size-exclusion chromatography. CRP_{MTB} was stored in CRP storage buffer (50 mM HEPES pH 7.6, 150 mM KCl, 1 mM EDTA pH 8.0, 10% glycerol) at -80ºC. The protein fractions of interest were confirmed using UV-Visible spectroscopy and SDS-PAGE (Protocol adapted from Sanuja Mohanaraj and Stephen Dokas of the Maillard Lab).

DNA Amplification

The four *whiB1* promoters were amplified by PCR using Taq polymerase under standard reaction conditions (50µL total volume per reaction, T_m of 66ºC, Eppendorf Mastercycler Nexus GX2 Thermocycler) with the forward and reverse primers seen below (**Table 1**). The DNA was purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and subsequently eluted to a volume of 40µL with elution buffer (10 mM Tris-Cl pH8.5). The purified DNA concentration was determined by absorbance at 260 nm. The size of the purified DNA was confirmed through gel electrophoresis (1% agarose gel, 1x Tris/Borate/EDTA buffer, 90 minutes, 100 V, EtBr staining), followed by DNA extraction using a QIAEXII Gel Extraction kit (Qiagen, Hilden, Germany) and subsequently eluted to a volume of 40 µL with ddH_2O. The final extracted DNA concentration was determined by absorbance at 260 nm.

Table 1. Sequences of the *whiB1* promoters used in DNA amplification.

Promoter	*FWD*	*REV*
WhiB1 CRP1	5'-GCCAGAGTGAGATAGCCCACGCGCTT-3'	5'-AAGCGCGTGGGCTATCTCACTCTGGC-3'
WhiB1 CRP2	5'-GCTTACGTAACACTATTGACATCTGT-3'	5'-ACAGATGTCAATAGTGTTACGTAAGC-3'
WhiB1 Full CRP1	Fl:5'-GCCAGAGTGAGATAGCCCACGCGCTTACGTAACACTATTGACATCTGT-3'	5'-ACAGATGTCAATAGTGTTACGTAAGCGCGTGGGCTATCTCACTCTGGC-3'
WhiB1 Full CRP2	5'-GCCAGAGTGAGATAGCCCACGCGCTTACGTAACACTATTGACATCTGT-3'	Fl:5'-ACAGATGTCAATAGTGTTACGTAAGCGCGTGGGCTATCTCACTCTGGC-3'

DNA Binding Monitored by Fluorescence Anisotropy

DNA-binding assays were performed with a 26 bp *whiB1* promoter (FWD: 5'-GCCAGAGTGAGATAGCCCACGCGCTT-3'), 26 bp *whiB1* promoter (FWD: 5'-GCTTACGTAACACTATTGACATCTGT-3'), 48 bp *whiB1* promoter (FWD: Fl:5'-GCCAGAGTGAGATAGCCCACGCGCTTACGTAACACTATTGACATCTGT-3'), and a 48 bp *whiB1* promoter (REV: Fl:5'-ACAGATGTCAATAGTGTTACGTAAGCGCGTGGGCTATCTCACTCTGGC-3'), all linked covalently to a fluorescein molecule (IDTDNA). Reaction mixtures were made containing 5 nM of fluorescein-labeled DNA and either 0 mM or 1 mM of cAMP in 4x DNA binding buffer (75 mM KCl, 50 mM HEPES, 1mM EDTA, pH 7.6) with water to volume of 2000 µL. CRP_{MTB} at concentrations of 0-200 µM was titrated in throughout

the binding assay. Fluorescence anisotropy measurements were then collected using time-based scans on a Photon Technologies International fluorimeter (Horiba Scientific). The excitation and emission wavelengths were set to 480 nm and 518 nm, respectively. Measurements were collected every 90 seconds at a slit setting of 9.8 nm at 25ºC. As described previously by Garaté *et al*, Heyduk and Lee, Lanfranco *et al*, the data was normalized to the first measured anisotropy value and analyzed. Experimental data points displaying anisotropy values with 2 standard deviations higher that the plateau overserved after the first DNA-binding phase were removed and the data was fit according to **Equation 1**,

$$A_{obs} = A_{DNA_F} + (A_{P-DNA} - A_{DNA_F}) \cdot \frac{K[DNA_T]+K[P_T]+1-\sqrt{(K[DNA_T]+K[P_T]+1)^2+4K^2[DNA_T][P_T]}}{2K[DNA_T]} \quad \textbf{(1)}$$

where A_{obs} is the observed anisotropy, A_{DNAF} and $A_{P\text{-}DNA}$ are the anisotropy values for free DNA and the protein–DNA complex, respectively, $[DNA_T]$ is the total DNA concentration, $[P_T]$ is the total protein concentration, and K represents the association constant for the protein and DNA.[1]

Stoichiometric Binding Assays

Stoichiometric DNA binding assays were also performed using fluorescence anisotropy on one of the two longer constructs, 48 bp *whiB1* construct (FWD: Fl:5'-GCCAGAGTGAGATAGCCCACGCGCTTACGTAACACTATTGACATCTGT-3'), linked covalently to a fluorescein molecule (IDTDNA). Reaction mixtures were made containing 200 nM of fluorescein-labeled DNA and either 0 mM or 1 mM of cAMP in 4x DNA binding buffer (75 mM KCl, 50 mM HEPES, 1 mM EDTA, pH 7.6) with water to volume of 2000µL. CRP_{MTB} at concentrations of 0-800 µM was titrated in throughout the binding assay. Fluorescence anisotropy measurements were then collected using time-based scans on the Photon Technologies International fluorimeter (Horiba Scientific). The excitation and emission wavelengths were set to 480 nm and 518 nm, respectively. Measurements were collected every 90 seconds at a slit setting of 4.5 nm at 25ºC. The data was again normalized to the first measured anisotropy value. (Protocol adapted from Heyduk and Lee[10] (1990) PNAS, 87, 1744-1748; Gárate (2021) JBC[1], 296, 100480; Lanfranco (2017), 292, 6086-6093.[11]).

Results

CRP_{MTB} Protein Purification

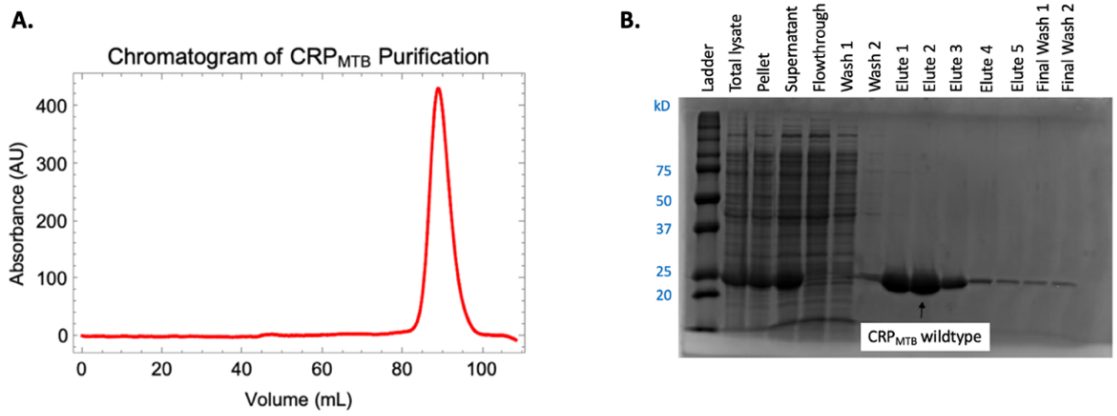

Figure 3. (A) Chromatogram of CRP_{MTB} protein purification. **(B)** SDS-PAGE gel utilized to confirm the presence of CRP_{MTB} wildtype following the size exclusion chromatography at the end of the protein purification process.

To perform studies of binding interactions between *whiB1* and CRP_{MTB}, CRP_{MTB} wild type (CRP_{MTB} WT) was expressed in BL21 competent cells (pLysY) through induction of the T7 promoter with 1mM isopropyl β-D-1thiogalactopyranoside (IPTG) for two hours. After isolating and resuspending the pellet, cell lysis was perfumed using the microfluidizer at 10,000 psi, followed by centrifugation. The supernatant was added to a nickel resin supplemented by 30 mM imidazole to induce His-tag binding of the CRP_{MTB} to the resin and allow for separation out from other proteins. Column elutions were then performed using 30 mM and 500 mM imidazole to elute CRP_{MTB}, after which size exclusion chromatography and a 10% SDS-PAGE gel was performed. The chromatogram is depicted in **Figure 3A** and the gel in **Figure 3B**, confirming the presence of CRP_{MTB} (25,000 kDa).

Nonstoichiometric Binding via Fluorescence Anisotropy

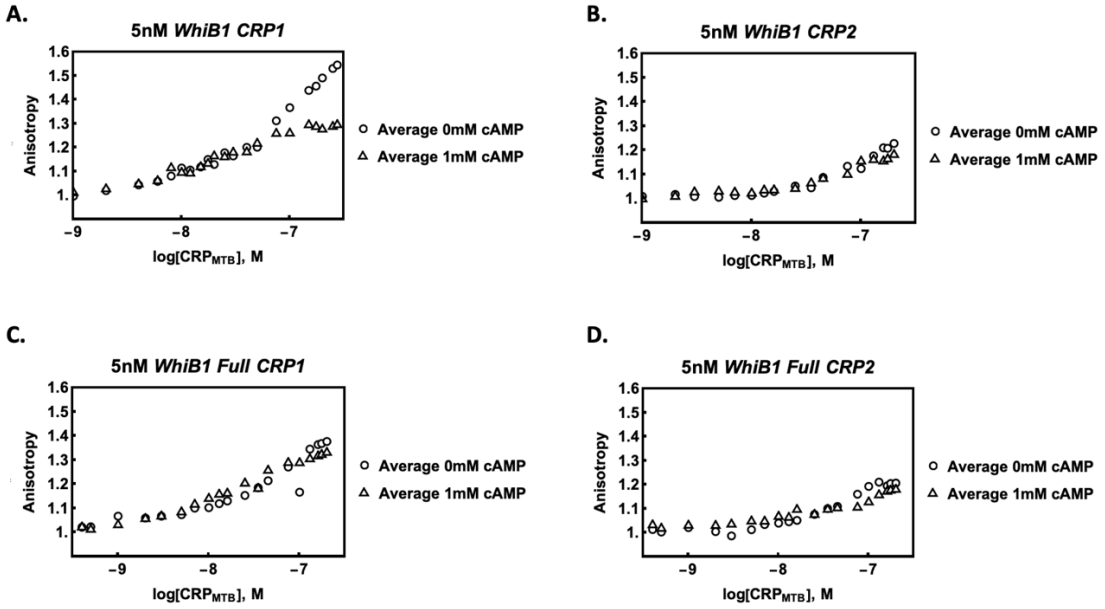

Figure 4. Average anisotropy values for four trials of nonstoichiometric titrations of CRP_{MTB} with and without cAMP for each *whiB1* construct (5nM). Four trials were performed utilizing 0mM cAMP (circles) and four utilizing 1mM cAMP (triangles) for each *whiB1* promoter. **(A)** Average anisotropy values for the *WhiB1 CRP1* (short) promoter. **(B)** Average anisotropy values for the *WhiB1 CRP2* (short) promoter. **(C)** Average anisotropy values for the *WhiB1 Full CRP1* promoter. **(D)** Average anisotropy values for the *WhiB1 Full CRP2* promoter.

For this experimentation, nonstoichiometric titrations were performed utilizing fluorescence anisotropy to measure binding affinities between CRP_{MTB} and four *whiB1* promoters. The primers utilized were two 26 bp promoters that contain one of each identified *whiB1* binding sites connected to a fluorescein molecule (*WhiB1 CRP1* and *WhiB1 CRP2*), and two 48 bp promoters that contain both identified *whiB1* binding sites, one with a fluorescein molecule attached to the upstream binding site, and the other with a fluorescein molecule attached to the downstream binding site (*WhiB1 Full CRP1* and *WhiB1 Full CRP2*). Eight titrations were performed for each of the *whiB1* promoters, four with 0mM cAMP and four with 1mM cAMP. The anisotropy values were then averaged and compared: the comparison for *WhiB1 CRP1* is depicted in **Figure 4A**, *WhiB1 CRP2* in **Figure 4B,** *WhiB1 Full CRP1* in **Figure 4C,** and *WhiB1 Full CRP2* in **Figure 4D.** This resulted in on average larger anisotropy values for *WhiB1 CRP1* and *WhiB1 Full CRP1* compared to the two constructs with the fluorophore on the downstream binding site. In **Figure 4A,** there is a significant decrease in anisotropy value with cAMP compared to without at around 10^{-7} M cAMP.

After the anisotropy values were collected, the averaged values of anisotropy for each of the four *whiB1* promoters with cAMP were fit to according to **Equation 1,** as previously

described by Garate *et al*[1]. The DNA-binding affinity constants were also mathematically calculated as previously described by Lanfranco *et al*.[11] The fitted data for the *WhiB1 CRP1* promoter is depicted in **Figure 5A,** *Whib1 CRP2* promoter in **Figure 5B,** *WhiB1 Full CRP1* promoter in **Figure 5C,** and *WhiB1 Full CRP2* in **Figure 5D**. Similar binding affinities for the two 26-bp constructs were calculated (k_{DNA} ~7.7-7.8), and additionally similar binding affinities for the two 48-bp constructs were calculated (k_{DNA}~8-8.5).

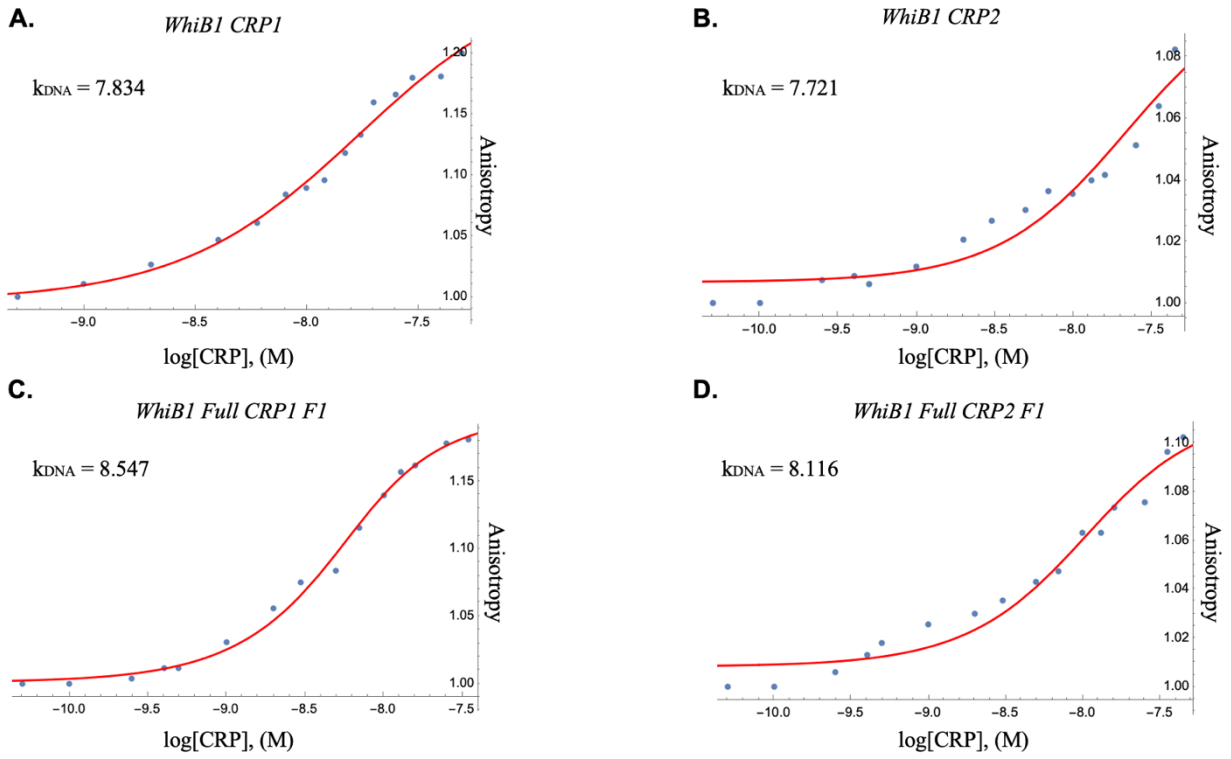

Figure 5. Quantification of the functional behavior of CRP_{MTB} when binding with each *whiB1* promoter in the presence of cAMP. Average anisotropy values of CRP_{MTB}-*whiB1* binding with 1mM cAMP are represented by blue circles, while solid red lines represent the mathematical fit created using **Equation 1.** DNA-binding affinities also included (k_{DNA}). **(A)** Fitted data for the *WhiB1 CRP1* promoter. **(B)** Fitted data for the *WhiB1 CRP2* promoter. **(C)** Fitted data for the *WhiB1 Full CRP1* promoter. (**D)** Fitted data for the *WhiB1 Full CRP2* promoter.

Preliminary Stoichiometric Binding Assay Results

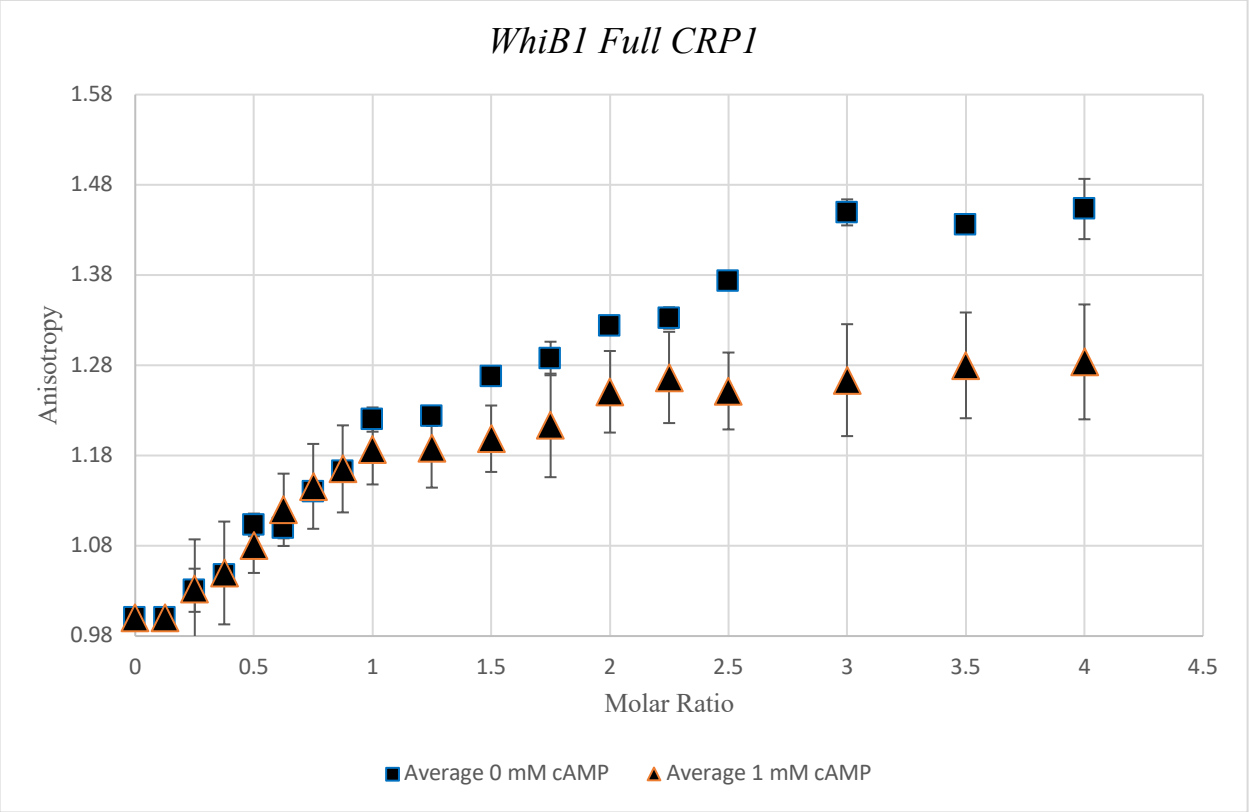

Figure 6. Preliminary stoichiometric binding assay results for the binding between CRP_{MTB} and the *WhiB1 Full CRP1* promoter with and without cAMP. Stoichiometric titration was performed utilizing 200 nM of the *WhiB1 Full CRP1* promoter and up to 800 µM of CRP_{MTB} for two trials with 0mM cAMP (blue squares) and two trials with 1mM cAMP (orange triangles). The anisotropy values from the trails were then averaged and normalized to the first point.

To confirm the results found from the nonstoichiometric titrations performed utilizing fluorescence anisotropy to draw conclusions regarding the binding affinity of CRP_{MTB} to the various *whiB1* promoters, stoichiometric titrations were also performed using fluorescence anisotropy. This was done using a concentration of 200 nM *whiB1* promoter with and without cAMP (0mM or 1mM). Although preliminary, this experimentation suggests that in the presence of cAMP, there is a linear increase in anisotropy signal until a molar ratio of 1 is reached, in which there is a plateau until a molar ratio of 2 is reached, after which there is a second plateau. In the absence of cAMP, there is an overlap in the linear increase until the molar ratio of 1, but rather than plateauing, the anisotropy signal continues to rise.

Discussion

Understanding the binding interactions between CRP_{MTB} and the multiple binding sites along the *whiB1* promoter is a relevant topic to approaching solutions for the treatment of TB due to its contribution to understanding the way in which *Mycobacterium tuberculosis* interacts with its host. Throughout this experimentation, it was necessary to elucidate the role of cAMP in these binding interactions, as many of the survival strategies utilized by *M. tuberculosis* are accomplished by raising cAMP levels inside the host cell. This includes blocking apoptosis-mediated killing, inhibiting inflammatory responses, and interfering with phagocytic acidification.[1]

In this study, we provide evidence for the role of the two binding sites in CRP_{MTB}-*whiB1* binding interactions, as well as recapturing cAMP regulation of DNA-binding specificity for CRP_{MTB}. Fluorescence anisotropy experiments show on average larger anisotropy values for the two constructs with the fluorophore on the upstream binding site (*WhiB1 CRP1* and *WhiB1 Full CRP1*) compared to the two constructs with the fluorophore on the downstream binding site (*WhiB1 CRP2* and *WhiB1 Full CRP2)* (**Figure 4,** A, B, C, D). This visually suggests that CRP_{MTB} binds with higher affinity at the upstream binding site, as larger anisotropy values signal slow tumbling, which occurs when larger complexes are formed (or as binding increases). While the anisotropy values are lower for the *WhiB1 CRP2* and *WhiB1 Full CRP2* promoters, binding still occurs, simply that CRP_{MTB} binds with lower affinity to the downstream binding site in relation to the upstream. This also suggests that while CRP_{MTB} does exhibit higher binding affinity to one binding site, it does not exhibit negative cooperativity from either site upon one another that halts binding. This is evident in that there are still linear increases in anisotropy values for the two 48bp constructs shown in **Figure 4C** and **Figure 4D** (*WhiB1 Full CRP1* and *WhiB1 Full CRP2*), These results are supported by previous experimentation utilizing EMSA performed by Stapleton *et al*[1] and Bai *et al*.[8] On average, the anisotropy values for the longer *whiB1* promoters (*WhiB1 Full CRP1* and *WhiB1 Full CRP2*) are lower than that of their shorter counterparts (*WhiB1 CRP1* and *WhiB1 CRP2*), which may be due to the fact that the polarized emissions are being overtaken by the size of the constructs (48 bp each), and true binding affinity cannot be determined as well.

The role of cAMP in regulating the binding between CRP_{MTB} and *whiB1* can also be visually determined utilizing the results of fluorescence anisotropy. As seen in **Figure 4A**, when analyzing the anisotropy values for the 26bp *WhiB1 CRP1* promoter, it is clear that the anisotropy values with cAMP are significantly lower than those without, suggesting that cAMP is promoting sequence-specific binding interactions and preventing the formation of high-ordered oligomers.[1] While not to a significant extent, this is visually the case for the other three *whiB1* promoters.

The binding interactions between CRP_{MTB} and the 48bp *WhiB1 Full CRP1* promoter were further studied through a stoichiometric fluorescence anisotropy binding assay, in which a high concentration of the promoter (200 nM) was utilized to determine the molar ratio between the promoter and protein. While only preliminary, the results revealed that there is a linear increase

in anisotropy signal until a molar ratio of 1 is reached, in which there is a plateau until a molar ratio of 2 is reached, after which there is a second plateau (**Figure 6)**. This suggests that two molecules of CRP_{MTB} will bind to one molecule of *WhiB1 Full CRP1*. This is in congruence with the fact that there are two binding sites and that neither expresses negative cooperativity on the other. In the absence of cAMP, there is an overlap in the linear increase until the molar ratio of 1, but rather than plateauing, the anisotropy signal continues to rise. This signals that high-order CRP_{MTB}-*WhiB1 Full CRP1* oligomers are being formed, and it can be drawn from this that cAMP enables the reduction of high-order nonspecific oligomers finding during such binding interactions.

After anisotropy values were collected and averaged, the values for anisotropy in the presence of cAMP were fit according to **Equation 1**[1]. As seen in **Figure 5C** and **5D**, for the two promoters that contain the fluorophore on the downstream binding site (*WhiB1 CRP2* and *WhiB1 Full CRP2*), from a visual standpoint, the computed equation is not a good fit for the averaged data. Although visually it seems as though it is a good fit for the promoters containing the fluorophore on the upstream binding site (*WhiB1 CRP1* and *WhiB1 Full CRP1*), the calculated DNA-binding affinity constants (k_{DNA}) between the four promoters suggests otherwise (**Figure 5A, D)**. Due to the difference in anisotropy values, there was expected to be much more difference between the binding affinity constants between the four promoters. However, based on the fit, all four binding affinity constants are between k_{DNA}= ~8. This may be attributed to the fact that the equation was adapted from Gárate *et al* and was originally based on a 32bp *SerC32* promoter with one binding site.[1] This discrepancy in promoter length between the original model for this equation and these four promoters, as well as the fact that the longer promoters would require an equation that accounts for two binding sites, will affect the mathematical fit of the equation to these promoters.

Future work would include performing repeated trials of the nonstoichiometric titrations utilizing fluorescence anisotropy. As the measurements for anisotropy rely heavily on the stabilization of the instrument, continuing repeated trials is beneficial to attain results most accurate to the actual biological mechanisms of binding. In addition, completing the stoichiometric titrations utilizing fluorescence anisotropy for not only the *WhiB1 Full CRP1* construct but also the *WhiB1 Full CRP2* construct is necessary to truly confirm the mechanisms through which CRP_{MTB} binds to *whiB1*. In addition, adjusting the equation for fitting the nonstoichiometric anisotropy values to account for the length of these promoters and the fact that the longer promoters contain two binding sites, unlike *SerC32*. To account for these two binding sites in *WhiB1 Full CRP1* and *WhiB1 Full CRP2*, the equation must result in a cubic function that will require estimates that are guided by the fitting for the single binding sites in *WhiB1 CRP1* and *WhiB1 CRP2*. Taking this into account as well as allowing for visual inspection to continue to guide our fitting is necessary in order to computationally confirm the biological interactions occurring in this study.

Concluding Remarks

In this study, we begin to investigate the binding interactions between CRP_{MTB} and *whiB1*, as well as the role of cAMP in this biological system. Most importantly, this study offers a visual interpretation as to the interactions occurring in regard to the two *whiB1* binding sites, as in **Figure 4**, it is clear that CRP_{MTB} binds with higher affinity to the upstream binding site, but the sites are not exhibiting negative cooperativity on one another, as binding still occurs at both sites. This is confirmed in **Figure 6** utilizing a stoichiometric titration. This study also confirms the previously studied role of cAMP on CRP_{MTB} binding interactions, in that in the presence of cAMP, the creation of high-ordered CRP-DNA oligomers are reduced. This study supplements the field of study regarding *Mycobacterium tuberculosis*, and by understanding these interactions with a gene so pivotal in development as *whiB1*, may prove to be fruitful in the search for potential treatment avenues for TB.

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