STUDY OF THE MECHANISTIC FEATURES OF DNA REPLICATION RESTART

IN NEISSERIA GONORRHOEAE

Thesis
Submitted to
The College of Arts and Sciences of the
UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for
The Degree of
Master of Science in Chemistry

By
Bharath Sunchu

UNIVERSITY OF DAYTON
Dayton, Ohio
August, 2012
STUDY OF THE MECHANISTIC FEATURES OF DNA REPLICATION RESTART
IN *NEISSERIA GONORRHOEAE*

Name: Sunchu, Bharath

APPROVED BY:

Matthew E. Lopper, Ph.D.
Faculty Advisor
Assistant Professor
Department of Chemistry

Madhuri Kango-Singh, Ph.D.
Committee Member
Assistant Professor
Department of Biology

Shawn M. Swavey, Ph.D.
Committee Member
Associate Professor
Department of Chemistry
ABSTRACT

STUDY OF THE MECHANISTIC FEATURES OF DNA REPLICATION RESTART
IN NEISSERIA GONORRHOEAE

Name: Sunchu, Bharath
University of Dayton

Advisor: Dr. Matthew E. Lopper, Ph.D.

*Neisseria gonorrhoeae* is the causative organism of gonorrheal infection. This pathogen shows remarkable resistance to the oxidative damaging agents released by the neutrophils. The Bacterial genome is one of the important targets of these agents and studies indicate that DNA replication restart pathways help in bypassing the hazardous effects of these oxidative agents. The DNA replication restart pathway helps to reload and restart the stalled or derailed replication machinery onto the DNA which is blocked by obstructions such as single stranded nicks, double stranded breaks, or oxidized bases. In *N. gonorrhoeae*, the primosome proteins PriA and PriB carry out the DNA replication restart process by forming a nucleoprotein complex with the DNA. PriA, a helicase protein, binds and partially unwinds the DNA at the fork where the replication machinery will be reloaded. PriB serves to stimulate PriAs DNA binding and unwinding activity. PriA, PriB and the DNA form a ternary nucleo-protein complex in this process. In, *E. coli*
the PriB has a high affinity interaction with ssDNA and a low affinity interaction with PriA, while N. gonorrhoeae PriB has a high affinity interaction with PriA and a low affinity interaction with ssDNA. The first part of my research contributed to understand the features of this affinity reversal. I performed pull down experiments, DNA unwinding assays and ATP hydrolysis experiments using single point alanine mutants of PriB. From my work, I have provided evidence that in N. gonorrhoeae, PriBs ssDNA binding activity is not required for PriB stimulation of PriA helicase, unlike what is seen in E. coli.

Evidence that PriA provides resistance against oxidative damaging agents bolsters the importance of DNA replication restart pathway for the survival of this disease causing bacteria. Developing antibiotics that target this pathway could be a vital event in the field of drug discovery research. In this process, we have developed an enzyme-based assay to use in high-throughput screening to identify inhibitors of the DNA replication restart pathway in N. gonorrhoeae. Initial screening of several thousand compounds from small molecule chemical libraries has produced several lead compounds. I worked with one of the lead compounds, CGS-15493, and here I report the mechanistic features of its inhibition of the DNA replication restart pathway.
Dedicated to my family and friends
ACKNOWLEDGEMENTS

First and foremost I would like to express my heartfelt gratitude to my advisor, Dr. Matthew Lopper. I attribute the successful completion of my thesis project and writing of my thesis to his invaluable guidance, effort, and encouragement. Starting life as a research student one simply could not wish for a better or friendlier supervisor. His enthusiasm for biochemistry, his communication and leadership skills will always be an inspiration to me.

I would like to thank my committee numbers Dr. Shawn M. Swavey and Dr. Madhuri Kango-Singh for their support. I would also like to thank Dr. Mark Masthay, Dr. Shawn Swavey and Dr. Garry Crosson for letting me use their U.V. spectrophotometer, silver staining chemical reagents and HPLC respectively. I am grateful to my instructors Dr. David Johnson, Dr. Shawn Swavey, Dr. Gary Crosson, Dr. Panagiotis tsonis and Dr. Matthew Lopper for their invaluable teaching and guidance. I specially thank Dr. Kevin Church for his help in selecting courses.

In addition, I would also like to offer my sincerest gratitude to Paula Keil, Dr. Kimberly Trick Dr. Rochael Swavey, and Rose Eckerle for helping me with my teaching duties. I sincerely appreciate Connie Schell and Margaret Goodrich for being patient with me and sending reminders whenever I forgot to complete any office formality. On the whole I would like to thank Chemistry Department at University of Dayton for
supporting me throughout my masters and Graduate school for offering me summer fellowship.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................ iii
DEDICATION ........................................................................................................... v
ACKNOWLEDGEMENTS ........................................................................................ vi
LIST OF FIGURES ................................................................................................. x
LIST OF TABLES ..................................................................................................... xii
CHAPTER I INTRODUCTION ................................................................................ 1
CHAPTER II METHODS .......................................................................................... 9
  II-1 Purification of *N. gonorrhoeae* PriA ......................................................... 9
  II-2 Purification of *N. gonorrhoeae* PriB ......................................................... 10
  II-3 Helicase assays ............................................................................................ 11
  II-4 ATP hydrolysis assays .................................................................................. 13
  II-5 FITC – labeling experiments ....................................................................... 15
  II-6 Pull-down experiments ................................................................................ 16
  II-7 Peptide-mapping experiments .................................................................... 18
CHAPTER III RESULTS ........................................................................................ 21
  III-1 PriB:K34A variant defective for ssDNA binding stimulates the PriA helicase activity ................................................................................................................. 21
III-2 Pull-down experiments.................................................................................................................. 24
III-3 Peptide-mapping experiments........................................................................................................ 25
III-4 Mechanism of the inhibition of DNA replication restart process of Neisseria gonorrhoeae by CGS-15943.................................................................................................................. 31
   III-4-1 PriA is the target of the CGS-15943 inhibition ................................................................. 31
   III-4-2 CGS-15943 affects PriA-catalyzed ATP hydrolysis.......................................................... 32
      III-4-2-a ATP hydrolysis experiments – DNA titrations .......................................................... 33
      III-4-2-b ATP hydrolysis experiments – ATP titrations............................................................. 36
CHAPTER IV CONCLUSIONS.................................................................................................................. 39
REFERENCES........................................................................................................................................ 41
LIST OF FIGURES

Figure 1: DNA replication process in *E. coli* ......................................................... 2
Figure 2: Ribbon diagram of the crystal structure of *E. coli* PriB complexed with ssDNA ................................................................. 5
Figure 3: DNA replication restart process in *E. coli* ......................................................... 5
Figure 4: A diagram showing the relative affinities between PriA, PriB and ssDNA in *E. coli* and *N. gonorrhoeae* ................................................................. 7
Figure 5: DNA unwinding assays .................................................................................. 12
Figure 6: ATPase assay regeneration system ............................................................... 14
Figure 7: Pull-down experiments .................................................................................. 17
Figure 8: Peptide-mapping experiments ....................................................................... 19
Figure 9: Structure of DSP (dithiobis [succinimidylpropionate]) .................................... 19
Figure 10 A) Ribbon diagrams of *E. coli* PriB:R34A and *N. gonorrhoeae* PriB:K34A variants ........................................................................................................ 23
Figure 10 B) Results of the DNA unwinding assays in presence of PriB:wild type and PriB:K34A ........................................................................................................... 23
Figure 10 C) Fork 3 DNA ............................................................................................ 23
Figure 11: Pull-down experiment to determine the affinities of PriB:E41A and PriB:K34A towards PriA ........................................................................................................ 25
Figure 12: Identification of Cross-linked PriA:PriB complex ........................................ 26
Figure 13: A secondary silver stained gel confirming the presence of cross linked PriA:PriB complex in the primary gel .................................................................27
Figure 14: Peptide-mapping experiments-HPLC chromatograms at 215 nm.................29
Figure 15: Peptide-mapping experiments-HPLC chromatograms at 280 nm..............30
Figure 16: PriA is the target of CGS-15943 inhibition .............................................32
Figure 17: PriA’s ATPase activity is affected by CGS-15943 – DNA titrations......... 35
Figure 18: PriA’s ATPase activity is affected by CGS-15943 – ATP titrations..........37
LIST OF TABLES

Table 1: Effect of a mixed inhibitor on apparent $V_{\text{max}}$ and $K_m$ values..........................34
Table 2: ATPase assays –DNA titrations- $V_{\text{max}}, K_m, k_{\text{cat}}$ values.................................35
Table 3: ATPase assays –DNA titrations- $K_l, K_l^', \alpha, \alpha^'$ values........................................36
Table 4: ATPase assays –ATP titrations -$V_{\text{max}}, K_m, k_{\text{cat}}$ values.................................37
Table 5: ATPase assays –ATP titrations- $K_l, K_l^', \alpha, \alpha^'$ values........................................38
CHAPTER I

INTRODUCTION

DNA replication involves duplication of the cellular genome and is essential for the survival and propagation of life. In bacteria, this process is continually disrupted by obstructions on the DNA template such as single stranded nicks, double stranded breaks, and oxidized bases which arise due to environmental or cellular factors\textsuperscript{21}. The progress of the DNA replication machinery (replisome) is hindered by these barriers, causing it to stall or derail from the DNA template\textsuperscript{2}. To ensure continuation of the DNA replication process, bacteria have evolved “DNA replication restart pathways” which reload and reactivate the DNA replisome onto a repaired DNA replication fork\textsuperscript{10}.

Replisome reloading at a repaired DNA replication fork has different mechanistic features when compared to replisome loading at an origin of replication during initiation of replication (see Ref 18 for review). In \textit{E. coli}, the DNA replication process initiates at a particular DNA sequence element referred to as \textit{oriC}, the origin of replication (Fig. 1). The first protein to participate in this process is DnaA, the initiator protein. It binds to the origin site, produces a loop of unwound DNA, and facilitates the loading of DnaB (the replicative helicase) onto each template strand of DNA. These two DnaB proteins travel
along the two single stranded DNAs in the 5’-3’ direction, unwinding the DNA as they travel. In this way they create two replication forks. As the DNA strands are separated at the fork, many molecules of single-stranded DNA-binding protein (SSB) bind to and stabilize the separated strands, and DNA gyrase relieves the topological stress induced ahead of the fork by the unwinding reaction. DnaG, a primase, synthesizes the RNA primers to which deoxyribonucleotides are added by DNA polymerase III.

DnaB helicase and DnaG primase constitute a functional unit within the replication complex, the primosome. This primosome travels on the template-lagging strand producing RNA primers to synthesize Okazaki fragments. Therefore DNA replication process is a coordinated and a step wise assembly process which synthesizes both the leading and lagging strands simultaneously.

**Figure 1: DNA replication process in E. coli.** DnaA binds to the DNA at the origin of replication and produces a loop of unwound DNA, followed by binding of DnaB helicase and establishment of two replication forks.

The replisome is particulary sensitive to imperfections in the template DNA, and DNA damage can occur anywhere on the chromosome. Therefore, the DNA replication machinery could potentially be blocked and derailed at a site far removed from the origin of replication. DnaA, the initiator protein of the DNA replication process, only works at
an origin sequence, and sites where the DNA replication process is blocked do not necessarily have any specific origin DNA sequences nearby. Therefore DNA replication restart pathways require a different set of proteins, referred to as DNA replication restart primosome proteins, to facilitate reactivation of disrupted replisomes at sites far removed from an origin sequence. DNA replication restart pathways typically involve recognition of specific DNA structures such as branched, fork-like structures or D-loop recombination intermediates (a D-loop or Displacement loop is formed due to the displacement of one strand of the duplex by an invading single strand of DNA during a strand pairing reaction). In *E. coli*, the DNA replication restart primosomal proteins that catalyze this process include PriA, PriB, PriC, DnaT, DnaB, DnaC, and DnaG.

These proteins were originally discovered during the bacteriophage ΦX174 complementary strand DNA replication *in vitro*. Studies in bacteriophage ΦX174 showed that PriA played a vital role in assembling the primosome, a multi-enzyme replication machine which includes PriA, DnaB, DnaG, DnaC, PriB, PriC, and DnaT. In the bacteriophage ΦX174, this primosome once formed can translocate along the DNA and synthesize short oligonucleotide primers that could be used to initiate the synthesis of the complementary strand.

After the function of PriA in the bacteriophage ΦX174 was studied, questions were raised about its role in the *E. coli* itself. *E. coli* strains which carried null mutations in PriA exhibit a complex set of phenotypes that includes reduced viability, sensitivity to UV irradiation, and induced stable DNA replication and constitutive stable DNA replication. All of these phenotypes can be suppressed when a *priA* allele encoding a PriA mutant protein no longer capable of acting as a DNA helicase, but still capable of
catalyzing primosome assembly is provided\textsuperscript{14}. This observation, and the requirement for recombination proteins during both induced and constitutive stable DNA replication, led to the proposal that the cellular function of PriA was to direct the assembly of replication forks at recombination intermediates such as D-loops\textsuperscript{15}.

PriA recognizes and binds to two types of DNA structures with high affinity: partially duplex DNA with a 3'-single stranded extensions or D-loop DNA,\textsuperscript{15,19}. Current biochemical data indicate that the functional form of PriA is a monomer, with a molecular mass of 81.7kDa\textsuperscript{12} and it has a DNA-binding and a helicase domain (citation needed here). It is a 3'-5' helicase which unwinds DNA while migrating from 3' end to 5' end of the DNA\textsuperscript{13}. This helicase activity of PriA is presumably required to unwind double stranded lagging strand template at stalled fork structures to facilitate loading of DnaB, the replicative helicase\textsuperscript{7,8}. PriA also forms a complex with PriB and DnaT to load the DnaB on to the DNA coated with SSB. DnaB cannot be loaded on to SSB coated DNA and this feature serves to prevent unnecessary loading of DnaB onto DNA sites other than \textit{oriC}. However, in replication restart this becomes a barrier for loading the DnaB helicase and it is circumvented by the activity of PriA:PriB:DnaT complex.

\textit{E. coli} PriB has been shown to stabilize binding of PriA to DNA\textsuperscript{16}. It is a single stranded DNA (ssDNA) binding protein\textsuperscript{5,9} and stimulates PriAs helicase activity by its interaction with ssDNA\textsuperscript{10}. \textit{E. coli} PriB was the first DNA replication restart primosome protein to be crystallized. Its crystal structure shows a homodimeric \(\beta\)-barrel with oligonucleotide/oligosaccharide binding (OB) folds with each monomer contributing one OB-fold in the dimer\textsuperscript{9,11,27,28} (Fig. 2). It is a single structural domain characterized by a \(\beta\)-barrel from which four \(\beta\)-hairpins extend.
Figure 2: Ribbon diagram of the crystal structure of *E. coli* PriB complexed with ssDNA. The red and blue colored chains represent the two monomers which contribute to the homodimeric β-barrel. The ssDNA is represented by the cyan tube.

It has been proposed that DnaT binds to the PriA: PriB: DNA complex causing the release of ssDNA that has been bound by PriB⁴. This is followed by the recruitment and reloading of the replicative helicase, DnaB, onto the lagging strand template DNA which results in reactivation of the repaired DNA replication fork.

Figure 3: DNA replication restart process in *E. coli*: It depicts the sequence of the events when DNA replication is stopped at a site far removed from the origin of replication. PriA, a 3’ to 5’ helicase, is the first protein to participate, followed by PriB. PriB stimulates the helicase activity of PriA by its interaction with DNA. DnaB, the replicative helicase, is loaded after the ssDNA has been freed of PriB, perhaps by interaction with DnaT with the nucleoprotein complex.

Although the DNA replication restart process has been well studied in *E. coli*, it has not received much attention in other bacterial species. The fact that many bacterial genomes do not share the same complement of DNA replication restart primosome genes found in
*E. coli* indicates that bacterial species might be different in the mechanistic details of the DNA replication restart process and in the extent to which cells rely upon it for growth and survival. For example the presence of only PriA and PriB homologs and absence of PriC and DnaT in *Neisseria gonorrhoeae* make it a good subject for studying possible differences among bacterial species in DNA replication restart process and their dependence on it.

In *Neisseria gonorrhoeae*, PriA's interaction with PriB is strong, whereas that of *E. coli* PriA with its PriB is weak. In addition, *N. gonorrhoeae* PriB interacts weakly with ssDNA, whereas *E. coli* PriB has a strong affinity for ssDNA. I used single point alanine mutants of PriB to understand the features of this affinity reversal, and the mechanistic features of DNA replication restart pathway in *N. gonorrhoeae*. 
Figure 4: A diagram showing the relative affinities between PriA, PriB and ssDNA in *Escherichia coli* and *Neisseria gonorrhoeae*: Relative affinities between the binding partners in nucleoprotein complex formed during the DNA replication restart in *E. coli* and *N. gonorrhoeae*. The thick arrows represent strong affinity between the binding partners and thin arrows indicate weak affinity. In *E. coli* PriB has strong affinity for ssDNA and weak affinity for ssDNA. In *N. gonorrhoeae* PriB interacts strongly with PriA and weakly with ssDNA.

In addition to helping in understanding the features of affinity reversal, we realized that studying DNA replication restart pathway in *N. gonorrhoeae* could also be used to develop novel antibiotics against this gonorrheal infection causing bacteria. Gonorrhea is a common sexually transmitted infection (STI) caused by *N. gonorrhoeae*. Studies have shown that this pathogen shows remarkable resistance to the oxidative damaging agents released by the neutrophils in the infected individuals\(^3\). The Bacterial genome is one of the important targets of these oxidative damaging agents and previous studies indicate that DNA replication restart pathway help in bypassing the hazardous effects of these oxidative agents\(^27\). Evidence that PriA plays an important role in DNA
repair in *N. gonorrhoeae* and it provides resistance against oxidative damaging agents bolster the importance of DNA replication restart pathway for the survival of this disease causing bacteria. We realized that developing antibiotics that target this pathway could be a vital event in the field of drug discovery research and developed an enzyme-based assay to use in high-throughput screening to identify inhibitors of the DNA replication restart pathway in *N. gonorrhoeae*. Initial screening of several thousand compounds from small molecule chemical libraries has produced several lead compounds. CGS-15493 is one of the lead compounds which showed potential signs of inhibition. I used helicase assays and steady state kinetic experiments to understand the mechanistic features of inhibition of DNA replication restart pathway in *N. gonorrhoeae* by CGS-15943.
II-1 Purification of *N. gonorrhoae* PriA:

*Neisseria gonorrhoae* PriA was purified from BL21 (DE3) *E.coli* harboring the pET28b: *Ngon* PriA plasmid. Cells were grown in Luria Bertani (LB) medium containing 50 μg/ml kanamycin at 37º C until an OD<sub>600</sub> of 0.6 was reached. 0.5 mM IPTG was used to induce the expression of PriA for over a period of 4 hours and cells were harvested by centrifugation at 5000×g for 25 min at 4ºC. Cell were lysed in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylflouride (PMSF) by sonication on ice, using 5× 30 sec pulsed bursts (pulse= 1 sec on, 1 sec off) at 70% amplitude using a Fisher scientific sonic dismembrator-500. The lysate was clarified by centrifugation at 40,000×g for 20 min at 4ºC. His-tagged PriA was bound to nickel-nitrilotriacetic acid (NTA) agarose beads and eluted in 10mM HEPES pH 7, 10% (v/v) glycerol, 0.1 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The eluate was dialyzed against 10 mM
HEPES pH 7, 10% (v/v) glycerol, 0.1 M NaCl, 1 mM β-mercaptoethanol and thrombin was used to remove the His-tag, leaving a Gly-Ser-His sequence at the amino-terminus directly preceding the first methionine residue. Residual His-tagged PriA that was not cleaved by thrombin, as well as contaminating E. coli proteins were filtered out by incubating the thrombin–cleaved PriA solutions with nickel-NTA agarose. The thrombin cleaved PriA solution was adjusted to pH 6 with 0.1M MES and PriA was purified with a HiPreP 16/10 Sepharose SPFF ion exchange column (GE Healthcare) using a linear gradient of NaCl from 0.1 to 1 M in 10 mM MES pH 6, 10% (v/v) glycerol, 1 mM β-mercaptoethanol. PriA fractions were pooled, and concentrated overnight by centrifugation in a Centriprep YM-10 concentrator at 2,643×g at 4°C, and stored at -80°C. For pull-down experiments, His-PriA was purified as described above except that thrombin was not added to the nickel-NTA agarose eluate.

II-2 Purification of N. gonorrhoeae PriB:

*Neisseria gonorrhoeae* PriB was purified from BL21(DE3) E.coli harboring the pET28b:Ngon PriB plasmid. Cells were grown in Luria Bertani (LB) medium containing 50 µg/ml kanamycin, and 50 µg/ml chloramphenicol at 37°C until an OD_{600} of 0.6 was reached. 0.5 mM IPTG was used to induce the expression of PriB for over a period of 4 hours and cells were harvested by centrifugation at 5000×g for 25 min at 4°C. Cell were lysed in 10 mM Tris-HCl pH 8.5, 10% (v/v) glycerol, 0.1 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice, using 5×30 sec pulsed bursts (pulse= 1 sec on, 1 sec off) at 70% amplitude using Fisher scientific sonic dismembrator-500. The lysate was clarified by centrifugation at 40,000×g for 20 min at 4°C. His-tagged
Pri-B was bound to nickel-NTA agarose beads and eluted in 10 mM Tris-HCl pH 8.5, 10% (v/v) glycerol, 0.1 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The eluate was dialyzed against 10 Mm Tris HCl pH 8.5, 10%(v/v) glycerol, 0.1 M NaCl, 1 mM β-mercaptoethanol and thrombin was used to remove the His-tag, leaving a Gly-Ser-His sequence at the amino-terminus directly preceding the first methionine residue. Residual His-tagged PriB that was not cleaved by thrombin, as well as contaminating E. coli proteins were filtered out by incubating the thrombin cleaved PriB solutions with nickel-NTA agarose. The thrombin cleaved PriB solution was adjusted to pH 6 with 0.1 M MES and PriA was purified with a HiPreP 16/10 Sephacryl S-100 size exclusion column (GE Healthcare) in 10 mM Tris-HCl pH 8.5, 10% (v/v) glycerol, 1 mM β-mercaptoethanol. PriB fractions were pooled and concentrated overnight by centrifugation in a Centriprep YM-10 concentrator at 2,643×g at 4°C, and stored at -80°C.

II-3 Helicase assays:

Helicase assays were performed to evaluate the helicase activity of PriA. In these assays, the lagging strand of DNA molecules are tagged with fluorescein and their fluorescence anisotropy is measured. Fluorescence anisotropy being directly proportional to the size of the molecule, it decreases as the DNA is unwound by the PriA helicase. Thus, the PriA helicase activity can be related to decrease in the fluorescence anisotropy of DNA molecules.
Figure 5: DNA unwinding assays: A) one of the strands of the fork DNA is tagged to a fluorescein molecule. PriA stimulated by PriB, uses energy from the ATP hydrolysis to unwind the fork DNA. B) As the DNA is unwound the fluorescence anisotropy values decrease and this can be related to the degree of DNA unwinding.

In these experiments 0 nM to 50 nM of PriB or its variants were used with 1 nM DNA and 2 nM PriA in the helicase assay buffer which consisted of 20 mM Tris HCl pH 8, 50 mM NaCl, 3 mM magnesium chloride, and 1 mM β-Mercaptoethanol. 1 mM ATP was added to initiate the reaction and the reaction mixture was incubated for 37°C for 10 min. The reaction was stopped by adding Sodium Dodecyl Sulfate (SDS) and the fluorescence anisotropy values (m_x) of the reaction samples were measured at 25°C in a Beacon TM 2000 variable temperature fluorescence polarization system. After the
measurements, the solution was incubated at 95°C for 25 sec and cooled in ice for 25 sec so that the entire DNA will be unwound and will reach the constant temperature quickly. The anisotropy values ($m_{ss}$) of the completely unwound DNA samples were then measured.

The fraction unwound of the DNA was then calculated using the formula below and a graph with fraction unwound DNA against the varying concentrations of PriB or its variants was plotted.

\[
\text{Unwound\%} = 1 - \frac{mx - [mss]}{mo - mss}
\]

$m_x$ = Fluorescence anisotropy values of the reaction mixtures in which the different concentrations of PriB or its variants was used.

$m_{ss}$ = Fluorescence anisotropy values of the completely unwound DNA after treating the reaction mixtures at 95°C.

$m_o$ = Fluorescence anisotropy values of the sample which consists of intact duplex DNA.

When studying the mechanism of the inhibitor (CGS-15943), helicase assays were performed to find out the primary target of the inhibitor between PriA and PriB. 5 nM PriA was used in these reactions with or without 114 µM of inhibitor, and 50 nM PriB.

**II-4 ATP hydrolysis assays:**

PriAs ATPase activity is the subject of investigation in these assays. These experiments were used to observe the trends in steady state rates of ATP hydrolysis.
catalyzed by PriA in different conditions. These assays use an ATP regeneration system in which the ATP hydrolysis is coupled with the oxidation of NADH to NAD$^+$. Spectrophotometry is used to measure the decrease in the absorbance at 340nm due to NADH oxidation and these Steady-state $\Delta A_{340 \text{ nm}}/\Delta t$ rates were converted to $\Delta [\text{ATP}]/\Delta t$ to determine the ATPase rates$^{23}$. 

![Figure 6: ATPase assay regeneration system](image)

To study the PriB-K34A dependent ATP hydrolysis profiles, different concentrations of PriB-K34A ranging from 0 nM to 100 nM were used with 10 nM of PriA and 200 nM of fork 3. The reactions were performed at 25°C by mixing PriA, PriB-K34A, and fork 3 with 20 mM HEPES pH 8, 50 mM NaCl, 7 mM $\beta$-mercaptoethanol, 0.1 mg/ml BSA, 2 mM Phosphoenol pyruvate, 0.1 mM NADH, 7 units of Pyruvate Kinase and 10 units of Lactate dehydrogenase and 2 mM Magnesium chloride in water. 1 mM ATP was added to initiate the reaction and was allowed to proceed for 1000 sec.

ATP hydrolysis assays were also performed to test the inhibitor CGS-15943. These experiments involved two variations in which the DNA dependent and ATP dependent profiles were measured in presence of the inhibitor. The reagents set used in these experiments are similar to that of the above mentioned in K34A dependent studies except for few changes. A single stranded oligonucleotide - poly dT 28, was used instead of fork
3 to avoid inconsistencies arising due to variations in different preparations of fork 3. 10 nM PriA, 100 nM PriB and 57 µM of inhibitor were used in this experiment and the reactions were run at 37°C.

The kinetic parameters $k_m$ and $k_{cat}$ values were calculated by applying the results from the ATPase assays to the Michaelis-Menten equation,

$$ \text{Rate} = \left( \frac{V_{max} \times [S]}{K_m + [S]} \right) $$

The $k_{cat}$ values were calculated by dividing $K_m$ values by the concentration of PriA used in the reaction.

**II-5 FITC-labeling experiments:**

These experiments were designed to study the interaction of single point alanine mutants of PriB with PriA. FITC-labeled PriB mutants were prepared for this purpose. The idea was to use fluorescence polarization spectroscopy and measure the fluorescence anisotropy of FITC-PriB wild-type and mutants in the presence of PriA. This data could be used to compare the affinities towards PriA of different PriB mutants with wild-type PriB. To measure PriA:FITC-PriB interactions, serial dilutions of *E. coli* PriA or *N. gonorrhoeae* PriA are made into 20 mM Tris–HCl pH 8, 4% (v/v) glycerol, 0.1 M NaCl, 1 mM β-mercaptoethanol, 0.1 g/l BSA and incubated with 10 nM FITC-PriB. Apparent dissociation constants are calculated by determining the concentration of titrant required to bind 50% of the FITC-PriB. The unbound state is reported by the fluorescence anisotropy of FITC-PriB in the absence of PriA. The fully bound state is
reported by the fluorescence anisotropy of FITC-PriB in the presence of a sufficient concentration of PriA to saturate the fluorescence anisotropy signal.

**II-6 Pull-down experiments:**

Pull-down experiments were planned to study the physical interaction of single point alanine mutants of PriB:E41A and PriB:K34A with PriA. These experiments could add more detail to the mechanism by telling which amino-acids in PriB was important for PriBs interaction with PriA.

The principle of these experiments uses two proteins - a bait protein and a prey protein. In these assays the tagged bait protein is incubated with a prey protein. This protein-protein complex is captured on an immobilized affinity ligand specific for the tag on the bait protein and any unbound prey protein is washed away. The putative protein-protein interaction complex is then eluted from the affinity ligand and the pull-down products will be resolved through a polyacrylamide gel and visualized by coomassie brilliant blue staining. After de-staining the gel shows if there is any interaction between two proteins and if present the degree of interaction.

His tagged PriA and tagless PriB wildtype, and PriB:E41A and PriB:K34A proteins were diluted to 1 g/l in 100 mM Tris-HCl pH 8, 10% (v/v) glycerol, 0.1 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 50 g/l BSA and incubated either separately or together at a 1:7 molar ratio of His-PriA:PriB for 1 hour at 4°C25. Nickel-NTA agarose beads, the affinity ligand were added to each sample and incubated for 10mins at 4°C, and collected by centrifugation at 21,130×g for 2min at 4°C. The beads were washed several times in 100 mM Tris-HCl pH 8, 10% (v/v) glycerol, 0.1 M NaCl, 10 mM
imidazole, 1 mM β-mercaptoethanol, 50 g/l BSA, and collected after each wash by centrifugation at 21,130×g for 2min at 4°C. The pull-down products were eluted by addition of SDS-PAGE sample buffer. The products were resolved through a 13.5% polyacrylamide gel and visualized by coomassie brilliant blue staining.

Figure 7: Pull-down experiments: step 1 and step 2: His-Tag PriA (violet rectangle) is incubated with PriB (red circle) during which they bind and form PriA:PriB complex. Step 3: This protein solution is then treated with nickel–NTA agarose beads (secondary affinity ligand) during which these beads interact with PriA through His-tag present on it. Step 4: In this step any unbound PriB is washed away Step 5: SDS-PAGE sample buffer is added to reverse the PriA-PriB interaction and the pull down products are evaluated by gel electrophoresis.
**II-7 Peptide-mapping experiments:**

These experiments were aimed to map the amino-acids at the PriA-PriB binding surface. These experiments involved using a chemical cross linker which can covalently bind and cross link proteins. The crosslinker makes sure that PriA-PriB binding surface which is made up of weak non-covalent bonds remains intact after proteolysis with trypsin. Our idea was to isolate the whole or part of a PriA:PriB binding surface fragment after trypsin digesting the crosslinked complex for mapping it.

The cross linker in these mapping experiments was DSP (Dithiobis[succinimidylpropionate]). DSP is a water-insoluble, homobifunctional N-hydroxysuccimide ester (NHS-ester). It is thiol-cleavable and reacts with primary amines to form covalent amide bonds that results in the release of N-hydroxysuccinimide$^{24}$.

In this experiment, PriA and PriB were incubated for 10min (step 1 & 2 –figure 6) and treated with the crosslinker in a buffer containing 0.1 M phosphate, 0.15 M NaCl at pH 7.2 for a duration of 30 min at room temperature (step 3-fig 6). 1M Tris pH 7.5 was added and incubated for 15mins to quench the reaction. The cross linking was reversed at necessary steps by reducing the disulfide bond in DSP using 50 mM DTT at 37°C for 30 mins. SDS-PAGE was used in conjunction with the cross linking in these experiments to identify and isolate desired cross-linked and trypsin digested products.
Figure 8: Peptide-mapping experiments: The idea of this experiment was to use a cross linker to covalently cross link the PriA and PriB proteins (step 3) and treat the protein:protein complex with trypsin. After trypsin digestion (step 4) the PriA:PriB binding surface which remains intact is isolated and mapped for the amino acids.

Figure 9: Structure of DSP (dithiobis[succinimidy]propionate)
After optimizing the cross linker concentration required for the experiment, the putative, cross linked PriA-PriB complex was identified and the piece of the polyacrylamide gel was isolated. A test to confirm the presence of the crosslinked PriA-PriB in the isolated band of gel was carried out by treating it with the reducing agent DTT and subjecting to analysis by another SDS-PAGE. The second gel showed two different bands at PriA and PriB locations assuring the presence of PriA-PriB complex in the earlier gel.

HPLC was also used in these peptide mapping experiments. SDS-PAGE was unable to show any more visible bands in the gel after trypsin digestion of the cross linked PriA-PriB complex and reduction of the putative PriA-PriB binding surface band. Then, we opted for HPLC which is a more sensitive technique than SDS-PAGE.

Two samples containing PriA and PriB were treated with a cross linker for 30 min. One of these samples was treated with 50 mM DTT (reducing agent) for 2 hrs. Then these two samples were subjected to proteolysis with 0.01 mg/ml trypsin. These samples were analyzed by HPLC -gradient elution in which the concentration of the acetonitrile in the mobile phase was slowly increased from 0.1% to 99.9% ACN. The idea was to observe few differences in the chromatogram of the reduced and the non-reduced samples. Peaks which stand out differently in the non reduced sample could be again reduced with DTT and checked if they are subjected to any change by running one more HPLC run. This could help to identify the PriA-PriB binding surface.
CHAPTER III

RESULTS

III-1 PriB:K34A variant defective for ssDNA binding stimulates the PriA helicase activity:

It is already known that *Escherichia coli* PriB stimulates PriA’s helicase activity and that PriB’s ssDNA binding activity is required for this stimulatory effect\(^\text{10}\). Specifically, if *E. coli* PriB is mutated to reduce its ability to bind ssDNA, the mutated variant no longer stimulates PriA at normal levels\(^\text{10}\). Our lab has found that this is not the case in *Neisseria gonorrhoeae*. The PriB in *N. gonorrhoeae*, despite having relatively weak ssDNA binding activity, stimulates its cognate PriA helicase activity\(^\text{26}\). In order to test the hypothesis that ssDNA binding by PriB is not important for its stimulation of its cognate PriA helicase activity, I examined PriA helicase activity on fork3 (fig 9C) in the presence of PriB:K34A. Amino acid residue K34A in *N. gonorrhoeae* PriB is structurally analogous to amino acid residue R34 of *E. coli* PriB, which is involved in binding ssDNA. PriB:K34A is defective for ssDNA binding, and the apparent dissociation constant for the interaction of PriB:K34A with ssDNA has been estimated at >3 \(\mu\)M compared with 475 nM for wild type *N. gonorrhoeae* PriB. Based on the value of the
apparent dissociation constant for the interaction of PriB:K34A with ssDNA, and assuming that it is a reliable indicator of the affinity of PriB:K34A for DNA in the context of a ternary PriA:PriB:DNA complex, the PriB:K34A variant should not be interacting with DNA to a significant degree under the condition of this DNA unwinding assay. Thus, under the experimental conditions of our helicase assay, we would not expect PriB:K34A to bind DNA to a significant extent.

When I tested the PriA helicase activity on fork 3 in the presence of PriB:K34A, similar levels of DNA unwinding to those seen when wild type is used to stimulate PriA were observed (Figure 9). These experiments show that PriA-catalyzed fork3 unwinding remains essentially the same in the presence of both wild type and K34A variant PriB. It is particularly noteworthy that in E. coli, a PriB variant with severely weakened ssDNA binding activity (the W47,K82A double mutant) fails to stimulate the DNA unwinding activity its cognate PriA to a significant degree. Therefore, my results suggest that ssDNA binding by N. gonorrhoeae PriB does not play a major role in N. gonorrhoeae PriB stimulation of its cognate PriA helicase.
Figure 10: A) Ribbon diagrams of *E. coli* PriB:R34A and *N. gonorrhoeae* PriB:K34A variants. The two monomers of PriB are colored and red and blue and the ssDNA is rendered as a cyan tube. Amino acid residues K34 of *N. gonorrhoeae* and structurally analogous residue R34 of *E. coli* are rendered as sticks. B) Results of the DNA unwinding assays in presence of PriB:Wild type and PriB:K34A. It shows unwinding of 1nM fork3 DNA by 2nM PriA in presence of PriB-wild type and PriB:K34A. It could be observed that degree of unwinding by PriA in presence of PriB-wild type and PriB:K34A are almost similar. Measurements are reported in triplicate and error bars represent one standard deviation of the mean. C) Fork 3 DNA. It is a forked DNA structure with a 40 base pair lagging strand arm.
III-2 Pull-down experiments:

The data from the above DNA unwinding assay suggests strongly that DNA binding by PriB is not required to stimulate PriAs helicase activity in \textit{N. gonorrhoeae}. We hypothesized that stimulation of PriBs ssDNA binding activity was compromised to establish a strong interaction with PriA and this is stimulating the PriAs helicase activity. To explore this further we thought of studying the PriA:PriB interactions and performed pull-down experiments, and peptide mapping experiments for this purpose.

As explained in the methods section the pull-down experiments were designed to identify if amino acids E41 and K34 of PriB are important for its physical interaction with PriA. His-tag PriA would work as a bait protein which is immobilized on NTA-agarose beads through the His-tag. To test PriAs interaction with PriB variants E41A and K34A, they are incubated together, immobilized on the beads, washed to remove any unbound PriB, treated with SDS-sample buffer and run through a polyacrylamide gel. The intensity of the PriB bands on the gel would help to determine the strength of the interaction between the His-PriA and the PriB variants. Unfortunately, the results in these experiments were not reproducible. When more than one gel was run the relative intensities of the pull down bands with respect to the inputs varied. The reasons for not being reproducible can be because of not able to acquire small and consistent volumes while injecting the samples into the lane. This could also be due to loss of sample while washing the NTA-agarose beads. It might also be due to over flow of the solution.
injected into the lanes and lane contamination. Acknowledging the technical limitations of these experiments they were stopped.

<table>
<thead>
<tr>
<th>PriA</th>
<th>Inputs</th>
<th>pull-down products</th>
</tr>
</thead>
<tbody>
<tr>
<td>PriB-WT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PriB-E41A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PriB-K34A</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 11: Pull-down experiment to determine the affinities of PriB:E41A and PriB:K34A towards PriA: The intensities of the bands in the PriB regions in lanes 9 to 11 indicate about the affinities of PriB variants towards PriA.

III-3 Peptide-mapping experiments:

The objective of these experiments was to map the amino-acids at PriA-PriB binding interface. As described in the methods section a covalent cross-linker, DSP, was used in these experiments. The idea was to covalently cross link the PriA and PriB proteins so that their binding surface remains intact after trypsin digestion and could be
isolated, cross-link reversed and mapped. The gel in figure 12 below shows PriA in lane 1, PriB in lane 2, PriA cross linked to itself in lane 3, PriB cross linked to itself in lane 4 and a mixture of PriA and PriB cross linked in lane 5. Lane 3 and lane 5 were compared to identify the cross linked PriA and PriB complex in lane 5. The cross linked PriA PriB complex is indicated in the figure 12 below. This gel shows that a high molecular weight complex can be trapped by the crosslinker when PriA and PriB are mixed.

Figure 12: Identification of cross-linked PriA:PriB complex. PriA and PriB were incubated together with the cross linker and SDS-PAGE was run. The bands in the PriA+PriB+cross linker in lane 5 was compared to that of PriA+cross linker in lane 3 to identify the cross linked PriA and PriB complex.

To make sure that the band identified in the gel above consists of the cross linked PriA and PriB complex, it was excised from the polyacrylamide gel and reduced with DTT to reverse the cross linker. Then, it was run through a secondary gel (Fig. 13) which was stained to visualize the proteins. As shown in figure 13, lane 3, after reversing the
cross linking of the band in lane 5 in the above gel in figure 12, it produced two bands at PriA and PriB molecular weight position in the gel below. This confirmed that band from the above gel consisted of cross linked PriA-PriB complex.

Figure 13: A secondary silver stained gel confirming the presence of cross-linked PriA:PriB complex in the primary gel: The cross linked PriA:PriB complex was confirmed to be present in the gel shown earlier in figure 11 when that portion of gel was treated with dithiothreitol (a reducing agent) to reverse cross linking and was run through this secondary gel. Bands showed up at PriA and PriB regions of this secondary gel, lane 3 confirming the presence of cross linked PriA and PriB complex in gel in figure 11. Silver staining was used to visualize the bands in this secondary gel.

In my experiments I was able to identify and isolate the cross-linked PriA-PriB complex using SDS-PAGE analysis. Next step was to trypsin digest and identify the
PriA-PriB binding interface before reversing the cross-linking and mapping the binding surface. However, I was unable to observe any protein bands in the tertiary gel after trypsin digesting the cross linked PriA-PriB complex. I believe that the amount of sample remaining at this stage of the procedure was below the threshold of detection for SDS-PAGE and silver staining. Therefore we opted to substitute HPLC – High performance liquid chromatography for SDS-PAGE.

As mentioned in the methods section, while using HPLC, two protein solutions containing PriA and PriB were incubated with the cross linker. One of these samples is reduced with DTT and the other was left untreated. Then these two samples are trypsin digested and were run through HPLC for gradient elution and the two chromatograms were compared (fig.14 & fig. 15). The eluate of these two samples was analyzed at two different wavelengths, 215 nm and 280nm. The 215 nm is used to detect the peptide bonds and the 280 nm is for the aromatic aminoacids. We were able to find some differences in the peaks of the chromatograms of these two samples. Later, the fractions of those different looking peaks in the non-reduced sample from 124 µl to 126 µl, 152.5 µl to 154 µl, and 168.0 µl to 169.0 µl were collected, reduced and run again through HPLC to check if they would split in to two distinct peaks indicating presence of a cross linked PriA-PriB binding interface. At this point we observed lot of noise in the chromatograms and were not able to get a desired result. Owing to time constraints these experiments were stopped. Future work has to be performed to trouble shoot the above mentioned problems.
Figure 14: Peptide-mapping experiments-HPLC chromatograms at 215 nm: In HPLC the eluate was analyzed using U.V. spectrophotometer at 215nm(channel A) and 280nm(channel B)figure 14 shows two chromatograms of the reduced and non reduced PriA + PriB cross linked samples respectively at 215nm. The retention volume is in µL.
Figure 15: Peptide-mapping experiments-HPLC chromatograms at 280 nm: In HPLC the eluate was analyzed using U.V. spectrophotometer at 215nm(channel A) and 280nm(channel B). Figure 14 shows two chromatograms of the reduced and non reduced PriA + PriB cross linked samples respectively at 280 nm. The retention volume is in µL.
III-4 Mechanism of the inhibition of DNA replication restart process of *Neisseria gonorrhoeae* by CGS-15943:

After the peptide mapping experiments we then moved on to investigate the inhibition of DNA replication restart pathway in this gonorrheal infection causing pathogen *N. gonorrhoeae*. Our lab started with screening around two thousand compounds from NIH clinical collection library through high through put screening (HTS) to identify potential lead compounds which could work as inhibitors of the DNA replication restart pathway of *N. gonorrhoeae*. CGS-15943 is one of the lead compounds identified in the screening. I worked to study the mechanism by CGS-15943 inhibits the DNA replication restart in *N. gonorrhoeae*. Below I report the results of my experiments with CGS-15943.

III-4-1 PriA is the target of the CGS-15943 inhibition:

Our initial screening assays were based on DNA helicase activity of PriA and both PriA and PriB were used in these assays. So, we wanted to know the prime target of CGS-15943 among PriA and PriB. In order to answer this question we designed an experiment based on the DNA unwinding assay. We examined the DNA unwinding activity of 5 nM PriA in the presence and absence of PriB and CGS-15943. There were two pairs of reaction mixtures in these experiments. The first pair had no PriB and received either CGS-15493 or a DMSO solvent control. The second pair had PriB and received or not received CGS-15493. In this way the percent inhibition by CGS-15943 in presence and absence of PriB was studied. As shown in figure 16 below we found that
CGS-15943 exhibited similar levels of inhibition in the presence and absence of PriB. This indicates that PriA is likely the main target of inhibition by CGS-15943.

![Graph showing inhibition of PriA](image)

**Figure 16: PriA is the target of CGS-15943 inhibition:** Unwinding of fork3 DNA by 5 nM PriA in the presence and absence of 50 nM PriB and 114 µm CGS-15943. Measurements are reported in triplicate and error bars represent one standard deviation of the mean.

### III-4-2 CGS-15943 affects PriA-catalyzed ATP hydrolysis:

PriAs ATPase activity applies energy gained from ATP hydrolysis for unwinding the duplex DNA. Therefore, we wanted to determine if the effects of CGS-15943 are due to perturbations that affect how PriA binds and hydrolyzes ATP. I used a coupled spectrophotometric assay to measure steady state rates of ATP hydrolysis catalyzed by PriA in the presence and absence of CGS-15943. In this reaction ATP hydrolysis is coupled to oxidation of NADH and absorbance of NADH is monitored at 340 nM. These ATPase assays were carried out by titrating with DNA and ATP. Both
DNA and ATP are substrates of the enzymes PriA and PriB and are necessary to elicit any ATP hydrolysis activity in PriA.

**III-4-2-a ATP hydrolysis experiments – DNA titrations:**

In this experiment the rate of ATP hydrolysis is measured while DNA is titrated. Since PriA has two substrates DNA and ATP, ATP is kept at maximum concentration while DNA is titrated. Poly dT₂₈ was used in these experiments. The steady state rates of ATP hydrolysis by 10 nM PriA were calculated in presence of 100 nM PriB and in the presence and absence of 57 µM CGS-15943 and while titrating with dT₂₈. The concentrations of PriA and PriB are similar to those used in ATP hydrolysis experiments performed previously in the lab. The 57 µM of CGS-15943 is half of its IC₅₀ (114 µM) and using this concentration allowed us to calculate the kinetic parameters of the ATP hydrolysis. When IC₅₀ was used in the experiments, the inhibition was too strong making it difficult to calculate the Kₘ and Vₘₐₓ values so we decided to work with 57 µM of CGS-15943. Different concentrations of dT₂₈ ranging from 0 nM to 100 nM were used and the kinetic parameters were calculated. The Vₘₐₓ decreased from 223.4 nm/sec to 140.5 nm/sec in presence of CGS-15943. The Kₘ value in absence of the inhibitor was 7 ± 1 nM and in presence of the inhibitor it increased to 42 ± 26 nM. This effect of CGS-15943 in the presence and absence of the inhibitor indicated a mixed mode of inhibition.

A mixed inhibitor binds at a site distinct from the substrate active site and it could bind either to PriA-PriB, enzyme complex or PriA-PriB:DNA:ATP, enzyme-substrate complex. The rate equation describing mixed inhibition is
\[ V_o = \frac{V_{\text{max}}[S]}{\alpha K_m + \alpha'[S]} \]

Where,

\[ \alpha = 1 + \frac{[I]}{K_I} \quad ; \quad K_I = \frac{[E][I]}{[EI]} \quad ; \quad \alpha = 1 + \frac{[I]}{K'_I} \quad ; \quad K'_I = \frac{[ES][I]}{[ESI]} \]

And the below table shows the effect of a mixed inhibitor on apparent $V_{\text{max}}$ and apparent $K_m$ values.

<table>
<thead>
<tr>
<th></th>
<th>Apparent $V_{\text{max}}$</th>
<th>Apparent $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Inhibitor</td>
<td>$V_{\text{max}}/\alpha$</td>
<td>$\alpha K_m/\alpha'$</td>
</tr>
</tbody>
</table>

**Table 1: Effect of a mixed inhibitor on apparent $V_{\text{max}}$ and $K_m$ values.**

Following the formulae mentioned above, the kinetic parameters and dissociation constants were calculated and are showed in table 2 and 3. The $V_{\text{max}}$ value decreases and $K_m$ value increases with the addition of CGS-15943, indicating that it is a mixed type of inhibitor with respect to DNA. $K_I$ being less than $K'_I$ suggests that the inhibitor has strong affinity towards the enzyme than the enzyme-substrate complex.
Figure 17: PriA’s ATPase activity is affected by CGS-15943 – DNA titrations: CGS-15943 exhibits a mixed mode of inhibition with respect to DNA: DNA dependent ATP hydolysis rates catalyzed by 10 nM PriA in presence of 100 nM PriB and in absence and presence of 57 µM of CGS-15943. The DNA used was poly dT$_{28}$. The measurements are reported in triplicate and the error bars represent one standard deviation of the mean.

<table>
<thead>
<tr>
<th></th>
<th>-CGS 15943</th>
<th>+ CGS 15943</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$, DNA, nM/sec</td>
<td>223.4 ± 5.4</td>
<td>140.5 ± 35.33</td>
</tr>
<tr>
<td>$K_m$, DNA, nM</td>
<td>7.1 ± 1</td>
<td>42.4 ± 25.6</td>
</tr>
<tr>
<td>$k_{cat}$, S$^{-1}$</td>
<td>22.3 ± 1</td>
<td>14.1 ± 3.5</td>
</tr>
</tbody>
</table>

Table 2: ATPase assays –DNA titrations- $V_{max}$, $K_m$, $k_{cat}$ values: Kinetic parameters for PriA ATPase activity in the presence and absence of CGS-15943
<table>
<thead>
<tr>
<th></th>
<th>$K_1$, $\mu$M</th>
<th>$K_1'$, $\mu$m</th>
<th>$\alpha$</th>
<th>$\alpha'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA titrations</td>
<td>8.6 ± 4.2</td>
<td>124.7 ± 86.7</td>
<td>9.0 ± 4</td>
<td>2 ± 0.5</td>
</tr>
</tbody>
</table>

Table 3: ATPase assays –DNA titrations- $K_1, K_1'$, $\alpha$, $\alpha'$ values: Dissociation constants, $\alpha$, and $\alpha'$ values for inhibitor interaction with the enzyme and enzyme substrate complex.

III-4-2-b ATP hydrolysis experiments –ATP titrations:

ATP is another substrate in the ATP hydrolysis reaction catalyzed by PriA, therefore, I also examined the steady state kinetics of ATP hydrolysis by PriA while using different concentrations of ATP in the presence and absence of 57 $\mu$M CGS-15943. The concentrations of PriA and PriB were similar to DNA titration experiments. 100 nM of dT$_{28}$ was used and different concentrations of ATP ranging from 0 $\mu$M to 800 $\mu$M were used. The Vmax decreased from 224.1 nm/sec to 143.9 nm/sec in the presence of CGS-15943. The $K_m$ value in the absence of inhibitor was 79 ± 10 $\mu$M and it increased to 117 ± 27 $\mu$M in the presence of inhibitor. This effect of CGS-15943 on the kinetic parameters suggests a mixed mode of inhibition with respect to ATP.
**Figure 18**: PriA’s ATPase activity is affected by CGS-15943 – ATP titrations: CGS-15493 exhibits a mixed mode of inhibition with respect to ATP: ATP dependent ATP hydrolisys rates catalyzed by 10 nM PriA in presence of 100 nM PriB and in absence and presence of 57 µM of CGS-15943. The DNA used was poly dT<sub>28</sub>. The measurements are reported in triplicate and the error bars represent one standard deviation of the mean.

<table>
<thead>
<tr>
<th></th>
<th>-CGS 15943</th>
<th>+ CGS 15943</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max, ATP, } \mu M/\text{sec}} )</td>
<td>224.1 ± 4.4</td>
<td>143.9 ± 2.2</td>
</tr>
<tr>
<td>( K_{\text{m, ATP, } \mu M} )</td>
<td>79.3 ± 9.9</td>
<td>116.6 ± 27.0</td>
</tr>
<tr>
<td>( K_{\text{cat}, S^{-1}} )</td>
<td>22.4 ± 1</td>
<td>14.3 ± 1.0</td>
</tr>
</tbody>
</table>

**Table 4**: ATPase assays –ATP titrations - \( V_{\text{max}}, K_{\text{m}}, k_{\text{cat}} \) values: Kinetic parameters for PriAs ATPase activity in the presence and absence of CGS-15943.
<table>
<thead>
<tr>
<th>ATP titrations</th>
<th>$K_I, \mu M$</th>
<th>$K'_I, \mu M$</th>
<th>$\alpha$</th>
<th>$\alpha'$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51.6 ± 23.6</td>
<td>102.4 ± 6.4</td>
<td>2.3 ± 0.8</td>
<td>1.6 ± 0.03</td>
</tr>
</tbody>
</table>

Table 5: ATPase assays – ATP titrations- $K_I, K'_I, \alpha, \alpha'$ values: Dissociation constants, $\alpha$, and $\alpha'$ values for inhibitor interaction with the enzyme and enzyme substrate complex.
CHAPTER IV
CONCLUSIONS

In *Escherichia coli* PriB's ssDNA binding is important for its stimulatory effect on PriA helicase activity. Therefore, it was surprising to find that in *Neisseria gonorrhoeae* in spite of its weak affinity towards ssDNA, PriB still stimulates PriA helicase significantly. I have used a single point alanine mutant of PriB:K34A to test the hypothesis that DNA binding by PriB is not important for PriB stimulation of PriA helicase activity in *N. gonorrhoeae*. K34 amino acid residue in *Neisseria gonorrhoeae* PriB is structurally analogous to amino acid residue R34 in *E. coli* which is important for ssDNA binding activity, and *N. gonorrhoeae* PriB:K34A has been shown to have a dissociation constant greater than 3 µM with respect to ssDNA. This indicates that PriB-K34A has a very weak ssDNA binding activity. Hence I used PriB:K34A to test its effect on PriAs helicase activity and the results were similar to that of wild type PriB. Thus corroborates the finding that in *Neisseria gonorrhoeae* PriB ssDNA binding activity is not required for its stimulation of PriA helicase activity. My mapping experiments were able to isolate the cross-linked PriA-PriB complex and future work is required in this regard to isolate the binding interface and map the amino acids present on it.
I also worked with CGS-15943, an inhibitor of the DNA replication restart pathway in *Neisseria gonorrhoeae* and studied mechanistic features of its inhibition. It was one of the lead compounds identified in our initial screening of compounds in the NIH clinical collection library. My work showed that PriA not PriB is the main target of CGS-15943. Through my steady state kinetic experiments based on ATP hydrolysis function of PriA, I was able to conclude that CGS-15943 follows a mixed mode of inhibition with respect to ATP and DNA.

Future work is required to study the other lead compounds and identify a potential candidate to use it to target the DNA replication restart mechanism in this gonorrheal infection causing pathogen.
REFERENCES


24) Thermo-scientific, Product information - DSP (dithiobis[succinimidylpropionate])


