FUNCTIONAL CONSEQUENCES OF PHYSICAL INTERACTIONS BETWEEN PRIA AND PRIB IN DNA REPLICATION RESTART PATHWAYS IN *NEISSERIA GONORRHOEAE*

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ABSTRACT

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DNA replication restart pathways enable bacterial cells to reinitiate DNA replication when replication has been disrupted due to encounters with DNA damage, thereby allowing complete and faithful duplication of the cell’s genetic information. Neisseria gonorrhoeae is a bacterium that is highly adapted to survive oxidative damage to its DNA incurred by attack from immune cells in infected individuals, suggesting that DNA replication restart pathways might play a critical role in N. gonorrhoeae pathogenicity. The bacterial helicase, PriA, is a key primosome protein that plays essential roles in DNA replication restart pathways. However, little is known of the mechanism by which PriA performs these roles in N. gonorrhoeae. I performed equilibrium DNA binding assays and DNA unwinding assays to provide insight into the mechanisms by which PriA functions in DNA replication restart pathways. I report that DNA binding by PriA is strongly dependent on the structure of the DNA. DNA substrates that resemble a DNA replication fork with a three-way branch are bound with higher affinity than partial duplex structures or single-stranded DNA. PriA-catalyzed DNA unwinding is also DNA structure-specific, and PriA-catalyzed unwinding decreases upon increasing the length of the duplex DNA, indicating that PriA is a low-processivity helicase. Another primosome protein, PriB, strongly stimulates the helicase activity of PriA, and this activity might facilitate reloading of the replication machinery.
by PriA at repaired replication forks. Stimulation of PriA by PriB appears to occur through a mechanism that is distinct from that used by the well-studied E. coli primosome proteins.
Dedicated to my parents
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CHAPTER I

INTRODUCTION

A cell’s genetic information is stored in DNA molecules. Replication of DNA must be highly accurate in order to maintain the integrity and stability of the cell’s genetic information. In *E. coli*, a large multi-enzyme complex called the replisome is responsible for duplicating the bacterial chromosome; it contains a single DNA polymerase as well as 20 or more functional enzymes and proteins. However, DNA replication replisome can be disrupted and stalled due to encounters with DNA damage resulting from both external effects including ionizing radiation and chemical treatment, and internal spontaneous processes, such as deamination and depurination. Therefore, the cells must use replication restart pathways to reinitiate DNA replication in order to maintain integrity and fidelity of the cell’s genetic information. Thus, the presence of such pathways appears to be fundamentally important for bacterial survival.

In *E. coli*, one of the several major pathways to activate stalled replication forks is DnaA catalyzed origin-dependent initiation of DNA replication (Figure 1). In *E. coli* cells, origin-dependent initiation of DNA replication is a carefully regulated sequence specific event, which is catalyzed by the initiator protein, DnaA. DnaA protein recognizes and binds to a replication origin site and recruits the replicative helicase, DnaB, and the helicase loader protein, DnaC. The replicative helicase unwinds the parental duplex DNA to facilitate replication of the template DNA strands (Figure 1).
Origin-independent initiation of DNA replication (known as DNA replication restart) requires a distinct cellular machinery to reload the replication machinery (replisome) at a repaired DNA replication fork. This replication process is initiated by an assembly of the primosome proteins, which collectively include PriA, PriB, PriC, DnaT, DnaB, DnaC, DnaG, and Rep proteins. These proteins operate through at least two distinct pathways, one that requires PriA, PriB, DnaT, DnaB, DnaC, and DnaG, and another that requires Rep, PriC, DnaB, DnaC, and DnaG.

Figure 1. Origin-dependent initiation of DNA replication. Initiator protein DnaA recognizes and binds to the origin, ori, and starts unwinding duplex DNA. The helicase DnaB protein is then loaded by the DnaC protein onto the unwound DNA strand, resulting in two replication forks, followed by DNA synthesis and this produces two duplicate chromosomes.

Figure 2. Origin-independent initiation of DNA replication (DNA replication restart). PriA recognizes and binds to the primosome assembly site, leading to the formation of a PriA-PriB-DNA complex. DnaT is then recruited into this complex, resulting in formation of PriA-PriB-DnaT-DNA complex, followed by recruitment of DnaB onto the lagging strand arm, where the DNA replication resumes.
Primosome protein A (PriA), known as 3’ to 5’ DNA helicase, serves to initiate the assembly of the primosome proteins. It binds to two types of DNA structures with high affinity, either a 3’-single stranded extensions from duplex DNAs or D-loop DNA, usually a bent DNA at three-strand junctions. PriB then joins in the PriA-DNA complex and is suggested to stabilize this complex and to stimulate PriA’s helicase activity via an interaction with single-stranded DNA. Association of DnaT on the PriA-PriB-DNA complex leads to the formation of PriA-PriB-DnaT-DNA complex. The function of DnaT has been explored recently, it has been proposed that DnaT physically interacts with PriB’s ssDNA binding site, thus leaving no access for ssDNA to bind to PriB. Dissociation of ssDNA from PriB could allow reloading of the DnaB/DnaC complex on the lagging strand template, where DnaG and DNA polymerase III holoenzyme are recruited to resume DNA replication. The process of loading DnaB requires ATP hydrolysis.

Reactivation of repaired DNA replication forks is essential for complete duplication of bacterial genomes. Given the broad conservation of PriA genes among sequenced prokaryotic genomes, it is likely that the general importance of DNA replication restart pathways extends throughout much of the bacterial world, although relatively little is known of the mechanistic underpinnings and biological importance of DNA replication restart in other bacterial species. Neisseria gonorrhoeae provides a prime example of how DNA replication restart pathways in some bacterial species might differ from those of the well-studied E. coli model organism. N. gonorrhoeae is a gram-negative bacterium which is responsible for the sexually transmitted infection gonorrhoeae. N. gonorrhoeae is highly adapted to survive oxidative damage to its genome incurred by neutrophil attack in infected individuals, suggesting that DNA replication restart pathways might play an expanded and essential role in N. gonorrhoeae pathogenicity.

However, N. gonorrhoeae does not encode homologues of all of the well-studied E. coli DNA replication restart primosome proteins. For example, N. gonorrhoeae lacks a clear homologue of dnaT in its genome, indicating that the N. gonorrhoeae PriA-PriB pathway might...
significantly differ from the *E. coli* PriA-PriB-DnaT pathway. In addition, physical interactions between primosome components in the PriA-PriB pathway show variation in their individual binary affinities: the physical interaction between PriA and PriB is weak in *E. coli*, but relatively strong in *N. gonorrhoeae*, and the physical interactions between PriB and ssDNA is strong in *E. coli*, but relatively weak in *N. gonorrhoeae*. Thus, the affinities of binary interactions between primosome components are reversed between the two species. This suggests that mechanistic details of DNA replication restart, its regulation, and the extent to which cells rely on it for growth and survival probably vary between *E. coli* and *N. gonorrhoeae*.

Since the ssDNA-binding activity of PriB is important for PriB-stimulation of PriA’s helicase activity in *E. coli*, there might be significant functional consequences for the variation in affinities of physical interactions within the *N. gonorrhoeae* PriA-PriB primosome. In my research, I investigated the functional consequences of the affinity reversal phenomenon by examining the helicase activity of *N. gonorrhoeae* PriA, and I determined how PriA-catalyzed ATP hydrolysis and DNA unwinding are affected by *N. gonorrhoeae* PriB.
CHAPTER II

METHODS

II-1. Purification of *N.gonorrhoeae* PriA

*N.gonorrhoeae* PriA was purified from BL21(DE3) *E. coli* harboring the pET28b:*N.gonorrhoeae*-PriA plasmid. Cells were grown in Luria-Bertani(LB) medium containing 50 mg/L at 37 °C until an OD$_{600}$ was reached. Expression of PriA was induced with 0.5 mM IPTG for 4 hours and cells were harvested by centrifugation at 5,500 × g at 4 °C for 25 min and stored at -80 °C. Cells were lysed in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethanesulphonylfluoride (PMSF) by Sonication on ice, using 5 × 30 sec pulsed bursts (pulse=1 sec on, 1 sec off) at 70% power,. The lysate was clarified by centrifugation at 40,000 × g for 20 min at 4 °C. His-tagged PriA was bound to nickel-Nitrilotriacetic Acid (NTA) agarose (Qiagen) and eluated in 10 mM HEPES pH 7, 10% glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol, 250 mM imidazole. The nickel-NTA agarose eluate was dialyzed against 10 mM HEPES pH 7, 10% glycerol, 100 mM NaCl and 1 mM β-mercaptoethanol and incubated with thrombin 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, was depleted by incubating the thrombin-cleaved PriA solution with nickel-NTA agarose. Thrombin-cleaved PriB was concentrated and incubated in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 6, and the protein solution was loaded onto a HiPrep™ SPFF 16/10 ion-exchange column pre-equilibrated with 10 mM MES pH 6, 10% (v/v) glycerol, 100 mM NaCl, 1mM β-mercaptoethanol. The SPFF column was resolved at 0.5 mL/min using a ten column volume linear gradient of 0%-100% Buffer B, which contains 10 mM MES pH 6, 10% (v/v) glycerol, 1 M NaCl, 1mM β-mercaptoethanol. Appropriate fractions containing PriA were collected and concentrated overnight by centrifugation in a CentriPrep YM-10 concentrator at 2,643 × g at 4°C. The concentrated protein solution was aliquoted and stored at -80 °C.

**II-2. Purification of *N. gonorrhoeae* PriB**

*N. gonorrhoeae* PriB was purified as described previously\(^1\), the detailed protocol is as follows. *N. gonorrhoeae* PriB protein was purified from BL21(DE3) *E. coli* harboring the pET28b:*N.gon*-priB plasmid. Cells were grown in LB medium containing 50 mg/L kanamycin at 37°C until an OD\(_{600}\) of 0.6 was reached. Expression of PriB was induced with 0.5 mM IPTG for 4 h and cells were harvested by centrifugation at 5000×g. Cells were lysed in 10mM Tris–HCl pH 8.5, 10% (v/v) glycerol, 0.1M NaCl, 10mM imidazole, 1mM β-mercaptoethanol, 1mM PMSF by sonication on ice. The lysate was clarified by centrifugation at 40 000×g. His-tagged PriB was bound to nickel-NTA agarose (Qiagen) and eluted in 10mM Tris–HCl pH 8.5, 10% (v/v) glycerol, 0.1M NaCl, 250mM imidazole, 1mM β-mercaptoethanol. The nickel-NTA agarose eluate was dialyzed against 10mM Tris–HCl pH 8.5, 10% (v/v) glycerol, 0.1M NaCl, 1mM β-mercaptoethanol and incubated with thrombin 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, was
depleted by incubating the thrombin-cleaved PriB solution with nickel-NTA agarose. Thrombin-cleaved PriB was concentrated and purified through a HiPrep HR 16/10 Sephacryl S-100 size exclusion column (GE Healthcare) in 10mM Tris–HCl pH 8.5, 10% (v/v) glycerol, 0.5M NaCl, 1mM β-mercaptoethanol. PriB fractions were pooled, concentrated and stored at –80°C.

II-3 Construction of DNA substrates

DNA substrates shown in Table 1 were constructed by annealing complementary single-stranded DNA (ssDNA) oligonucleotides. Oligonucleotides were suspended at a 2:1 molar ratio of non-labeled DNA with fluorescein-labeled DNA in Sodium-Tris-EDTA (STE) Buffer containing 10 mM Tris·HCl pH 8, 50 mM NaCl. After incubation at 95 °C for 5 min, the solution was slowly cooled to 70 °C and then incubated for one hour. Following the 1 hour incubation, the solution was slowly cooled to 25 °C, and was stored at 4 °C. The DNA was purified through a 6% polyacrylamide gel in the dark in 100 mM Tris borate pH 8.3, 2 mM EDTA as the electrophoresis buffer. The gel slice containing the fluorescent DNA was excised from the gel, and the DNAs were electroeluted in the same electrophoresis buffer, and dialyzed against 10 mM Tris·HCl pH 8.0 and 5 mM MgCl₂. After dialysis, the DNA substrate was aliquoted and stored at -20 °C.
Table 1 DNA substrates

<table>
<thead>
<tr>
<th>ssDNA</th>
<th>oML228 (18)</th>
<th>oML228: 5’-AAG CAC AAT TAC CCA CGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA</td>
<td>oML230 (36)</td>
<td>oML230: 5’-GCC GTG ATC ACC AAT GCA GAT TGA CGA ACC TTT GCC</td>
</tr>
<tr>
<td></td>
<td>oML233 (36)</td>
<td>oML233: 5’-GGG AAA GGT TCG TCA ATC TGC ATT GGT GAT CAC GGC</td>
</tr>
<tr>
<td>3’</td>
<td>oML276 (50)</td>
<td>oML276: 5’-AAC GTC ATA GAC GAT TAC ATT GCT ACA TGG AGC TGT CTA GAG GAT CCG AC</td>
</tr>
<tr>
<td>Overhang</td>
<td>oML277 (25)</td>
<td>oML277: 5’-TAG CAA TGT AAT CGT CTA TGA CGT T</td>
</tr>
<tr>
<td>Fork 1</td>
<td>oML211 (50)</td>
<td>oML211: 5’-GTC GGA TCC TCT AGA CAG CTC CAT GAT CAC TGG CAC TGG TAG AAT TCG GC</td>
</tr>
<tr>
<td></td>
<td>oML212 (25)</td>
<td>oML212: 5’-GCC GAA TTC TAC CAG TGC CAG TGA T</td>
</tr>
<tr>
<td></td>
<td>oML287 (15)</td>
<td>oML287: 5’-ACG ATT ACA TTG CTA CAT GGA GCT GTC TAG AGG ATC CGA C</td>
</tr>
<tr>
<td></td>
<td>oML288 (25)</td>
<td>oML288: 5’-TAG CAA TGT AAT CGT</td>
</tr>
<tr>
<td>Fork 2</td>
<td>oML211 (50)</td>
<td>oML213: 5’-ACG TAG GCC GGA AAC AAC GTC ATA GAC GAT TAC ATT GCT ACA TGG AGC TGT CTA GAG GAT CCG AC</td>
</tr>
<tr>
<td></td>
<td>oML212 (25)</td>
<td>oML278: 5’-TAG CAA TGT AAT CGT CTA TGA CGT TGT TTC CGG CCT ACG T</td>
</tr>
<tr>
<td>Fork 3</td>
<td>oML211 (65)</td>
<td>oML213: 5’-ACG TAG GCC GGA AAC AAC GTC ATA GAC GAT TAC ATT GCT ACA TGG AGC TGT CTA GAG GAT CCG AC</td>
</tr>
<tr>
<td></td>
<td>oML212 (40)</td>
<td>oML278: 5’-TAG CAA TGT AAT CGT CTA TGA CGT TGT TTC CGG CCT ACG T</td>
</tr>
</tbody>
</table>

The 3’ end of each DNA oligonucleotide is denoted by a half arrow. The asterisk denotes the position of the fluorescein label. Numbers in parentheses denote the number of bases in the oligonucleotide.
II-4. Equilibrium DNA binding assays

DNA substrates were fluorescently labeled on the lagging strand arm, which enabled me to measure the increase in fluorescence polarization of the DNA:PriA complex relative to the unbound DNA due to PriA binding. The fluorescence anisotropy of these DNAs was measured in the presence of serial dilutions of PriA, which was prepared with different concentrations ranging from 0.001 nM to 3000 nM in 20 mM Tris·HCl pH 8, 10% glycerol, 1 mM 1mM β-mercaptoethanol, 50 mM NaCl and 0.1 g/L bovine serum albumin (BSA). Dilutions of PriA proteins were incubated for 5 min at room temperature with 1 nM DNA substrates in a total volume of 100 μL.

![Diagram](image.png)

**Figure 3. FP-based DNA binding Assays.** The anisotropy of fluorescein-labeled DNA alone is very low, while when it binds a protein, the anisotropy would be increased due to increment in the size of the protein:DNA complex.

The fluorescence anisotropy was measured in a Beacon™ 2000 variable temperature fluorescence polarization system at 25 °C and plotted versus the concentration of PriA. The binding fraction was calculated with this equation,
BindingFraction \(= (m_x - m_0)/(m_A - m_0)\) \hspace{1cm} \text{Equation 1.1}

where \(m_x\) represents the experimental anisotropy for each measurement, \(m_0\) represents the anisotropy when DNA binds no PriA, and \(m_A\) represents the anisotropy when there is the highest concentration of PriA. Apparent dissociation constants \(K_{d,app}\) were referred to as the concentration of the substrates when half of the ligand-binding sites were occupied.

\section*{II-5. Helicase assays}

DNA helicases are proteins that use the energy of ATP hydrolysis to processively unwind duplex DNA. A helicase assay is used for the detection of PriA’s helicase activity. This assay involves the unwinding of a forked DNA substrate by PriA on the lagging strand arm, which was labeled by fluorescein. The unwound ssDNA and the intact DNA fork differ greatly in size, resulting in a sharp decrease in fluorescence polarization value, which in turn, reveals PriA’s helicase activity (Figure 4).

\textbf{Figure 4. Helicase Assays.} A) Duplex DNA was unwound by helicase PriA using energy released by ATP hydrolysis. B) As the unwinding reaction proceeds, the intact DNA substrates have been unwound to smaller molecules, resulting in a sharp decrease in fluorescence polarization value.
To measure DNA unwinding, a reaction mixture containing different concentrations of PriA ranging from 0-15 nM and approximately 1 nM DNA substrates was diluted into Helicase Assay Buffer which contains 20 mM Tris-HCl pH 8, 50 mM NaCl, 3 mM MgCl2 and 1 mM 2-mercaptoethanol. 1 mM ATP was added to the mixtures to start the duplex DNA unwinding reaction by providing the chemical energy needed for PriA to unwind the duplex DNA substrates. Reaction mixtures solutions were incubated at 37 °C for 10 min before the reaction is terminated by adding 10% sodium dodecyl sulfate (SDS) (to denature PriA). The fluorescence anisotropy values of the reaction were measured at 25 °C in a BeaconTM 2000 variable temperature fluorescence polarization system. After the measurements, the reaction solution was incubated in 95 °C for 20 sec to denature all duplex DNA substrates and then immediately shifted to ice to cool down for 10 sec until the anisotropy of all reactions is constant which demonstrates that all the DNAs are completely unwound. Fraction unwound was plot versus the concentration of PriA, where the fraction unwound was derived by this equation:

\[
\text{unwound} \% = 1 - \frac{m_x - m_{ss}}{m_0 - m_{ss}}
\]

Equation 1.2

where mx represents the experimental anisotropy for each measurement, mss represents the anisotropy when DNA was heated to 95 °C and then suddenly cooled on ice (completely unwound) and m0 represents the anisotropy in the absence of PriA in the measured reaction solution (fully intact DNA duplex substrates). For some experiments, PriB was included in the reaction mixture at concentrations ranging from 0 to 50 nM.

II-6. ATP hydrolysis assays

A spectrophotometric assay was used to measure the steady state rate of ATP hydrolysis catalyzed by PriA. The ATPase assay included an ATP regeneration system that converts ADP-ATP in a reaction that is coupled to the conversion of NADH to NAD\(^+\). This coupled reaction
can be detected spectrophotometrically by measuring the decrease of absorbance at 340 nm due to NADH oxidation.

Based upon the Beer-Lambert Law,

\[ A = \varepsilon bc \]

Equation 1.3

whereas the molar extinction coefficient of NADH is 6,220 M\(^{-1}\) cm\(^{-1}\), the initial rate of ATP hydrolysis was calculated by converting steady-state \( \Delta A_{340 \text{ nm}}/\Delta t \) rates to \( \Delta [\text{ATP}] / \Delta t \) from the linear region in the time courses. To compare DNA-dependent ATP hydrolysis profiles, purified PriA at 10 nM was mixed with certain concentrations of Fork 3 ranging from 0-200 nM in 20 mM HEPES (pH 8.0), 50 mM NaCl, 7 mM \( \beta \)-mercaptoethanol, 0.1 mg/ml BSA, and 1 mM ATP in the presence and absence of 100 nM PriB.

To compare ATP-dependent ATP hydrolysis profiles, purified PriA at 10 nM was mixed with 0-1000 µM ATP in the absence and presence of 100 nM PriB and 100 µL Fork 3, the mixture solution also contained 20 mM HEPES (pH 8.0), 50 mM NaCl, 7 mM \( \beta \)-mercaptoethanol, 0.1 mg/ml BSA. For the ATP regeneration system, 2 mM phosphoenolpyruvate...
(PEP), 0.1 mM NADH, 7 units of pyruvate kinase, and 10 units of lactate dehydrogenase (LDH) were also included in each reaction mixture. Reactions were incubated at 25°C and the absorbance at 340 nm was measured over a period of 800±200s. The kinetic parameters, $K_m$ and $k_{cat}$ values, were derived by fitting ATPase activity resulting from ATP titrations to the Michaelis-Menten equation,

\[
rate = \frac{(V_{max} \times [S])}{(K_m + [S])}
\]  

Equation 1.4

The $k_{cat}$ values were calculated by dividing $V_{max}$ values by the concentration of PriA in the reaction. $K_{DNA}$ values are the determined by calculating the concentration of DNA at which each enzyme was half-maximally stimulated.
III-1. Fork structures are the most relevant DNA substrates for *N. gonorrhoeae* PriA binding activity and unwinding activity

In order to test which DNA substrate is the most biologically relevant for PriA helicase, fluorescence polarization spectroscopy was used to perform equilibrium DNA-binding assays and helicase assays to examine PriA’s DNA binding and unwinding activities on various types of DNA substrates. The DNA substrates that I analyzed include 1) a fork structure that has a three-way branch which is fully duplexed (analogous to what is thought to exist at a stalled replication fork in living bacterial cells), 2) 3’ overhang, 3) a segment of ssDNA, and 4) a segment of duplex DNA (Figure 6).

![Figure 6. Structures of DNA substrates investigated in the binding and helicase experiment.](image)

DNA substrates and the single stranded DNA oligonucleotides used to construct the substrates are shown above. Numbers near each arm of the DNA substrates indicate the number of bases. The green circle represents the fluorescein tag that was covalently linked to one of the ssDNA oligonucleotides in each structure.
III-1-1. Theory of fluorescence polarization

Fluorescence polarization (FP) spectroscopy is commonly used to measure macromolecular interactions between protein and its binding DNA substrates and interactions between protein and nucleic acids by monitoring changes in molecular volume. The FP theory was first introduced by Perrin in 1926\(^\text{17}\), based on the observation that when fluorescent molecules are excited with plane-polarized light, the more rapidly the molecules tumble, the more depolarized the emitted light is during its fluorescence lifetime\(^\text{18}\).

The Polarization value of a molecule is related to the molecule’s rotational relaxation time, as shown in equation 3.1:

\[
polarization \propto relaxation \text{ time} = \frac{3\eta V}{RT} \tag{Equation 3.1}
\]

Where \(\eta\) is viscosity, \(T\) is absolute temperature, \(V\) is molecular volume and \(R\) is the gas constant. Accordingly, the polarization would be directly proportional to the molecular volume if viscosity of the solution and absolute temperature are fixed. Thus, association results in a slower rotation of the molecule, which in turn, leads to an increase in the polarization.

Two methods are commonly used for measurement of fluorescence anisotropy\(^\text{17}\): the L-format method, in which either the intensity of vertically or horizontally polarized light is measured, and the T-format method, in which both of these two are measured simultaneously. These two methods differ in efficiency on detecting various polarized components of fluorescent emission. I used the L-format method in my study which is the most frequently used method (Figure 7).

In the L-format, or single channel method, the emission is detected through a monochromator which has different efficiencies in transmitting vertically polarized and horizontally polarized
light. This depends on whether the excitation polarizer is placed horizontally or vertically. When the emission polarizer is parallel to the direction of the polarized excitation the observed intensity is called $I_\parallel$. Similarly, when the polarizer is oriented perpendicular to the excitation the intensity is called $I_\perp$. These intensities provide the definition of anisotropy and polarization:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$  \hspace{1cm} \text{Equation 3.2}

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$  \hspace{1cm} \text{Equation 3.3}

Recently, the term “fluorescence anisotropy” is used in the literature. For consistency, I used this term in my study. The mathematical relationship between anisotropy and polarization is illustrated in equation 2.4, where $r$ is anisotropy and $P$ is polarization.

$$T = \frac{2xP}{3-P}$$  \hspace{1cm} \text{Equation 3.4}

In my FP-based experiments the DNA substrates are fluorescently labeled, and the degree of the difference in fluorescence anisotropy between the DNA substrates and PriA:DNA complexes allows me to determine the binding affinity between DNA and PriA.
III-1-2. *N. gonorrhoeae* PriA has the greatest binding affinity with a 3 way branched DNA structure

To determine which DNA substrate is bound by PriA with the highest affinity, I incubated each DNA with serial dilutions of PriA protein and measured the fluorescence anisotropy of this sample. If PriA binds to the DNA, I should see a PriA dependent increase in the fluorescence anisotropy of the DNA due to the larger size of the complex relative to the unbound DNA. Both the data and graph in Figure 8 suggests that *N. gonorrhoeae* PriA binds all these three DNA substrates that I have investigated, as expected from studies using *E. coli* PriA.\(^8,20,21,22\). Equilibrium DNA binding constants are calculated and shown in Table 2. The lower the apparent dissociation constant \(K_d\) value is, the higher the binding affinity is between PriA and DNA substrates.

Among these various DNA substrates, a forked DNA structure is found to be recognized by PriA with the highest binding affinity with an apparent dissociation constant of 134±22 nM. This result is consistent with the observations in *E. coli*, in which PriA binds preferentially to a

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**Figure 7. Diagram of L-Format fluorescence polarization\(^19\).**
three way branched DNA structure. However, there is an alternative possibility that the observed difference in $K_d$ values results from the different number of nucleotides in each DNA substrate. In order to reject this possibility, I performed equilibrium binding experiment on a fully duplex DNA substrate, Duplex 1, which has the same number of nucleotides with the other DNA substrates on lagging strand, but a lower overall number of nucleotides than the fully duplex fork or the partial duplex fork. Duplex 1 has a very high binding affinity with $N. gonorrhoeae$ PriA with an observed $K_d$ of 35±5 nM. This suggests that the structure of the DNA substrate is likely more important than the number of nucleotides among the DNA substrates tested.

![Figure 8. DNA binding activity of $N. gonorrhoeae$ PriA. PriA was serially diluted and incubated with 1nM fluorescein-labeled ssDNA(squares), 3’ Overhang(circles), or Fork 2(triangles). Measurements are reported in triplicate and error bars represent one standard deviation of the mean.](image)

<table>
<thead>
<tr>
<th>DNA substrates</th>
<th>Apparent $K_d$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ss-DNA</td>
<td>306±43</td>
</tr>
<tr>
<td>3’-OH</td>
<td>234±62</td>
</tr>
<tr>
<td>Fork 2</td>
<td>134±22</td>
</tr>
<tr>
<td>Duplex 1</td>
<td>35±5</td>
</tr>
</tbody>
</table>

Table 2. Equilibrium DNA Binding Constants
As shown in Table 2, the apparent dissociation constants for the partial duplex DNA with a 3’ ssDNA overhang and the ssDNA substrate are 234±62 nM and 306±43 nM, respectively, which demonstrates that this binding affinity of PriA on these two substrates is relatively lower than on a 3-way forked structure. The partial duplex DNA and the ssDNA substrates might lack significant structures that are required for the *N. gonorrhoeae* PriA to recognize and bind with high affinity.

**III-1-3. PriA has the greatest unwinding activity when the lagging strand arm is short**

PriA functions as a helicase, unwinding double stranded DNA using the energy provided by hydrolysis of ATP. In order to explore the functional consequences of *N. gonorrhoeae* PriB’s weak DNA binding activity, it was necessary to study *N. gonorrhoeae* PriA’s helicase activity first. To do this, I used a FP-based assay to measure PriA-catalyzed unwinding of duplex DNA. Helicase activity and DNA substrates preference of *E. coli* PriA have been analyzed previously.8,24,25 I tested several forked structures and the partial duplex DNA to determine which DNA substrate would be unwound by *N. gonorrhoeae* PriA.

Of all these three forked structures, fork 1 has 15 bases on the lagging strand, fork 2 has 25 bases on the lagging strand, and fork 3 has 40 bases on the lagging strand. The maximal fraction unwound by PriA is approximately 89% for Fork 1, 65% for fork 2, 42% for fork 3 (Figure 9). Of all these forked DNA substrates, PriA has the greatest unwinding activity on Fork 1 while it has the least unwinding activity on Fork 3. Clearly, PriA-catalyzed unwinding activity decreases significantly upon increasing the length of the duplex DNA on the lagging strand arm. This implies that PriA has a low processivity.
The level of PriA unwinding is markedly lower on a partial duplex with a 3’ overhang (3’-OH), with a 20% maximal unwinding degree, which also agrees with *E. coli* PriA\textsuperscript{7,24}, indicating that a three-way branched DNA substrate is required for efficient PriA-catalyzed unwinding. In addition, as shown in Figure 9, unwinding of the 3’Overhang does not markedly increase upon increasing the concentration of PriA from 5 nM to 15 nM, suggesting that binding of PriA to the DNA is not the limiting factor.

### III-2. PriB stimulates the helicase activity of PriA

It is proposed that *E. coli* PriB stimulates *E. coli* PriA’s helicase activity via an interaction with single stranded DNA\textsuperscript{7}. To test if *N. gonorrhoeae* PriB stimulates the helicase activity of PriA as it does in *E. coli*, I used FP-based helicase assays to measure PriA-catalyzed unwinding on Fork 3 in the presence and absence of PriB. In *E. coli*, PriB stimulation of PriA helicase requires PriB’s ssDNA binding activity\textsuperscript{7}. While in my studies, I found that *N. gonorrhoeae* PriB can stimulate PriA-catalyzed DNA unwinding activity on Fork 3 despite PriB’s...
weak ssDNA binding activity. This could indicate that the mechanism of PriB’s stimulation of PriA helicase activity is varied from different species. According to Figure 10, the fraction unwound is 31% in the absence of PriB while the degree is increased to 74% with addition of 20 nM PriB. These experiments show that PriA-catalyzed Fork 3 unwinding significantly increases (almost doubled) in the presence of PriB, indicating that PriB stimulates the helicase activity of PriA. And the degree of stimulation is increased upon increasing the concentration of PriB. While PriB alone (data not shown here) does not unwind Fork 3, demonstrating that the helicase activity resides within PriA.

![Graph showing PriB stimulation on PriA helicase activity](image)

**Figure 10. PriB stimulates the helicase activity of PriA.** Unwinding of 1 nM Fork 3 by 2 nM PriA in the presence of N. gonorrhoeae PriB (circles) or E. coli PriB (triangles). Measurements are reported in triplicate and error bars represent one standard deviation of the mean.

The strong stimulation effect of PriB on PriA’s helicase activity in *N. gonorrhoea* is impressive since in *E. coli*, PriB stimulation on PriA requires PriB’s ssDNA binding activity. However, in *N. gonorrhoeae*, the binding affinity between PriB and ssDNA is relatively weak. The mechanism by which *N. gonorrhoeae* PriB interacts with and stimulates its cognate PriA helicase might be different from the ssDNA product binding mechanism proposed for how *E. coli*
PriB stimulate its cognate PriA helicase. Therefore, I examined the effect that PriB has on PriA’s other catalytic functions.

III-3. PriB stimulates PriA’s ATPase activity

It is thought that PriA helicase couples the energy gained from ATP hydrolysis to the unwinding of duplex DNA. Therefore, I investigated whether PriB stimulates PriA’s ATPase activity. In *E. coli*, PriB fails to affect the rates of ATP hydrolysis on a fork structure. *E. coli* PriB shows no significant effect on the $k_{cat}$ or $K_m$ values with respect to ATP, but it was found to reduce the $K_m$ values with respect to DNA. In my research, a coupled spectrophotometric assay was used to measure steady-state rates of ATP hydrolysis by *N. gonorrhoeae* PriA helicase in the presence and absence of its cognate PriB. In this reaction, ATP hydrolysis is coupled to oxidation of NADH to NAD$^+$. The overall rate of ATP hydrolysis is increased by the presence of PriB (Figure 12, 13). However, I found that PriB does not significantly affect $K_m$ values with respect to DNA or to ATP (as shown in Table 2).

III-3-1 Titrations in DNA

Fork 3 was used in this experiment. According to Figure 11, PriA’s ATP hydrolysis activity is dependent upon the presence of DNA, as expected from work done in *E. coli*. In the absence of DNA, almost no ATP hydrolysis activity was observed, and as the concentration of DNA is increased from 0 to 50 nM, the rate of ATP hydrolysis is greatly increased and reaches to the maximal rate when DNA is approximately 10 nM. When DNA exceeds 50 nM, the ATP hydrolysis activity of PriA is not largely changed upon increasing the concentration of DNA both in the presence and absence of PriB. The kinetic parameters are also calculated. Under these conditions, $K_m$ with respect to DNA is 1.6±0.2 nM. Furthermore, the maximum rate of ATP
hydrolysis is greatly enhanced from 76±9 to 126±4 with addition of 100 nM PriB. The $K_m$ value is 3.3±1.6 nM, which is not significantly affected by PriB.

![Graph](image.png)

**Figure 11. PriA’s ATPase activity is stimulated by DNA.** DNA-dependent ATP hydrolysis was catalyzed by 10 nM PriA in the presence or absence of 100 nM PriB (as monomers). The DNA substrate is Fork 3. Measurements are reported in triplicate and error bars represent one standard deviation of the mean.

III-3-2 Titrations in ATP

I also performed PriA’s ATP hydrolysis activity reactions at different concentrations of ATP, as shown in Figure 12, PriA’s ATPase activity is dependent on concentrations of ATP. The maximal rate of ATP hydrolysis of PriA is increased from 102±16 nM/s to 137±7 nM/s upon addition of 100 nM PriB with respect to ATP (Figure 12 and Table 2). Under these conditions, the $K_m$ with respect to ATP is 83±17 nM, which is not significantly different compared with 64±28 nM in the absence of PriB.
Figure 12. PriA’s ATPase activity is stimulated by PriB. Effect of ATP concentration on rates of ATP hydrolysis catalyzed by 10 nM PriA in the presence of 100 nM Fork 3 and in the presence (circles) or absence (squares) of 100 nM PriB (as monomers). Measurements are reported in triplicate and error bars represent one standard deviation of the mean.
CHAPTER IV

CONCLUSIONS

Physical interactions between DNA replication restart primosome proteins in *N. gonorrhoeae* and DNA substrates are studied in my research. My work shows that a 3-way branched fork DNA structure is the preferred substrate for PriA’s binding and unwinding actions in both *N. gonorrhoeae* and *E. coli*. This feature has been conserved between these two species.

PriB in *N. gonorrhoeae* has a stronger interaction with PriA than ssDNA compared with PriB in *E. coli* which instead possesses a stronger interaction with ssDNA than with PriA. Interestingly, my studies show that *N. gonorrhoeae* PriB can stimulate the DNA unwinding activity of its cognate PriA helicase as it does in *E. coli*, despite *N. gonorrhoeae* PriB’s low binding affinity for ssDNA. In *E. coli*, ssDNA binding by PriB plays a vital role in PriB’s ability to stimulate PriA helicase. Thus, the mechanistic details on how PriB stimulates PriA’s helicase activity in *N. gonorrhoeae* appear to be different from that in *E. coli*. I also found that the interaction between *N. gonorrhoeae* PriB and PriA could serve to simulate PriA-catalyzed ATP hydrolysis, which is not observed in *E. coli*.

Based on these observations, I conclude that the mechanism of PriB’s stimulation on PriA’s helicase activity might involve allosteric regulation. Association of PriA and PriB could lead to a conformation change in PriA, which could enhance its ATPase activity and account for the stimulation of PriA-catalyzed DNA unwinding. This allosteric activation model could also explain why *N. gonorrhoeae* PriB has a stronger binding affinity with its cognate PriA than do
the *E. coli* PriA and PriB proteins. In the case of *N. gonorrhoeae*, a strong physical interaction between PriB and ssDNA might have been sacrificed throughout evolution for a strong interaction between PriB and PriA, resulting in allosteric activation of PriA helicase activity.
REFERENCES


19. 2007 HORIBA JOBIN YVON Fluorescence Technical Note FL-3, Version 1.0


APPENDIX

A. Introduction to X-ray crystallography

Solving the three dimensional structure of a macromolecule is helpful to study its biological functions. A crystallized protein can serve to determine the protein’s three-dimensional structure, which in turn, helps to investigate its relative biological functions. X-ray crystallography is a method that is commonly used for this purpose.

So far, several crystal structures of *E. coli* PriB\[^{11,27,30}\] and *N. gonorrhoeae*\[^{15}\] PriB have been solved and published. However, no high resolution models of PriA are available at present. Therefore, we attempted to solve the crystal structure of PriA. Numerous investigators over the years have failed to produce crystals of PriA alone, so we attempted to crystallize a complex between PriA and DNA. Initial trials using the hanging drop vapor diffusion method failed, and I soon learned that another research group had made considerable progress crystallizing a PriA homolog from *Klebsiella pneumoniae* and that a high resolution model would soon be published. Therefore, we established a collaboration with the other research group to solve the structure of *K. pn* PriB so that the scientific community would have PriA and PriB structures from a single organism. My goal was to elucidate the crystal structure of the *K. pn* PriB-ssDNA complex or the apo form of *K. pn* PriB.
In my research, the protein:DNA complex crystals were grown using the hanging-drop vapor diffusion method, which is one of the most commonly used methods for protein crystallization. The principle of this method is shown in Figure 13. A drop composed of a mixture of sample and reagent is placed above a liquid reservoir of reagent. The drop contains a lower reagent concentration than the reservoir. To achieve equilibrium, water vapor leaves the drop and diffuses into the reservoir. Thus, the protein in the drop will eventually become supersaturated, resulting in growth of crystals.¹

Figure 13. Hanging drop vapor diffusion method of protein crystallization. The well is sealed with high vacuum grease and cover to prevent water vapor loss to the atmosphere which would dry the drop.

B. Purification of E.coli PriA

_E. coli_ PriA was purified from BL21(DE3) _E. coli_ harboring the pET28b:E. _coli_-PriA plasmid. Cells were grown in Luria-Bertani(LB) medium containing 50 mg/L at 37 °C until an OD<sub>600</sub> was reached. Expression of PriA was induced with 0.5 mM IPTG for 4 hours and cells were harvested by centrifugation at 5,500 x g at 4 °C for 25 min and stored at -80 °C. Cells were lysed in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethanesulphonylfluoride (PMSF) by Sonication on ice, using 5 × 30 sec pulsed bursts (pulse=1 sec on, 1 sec off) at 70% power,. The lysate was clarified by centrifugation at 40,000 x g for 20 min at 4 °C. His-tagged PriA was bound to nickel-Nitrilo-triacetic Acid (NTA) agarose
(Qiagen) and eluated in 10 mM HEPES pH 7, 10% glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol, 250 mM imidazole. The nickel-NTA agarose eluate was dialyzed against 10 mM HEPES pH 7, 10% glycerol, 100 mM NaCl and 1 mM β-mercaptoethanol and incubated with thrombin 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, was depleted by incubating the thrombin-cleaved PriB solution with nickel-NTA agarose. Thrombin-cleaved PriB was concentrated and incubated in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 6, and the protein solution was loaded onto a HiPrep™ SPFF 16/10 ion-exchange column pre-equilibrated with 10 mM MES pH 6, 10% (v/v) glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol. The SPFF column was resolved at 0.5 mL/min using a ten column volume linear gradient of 0%-100% Buffer B, which contains. Appropriate fractions containing PriA were collected and concentrated to < 2 mL overnight by centrifugation in a CentriPrep YM-10 concentrator at 2,643 × g at 4°C. The concentrated protein solution was purified through HiPrepTM 16/60 Sephacryl S-300 High Resolution size exclusion column in 10 mM MES pH 6, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. Appropriate PriA fractions were pooled, concentrated and stored at -80 °C.

C. Purification of Klebsiella pneumonia PriB

*K. pneumonia* PriB was purified from BL21(DE3) *E. coli* harboring the pET28b:(*K. pneumonia*) PriB plasmid. Cells were grown in Luria-Bertani medium containing 50 mg/L kanamycin at 37 °C until and OD₆₀₀ was reached. Expression of PriA was introduced with 0.5 mM IPTG for 4 hours and cells were harvested by centrifugation at 5,500 × g at 4 °C for 25 mins and stored at -80 °C. Cells were lysed in 10 mM tris(hydroxymethyl) amino methane (Tris-HCl) pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethanesulphonylfluoride (PMSF) by sonication using 5 × 30 sec pulsed bursts (pulse=1 sec on, 1 sec off) at 70% power, and the lysate was clarified by centrifugation at 40,000 × g for
20 min at 4 °C. His-tagged PriB was bound to nickel-Nitrilo-triacetic Acid (NTA) agarose (Qiagen) and eluted in 10 mM Tris-HCl pH 8, 10% glycerol, 300 mM NaCl, 1 mM β-mercaptoethanol, 250 mM imidazole. The Ni-NTA agarose eluate was dialyzed against 10 mM Tris-HCl pH 8, 10% glycerol, 300 mM NaCl and 1mM β-mercaptoethanol and incubated with thrombin 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, was depleted by incubating the thrombin-cleaved PriB solution with nickel-NTA agarose. Thrombin cleaved PriB was concentrated in a CentriPrep YM-3 concentrator to < 2 mL overnight at 4 °C. Then the protein solution was purified through a HiPrep HR 16/10 Sephacryl S-100 size exclusion column (GE Healthcare) in 10 mM Tris-HCl pH 8, 10% glycerol, 500 mM NaCl and 1mM β-mercaptoethanol. Appropriate fractions containing PriB were collected and concentrated overnight by centrifugation in an Amicon Ultracel-3K concentrator at 2,643 × g at 4°C. The concentrated protein solution was aliquoted and stored at -80 °C.

**D. Construction of double stranded DNA**

Oligonucleotide oML299 was heated 95 °C for 5 min, then slowly cooled to 70 °C and incubated for one hour. After that, the solution was slowly cooled to room temperature and stored at 4 °C.

**E. Crystallization of *E.coli* PriA:dsDNA complex**

*E.coli* PriA was purified as described previously and dialyzed against 10 mM MES pH 6, 0.5 M ammonium acetate overnight at 4 °C. Double stranded DNA was added to PriA at a molar ratio of 1:1 and the mixture was heated at 37 °C for 30 min.

To grow crystals, 1µL of PriB: dsDNA was mixed with 1µL well solution. Hampton Research Index Screen Reagents were used to optimize and design follow-up screening
experiments. These screens failed to provide any crystals or any structured material that could be suitable for follow-up studies.

F. Crystallization of *K. pn* PriB:dC15 complex and *K. pn* PriB:dT15 complex

*K. pn* PriB was purified as described previously and dialyzed against 20 mM sodium citrate, 50 mM sodium chloride(pH 5), 50 mM ammonium acetate (pH 5) overnight at 4 °C. Then *K. pn* PriB was mixed with ssDNA at a molar ratio of PriB dimer:DNA=1:2.5. The PriB:DNA mixture was then incubated at 37 °C for 30 min.

I started with Index Screen Reagents to design optimization experiments and to identify follow on screens. Index screen is a primary, diverse reagent system crystallization screen for proteins, complexes, peptides, nucleic acids, & water soluble small molecules. It is designed as a 96 reagent crystallization, it utilizes a broad, yet refined portfolio of crystallization reagent systems, which include the following: (1) traditional salts such as Ammonium sulfate and Sodium chloride versus pH; (2) neutralized organic acids such as Sodium malonate and Tacsimate; (3) High salt concentration mixed with low polymer concentration as well as high polymer concentration mixed with low salt concentration and; (4) Low ionic strength using polymers such as PEG, MPD, Pentaerythritols versus pH. These reagent systems are formulated across a sparse matrix and incomplete factorial of concentration ranges, sampling a pH range of 3 to 9.

Based on my observations, I followed up with further screening experiments using this condition, 0.2 M CaCl$_2\cdot$2H$_2$O, 0.1 M Bis-Tris pH 6.5, 45% (v/v) (+/-)-2-methyl-2, 4-pentanediol (MPD). The screening experiments were set up as follows, of which pH, concentration of CaCl$_2$ was varied every two columns and pH was varied across every row, concentration of MPD (v/v) was varied over every column. Generally speaking, the pH is increased, more spherulites formed
or the more pure the spherulites are (shown in a darker color); a relatively more concentrated 
CaCl$_2$ is preferred (dark green or red), and results from a higher concentration of MPD are 
favorable (shown in red). To sum up, the condition of Tris-HCl pH 8 and 0.2 M CaCl$_2$ with 45% 
MPD (v/v) gave me promising results, which I used as reservoir solution. I then added additives 
to perform the next screening experiments.

<table>
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<tr>
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<th>0.05 M CaCl$_2$</th>
<th>0.2 M CaCl$_2$</th>
<th>0.3 M CaCl$_2$</th>
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<tbody>
<tr>
<td>0.1 M Bis-Tris pH 6.5</td>
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<td>Green</td>
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<td>0.1 M HEPES pH 7</td>
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<td>0.1 M HEPES pH 7.5</td>
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<td>0.1 M Tris-HCl pH 8</td>
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<td>Green</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

(+/-)-2-methyl-2, 4-pentanedial (MPD) (v/v)

Table 3. Screening experiments of PriB:dT15. The pH, concentration of CaCl$_2$ and 
MPD (v/v) was varied. The darker the color is, the more regular the crystals are or the 
higher the degree of the precipitates is. Yellow represents precipitates. Green represents regular spherulites. Red represents conditions worth being followed up on.

Additive Screen$^{33}$ is a library of small molecules that can affect the solubility and 
crystallization of biological macromolecules, including both soluble and membrane proteins. 
These small molecules include multivalent cations, salts, amino acid, dissociating agents, linkers, 
polyamines, chaotropes, cofactors, reducing agents, polymers, chelating agent, carbohydrates, 
polyols, non-detergents, amphiphiles, detergents, osmolyte, organic (non-volatile) and organic 
(volatile) reagents. They can perturb sample-sample and sample-solvent interactions, as well as 
perturb water structure which can alter and improve both the solubility and crystallization of a 
Sample$^3$. 

35
K. *pn* PriB was dialyzed against 20 mM sodium citrate, 50 mM sodium chloride (pH=5), 50 mM ammonium acetate (pH 5) overnight at 4 °C. Then K. *pn* PriB was mixed with ssDNA at a molar ratio of PriB dimer: dT15=1:2.5. The PriB:dT15 mixture was then incubated at 37 °C for 30 min. The reservoir solution contains Tris-HCl pH8 and 0.2 M CaCl2 with 45% MPD (v/v). To grow crystals, 1µL of PriB: dT15 was mixed with 1µL well solution with addition of 5 mM of each additive. Plate-like crystals grew in the presence of 5 mM of additive NiCl2 with two weeks, see Figure 2.

**Figure 14. Formation of plates.** Plates formed in these conditions: ratio of His-tag removed PriB dimer:dT15=1:2.5, 45% MPD(v/v), 0.1 M Tris-Bis pH8, 0.2 M CaCl2, 5 mM additive NiCl2.

Based on these observations, the next screening experiment was set up in three different concentrations of NiCl2, two different concentrations of MPD (v/v), and the molar ratio of PriB dimer:dT15 or PriB dimer:dC15 was varied across each row as follows:
Crystals grew in two weeks as follows:

**Figure 15. Formation of crystals.** Crystals formed in: PriB dimer:dT15=1:2.5, 20% MPD(v/v), 0.1 M Bis-Tris pH8, 0.2 M CaCl2, 2 mM NiCl2.
Crystals were harvested and stabilized by transferring into cryoprotectant solution comprising 25% MPD (v/v), 0.1 M Bis-Tris pH 8, 0.2 M CaCl₂, 2 mM NiCl₂, 25% glycerol (v/v) (and or 25% PEG 400). Our collaborators at the University of Wisconsin-Madison analyzed these crystals for X-ray diffraction, but unfortunately, none of the crystals diffracted X-rays.
G. Crystallization of *K.pn* PriB alone

Crystal Screen reagents were used to optimize the conditions. The Crystal Screen and Crystal Screen 2™ reagent kits are designed to provide a highly effective and rapid screening method for the crystallization of macromolecules. The screens are simple and practical for finding initial crystallization conditions. The initial crystallization conditions for more than 1,000 proteins, peptides, oligonucleotides, and small molecules have been determined using Crystal Screen.

Based upon the observations, I followed up on screening experiments using different concentration of PEG 8000 in various pH conditions. Eventually, thin blade-like crystals formed (indicated by red in Table 5). These conditions will require further refinement and optimization in order to grow diffraction-quality apo crystals.

<table>
<thead>
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</table>

**Table 5. Crystallization of PriB alone.** Using different pH and various concentrations of PEG8000 (w/v). Precipitates formed (green and light green), some twin crystals grew(red or organge), the darker the color is, the more precipitate formed or the more regular the crystals are.