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The Effects of Structural Modification of the Wing Helix Domain of PriA on Bacterial DNA

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Abstract

During a cell's life it must copy itself or face death. For the cell to form a new copy it must replicate its DNA, this DNA is the blueprint for the essential components of the cell. The replication process does not always go to completion, which is necessary for the cell to survive, so there are mechanisms to keep the replication process running smoothly. In bacteria, PriA is used to restart replication after damage to the DNA. By looking at the structure of PriA we can start to determine how exactly it works with the damaged DNA and restart the replication process. Specifically looking at the portion of PriA that attaches to DNA we are attempting to determine why this method of attachment is beneficial to the entire replication process. My hypothesis was that by modifying the structure of PriA there would be some change in its binding and helicase abilities. The results showed that the modification of the N-terminal tether of the wing helix domain had no effect on PriA's activity within the DNA replication restart pathway.

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Introduction

In order for a cell to survive and make copies of itself it needs to first replicate its chromosomal DNA. This replication process has three main steps: initiation, elongation, and termination. The *Escherichia coli* chromosome has 4.7 million base pairs, 260 of these base pairs code for a replication origin site, oriC. The oriC contains multiple copies of base pairs that are recognized by the replication initiator protein DnaA [1]. DnaA recruits histone-like proteins and together they are able to destabilize ds-DNA close to oriC creating a bubble-like structure [2]. This bubble in DNA allows for the DNA helicase $DnaB_6$ to join with DnaA on each single strand portion of DNA (ss-DNA). $DnaB_6$ then uses ATP to move in the 5'-3' direction down both strands of the parental DNA, further separating the double stranded DNA. $DnaB_6$ then interacts with DnaG, a RNA polymerase that places RNA primers on the single strands of DNA. The RNA primers are required for the replicative polymerase (Pol III) to bind to the ss-DNA; this polymerase then places complementary base pairs on the ss-DNA forming new ds-DNA. DNA polymerase I and DNA ligase then move down the newly synthesized ds-DNA removing the RNA primers and joining together segments of non-continuous base pairs [1]. At the end of this process there will be two identical copies of the original DNA, each copy containing half of its DNA from the original (Figure 1).

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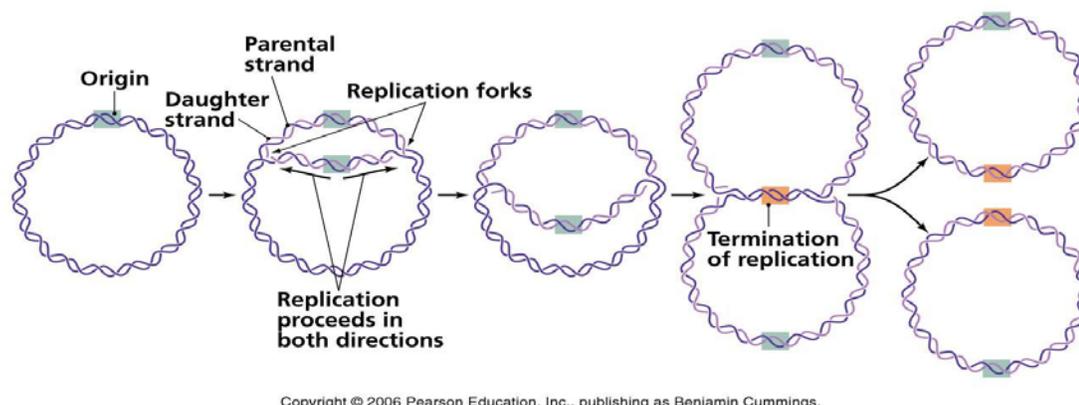
Figure 1: DNA replication, the origin in *E. coli* is OriC, replication then proceeds to completion creating two identical copies of DNA.

During the replication of the chromosome it is extremely common for the replication machinery to encounter a problem in the parental DNA. These problems can include: breaks in ds-DNA, DNA lesions, and frozen protein complexes. If one of these problems is present DNA replication will stop, creating a forked structure in the DNA. This occurs about once every generation in each cell [3]. Due to how common the replisome stalls during replication, *E. coli* has developed multiple pathways of origin-independent replication restart that are non-mutagenic [4]. The first restart pathway, which was the focus of my research, is used when there are few-to-no gaps in the leading strand of DNA at the stalled fork. This pathway involves the recruitment of PriA to the fork or the d-loop structure in DNA, PriA then recruits PriB and DnaT [5, 6]. This pathway favors DNA with very few gaps in the leading strand because of the structure of PriA. There is a 3' terminal binding pocket that is used for increased specificity for the forked structure of DNA. For this binding pocket to be filled there needs to be a 3'-OH near the fork. Without it the PriA will not bind to this location, thus leading to the second possible pathway [7]. This next pathway is favored when there are gaps in the leading strand of DNA, the pathway uses PriC in addition to either PriA or Rep helicase [5, 6].

PriA has two domains that establish its structure. The first is the DNA binding domain (DBD). This domain allows PriA to have great specificity to forked DNA and D-loop DNA, including the ability to bind to all three strands of DNA involved in the D-loop [5, 8]. The second domain is the helicase domain (HD). Unlike normal DNA helicases used in replication, PriA moves in a 3'-5' direction. This reversed direction allows PriA to remove base pairs added before the replication process stopped. This is potentially important so that the other proteins required for replication restart can properly bind to the DNA. This helicase uses ATP hydrolysis to move the helicase along DNA in order to unwind the DNA [5].

The DBD is also split into two portions, the first is the 3'BD. This portion of the DBD binds to the 3' end of the leading strand that is present in the forked DNA structure. This portion allows PriA to recognize stalled DNA forks and bind to them to restart replication. The second portion of the DBD is the Wing Helix (WH) domain, the WH domain is present in other DNA binding proteins. The WH of PriA differs slightly from traditional WHs in its structure: one of the β-hairpin wings that is usually present is opened up and connects the WH to the rest of the protein structure. There are two tethers that connect the WH to PriA: the N-terminal and the C-terminal tether. This structure allows the WH to bind to partial duplex DNA that is present in forked DNA allowing PriA to have greater specificity to these structures [9].
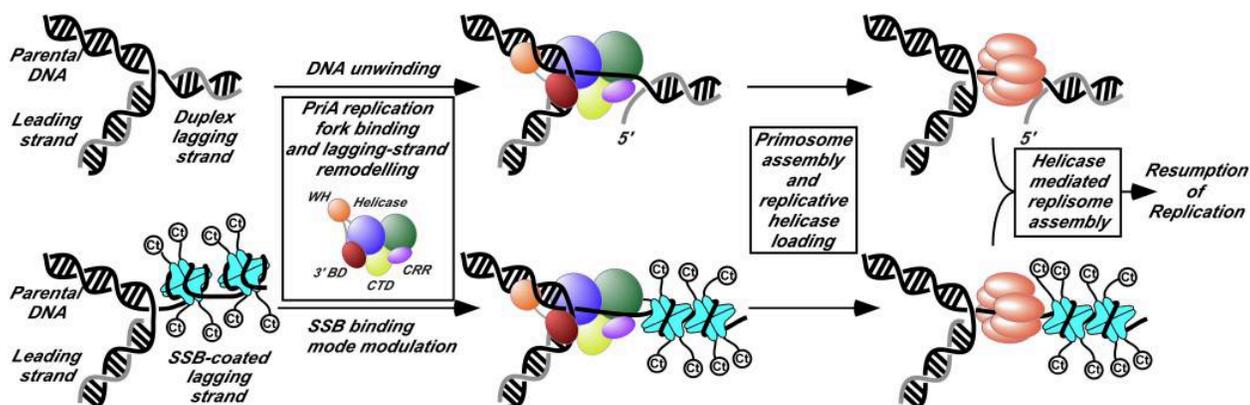
Figure 2: Attachment of PriA to the fork structure of stalled DNA replication [9].

The purpose of this research was an in depth study of the structure of the WH domain of PriA (shown in Figure 3) and its relation to the activity of the protein. The tethers that connect the WH domain to the rest of PriA were the main focus of this study. This was chosen due to the lack of information regarding how they are structured the way they are and how they help the WH to bind to DNA. The idea was that the tethers allow the WH to have the flexibility to move and form around DNA at the stalled fork structure. My hypothesis was that by modifying the length of the N-terminal polypeptide chain, connecting the Wing Helix domain to PriA, that there would be a change in the ability of PriA to restart replication. To test this hypothesis, I used site directed mutagenesis to insert DNA coding for a longer N-terminal tether, which transformed this DNA into *E. coli* cells which were then used to overexpress the protein. The protein was then isolated and used to test the unwinding ability and the binding affinity of the structurally modified PriA.
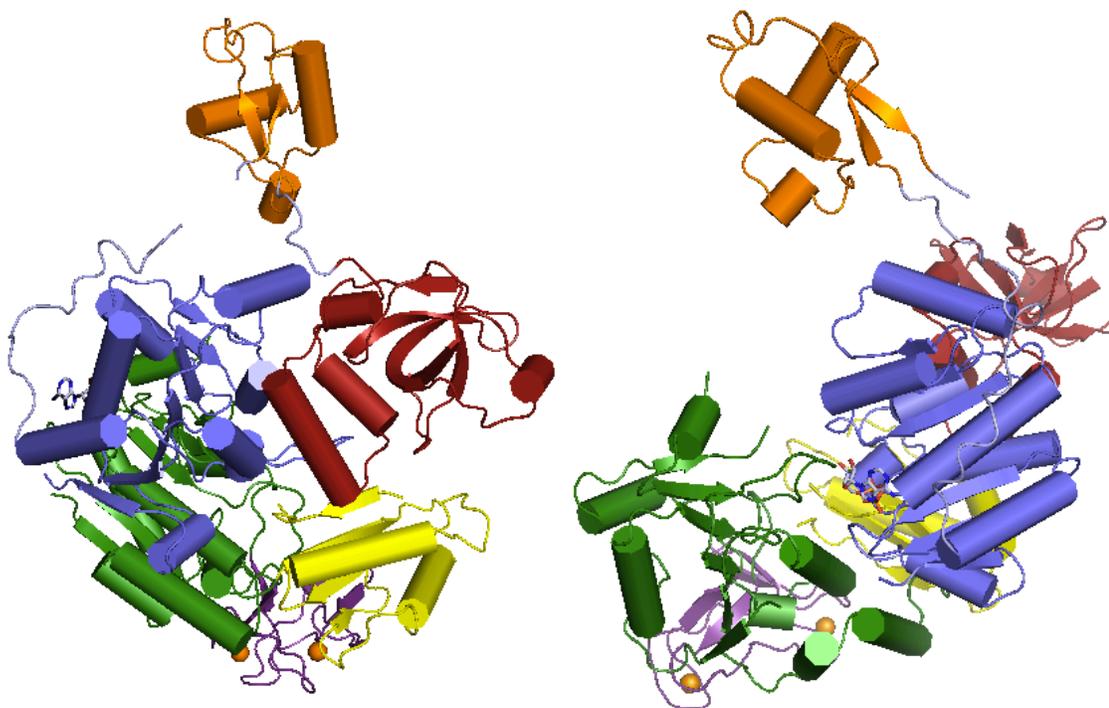
Figure 3: Cartoon ribbon structure of PriA with a bound ADP molecule (PDB code: 4NL4). The WH domain is represented by the orange portion of the structure separated at the top of the structure.

Experimental Methods

Cloning strategy: Using site-directed mutagenesis a Sca I restriction site was inserted into pET128 plasmid, which codes for Kpn PriA, at the location coding for the N-terminal tether of the WH. The Sca I site was then used to linearize the mutant plasmid, the blunt-end DNA insert OML 170 (5' –GAG AAC TTG TAT TTC CAA GGT- 3') was then used to ligate the plasmid.

PCR and agarose gel used to verify insert: 10 samples were prepared for PCR containing: 401.5 μL H_2O, 110 μL 5x Buffer, 11 μL OML 170, 11 μL OML 259 (5' – GCT AGT TAT TGC TCA GCG G), 11 μL dNTPs, and 5.5 μL of Pol I. 10 samples that did not contain the insert were also made for PCR. The PCR was as follows: 95 °C for 5

minutes, 25 x [95 °C for 1 minute,   55 °C for 1 minute, 72 °C for 3 minutes], 72 °C for

10 minutes, 4 °C until ready to be run on the DNA gel.

20 μL of each sample + 4 μL of 6x DNA loading dye were loaded onto a 0.8%

(w/v) agarose in 1x TAE. The gel was run at 80V for 4 hours to make sure that the

plasmid contained the insert intended.

Transformation into E.coli cells: The mutated plasmid from PCR was then

transformed into *E. coli* cells by suspending the plasmid DNA in 2 μL of EB buffer and

combining this with 40 μL of *E. coli* cells incubated on ice for 30 minutes. The cells were

then heat shocked for 45 seconds at 42 °C, placed on ice for 2 minutes, 200 μL of SOC,

incubated for 1 hour at 37 °C. The cells were then plated on agarose gels and incubated at

37 °C overnight.

Glycerol stocks: 12 colonies from the agarose gels were taken and placed into LB

medium containing 3 μL kanamycin and incubated at 37 °C. Only 6 samples grew

bacteria. The DNA in these cells was then transformed into Rosetta BL21 (DE3) *E. coli*

cells following the procedure used for transformation into *E. coli* cells.

Colonies from each plate were then grown in LB, kanamycin, and

chloramphenicol.   850 μL of cells and 150 μL of glycerol were added and put in 6

separate tubes and placed in the -80 °C freezer. These cells were labeled ML344 1-6.

TEV Protease Purification: To test whether the plasmid DNA contained the

intended insert, the plan was to perform a TEV protease digest on PriA which cleaves the

amino acid sequence (ENLYFQG) coded by the plasmid DNA insert. ML 172 glycerol

stock was used to grow BL21 (DE3) *E. coli* cells containing TEV protease in LB

medium. To induce protein expression: cells were grown in LB medium in a moving

incubator at 37 °C for 2 hours. The optical density between 0.4 and 0.6 was obtained for the growth, this value was measured at 600nm absorbance. 1000 μL of 0.5 mM IPTG was added to the cells, which were then allowed to continue growth in the incubator for 3 hours. The cells were then spun down in a centrifuge at 5,000xg at 4 °C for 20 minutes, the cells were then placed in a freezer at -80 °C.

The pellets were then suspended in 250 mL of lysis buffer consisting of: 10mM Hepes pH7, 10% glycerol, 0.5M NaCl, 100 mM imidazole, 1 mM β-mercaptoethanol, and 1 mM PMSF. The cells were suspended using approximately 10 mL of lysis buffer per gram of cells. Once suspended the cells were then sonicated at 70% amplitude with a 1 second on 1 second off pulse scheme for 30 seconds 5 times. The sonicated cells were then centrifuged at 40,000xg at 4 °C for 20 minutes. The supernatant was removed and 400 μL of Ni-NTA beads were added and placed on a moving platform for an hour at 4 °C. The solution was then placed in a nickel-affinity chromatography column. Once all of the supernatant+ Ni bead complex was poured through the column was washed with the additional lysis buffer not previously used. 10 mL of elution buffer was then poured through the column containing: 10 mM Hepes pH 7, 10% glycerol, 50 mM NaCl, 250 mM imidazole, and 1 mM β-mercaptoethanol. The eluted protein was then placed into vascular tubing and underwent dialysis in 1L of dialysis buffer containing: 10 mM Hepes pH 7, 10% glycerol, 50 mM NaCl, and 1 mM β-mercaptoethanol. Dialysis occurred overnight at 4 °C. The TEV protease was then placed in 100mL of storage buffer consisting of: 10 mM Hepes pH 7, 50% glycerol, 50 mM NaCl, and 1 mM β-mercaptoethanol. This was then placed in the -80 °C freezer.

A protein gel was performed to test for the purity of the TEV protease. Each sample contained 5 μL of 4x +SB and the gel was run for 45 minutes at 200V. The gels showed a good level of purity in the TEV.

Mutated PriA Verification: The ML 344 glycerol stocks were used to grow cultures in LB medium so as to express the N-tev-PriA. Small amounts of the cells were placed in autoclaved LB medium and grown overnight at 37 °C in a shaking incubator. The overnight cultures were then placed in new LB and grown and induced using 0.5 mM IPTG and grown for 3 hours. The cells were then spun down in a centrifuge at 5,000xg. The pellets were then massed and suspended in 5 mL/g of BugBuster protein extraction reagent and 1μL of Benzonase, a DNA nuclease, to cleave chromosomal DNA. The solutions were then incubated for 20 minutes at room temperature and centrifuged at 4 °C for 10 minutes at 21,130xg. 100 μL of Ni-NTA beads were added to each sample and placed on a rotating platform at 4 °C for 1 hour. The samples were then centrifuged at 21,130xg for 1 minute. The supernatant was removed and placed into tubes labeled ML 344 1-6 and placed in the -20 °C freezer.

PriA was then tested for the TEV protease location. All 6 candidates that could contain the desired protein structure were run on a protein gel, each sample was tested with and without TEV protease present. Prior to being placed on the gel the samples were incubated at room temperature with the protease for 1 hour. Each sample on the gel contained 3 μL of 4x +SB so the proteins could be visualized.

To ensure that the samples actually contained PriA. ML 344-2 was run on a protein gel along with ML 313, the control known to contain Kpn PriA. LB medium cultures were allowed to grow overnight at 37 °C in the moving incubator. They were

then allowed to grow and were induced using 50 μL of 0.5 mM IPTG. At this point the cells were allowed to grow for three hours, however every hour 50 μL were removed to be tested on the protein gel. The cells were spun down at 21,130xg for 1 minute and stored in the -20 °C freezer. All of the samples were then suspended in 50 μL of 1x +SB. The gels were then run at 200V for 45 minutes.

A second TEV protease test was done, this time using ML 172 which also has TEV cleavage site on the PriA protein. This was done to ensure that the TEV was working correctly.

The samples ML344 1, 2, and 5 were also tested using TEV protease to see if there was PriA present in the samples. These samples were taken from the glycerol stocks and grown in LB medium, induced with 0.5mM IPTG, and broken open using sonication. The samples were then spun down, the supernatant was removed, and the cells were re-suspended in 10mL of lysis buffer. The samples were then sonicated for 6 cycles at 40% amplitude, the samples were then centrifuged for 20 minutes at 5,000xg at 4 °C. The supernatant was removed and 100 μL of Ni-NTA beads were added to each sample and were allowed to incubate for 1 hour at 4 °C on a rotating platform. The samples were then spun down for 7 minutes at 4,128xg. 500 μL of elution buffer was added suspending the pellets, the solution was centrifuged again and the supernatant was stored in the -20 °C freezer. A protein gel was then run, containing all of the samples with and without the TEV protease, for 45 minutes at 200V. Another protein gel was also run with the same setup, this time the samples were incubated at 4 °C overnight with and without the TEV protease. A third gel was run with the samples incubating overnight at 34 °C with and without the TEV protease. A fourth gel was run with the samples going through micro-

dialysis overnight, this was done by placing 60 µL of protein + 10 µL of TEV protease in a beaker with 100 mL of dialysis buffer containing: 10 mM Tris-HCL pH 8, 50mM NaCl and 1 mM β-mercaptoethanol.

Sca I digest: To determine whether or not ML344 had the N-Tev-PriA insert the samples were tested with Sca I, if the Sca I site was still present then the insert was not in the sample. Each candidate from the first transformation into *E. coli* was run on a 0.8% DNA agarose gel. Each sample was tested with and without the Sca I-HF. The positive control pML 130 was used and all samples were incubated for 1 hour at 37 °C before running the DNA gel.

Protein Growth and Purification: To be able to make any comparisons between the   N-tev-PriA and the wildtype PriA, larger amounts of both types of PriA had to be grown and purified. ML 313 was used as the wildtype and ML 344 #2 was used as the N-tev-PriA sample. Overnight cultures were grown overnight in LB medium in a moving incubator at 37 °C. The cultures were then induced with 0.5 mM IPTG and allowed to grow for 3 hours. The cells were then centrifuged and the pellets were then suspended in lysis buffer and sonicated at 70% amplitude for 10 cycles of 1 second on and 1 second off. The cells were then centrifuged for 20 minutes at 5,000xg and 4°C.

Nickel-affinity Column Chromatography: The supernatant from cracking open the cells was placed in 50mL conical tubes and 333 µL of Ni-NTA beads were added. The conical tubes were then incubated on a moving platform for 1 hour at 4 °C. The samples were then run through columns, once the solution had passed through the column lysis buffer was passed through to wash the beads. 10 mL of the elution buffer was added to

the beads and allowed to incubated. The run through of each column was collected for future purification processes.

Size-Exclusion Chromatography: The nickel column products for ML 313 and ML344 #2 went through size-exclusion chromatography to purify PriA from the other proteins contained in the cells. The column ran overnight using 1L of vacuum filtered S-300 buffer which contained: 10 mM 2-(N-morpholino)ethanesulfonic acid pH 6, 10% glycerol, 0.5M NaCl, and 1 mM β-mercaptoethanol. Samples were placed into 3mL portions that were separated by the size of the proteins. The samples corresponding to PriA for both ML 313 and ML 344 #2 were placed into separate concentrating tubes and were centrifuges for approximately three hours until they reached a minimal volume. The samples were collected and distributed into 15 μL aliquots and placed in the -80 °C freezer. Each sample was also run on a protein gel to determine the purity level of each.

Helicase Assays: Helicase assays were performed to test the unwinding ability of the helicase domain for both proteins. Using a UV/vis spectrophotometer set to 280 nm the concentrations of both the PriA and the N-tev-PriA were determined so that the correct concentrations could be used moving forward.

The Helicase buffer was made using: 20 mM Tris-HCl pH 8, 50 mM NaCl, 3 mM MgCl, and 1 mM β-mercaptoethanol. The helicase assays had to be performed very carefully because each step is extremely important to achieve useful data. Helicase buffer was added to fork 2 DNA to achieve a 1:40 dilution. 1 μL of the diluted DNA was placed in a 1.5mL microcentrifuge tube, making sure to keep the tubes on ice. 89 μL of serially diluted PriA was added to each tube and vortexed for 0.5 seconds directly after the addition and then the tube was placed back on ice. The concentrations of PriA used were

0.25 nM, 0.5 nM, 1.0 nM, 2.0 nM, 5.0nM, 10.0 nM, 15.0 nM and 20.0 nM. 10 µL of 10

mM ATP was then added to the tubes vortexed for 0.5 seconds and incubated for 10

minutes at 37 °C. At the end of the incubation 10 µL of 10% SDS was added to each

sample vortexed for 0.5 seconds and placed on the benchtop, terminating the reaction.

100 µL of each solution was placed in fresh FP tubes, each tube was then read in

the FP spectrophotometer. The samples were then vortexed for 0.5 seconds and read for a

second time and an average of the values was then taken. The tubes were then incubated

at 95°C for 20 seconds, the tubes were then shifted onto ice for 10 seconds. Each sample

was then read in the FP spectrophotometer, vortexed for 0.5 seconds and read again. If

there were any major deviations in the readings, then a third measurement was taken.

DNA Binding Assays: In order to measure the binding ability of PriA and N-tev-

PriA to DNA forked structures, DNA binding assays were performed. 50 mL of the DNA

binding assay buffer was made containing: 20 mM Tris-HCl pH 8, 10% glycerol, 50 mM

NaCl, 1 mM β-mercaptoethanol, 0.1 g/L Bovine Serum Albumin. Serial dilutions of each

protein were made of the following concentrations: 0.01 nM, 0.1 nM, 0.3 nM, 1.0 nM,

2.25 nM, 5.0 nM, 10.0 nM, 22.5 nM, 50.0 nM, 100.0 nM, 225.0 nM, 500 nM, 1000.0

nM, 2250.0 nM, 5000.0 nM, and 10000.0 nM. Using the 1:40 dilution of Fork 2 DNA

used in the helicase assays, place 1µL of DNA into fresh FP tubes. 99 µL of the diluted

protein was added to the appropriate tubes and vortexed for 0.5 seconds. Each tube was

then read by the FP spectrophotometer, vortexed for 0.5 seconds and read a second time.

ATPase Activity determination: To determine whether there was any

contamination in the ATPase activity of PriA we tested the ATP activity. The helicase

assay protocol was followed for timed ATP incubations for 2.0 nM and 5.0 nM PriA and N-tev-PriA.

Results and Discussion

Cloning strategy: A Sca I cleavage site was introduced into the gene that codes for PriA and the location representing the N-terminal tether of the wing helix domain using site directed mutagenesis. The pET28b contained this Sca I site, it was cleaved and linearized so that a small portion of DNA coding for the amino acid sequence, ENLYFQG, could be ligated. The insert was ligated in-frame so that the resulting N-terminal tether would have the amino acid sequence now present. This amino acid sequence also codes for a TEV protease cleavage and was inserted between PriA amino acids L114 and W115, this ensured it was both lengthened and had a cut site.

PCR and agarose gel used to verify insert: The resulting gels that were run to test for the presence of the insert after PCR amplification did not show if the insert was present or not. Since the colonies grew on the petri dishes as expected, the conclusion was made that something went wrong during PCR. The project moved forward and it was decided that the protein would be tested for the TEV cleavage site later, to determine whether or not the plasmid contained the insert.

Mutated PriA Verification: The protein gels that were run to test the ability of TEV protease to cleave PriA did not show that there was any cleavage. It was determined that if there was no PriA to begin with then there would be no site for TEV to act on. The presence of PriA was tested by comparison between ML313, the control, and ML344 #2.

The results showed that there was definitely PriA present, it also showed that there was PriA in ML344 #1 and #5.

The next potential problem that could have impacted the ability of TEV to cleave PriA was the TEV protease. A quality test of TEV was run using the ML344 samples and ML172, PriA known to be cleaved by TEV protease. The protein gel showed that ML172 was not cut completely by the TEV. The same procedure was repeated except this time the samples were incubated in the presence of TEV overnight at 4 °C, the results once again showed that the PriA samples were not digested. The procedure was repeated at the TEV proteases optimal temperature of 34 °C overnight, once again the protein gels did not show cleaved PriA. There was a possibility that the presence of imidazole was stopping TEV from cleaving PriA. To see if this was occurring the PriA went through micro-dialysis overnight to remove a majority of the imidazole. The results of the gel run with these proteins showed a cleaved PriA but the PriA band could not be visualized.

Sca I digest- To make sure that the insert was present in the *E. coli* cells Sca I was added to each candidate. If the DNA of the cells were cut then the insert had not been successfully transformed into *E. coli,* the inserted segment removed the Sca I site, if the DNA was not cut then the insert was present in *E. coli.* All of the candidates were tested and ML344 #2 was selected as the best; however there was still some uncertainty in the orientation in which the insert was in the plasmid. Candidate #2 was also a good choice because it had already been transformed into the Rosetta 2 BL21 (DE3) cells.

Size-Exclusion Chromatography- The resulting chromatograph following the size exclusion chromatography of the wildtype PriA, showed a peak at 76.15mL as demonstrated in Figure 4. This peak was recognized as the Kpn PriA based off of

previous studies that had been performed. The fractions containing this protein were 23, 24, and 25, these fractions were then combined and concentrated in a Centerprep YM3. The concentrated protein was read using a UV/vis spectrophotometer at 280nm and the concentration was determined to be 7407 nM.
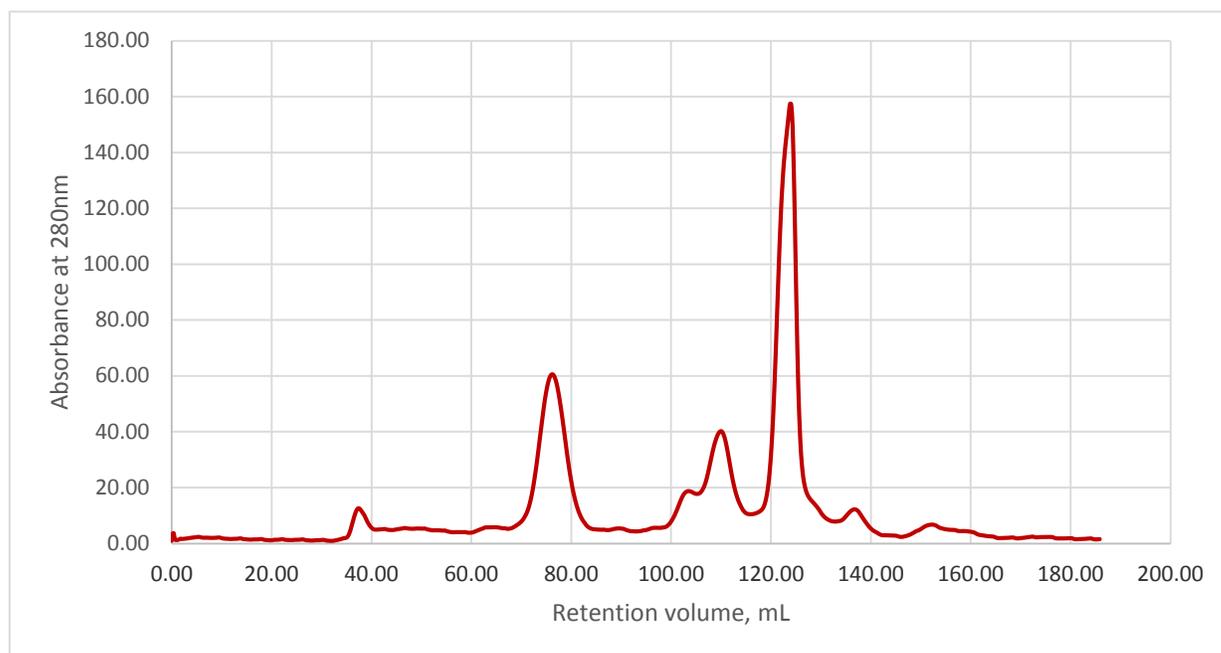

Figure 4: ML313 wildtype PriA Chromatograph from size-exclusion chromatography

Another size exclusion chromatography column was performed on ML344, the peak representing PriA was found at 75.20mL. The fractions 23, 24, and 25 were pulled and concentrated. The concentration of the purified protein was found to be 25,054 nM using the Beer-Lambert law and an extinction coefficient of 110,160 $M^{-1}cm^{-1}$.

The proteins were then run on a protein gel to determine the relative purities of both of the proteins. The protein gel showed that both PriA and N-tev-PriA had a purity of approximately 95%. This was determined by comparison to a 10 fold dilution of each protein in the same gel.

Helicase Assays- Based on the concentrations of the proteins found using UV spectrophotometry, serial dilutions of each protein were made in accordance to the helicase assay protocol. For the wildtype PriA it was expected that as the concentration of the proteins increased the DNA would unwind more until it reached a maximum level of unwinding. The actual results from the helicase assays showed gradual unwinding as the concentration increased, however the fraction unwound was never as high as suspected. The original plan was to perform three trials of the helicase assays for the wildtype; however the data deviated too drastically from trial to trial, so 10 trials were performed to get a good average for the unwinding ability of the wildtype PriA.

Just like the wildtype the concentration of N-tev-PriA was used to make appropriate serial dilutions to ensure that the same concentrations were used for all helicase assays. This was done so that the data could accurately be compared. Only three trials were necessary for the N-tev-PriA helicase assays because the data was consistent enough across the trails that triplicate data was sufficient. The results of both sets of helicase assays can be seen in Figure 5. The results showed that there was some difference in the trend associated with N-tev-PriA when using PriA as a control comparison.
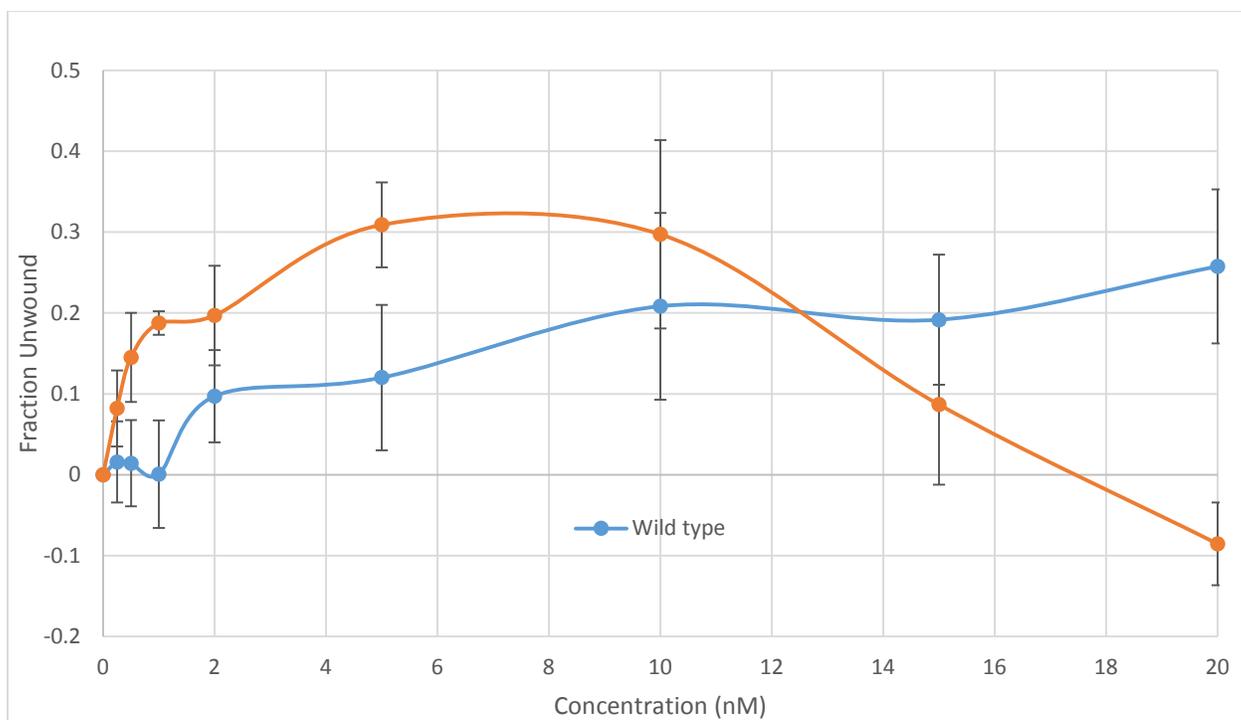
Figure 5: Helicase assay data for the wildtype PriA and N-tev-PriA

 DNA Binding Assays- Due to the disagreement between the helicase activity of

wildtype PriA and N-tev-PriA DNA binding assays were used to monitor the binding

activity of each protein and compare their bind affinities. As the concentration of protein

increases there should be increased anisotropy, meaning that more of the DNA should be

binding to the protein, until it reaches a maximum anisotropy. Once again the

concentrations determined using UV spectrophotometry were used to create the correct

serial dilutions of both proteins for the DNA binding assay protocol. Three trials of DNA

helicase assays were performed for each of the proteins. The data recorded showed that

both proteins followed the expected trend of increased binding affinity until a certain

maximum value (Figure 6). Due to the agreement between the wildtype PriA and N-tev-

PriA in the DNA binding assays, no conclusions about why the helicase assay data

differed could be reached.
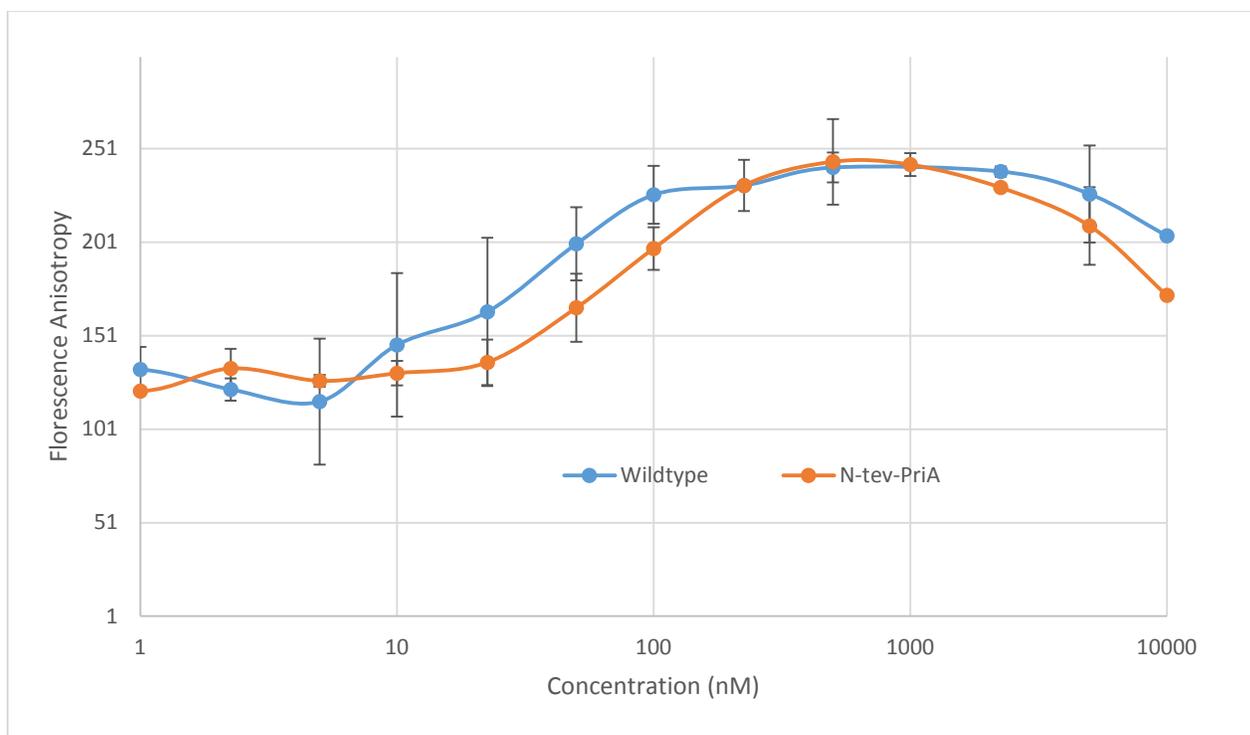
Figure 6*:* DNA binding assay data

ATPase Activity determination- Since the data for the DNA binding assays did not provide any insight into why the helicase assays differed for the two proteins the ATPase activity of each version of PriA was tested. The results of these assays showed that both PriA and N-tev-PriA had sufficient ATPase activity to support the role of the helicase domain of PriA.

Even though there was a significant difference between the helicase activity of PriA and the N-tev-PriA, it was determined that lengthening the N-terminal tether of the wing helix domain had no effect on the overall activity of PriA. This goes against the hypothesis that there would be a change in PriA's ability to restart DNA replication. It was previously determined that lengthening the C-terminal tether had no effect on the role of PriA. This made sense because the C-terminal is greater in length than the N-terminal and it was thought that the N-terminal was holding the winged helix in place.

This data shows that there must be something else that forces the wing helix to bind to a forked DNA structure.

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