4-9-2016

Usability for the AARP Facebook User Guide,

The Use of a Molecular Probe to Investigate the Details of PriA Helicase Function

Luke F. Bugada[1,2,3] and Matthew E. Lopper, Ph.D.[4]

[1]Department of Chemical Engineering, [2]Berry Summer Thesis Institute, [3]University Honors Program, [4]Department of Chemistry

Abstract

During DNA replication in both eukaryotic and prokaryotic cells, the replication machinery (replisome) invariably encounters structural DNA damage, an event that can result in disbanding of the replisome and the creation of a collapsed replication fork. In order for DNA replication to continue, the replisome must be reloaded onto the DNA strand, a process that often begins with unwinding of double-stranded DNA (dsDNA) by the primosome protein PriA. Little is known about the mechanism through which PriA unwinds dsDNA and begins replisome recruitment. We seek to shed new light on this mechanism through the use of a PriA inhibitor, compound 0207. In our study, we attempt to determine the method of inhibition, the three-dimensional structure of the PriA•0207 complex, and the 0207 binding site through steady-state kinetics experiments, x-ray crystallography experiments, and mutagenesis assays. Data from the steady-state kinetics titrations show that compound 0207 acts through a mixed mode of inhibition and binds to the PriA•ATP, PriA•DNA, and PriA•ATP•DNA complexes with equal affinities. PriA crystals have been grown in the presence of compound 0207 in an attempt to solve the three-dimensional structure of the PriA•0207 complex using x-ray crystallography. Finally, a docking simulation based on steric interactions was used to identify possible 0207 binding sites. To verify these results, single alanine substitutions of PriA were generated, each with an alteration designed to inhibit the binding of compound 0207. With these results, we seek to provide a more complete understanding of the interactions between PriA and compound 0207, which will contribute to the overall goal of understanding the detailed mechanisms through which PriA catalyzes dsDNA unwinding to initiate replication restart.

Background

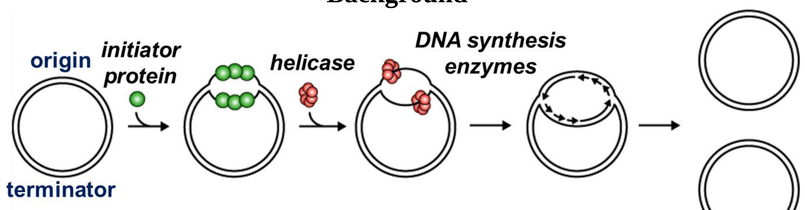


Figure 1: Replication of the bacterial chromosome begins with helicase recruitment at the origin. Replication proceeds along the circular chromosome in both directions until replication is complete.

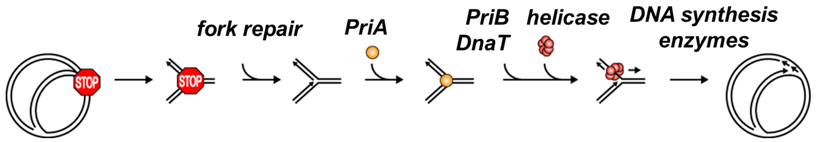


Figure 2: Often, the replication machinery (replisome) dissociates from the DNA, resulting in a collapsed replication fork (represented by the stop sign). In order for DNA replication to continue, the replisome must be reloaded back onto the chromosome. PriA serves as a key initiator of this pathway as it binds to the collapsed fork and unwinds a portion of double-stranded DNA. The protein then recruits PriB and DnaT, forming a complex that reloads the replisome onto the collapsed replication fork so DNA replication can continue.

Goal

Helicases are incredibly small and dynamic protein machines that move at very high speeds while unwinding DNA. This makes studying these proteins challenging. We are using a small molecule to "freeze" PriA in the process of unwinding DNA, so that we can more easily study PriA's DNA unwinding mechanism.

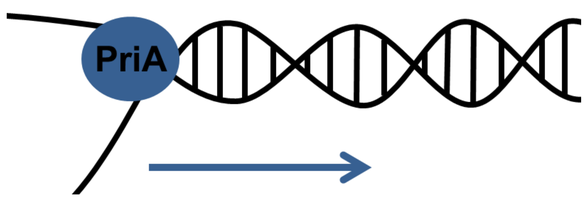


Figure 3: PriA initiates replisome reloading by unwinding a short segment of DNA. PriA changes conformations at very high rates during this process.

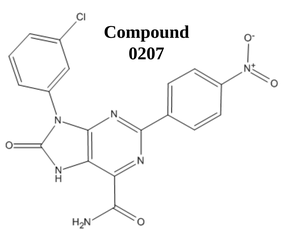
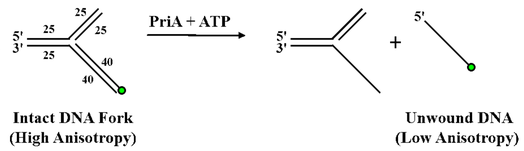


Figure 4: Compound 0207 is a small molecule capable of inhibiting the helicase action of PriA. Compound 0207 was identified using a high throughput screen that scanned for compounds able to inhibit the helicase activity of PriA. This inhibitor is being used to lock PriA in an intermediate conformation in order to better study PriA's helicase action.

Results

Compound 0207 inhibits PriA helicase activity with an IC_{50} of 2.8 µM

Figure 5: Helicase Assays were used to indirectly measure the fraction of DNA unwound through fluorescence anisotropy. The green circle represents the fluorescein label, and the numbers represent the number of base pairs that comprise each oligonucleotide.



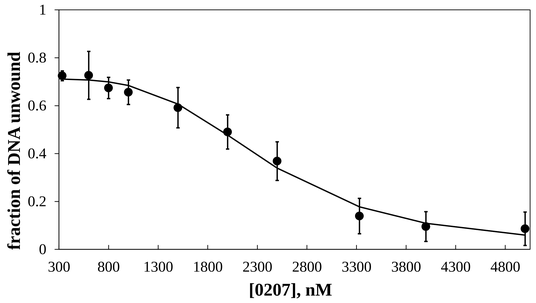


Figure 6: Compound 0207 inhibits PriA with an IC_{50} of 2.8 µM. Helicase assays were used to determine the IC_{50} of compound 0207. PriA was incubated with duplex DNA in the presence of varying concentrations of 0207, and the fraction of double-stranded DNA unwound by PriA was measured.

Compound 0207 does not bind to the ATP binding site of PriA

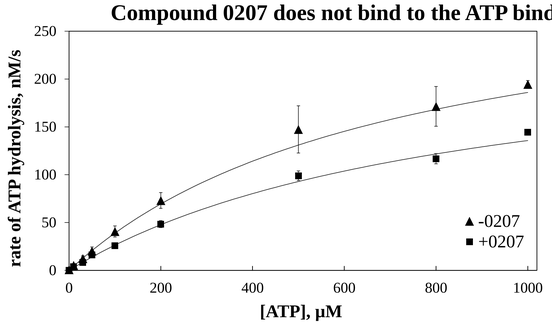


Figure 7: Compound 0207 acts through a mixed mode of inhibition with respect to ATP binding. ATPase assays were used to determine the mode of inhibition. PriA, single-stranded DNA, and compound 0207 were incubated with varying concentrations of ATP, and the rate of ATP hydrolysis was measured.

Compound 0207 does not bind to the DNA binding site of PriA

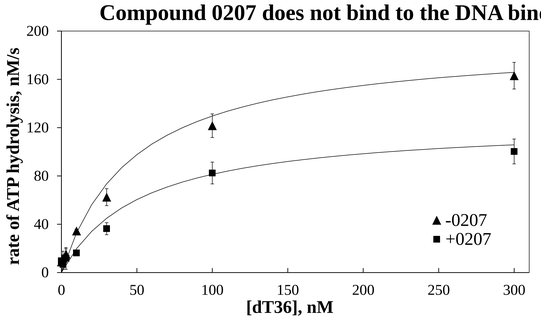


Figure 8: Compound 0207 acts through a mixed mode of inhibition with respect to DNA binding. ATPase assays were used to determine the mode of inhibition. PriA, ATP, and compound 0207 were incubated with varying concentrations of single-stranded DNA, and the rate of ATP hydrolysis was measured.

0207 Dissociation Constants	
Complex	**K_d value (µM)**
PriA•ssDNA	16.6
PriA•ATP	15.9
PriA•ssDNA•ATP	18.0

Table 1: Dissociation constants for 0207 inhibition of PriA. Compound 0207 acts through a mixed mode of inhibition with respect to both ATP and ssDNA binding and binds to the PriA•ssDNA, PriA•ATP, and PriA•ssDNA•ATP complexes with approximately equal affinities.

Crystallography Experiments

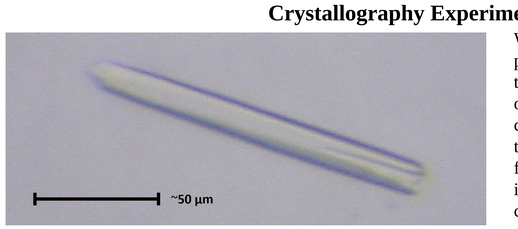


Figure 9: PriA crystals grown in the presence of compound 0207

We have grown PriA crystals in the presence of compound 0207. We hope to find the three dimensional structure of the PriA•0207 complex and are currently refining the structure based on the electron density maps generated from our crystals. Unfortunately, there is not yet any evidence of either compound 0207 or ADP in the electron density maps.

Crystallography Experiments

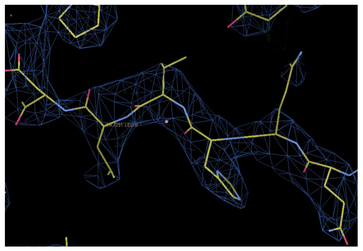


Figure 10 (left): The PriA structure is built by fitting atoms into the electron density maps. The electron density maps were generated by passing an x-ray beam through PriA crystals grown in the presence of compound 0207 and measuring the diffracted beams.

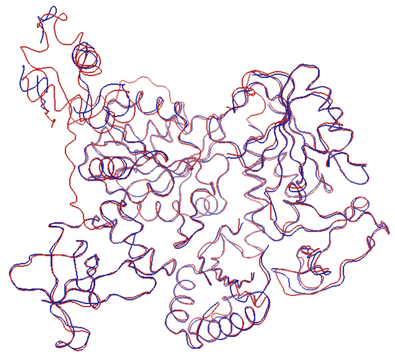
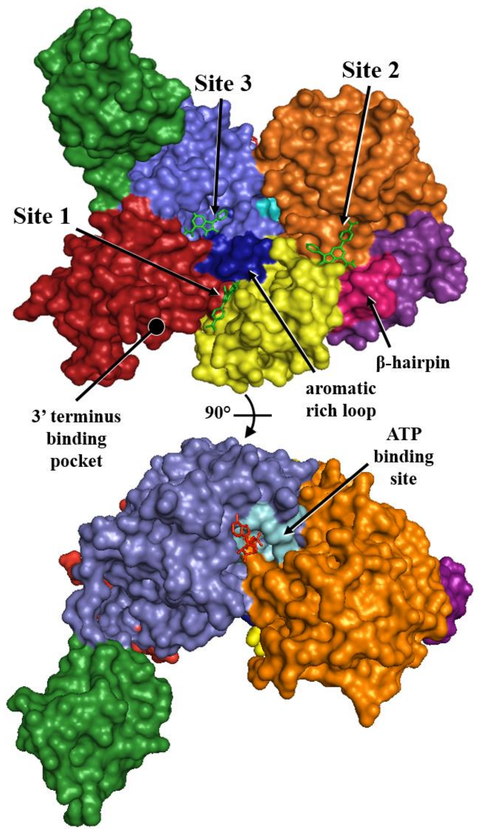


Figure 11 (right): Comparison of the apo structure of PriA and the structure in the presence of compound 0207. The apo structure is shown in red and the 0207 PriA structure in blue. The current model for PriA crystals grown in the presence of compound 0207 does not exhibit any major structural differences compared to the apo structure. The RMSD for the backbone atoms is 0.594 Å.

Mutagenesis Assays



A docking simulation was run to find likely 0207 binding sites. The amino acid residues at these locations have been altered to prevent 0207 binding. Helicase assays will be used to see if the mutant PriA proteins are resistant to the inhibitor.

Figure 12: PriA helicase structure and possible 0207 binding sites. ADP is shown as red sticks and compound 0207 as green sticks. The DNA binding-domain consists of the winged helix domain (green) and the 3' binding domain (red). The helicase domain is made of helicase lobes 1 (blue) and 2 (orange), the Cys-rich region (purple), and the C-terminal domain (yellow). The aromatic rich loop is shown in dark blue, the β-hairpin in pink, and the ATP binding site in light blue. Compound 0207 is shown in the three most promising binding sites.

Conclusions

- Compound 0207 is an excellent inhibitor of PriA helicase activity.
- Compound 0207 binds to the PriA•ATP, PriA•DNA, and PriA•ATP•DNA complexes with equal affinities.
- Compound 0207 does not inhibit the helicase activity of PriA by directly competing with the binding of ATP or DNA.
- Therefore, compound 0207 is binding to PriA at a site separate from the ATP and DNA binding sites and could be inhibiting the helicase action of PriA by locking the protein in an intermediate conformation.
- Further investigating how compound 0207 inhibits PriA helicase action could shed light on the mechanism of PriA catalyzed DNA unwinding.