INVESTIGATION OF THE STRUCTURAL MECHANISMS OF A BACTERIAL CLAMP LOADER

A Dissertation Presented

By

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This work was undertaken in the Morningside Graduate School of Biomedical Sciences

Program of Biochemistry and Molecular Biotechnology

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rough days, you have helped me through so many things. You enable me to be a true version of myself, laughing at my jokes, supporting my hobbies, and dealing with my inability to plan. Thank you for taking all my calls as I leave work and talking to me the whole ride home. Thank you for agreeing that my life is so hard, even though you wake up before me, deal with legendarishly challenging people (Surgeons), and occasionally, need to literally restrain people. You really have done more for me than you can imagine, and I would not have made it to this point without you.

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Abstract
The sliding clamp is an integral protein in DNA replication and repair, where it increases the speed of DNA synthesis and serves as a scaffold for repair proteins. Since the sliding clamp is a closed ring, clamp loaders must open the ring and then close it around DNA. The clamp loader and sliding clamp are the only two components of the replisome that are conserved across all three domains of life. However, differences in their structures and biochemical activities suggest that the mechanisms of opening and loading differ between prokaryotes and eukaryotes. Structures of the eukaryotic clamp loader clearly illustrate its clamp loading mechanism, but there were no comparable structures for bacterial clamp loaders. To understand how mechanisms of bacterial clamp loaders compare to their eukaryotic counterparts, I determined a series of structures of the *E. coli* clamp loader at distinct stages in clamp loading. To understand how ATP binding enables the clamp loader to bind to the sliding clamp, I determined a structure of the *E. coli* clamp loader bound to a non-hydrolyzable ATP analog. I found that the *E. coli* clamp loader opens its sliding clamp at a single pivot point into a planar conformation, but transitions to a helical conformation upon binding primer-template (p/t)-junctions. This behavior contrasts with eukaryotic clamp loaders, which open their sliding clamp through multiple pivot points into a helical conformation before binding p/t-junctions. My work also revealed that like the eukaryotic clamp loader, the *E. coli* clamp loader does not need to undergo a conformational change to close the sliding clamp on p/t-junctions. Furthermore, I
explored how the *E. coli* clamp loader is inhibited by the bacteriophage protein gene product 8. This dissertation explores the structural mechanisms used by the bacterial clamp loader and illuminates similarities and differences between clamp loaders across the domains of life.
# Table of Contents

Acknowledgements ........................................................................................................ i
List of Tables .................................................................................................................. viii
List of Figures ................................................................................................................ viii

## Chapter I: Introduction
- Preface ......................................................................................................................... 1
- DNA Replication ........................................................................................................... 3
- Structure of DNA .......................................................................................................... 3
- DNA replication mechanism ....................................................................................... 4
- The clamp loader and sliding clamp ........................................................................... 6
- Clamp Loaders are AAA+ ATPases ............................................................................ 15
- Outstanding questions and research scope ............................................................... 27

## Chapter II: Differences in clamp loader mechanism between bacteria and eukaryotes
- Introduction: .................................................................................................................. 32
- Materials and Methods ............................................................................................... 38
- Results ........................................................................................................................... 57
- Discussion: .................................................................................................................... 81

## Chapter III: Inhibition of the E. coli clamp loader by N4 phage protein gene product 8
- Preface ........................................................................................................................... 96
- Introduction: .................................................................................................................. 97
- Materials and Methods ............................................................................................... 98
- Results ........................................................................................................................... 100
- Discussion: ................................................................................................................... 138

## Chapter IV: Discussion
- Preface ........................................................................................................................... 144
- Thesis Summary: .......................................................................................................... 146
- Summary: Mechanism of the E. coli clamp loader .................................................... 147
Open Questions: *E coli* clamp loading mechanism...........................................148
How does gp8 inhibit the *E. coli* clamp loader?..............................................162
Concluding Remarks................................................................................................168
Appendix I: Cryo-EM study of the *E. coli* clamp loader bound to ADP•BeF₆......169
Preface....................................................................................................................170
Introduction:............................................................................................................171
Materials and Methods........................................................................................172
Results....................................................................................................................174
References..............................................................................................................199

List of Tables

Table 1.1 Conservation of different components of the replisome
Table 2.1 Oligonucleotides used in this study
Table 2.2 Cryo-EM sample preparation condition
Table 2.3 Cryo-EM data collection, processing, and model statistics
Table 3.1 Buffer compositions used to wash the His-SUMO-gp8

List of Figures

Figure 1.1 Comparison of bacterial and eukaryotic sliding clamps
Figure 1.2 General architecture of clamp loaders and sliding clamps
Figure 1.3. Architecture of the Bacterial and Eukaryotic Replication Fork
Figure 1.4. Schematic of an ATPase motor
Figure 1.5 Schematic of the clamp loading mechanism
Figure 1.6. Schematic comparing the phage lytic and lysogenic cycles of replication.
Figure 2.1. General architecture and mechanism of clamp loaders and sliding clamps
Figure 2.2. Characterization and cryo-EM map validation of the bacterial clamp loader

Figure 2.3. Structures of the clamp loader/sliding clamp complex throughout the process of clamp opening

Figure 2.4. Schematic of the Cryo-EM processing workflow for the Clamp Loader, Sliding Clamp, ATPγS dataset

Figure 2.5. Schematic of the Cryo-EM processing workflow for the Clamp Loader, Sliding Clamp, ADP•BeF₆ dataset

Figure 2.6. Clamp loader/sliding clamp complex bound to p/t-DNA or p/t-RNA

Figure 2.7. Schematic of the Cryo-EM processing workflow for the Clamp Loader, Sliding Clamp, p/t-RNA, and ADP•BeF₆ dataset

Figure 2.8. Schematic of the Cryo-EM processing workflow for the Clamp Loader, Sliding Clamp, p/t-DNA, and ADP•BeF₆ dataset

Figure 2.9. Conformational change induced by p/t-junction binding

Figure 2.10. Structure of the Altered-Collar conformation of the bacterial clamp loader

Figure 2.11. Closing the sliding clamp

Figure 2.12. Examination of ATPase activation mechanism

Figure 2.13. Updated bacterial sliding clamp loading mechanism

Figure 2.14. Comparing Bacterial and Eukaryotic clamp loader mechanisms

Figure 3.1. Protein and DNA imaging of gp8 purified under different conditions.

Figure 3.2. Untreated gp8 agarose gel migration

Figure 3.3. Purified gp8 treated with nuclease

Figure 3.4. Effect of treating clamp loaders with the purified Gp8 sample

Figure 3.5. Cryo-EM data processing Clamp loader, sliding clamp, Gp8, ATPγS sample

Figure 3.6. 3D Reconstructions from the Clamp Loader from the Clamp Loader:Gp8:ATPγS dataset

Figure 3.7. Extraneous density on the sliding clamp reconstructions only in the gp8 dataset
Figure 3.8. Alpha and Alphafold Multimer Prediction of gp8 and gp8 in complex with the delta subunit.

Figure 3.9. PydockDNA models of gp8 bound to dsDNA and composite gp8:clamp loader:DNA models.

Figure 4.1. All states of the clamp loader, sliding clamp, ADP•BeF₆ identified in the clamp loader, sliding clamp, ADP•BeF₆ dataset.

Figure 4.2. Comparison of potential antibiotic target sites between bacterial and eukaryotic clamp loaders.

Figure A1.2. 3D-Reconstruction of the Clamp Loader ADP•BeF₆ complex.

Figure A1.3. Fit of the Clamp Loader ADP•BeF₆ model into the 3D reconstruction.

Figure A1.4. Conformational differences between the ADP•BeF₆ bound clamp loader and previously determined structures.
Chapter I: Introduction
Preface

Contributors: Jacob Landeck

I prepared the content and figures in this chapter.
DNA Replication

The faithful replication of genetic information is essential across all forms of life. It is not only necessary for this process to occur rapidly but with extremely high fidelity. To ensure this fidelity, there is a highly coordinated repertoire of DNA replication and repair proteins responsible for this process. These proteins enable cells to produce, proofread, and edit the millions of base pairs necessary in each round of replication.

Structure of DNA

The DNA stored in cells is stored as double stranded molecules, where the two strands of the molecule twist around each other forming a helix (Franklin & Goslino, 1953; Watson & Crick, 1953). The DNA strands have directionality as one end has a terminal 3’ hydroxyl group and the other end has a 5’ phosphate. The two strands bind to each other in opposite directions and so they are called antiparallel. The 5’ hydroxyl group is necessary for the addition of nucleotides, forcing DNA to be synthesized 5’ to 3’. DNA replication must work around these structural elements of DNA.

DNA replication does not begin at the ends of a DNA molecule, but in the middle of it. This site is called the Origin of Replication, where the two DNA strands are separated, forming a replication bubble where the middle of the bubble consists of single-stranded DNA (ssDNA) and the ends of the bubble are double stranded DNA (dsDNA) (Ekundayo & Bleichert, 2019; Méchali, 2010). As DNA replication progresses, the dsDNA ends of the replication bubble are
continuously separated producing new ssDNA for DNA synthesis. Where the
dsDNA is separated into ssDNA is called a replication fork. Since DNA is
antiparallel, each fork has one strand of ssDNA going 5’ to 3’ and the other going
3’ to 5’. This situation creates a challenge during replication since DNA can only
be synthesized on the 3’ end of the molecule. The strand in the 5’ to 3’ direction
can be synthesized and is called the leading strand. However, the strand in the 3’
to 5’ direction cannot be continuously synthesized and is called the lagging
strand. As the replication fork progresses, sections of ssDNA on the lagging
strand and are synthesized in short discontinuous fragments called Okazaki
fragments (Okazaki et al., 1967), which vary in length from 100-300 base pairs
(bp) in eukaryotes to 100-2000 base pairs in bacteria (Balakrishnan & Bambara,
2013; Reha-Krantz, 2013). The replication bubble progresses along the entire
length of the DNA molecule continuously producing ssDNA to serve as a
template for DNA replication. Eventually the replication forks progress to the ends
of the DNA molecules and produce double helices of DNA, each containing one
of the original strands of DNA and one newly synthesized strand of DNA.

**DNA replication mechanism**

The progression of the replication fork and synthesis of new DNA requires
many proteins which are collectively called the replisome. Replisomes in all
domains of life have the central components: 1) helicase, 2) primase, 3) DNA
polymerase, 4) sliding clamp, 5) sliding clamp loader, and 6) single-stranded
the two strands of dsDNA and are responsible for progressing the replication fork (ABDEL-MONEM et al., 1976; Arias-Palomo, O’Shea, et al., 2013; Lebowitzs & Mcmackeng, 1986). Primases begin the new strand of DNA by placing a short fragment of RNA onto ssDNA (Frick & Richardson, 2001). Polymerases synthesize new DNA extending from the RNA primer (Wu et al., 2017). Sliding clamps are protein complexes that act as scaffolds for DNA polymerases and other DNA replication and repair proteins (Kong et al., 1992). Clamp loaders are responsible for loading sliding clamps onto DNA (Stukenberg et al., 1991). Finally, ssDNA binding proteins bind to the exposed ssDNA and prevent the ssDNA from creating structures that prohibit DNA replication (Meyer & Laine, 1990).

*Differences between Bacterial and Eukaryotes/Archea*

Previous work has revealed differences between DNA replication in bacteria, eukaryotes, and archaea. Bacterial DNA replication requires fewer individual proteins and is simpler than the eukaryotic DNA replication system (N. Y. Yao & O’Donnell, 2016). Although both systems contain proteins that perform the same basic functions within the DNA replication regime, it is surprising that most of these core components are not conserved or are only poorly conserved.
<table>
<thead>
<tr>
<th>Replication Stage</th>
<th>Protein</th>
<th>Bacterial</th>
<th>Eukaryotes</th>
<th>Conservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation</td>
<td>Helicase Loader</td>
<td>DnaA</td>
<td>Orc1-6</td>
<td>Non-orthologous</td>
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<td>DnaB</td>
<td>MCM2-7</td>
<td>Non-orthologous</td>
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<td>Polymerase</td>
<td>Pol III</td>
<td>Pol ε, Pol δ</td>
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<tr>
<td></td>
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<td>Hola, Holb, Holc, Hold, DnaX</td>
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<td>PCNA</td>
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<td>ssDNA-binding protein</td>
<td>SSB</td>
<td>RPA</td>
<td>Non-orthologous</td>
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Table 1. Conservation of different components of the replisome. (Leipe et al., 1999; Stephen et al., 2017)

**The clamp loader and sliding clamp**

*The sliding clamp*

While DNA polymerases have the glamorous job of synthesizing new DNA as well as and repairing damaged DNA, they are not capable of performing these functions on their own because of their low speed and processivity. However, the rate of DNA synthesis by the bacterial replicative polymerase increases by up to ~100 fold when the polymerase is associated with the sliding clamp (Fay et al., 1982; Tanner et al., 2008; N. Y. Yao et al., 2009).

Sliding clamps form stable closed rings in solution which are only active when they encircle DNA (Georgescu et al., 2008; Kong et al., 1992; TS et al.,
1994; N. Yao et al., 1996). Although the amino acid sequence of sliding clamps is not conserved across the domains of life, their overall structure is (Figure 1.1). Sliding clamps are composed of six globular domains, which form a ring with a pore ~40 Å wide (Kong et al., 1992; R Krishna et al., 1994). In eukaryotes, the replicative sliding clamps (Proliferating Cell Nuclear Antigen or PCNA) are homotrimers where each subunit has two globular domains (Moarefi et al., 2000; R Krishna et al., 1994). In bacteria, the sliding clamp (β-clamp) is a dimer where each subunit has three globular domains (Kong et al., 1992). The subunits are arranged in a head-to-tail arrangement such that the C-terminal domain of one subunit is adjacent to the N-terminal domain of its neighboring subunit. The head-to-tail arrangement ensures that the two faces of the sliding clamp are distinct. For both PCNA and the β-clamp the C-terminal face interacts with the majority of its binding partners (Indiani et al., 2005).
Figure 1.1 Comparison of bacterial and eukaryotic sliding clamps. A. The bacterial sliding clamp is a homodimer where each subunit has three domains. B. The eukaryotic sliding clamp is a homotrimer, where each subunit has two domains.
The clamp loader

The clamp loader complex is responsible for loading sliding clamps onto primer-template (p/t)-junctions during DNA replication (Stukenberg et al., 1991). Although only one sliding clamp is loaded onto the leading strand during replication, the clamp loader must continuously load sliding clamps onto the lagging strand to ensure the two strands are replicated in unison. The clamp loader binds to closed sliding clamps and must open and load them onto p/t-DNA (Turner et al., 1999a). However, even sliding clamps that are primarily open in solution require clamp loaders to be loaded onto DNA (Alley et al., 1999).

Clamp loaders are pentameric complexes with a generally well conserved architecture. They are members of the ATPases associated with diverse cellular activities (AAA+ proteins) protein family, a superfamily of proteins that functions in all domains of life (Khan et al., 2022). The five subunits of the clamp loader can universally be called A-E in a counter-clockwise direction around the complex (Figure 1.1B). The complex has two regions, the collar domain and the AAA+ module. The collar domain serves as an oligomerization domain holding the complex together (Bowman et al., 2004a; Jeruzalmi, O’Donnell, et al., 2001). The AAA+ module is responsible for both binding and opening the sliding clamp as well as binding and hydrolyzing ATP (Bowman et al., 2004a; Simonetta et al., 2009). The ATP module is composed of two separate domains, the Rossmann domain and the Lid domain. Unlike many hexameric AAA+ ATPases, the clamp loader does not form a complete ring, instead there is a gap between the AAA+
modules of the A and E subunits called the A-Gate. The A-gate allows DNA to enter the central chamber of the clamp loader, which triggers ATPase activity. ATP hydrolysis causes closure of the sliding clamp and ejection of the clamp loader, resulting in a sliding clamp loaded onto DNA.

Although the general architecture of clamp loaders is conserved, clamp loaders from different domains of life have differing features (Figure 1.2). The T4 phage clamp loader has the simplest composition where it is composed of two different proteins, gp44 in the A position and gp62 in positions B-E. The primary bacterial clamp loader is composed of four distinct proteins: Tau (τ)/gamma (γ), delta (δ), and delta prime (δ’) (Onrust et al., 1995). Two other accessory subunits, Psi (ψ) and Chi (χ), are dispensable for clamp loading and are not present in many bacteria. Both τ and γ are products of the dnaX gene, where γ is produced by a programmed post translational frame shift that truncates 212 residues on the C terminal region of the protein (Blinkowa & Walker, 1990; Tsuchihashi & Kornberg, 1990). The stoichiometry of this complex is (τ/γ):δ:δ’:ψ:χ. The τ, χ, and ψ subunits are dispensable for the clamp loading activity in vitro and so the γ3:δ:δ’ complex (often called the gamma complex) is the minimal functional assembly (Jeruzalmi, O’Donnell, et al., 2001; Onrust et al., 1995; Xiao et al., 1995). The complex is assembled such that δ is in position A, γ or τ is in positions B through D, and δ’ is in position E (Jeruzalmi, O’Donnell, et al., 2001). The ψ protein directly binds to the collar of the clamp loader between the A and B subunits and the χ subunit binds to ψ (Gulbis et al., 2004; Simonetta
et al., 2009). The eukaryotic clamp loader complex is called Replication Factor C (RFC), and each subunit is called RFC1-5. Each of the proteins that comprise RFC is unique.

Amongst the different subunits, there are the most differences between the A subunits across clamp loaders. In T4 and eukaryotes the A subunit has an additional domain compared to the bacteria subunit called the A' domain (Figure 1.2) (Bowman et al., 2004b; Kelch et al., 2011). This domain extends from the collar across the A-gate and contacts the E subunit. An additional difference between the bacterial and eukaryotic clamp loaders is how these complexes bind ATP. In the bacterial clamp loader only the γ subunit (positions B through D) binds to ATP, and so the gamma complex binds to three ATP molecules (Jeruzalmi, O’Donnell, et al., 2001). In contrast, RFC binds to four ATP molecules and, surprisingly, one GDP molecule in subunit E (Bowman et al., 2004b; Schrecker et al., 2022). It was unknown whether these differences in subunit stoichiometry result in altered clamp loading mechanisms. Part of the goal of this Dissertation is to examine this possibility.
Figure 1.2 General architecture of clamp loaders and sliding clamps. Comparison of bacterial, eukaryotic, and bacteriophage clamp loaders. A representative cartoon representation (Top) and a Model (Bottom) is shown of a bacterial, eukaryotic, and bacteriophage clamp loader. Clamp loaders are pentamers with each subunit consisting of a AAA+ module and a collar domain. The “A-gate”, a gap between the A and E subunits, is where p/t-junctions enter. The A' domain is unique to eukaryotic and bacteriophage clamp loaders.
Architecture of the *E. coli* clamp loader at the DNA replication fork

The *E. coli* clamp loader is distinct from eukaryotic clamp loaders since it plays a structural role in organizing the replication fork (Figure 1.3) (Maki et al., 1988; N. Y. Yao & O'Donnell, 2016). The DNA polymerase III holoenzyme consists of two copies of a core polymerase III composed of the α, ε, and θ proteins, and single clamp loader complex composed of (τ/γ)_3, δ, δ', ψ, χ, the DNA helicase, and two sliding clamps (Maki et al., 1988). The τ subunits and ψ:χ complex help coordinate the topology of the replication fork (Cull & McHenry, 1995; Dohrmann et al., 2016; Gulbis et al., 2004). The τ subunit binds to the α subunit of the polymerase and the DNA helicase dnaB (D. Gao & McHenry, 2001a, 2001b). This allows the clamp loader to directly couple the progression of the replication fork with the polymerases responsible for the synthesis of the lagging and leading strands. The χ:ψ heterodimer interacts with single-stranded DNA-binding proteins (SSB), thus tightly tethering the clamp loader to the lagging strand (Kelman et al., 1998).
Figure 1.3. Architecture of the bacterial and eukaryotic replication fork. 
A. Cartoon representation of a bacterial replication fork. B. Cartoon representation of a eukaryotic replication fork.
Clamp Loaders are AAA+ ATPases

Clamp loaders are members of the AAA+ (ATPases Associated with various cellular Activities) family of ATPases. AAA+ proteins bind and hydrolyze ATP to perform their various activities (Hanson & Whiteheart, 2005). AAA+ ATPase are members of the larger Addition strand conserved E family (ASCE) of ATPase (Hanson & Whiteheart, 2005). These proteins are involved in almost every process within the cell and are found across the different domains of life.

The core structural features that allow clamp loaders to bind and hydrolyze ATP are highly conserved and are thought to predate that last universal common ancestor. The P-loop NTPase superfamily of proteins includes kinases and GTPase as well as AAA+ proteins (N. Y. Yao & O'Donnell, 2016). P-loop NTPases have two key structural features, the Walker A and Walker B motifs. The Walker A loop is responsible for binding nucleotide and the Walker B motif coordinates an Mg$^{2+}$ ion, which is essential for hydrolysis (Miller & Enemark, 2016; Wendler et al., 2012). AAA+ proteins have additional elements in their catalytic core. The Second Region of Homology (SRH) contains the sensor 1 motif which helps to orient the activated water molecule to cleave the γ-phosphate (Miller & Enemark, 2016; Wendler et al., 2012). The sensor I motif also contains an “arginine finger” residue (Miller & Enemark, 2016; Wendler et al., 2012). This residue acts in the ATP binding pocket of the adjacent molecule in the complex and is necessary for ATP hydrolysis. The sensor 2 residue (usually
an arginine) mediates conformational changes related to nucleotide binding and hydrolysis. The sensor 3 feature likely plays a role in stabilizing the ATP bound state (Gates & Martin, 2020a). The features either act in cis (i.e. functioning in the active site on the same subunit) or in trans (i.e. functioning in the active site of an adjacent subunit). The Walker A, Walker B, sensor 1 features all act in cis, whereas the arginine finger acts in trans. Sensor 2 and sensor 3 can act in either cis or trans.

Recent structural studies of AAA+ proteins have brought new insight into the mechanism behind how these proteins utilize ATP hydrolysis to perform their various functions (S. Li et al., 2021; Qiao et al., 2022; Santosh et al., 2020; Villarreal et al., 2013). AAA+ proteins function as either motors or switches (Hanson & Whiteheart, 2005; Kelch et al., 2012), although the mechanistic underpinnings of these different functions remain unknown. A unifying feature of AAA+ proteins is to link conformational changes induced by the binding and/or hydrolysis of ATP to mechanical work. In the case of the clamp loader, the major mechanical step is the opening of the sliding clamp.

**AAA+ Motors**

Many helicases, unfoldases, and viral genome packaging motors are AAA+ proteins that function as molecular motors. These complexes, form closed rings are either pentamers, like the viral DNA packaging protein TerL (Hilbert et al., 2015), or hexamers like Lon protease (S. Li et al., 2021). Most motors pull the
substrate through the center of the AAA+ ring using ATP binding, hydrolysis, and exchange to coordinate the translocation of the substrate with gripping and release of the substrate to ensure the substrate moves unidirectionally (Hanson & Whiteheart, 2005). This mechanism is called a “spiral-staircase” and is similar to pulling a rope with a hand-over-hand motion (Figure 1.4) (Puchades et al., n.d.). The six subunits of the complex are in one of four states: ATP-bound, ADP+P\(_i\)-bound, ADP-bound, or apo (Gates & Martin, 2020b). When the subunits are bound to ATP, they grip the substrate tightly; when the subunits are either in an ADP bound or apo state, they bind the substrate weakly or not at all. The nucleotide state correlates with the vertical positioning of the subunits in the spiral staircase. The two highest subunits relative to the substrate are the apo and the adjacent ATP bound subunit. The three consecutive ATP-bound subunits are in descending height relative to each other. Finally, the ADP bound subunit is the lowest in the complex. It is necessary for both the ADP-bound and apo states to be released from the substrate to allow these subunits to rise to the top of the complex without translocating the substrate backwards through the pore.
Figure 1.4. Schematic of an ATPase motor. ATP hydrolysis occurs in a counterclockwise direction around the complex. An arginine finger acts in trans to activate ATP hydrolysis. ATP hydrolysis coordinates substrate binding and release which translocate a substrate through the central pore.
**AAA+ proteins as molecular-switches**

Although AAA+ motors continuously use ATP hydrolysis to translocate along a substrate, AAA+ proteins that serve as molecular switches use ATP hydrolysis to regulate the timing of a cellular event. Thus, AAA+ switches can be thought of as “one-and-done” machines, where one round of ATP hydrolysis triggers a singular event. Therefore, these proteins act as gateways in cell fate since their activity triggers downstream events. Examples of AAA+ proteins that function as molecular switches are DnaC, a helicase loader (Arias-Palomo, O’Shea, et al., 2013), sliding clamp loaders, and bacterial Enhancer-Binding Proteins and transcription activators (bEBPs) (F. Gao et al., 2020). Because these machines do not continuously perform mechanical motion, the energy from ATP hydrolysis does not need to provide motive force, but can be used to control binding states in these proteins. Therefore triggering ATP hydrolysis, and regulating the conformation or assembly of a cellular structure.

**Mechanism of loading a sliding clamp**
Clamp loaders coordinate the loading of sliding clamps onto DNA. This process is highly coordinated to ensure that clamp loading is accomplished.
efficiently. The clamp loading cycle can be broken up into five different phases: (i) sliding clamp binding, (ii) clamp opening, (iii) DNA binding, (iv) clamp closure, and (v) clamp loader recycling (Figure 1.5). Performing any of these steps out of order would lead to unsuccessful clamp loading and would disrupt the progression of DNA replication.

**ATP Binding**

Although the clamp loading cycle is cyclic, and so it has no beginning or end, we can consider the apo clamp loader as the first stage of the cycle. In the apo state, the clamp loader cannot bind to the sliding clamp or p/t-DNA (Gomes & Burgers, 2001; Hingorani & O’Donnell, 1998; Turner et al., 1999a). ATP binding allows the clamp loader to bind both the sliding clamp and p/t DNA. In *E. coli*, the A subunit (delta protein) has high affinity for the sliding clamp in the absence of other clamp loader subunits (Naktinis et al., 1995). The E subunit (delta’) competes with the sliding clamp for binding with the A subunit (Onrust et al., 1995). This finding led to the hypothesis that the E subunit modulates the A subunit’s binding to the sliding clamp by directly interacting with the A subunit and blocking the interface for binding the sliding clamp (Onrust et al., 1995). Furthermore, it was thought that ATP binding would initiate a conformational change within the clamp loader that would relieve A:E subunit interaction, allowing the A subunit to bind to the sliding clamp.

The first crystal structure of the *E. coli* clamp loader was determined of the complex in the apo-state. In this structure, the N-terminal domains of the A and E subunits are not in contact with each other (Jeruzalmi, O’Donnell, et al., 2001). At
the time, this was believed to be a result of crystal packing interactions that artificially separated the A and E subunits (Jeruzalmi, O’Donnell, et al., 2001). In a subsequent study, the change in the distance between the A and E subunits of the *E. coli* clamp loader was measured using fluorescence resonance energy transfer (FRET) (Goedken et al., 2004a). In the presence and absence of ATP, the distance between the two probes were essentially unchanged, and the calculated distance between the FRET probes was roughly consistent with the crystal structure of the apo-state (Goedken et al., 2004b). To further explore this conformational change, another group truncated the N-terminal domain of the E subunit and assessed how this affected clamp loading (Indiani & O’Donnell, 2003). It was thought that if this domain prevents the clamp loader from binding to the sliding clamp in the absence of ATP, then a complex lacking this domain would bind to the sliding clamp in the absence of ATP. However, the clamp loader with the E subunit truncation was unable to bind the sliding clamp, suggesting that subunits B through D also play a role in regulating binding to the sliding clamp. It is unclear how ATP activates the clamp loader and allows it to form a stable complex with the sliding clamp. I will explore this question in the Discussion (Chapter IV) and in Appendix I.

*Clamp loader sliding clamp complex formation and opening*

After binding to ATP, clamp loaders bind and open sliding clamps. Surprisingly, clamp loaders do not hydrolyze ATP when they open the sliding clamp; instead, the energy for clamp opening comes from binding energy alone
(Turner et al., 1999b). Two primary mechanisms have been proposed for how clamp loaders open sliding clamps. The limited-change model proposes that the clamp loader traps spontaneously open sliding clamp in the open state (Goedken et al., 2004a). In contrast, the crab-claw model proposes that the clamp loader binds a closed sliding clamp and that the sliding clamp and clamp loader undergo a concomitant conformational change transitioning into the open state (Goedken et al., 2004a). Although several structures of the yeast clamp loader (Replication Factor C or RFC) bound to sliding clamp (PCNA) and p/t-DNA illustrate the mechanism behind how RFC opens its sliding clamp (Gaubitz et al., 2022), a clear understanding of how the bacterial clamp loader opens the β-clamp was unavailable until this thesis (Chapter II).

The limited-change model has been favored for the *E. coli* following a pivotal study, monitoring the distance between the A and E subunits using FRET (Goedken et al., 2004a). They found that the distance between the A and E subunits did not change significantly when the clamp loader was in the apo-state, ATP-bound, or bound to both ATP and the sliding clamp (Goedken et al., 2004b). Previous structural studies found that when the A subunit binds to the sliding clamp, it distorts the sliding clamp at the dimer interface and the sliding clamp adopts a less curved conformation that would result in clamp opening (Jeruzalmi, Yurieva, et al., 2001). These studies led to a proposal where clamp loader binding destabilizes the sliding clamp dimerization interface and promotes clamp
opening. Then, subunits B-E engage with the sliding clamp and stabilize the open conformation.

A collection of biochemical data on the *E. coli* clamp loader supports that clamp opening is a multistep process with a minimum of three steps (Douma et al., 2017). The group used rapid kinetic techniques to monitor the assembly of the clamp loader sliding clamp complex and the opening of the sliding clamp in real time (Douma et al., 2017). This study used a sliding clamp variant, R103S, that is a less stable ring and is more prone to spontaneous opening events (Purohit et al., 2017). If the primary mechanism driving the opening of the sliding clamp by the clamp loader was destabilizing the closed clamp and trapping the open clamp, it would be expected that the less stable sliding clamp variant would be opened faster than the wild-type clamp in this study. However, they found that the wild-type and variant sliding clamps were opened at the same rate, and so clamp opening was likely an active process by the clamp loader (Douma et al., 2017). Additionally, clamp binding preceded clamp opening and clamp opening occurred in two stages. Thus, clamp binding and opening were proposed to occur in three stages; initial-binding, opening, and locking, where the clamp loader binds a closed sliding clamp, opens the clamp, then undergoes a conformational change that locks the complex in an open state.

Because clamp loader and sliding clamps are the most conserved DNA replication machinery and have similar overall architectures, it was assumed that they would have similar mechanisms for opening their sliding clamps (N. Y. Yao &
O'Donnell, 2016). Recent structures from the Kelch lab of the yeast clamp loader opening its sliding clamp were consistent with the crab-claw mechanism (Gaubitz et al., 2022). RFC first binds PCNA with subunits A through C, and arranges into a conformation where the A' domain blocks the A-gate and the ATP module’s conformation does not support ATP hydrolysis (Bowman et al., 2004a; Gaubitz et al., 2020). Upon clamp opening, subunits D and E bind to PCNA and the clamp loader and sliding clamp undergo a concomitant conformational change, resulting in the opening of the clamp loader’s A-gate. This work provided a clear mechanism as to how RFC opens its sliding clamp, and clearly demonstrated eukaryotic clamp opening is best described with a crab-claw mechanism.

DNA binding

Once the clamp loader and sliding clamp are opened, the complex can then bind to p/t-junctions. The clamp loader preferentially loads sliding clamps onto 3’ primer/template DNA over 5’ p/t DNA or ssDNA (Park & O’donnell, 2009). A crystal structure of the E. coli clamp loader bound to p/t DNA revealed that duplex DNA binds to the interior of the ATP module and the 5’ overhang extends out the A-gate (Simonetta et al., 2009). This structure was consistent with a “screw-cap” mechanism, where the open clamp loader:sliding clamp complex can bind to ssDNA and then slide along the ssDNA until it encounters p/t DNA which sterically blocks further translocation (Bowman et al., 2004a). Upon binding DNA, the AAA+ modules of the clamp loader could reconfigure into a helical conformation that matches the helicity of dsDNA. This was thought to
align the AAA+ modules and stimulate ATP hydrolysis. Structures of the
eukaryotic clamp loaders show that upon opening the sliding clamp, the complex
reconfigures into a helical arrangement and the ATP binding pocket appears
competent for ATP hydrolysis, which is inconsistent with this model (Gaubitz et
al., 2022).

Clamp loading in bacteria and eukaryotes is primarily directed onto
different p/t-junctions. During active DNA replication in bacteria, sliding clamps
are loaded onto the RNA primer, and so the duplex region is an RNA-DNA hybrid
(Naktinis et al., 1995; Waga & Stillman, 2003). Clamp loading in eukaryotes
occurs after DNA polymerase alpha has extended the primer strand with DNA,
and so it occurs on DNA-DNA hybrids (Maga et al., 2000; Yuzhakov et al., 1999).
Thus, the bacterial and eukaryotic clamp loaders have evolved to primarily use
different nucleic acid substrates. Whether this results in different mechanisms of
clamp loading remains unknown.

*Clamp Closure and Release*

After binding to p/t-DNA the next requirement for clamp loading is the
closure and release of the sliding clamp. P/t-DNA binding triggers ATP hydrolysis,
which decreases the clamp loader’s affinity for DNA and the sliding clamp (Ason
et al., 2000; Bertram et al., 2000; Hingorani et al., 1999; Hingorani & O'Donnell,
1998). An major unanswered question is whether the clamp loader actively
closes the sliding clamp or if the clamp loader releases an open clamp onto DNA,
and allows the sliding clamp to spontaneously close. Kinetic studies show that both ATP hydrolysis and clamp closure occurs prior to clamp release, supporting that the clamp loader dissociates away from a closed sliding clamp (Hayner & Bloom, 2013). It is not understood whether closure of the sliding clamp requires a concomitant conformational change with the clamp loader which aids in clamp closure. Potentially, the clamp loader releases one subunit of the sliding clamp which allows the sliding clamp to close prior to its release.

Cryo-EM structures of the human and yeast clamp loaders reveal these clamp loaders bound to p/t-DNA and a closed sliding clamp (Gaubitz et al., 2022; Schrecker et al., 2022). In both of these complexes the D and E subunits of the clamp loaders have disengaged from PCNA (Gaubitz et al., 2022; Schrecker et al., 2022). Furthermore, RFC is bound to a slowly-hydrolysable ATP analog, suggesting that while ATP precedes clamp release it is not required for clamp closure. Clamp release is the rate-limiting step in both bacterial and eukaryotic clamp loaders and illustrates a commonality between the two systems (S. Chen et al., 2009; Hayner & Bloom, 2013).

**Outstanding questions and research scope**

Clamp loaders play a central role in DNA replication and repair in all domains of life and so their mechanisms are important to understand. While clamp loaders have a largely conserved architecture, there are several differences between bacterial, viral, and eukaryotic clamp loaders (Bowman et
A key question remains, how do these structural differences result in changes in the clamp loading mechanism? Structures of the eukaryotic clamp loaders have been determined at different stages throughout clamp loading, which provide a clear understanding of its mechanism (Gaubitz et al., 2020, 2022; Schrecker et al., 2022; Zheng, Georgescu, Yao, O'Donnell, et al., 2022a). However, prior to this thesis, there were no structures that could provide the same clarity into the mechanisms of bacterial clamp loader. How does a bacterial clamp loader initially bind to its sliding clamp? Does it open its sliding clamp with a crab-claw or limited-change like mechanism? Are there differences between binding RNA/DNA or a DNA/DNA primer template substrate? How does p/t-DNA binding trigger DNA binding? In this work, we sought to answer these questions by determining novel structures of the *E. coli* clamp loader. The results of these studies are discussed in Chapter II as well as in Appendix I.

**Viruses and bacterial infection**

Many well-known viruses cause human diseases, such as acquired immunodeficiency syndrome (AIDS), chicken pox, and COVID-19. However, human disease-causing viruses only make up a small subset of viruses as they infect all cellular organisms (Koonin et al., 2006). In fact, viruses that infect bacteria (known as bacteriophage or phage) are the most abundant biologic entity on Earth (Hendrix et al., 1999).
Compositionally, phage particles (virions) are primarily composed of structural proteins and a genome that can be either double-stranded or single-stranded DNA or RNA (Louten, 2016). Compared to cells, most phages have small genomes, which encode for structural proteins as well as proteins that promote their replication when infecting their host. Inside the virion, the phage genome is protected by a protein shell called a capsid (Louten, 2016; Stone et al., 2019a). To infect a host, the genome is ejected out of the capsid and through a tail tube (Fokine & Rossmann, 2014). The tail tube is attached to the capsid via a protein complex called the portal (Fokine & Rossmann, 2014). At the base of the tail is a tail-tip complex, which recognizes and binds to host cells prior to genome ejection (Xiao et al., 2023). Phages have diversity in their morphology, with different sized capsids, tails, and tail-tip complexes (Agnello et al., 2023; Pell et al., 2009; Stone et al., 2019b).

Viruses must infect host cells since they cannot replicate on their own. They lack metabolites (e.g. nucleotides) and machinery (e.g. ribosomes) that are necessary for replication (Sanchez & Lagunoff, 2015). Therefore, when a phage infects a host, they hijack the host resources to replicate. To more efficiently replicate the viral genome, phages code for their own replication proteins such as clamp loaders, sliding clamps, or polymerases or proteins that target the host (Jarviss et al., 1989; Marino et al., 2020).

To infect a host, phage must attach to their host and inject their genome into it. Following genome injection, a phage will either start the lytic or lysogenic
life cycle (Figure 1.6). In the lytic life cycle, immediately following infection the host will replicate the virus’s genome and produce the proteins encoded by the viral genome (Traylen et al., 2011). Next, new viruses assemble from the structural proteins and the viral genome is packaged into the viral capsid as well as any viral proteins that must be injected into a host during infection (Hepp & Robb, 2018; Lindenbach, 2013). Finally, the assembled virions exit the cell, which usually occurs through lysis of the host cell (Fernandes & São-José, 2018; Hoffmann-Beriing, 1964). In the lysogenic life cycle, the phage genome is integrated into the host’s genome after injection, which is then called a “prophage” (Freed, 2015; Howard-Varona et al., 2017). The viral genome may remain dormant in the host’s genome and is replicated by the host’s DNA replication machinery. Occasionally, a prophage will be triggered to enter the lytic cycle. At this point, the viral genome is excised from the host’s genome and the lytic cycle begins producing new virions.
Viral genomes do not only code for structural proteins but a myriad of proteins that target the host to optimize their own replication. Some viruses, such as Human Papilloma Virus (HPV), downregulate host transcription by inhibiting the formation of host initiation complexes or even degrading them (Rampersad & Tennant, 2018). Viruses can produce endonucleases that specifically degrade either host RNA or DNA (Rampersad & Tennant, 2018). It is thought that inhibiting host processes conserves precious cellular resources that can then be funneled toward viral replication. It is becoming increasingly clear that the
warfare between phage and their bacterial host is a rich source for the identification of new interesting biology, as well as the development of important biological tools such as clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing (Cong et al., 2013; Jinek et al., 2012). Thus, understanding the mechanisms that viruses use to hijack host resources is not only necessary for understanding phage and bacterial biology, it also can provide tools and technology for various applications.
Outstanding questions and research scope

N4 bacteriophage infect *E. coli* and inhibits the host’s DNA replication within minutes of infection (Schito, 1974). This effect has been attributed to the production of the N4 phage protein gene product 8, gp8 (Yano & and Rothman-denes, 2011). Gp8 stalls the host’s DNA replication by inhibiting the clamp loader. The mechanism behind how gp8 inhibits the *E. coli* clamp loader remains unknown. In chapter III, I will discuss my research investigating this question. I tried to determine its mechanism using *in vitro* biochemistry, and I tried to determine the structure of gp8 bound to the clamp loader and sliding clamp using cryo-EM. While neither of these goals were entirely met, I did make several surprising finding that potentially illuminate how gp8 functions. This work sets the groundwork for additional studies of gp8 and will hopefully prove to be useful to future researcher(s).
Chapter II: Differences in clamp loader mechanism between bacteria and eukaryotes
Preface

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Introduction:

Sliding clamps and clamp loaders are integral components of DNA replication machinery throughout all known life. Sliding clamps are ring-shaped protein complexes that encircle DNA in order to tether proteins to the genome (Kong et al., 1992). Because the sliding clamp is a closed ring in solution, it needs to be opened and placed onto DNA by a separate ATPase complex called the clamp loader. Sliding clamps and clamp loaders are necessary for numerous aspects of DNA replication and repair, thus these protein complexes are important for human health (N. Y. Yao & O'Donnell, 2016).

All clamp loaders are pentameric ATPases of the AAA+ family (ATPases associated with various cellular activities) (Hedglin et al., 2013; Kelch et al., 2012). In the presence of ATP, clamp loaders bind to the sliding clamp and open the ring (Figure 2.1C) (Gomes & Burgers, 2001; Latham et al., 1997; Turner et al., 1999a). This open binary complex can then attach to a primer-template (p/t)-junction, which triggers ATPase activity (Ason et al., 2003; Berdis & Benkovic, 1996; Gomes et al., 2001; Jarviss et al., 1989). ATP hydrolysis results in closure of the sliding clamp and ejection of the clamp loader (Hayner & Bloom, 2013; Marzahn et al., 2015; Sakato et al., 2012; Trakselis et al., 2003). Therefore, clamp loaders can be thought of as protein-remodeling switches (Kelch, 2016; Kelch et al., 2011; Marzahn et al., 2014), in contrast to most AAA+ proteins which act as processive motors (Hanson & Whiteheart, 2005).

Despite the overall conservation of this machinery, there are key differences
in subunit stoichiometry and structure across different domains of life. While all sliding clamp proteins have six domains arranged into a ring, bacterial sliding clamps (also known as β-clamps) are dimers of three domains (Kong et al., 1992), and eukaryotic clamps (Proliferating Cell Nuclear Antigen or PCNA) are trimers of two domains (Figure 2.1B) (Gulbis et al., 1996; R Krishna et al., 1994). Moreover, clamp loader subunit stoichiometry is distinct between eukaryotes and bacteria, despite the fact that they are all pentamers (with the subunits designated A through E). Eukaryotic loaders have five distinct proteins (RFC1 through RFC5), while bacteria have three proteins that are arranged in a 3:1:1 ratio (γδδ', referred to as γ-complex hereafter; Figure 2.1A). Moreover, the A subunit of eukaryotic clamp loaders contains a region called the A’ domain that is not present in bacterial clamp loaders (Bowman et al., 2004a; Castaneda et al., 2022a; Day et al., 2022; Zheng, Georgescu, Yao, O'Donnell, et al., 2022b). It has remained unclear how or if these differences in composition and structure affect the mechanism of clamp loading.

Structural studies of clamp loaders in action have revealed important insights into their mechanism. Numerous structures indicate that the eukaryotic loader (Replication Factor C or RFC) binds to its cognate clamp PCNA in a closed form, and then opens the clamp with a concomitant opening of the RFC structure (Bowman et al., 2004a; Gaubitz et al., 2020, 2022; Schrecker et al., 2022; Zheng, Georgescu, Yao, O’Donnell, et al., 2022b). The key motion is the opening of the “A-gate” at the A subunit, which allows p/t-DNA to enter into the complex. This
conformational change has been described as a “crab-claw” mechanism for clamp opening. However, earlier fluorescence resonance energy transfer (FRET) studies of the *E. coli* γ-complex predicted that the dimensions of the A-gate do not substantially change during clamp opening, which is inconsistent with the crab-claw mechanism (Goedken et al., 2004b). In RFC, the A’ domain makes important interactions that control the opening of the A-gate (Gaubitz et al., 2022; Goedken et al., 2004b), but bacterial clamp loaders lack an A’ domain (Jeruzalmi, O’Donnell, et al., 2001). These observations raise the possibility that bacterial and eukaryotic clamp loaders open clamps using different mechanisms.

Here we describe a series of structures of the *E. coli* clamp loader in the process of placing the clamp onto a p/t-junction. These structures show that the bacterial clamp loader also uses a crab-claw mechanism, but it is quite different from what we observed in the eukaryotic system. The γ-complex binds to the β-clamp and, through a multi-step process, opens the clamp into a planar configuration. The planar opening requires a single pivoting motion in between the B and C subunits, which disrupts the interfaces at the ATPase active site, preventing ATP hydrolysis. Subsequent binding of a p/t-junction activates the ATPase through tight interactions between adjacent AAA+ modules. Our results reveal a distinction in clamp loading between bacteria and eukaryotes, potentially opening the door for specific inhibition of bacterial clamp loaders as a new target for antibiotic development.
Materials and Methods
Expression Plasmid Cloning

\[ \delta \text{pET28PP}: \] All primers used for cloning are listed in Supplemental Table 1. To
make the expression plasmid for δ protein, we transferred the δ coding region from pET3c\textsuperscript{30} to pET28PP for higher expression using standard restriction enzyme cloning. We generated the δ coding region with flanking cleavage sites for the restriction enzymes XbaI and BamHI using PCR with Q5\textsuperscript{®} High-Fidelity DNA Polymerase and DNA oligonucleotides (Integrated DNA Technologies). PCR products were purified with the Monarch PCR&DNA Cleanup kit (New England Biolab). The pET28 vector was purified from DH5α E. coli (Thermo Fisher Scientific) using a Qiagen miniprep kit (Thermo Fisher Scientific). Both the δ insert and pET28 plasmid were digested with the restriction enzymes XbaI and BamHI (New England Biolabs) and then purified by gel extraction using the QiAquick Gel Extraction kit (Qiagen). The δ coding region was ligated into pET28PP using T4 DNA ligase in T4 DNA ligase buffer (New England Biolabs). This mixture was transformed into Mix & Go competent DH5α E. coli (Zymo Research) and plasmids were recovered by miniprep (Qiagen). Plasmids were checked for proper gene insertion by Sanger sequencing (Genewiz).

**His-tagged δ' pET28PP:** Gibson assembly was used to generate a 6x-His tag δ' pET28PP plasmid for expression of the δ' protein. The pET28 backbone was generated by PCR. The δ' insert was amplified by PCR from a δ' containing pET3a vector. After purification of the PCR products, the δ' fragment was inserted into the pET28PP backbone via Gibson assembly (2x NEBuilder Hifi DNA Assembly Mix, New England Biolabs). Expression plasmids were transformed into Mix & Go competent DH5α E. coli and plasmids were recovered by miniprep
Plasmids were checked by Sanger sequencing (Genewiz). 

\( \delta'-K130A \) and \( \gamma-K141A \) pET28PP: Inserts with the coding region for \( \delta'-K130A \) and \( \gamma-K141A \) and flanking regions containing restriction digest sites, were purchased from Twist Bioscience. We treated the inserts and the pET28PP backbone with the restriction enzymes XbaI and XhoI and then purified the products by gel extraction using the QiAquick Gel Extraction kit (Qiagen). The coding regions were ligated into pET28PP using T4 DNA ligase in T4 DNA ligase buffer (New England Biolabs). These mixtures were transformed into chemically competent 10-\( \beta \) E. coli (New England Biolabs) and plasmids were recovered by miniprep (Qiagen). Plasmids were checked for proper gene insertion by Sanger sequencing (Genewiz).

**Expression and Purification of E. coli Clamp Loader**

The \( \gamma, \gamma-K141A, \delta, \delta', \) and \( \delta'-K130A \) proteins of the clamp loader and the \( \beta \)-clamp were expressed individually in the BLR21(DE3) strain of E. coli. Plasmids for expression of the \( \beta \) and \( \gamma \) proteins are as described in (Simonetta et al., 2009). We used a truncated form of the \( \gamma \) subunit (residues 1-373), which removes the segments that bind to the polymerase and helicase (D. Gao & McHenry, 2001b, 2001a) and are dispensable for clamp loading (Onrust et al., 1991). The \( \gamma, \delta', \gamma-K141A, \delta'-K130A, \) and \( \beta \) proteins each contain a PreScission Protease cleavable 6x-His tag at their N-terminus.

**Protein Expression.** The expression plasmids were separately transformed into BLR21(DE3) E. coli cells (Millipore) and grown overnight at 37°C and then
transferred into 100 ml Luria Broth media supplemented with 100 µg/ml Kanamycin pre-warmed to 37°C. These cultures were grown for 4 hours while shaking at 37 °C. Then 20 mL of the starter cultures were used to inoculate 1 L of Terrific Broth media pre-warmed to 37°C. The cultures were grown until they had an optical density at 600 nm of 0.6-0.8 and then were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM final concentration. Following induction, the cultures were grown overnight at 18°C. Cells were then harvested by centrifugation at 5,000 x g for 30 minutes. Cell pellets were flash frozen in liquid nitrogen and then stored at -80°C.

Protein purification. To purify the His-tagged proteins (γ, δ’, and β), the frozen pellets were thawed on ice and then resuspended in 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole (Buffer Ni-A) and lysed with a cell homogenizer (Microfluidics Inc, Westwood, MA). Cell lysates were then clarified by centrifugation at 20,000 x g for 20 minutes. Lysates were then loaded onto HisTrap columns (Cytiva) pre-equilibrated with Buffer Ni-A. The loaded HisTrap columns were washed with at least 5 column volumes of Buffer Ni-A. The protein was then eluted with 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM BME, and 250 mM imidazole (Buffer Ni-B). Fractions were then checked for protein by SDS-PAGE and pooled.

δ’ protein: The His-tag on δ’ was left intact. His-δ’ was dialyzed into buffer Ni-A overnight, then concentrated to ~15 mg/ml, flash frozen, and stored at -80°C.
γ protein:  His-γ was treated with PreScission Protease to remove the His-tag, while dialyzing into Buffer Ni-A overnight at 4°C. The treated protein was then passed over a HisTrap column, and the flowthrough was collected. Flowthrough fractions containing purified γ were pooled and dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 10% glycerol, and 2 mM dithiothreitol (DTT) (Storage Buffer) overnight at 4°C. Finally, γ protein was concentrated to ~6 mg/ml, flash frozen, and stored at -80°C.

β protein:  Fractions containing His-β protein were pooled and dialyzed into 50 mM Tris pH 8.0, 10% glycerol, 2mM DTT overnight at 4°C while being treated with PreScission Protease. The protein was then loaded onto a 5-ml HiTrap Q column (Cytiva). A gradient of 50 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 2mM DTT was used to elute β. Fractions containing β were pooled and dialyzed into Storage Buffer overnight. Purified β protein was concentrated to ~16 mg/ml, flash frozen, and stored at -80°C.

γ-K141A and δ'-K130A:  To purify the His-tagged γ-K141A and δ'-K130A mutants, the frozen pellets were thawed on ice and then resuspended in Buffer Ni-A, and then lysed with a LM10 Microfluidizer® Processor (Microfluidics). Cell lysates were then clarified by centrifugation at 20,000 x g for 20 minutes. 10 ml of Nickel NTA Agarose Beads (Gold Biotechnology) was added to each clarified lysate. The nickel resins were incubated with the supernatants for 1 hour at 4 °C while mixing. The resins were then collected by centrifugation and washed with at least 5 CV of Buffer Ni-A. 10 ml of Buffer Ni-B was added to the resins and the resins
were incubated for 30 minutes at 4°C while constantly mixing. After the incubation, the supernatants were collected and checked for protein by SDS-PAGE. Both the γ-K141A and δ'-K130A supernatants contained protein and the proteins were dialyzed into Buffer Ni-A overnight while being treated with PreScission Protease.

After PreScission Protease treatment, the proteins were collected and loaded onto HisTrap columns (Cytiva) pre-equilibrated with Buffer Ni-A. A 0-100% gradient of Ni-B was applied to the columns. γ-K141A and δ'-K130A eluted between 7-20% and 20-40% Buffer Ni-B, respectively. To check for cleavage of the His-tag, protein fractions were assessed by SDS-PAGE and near complete cleavage of the tag was observed. The purified γ-K141A and δ'-K130A were then dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 10% glycerol (v/v), and 2 mM DTT (Buffer Q-A) at 4°C overnight. Following dialysis, γ-K141A and δ'-K130A were concentrated to ~5 and ~2 mg/ml, respectively, flash frozen, and stored at -80°C.

δ protein: To purify δ protein, the frozen cell pellets were resuspended in 20 mM Tris pH 7.5, 10% glycerol, 0.5 mM Ethylenediaminetetraacetic acid (EDTA), and 2 mM DTT and lysed using a cell homogenizer (Microfluidics Inc, Westwood, MA). Cell lysate was clarified by centrifugation at 20,000 x g for 20 minutes. The clarified lysate was then loaded onto a 5 ml HiTrap SP-sepharose column (Cytiva). δ protein was eluted with a gradient of 20 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol (v/v), 0.5 mM EDTA, and 2 mM DTT over a 10 column volume gradient. Fractions containing δ protein were pooled and concentrated.
Purified δ protein was then dialyzed overnight into 50 mM Tris pH 7.5, 300 mM NaCl, 10 % glycerol (v/v), 2 mM DTT, and 0.5 mM EDTA. Following dialysis, the protein was concentrated to ~2.0 mg/ml, flash frozen, and stored at -80°C.

γ complex: To reconstitute the γ-complex, we combined purified His-δ', δ, and γ proteins in a stoichiometric ratio of 0.75:1:3, and the protein mixture was then dialyzed overnight into Buffer Ni-A. We then loaded the protein onto a 5 mL HisTrap column (Cytiva), then eluted with Buffer Ni-B. Fractions containing the assembled complex were combined with PreScission Protease, and were dialyzed overnight into Buffer Ni-A. The γ-complex was then passed over a 5 mL HisTrap column, and the flowthrough was collected and dialyzed into Buffer Q-A. The dialyzed protein was then loaded onto a 5 mL Q-sepharose column (Cytiva) and eluted with a 10 column volume gradient of 50 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol (v/v), and 2 mM DTT (Buffer Q-B). Fractions containing the intact γ-complex were then pooled and dialyzed into Storage Buffer. We then concentrated the purified γ-complex to ~2.5 mg/ml using an Amicon Ultra-15 centrifugal filter with a 30,000 kDa molecular weight cutoff. Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

γ-K141A and δ'-K130A γ complexes: To study the effect of the γ-K141A and δ'-K130A mutations on the γ complex, we purified WT γδδ', γ_K141Aδδ', γδδ'K130A, and γ_K141Aδδ'K130A complexes. The γWT/K141A, δ, and δ'WT/K130A proteins were combined in a 2.5:1:1 stoichiometric ratio and dialyzed into Q-A overnight at 4°C. The next day, the protein was loaded onto a 1 ml Hitrap Q column (Cytiva). The column
was washed with at least 5 column volumes of Buffer Q-A, and then, the protein was eluted with a 10 column volume gradient of Buffer Q-B. Fractions containing the intact γ-complex were then pooled and concentrated. Aliquots of the purified complex were flash frozen in liquid nitrogen, and stored at -80°C.

**DNA/RNA oligonucleotide preparation**

DNA and RNA oligonucleotide sequences are listed in Table 2.1. The RNA primer sequence was the preferred length and sequence of the *E. coli* primase. Oligonucleotides were resuspended to a concentration of 100 µM in nuclease free water. To assemble the p/t substrates, the oligonucleotides were combined at equimolar concentrations (final stock concentration of 20-30 µM) in 25 mM Tris pH 7.5 and 5 mM MgCl₂. To promote proper annealing, the equimolar oligonucleotide solutions were heated to 95°C for 2 minutes, then 85°C for 3 min, then 75°C for 5 minutes, then 65°C for 5 minutes, then 55°C for 10 minutes, then 50°C for 10 minutes, then 45°C for 10 minutes, then 40°C for 10 minutes, then 35°C for 10 minutes, then 30°C for 10 minutes, and 20°C in a BioRad T100 Thermal Cycler until they were removed and stored at -20°C.

**Cryo-EM sample preparation**

Before preparing the samples, the purified clamp loader and sliding clamp proteins were buffer exchanged into 25 mM HEPES-NaOH, pH 8.0, 200 mM NaCl, 4 mM MgCl₂, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) using Amicon Ultra-0.5ml centrifugal filters with a 30,000 kDa molecular weight cutoff (Millipore). We then combined clamp loader and sliding clamp. Next, we added
the p/t-junction, if present. We then added the ATP analog, which is either ATPγS or ADP•BeF$_x$. If the sample is cross-linked, we then treat the sample with 1 mM of bis(sulfosuccinimidyl)suberate (BS3, Thermo Scientific Pierce) for 15 minutes at room temperature. To neutralize the cross-linking reaction, the samples were treated with Tris-HCl to a final concentration of 25 mM. Samples were stored on ice until grid preparation.

**Clamp loader, sliding clamp, ATPγS sample:** We combined equimolar clamp loader and sliding clamp to a final concentration of 3 µM each, then ATPγS was added to a final concentration of 1 mM. The sample was crosslinked according to the method above.

**Clamp loader, sliding clamp, ADP•BeF$_x$ sample:** We made a solution 2.5 µM of clamp loader and 2.2 µM of sliding clamp. 1 mM of ADP•BeF$_x$ was prepared in the sample according to (Kelch et al. 2011) (Kelch et al., 2011). The sample was then crosslinked according to the method above.

**Clamp loader, sliding clamp, RNA/DNA p/t-junction, ADP•BeF$_x$ sample:** The sample was prepared such that the final concentration was 2.5 µM of clamp loader, 2.2 µM of sliding clamp, and 7 µM of either RNA/DNA p/t substrate or DNA/DNA p/t substrate. The oligonucleotide sequences are listed in Table 2.1 1 mM of ADP•BeF$_x$ was prepared as described above. The p/t-RNA sample was then crosslinked according to the method above.
### Table 2.1 Oligonucleotides used in this study

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<tr>
<th>Template Name</th>
<th>Sequence</th>
<th>Description</th>
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<td>DeltaForward_XbaI</td>
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<td>Reverse primer for the delta coding region fragment insert with a BamHI restriction digest site</td>
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<td><strong>Cryo-EM and assay substrates</strong></td>
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<td>Used as the primer strand for the RNA/DNA substrate used in the cryo-EM data and anisotropy experiments</td>
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Cryo-EM grid preparation

All grids were first washed with ethyl acetate. The grids were then glow discharged on a PELCO easiGlow for 20-45 s at 10-25 mA (Ted Pella) (negative polarity). 3.5-4.5 µL of sample was applied to a grid and the grid was then blotted on both sides with a blot force of 5, a blot time between 5-7 seconds, and wait time between 0-2 seconds. The grids were then vitrified by plunging into liquid ethane using a Vitrobot Mark IV (FEI) at 10°C and 95% humidity. For the specific grid type, glow discharge conditions, sample volume, blot force, and blot time for each sample see Table 2.2.
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Table 2.2. Cryo-EM sample preparation conditions
Cryo-EM data collection

Clamp loader, sliding clamp, and ATPγS sample: This sample was imaged on a Talos Arctica operated at 200 kV equipped with an GIF energy filter at x45000 magnification and a pixel size of 0.435 Å (bin=0.5) using a K3 Summit direct electron detector (Gatan) in superresolution counting mode. 3,664 micrographs were collected at a target defocus range of -1.1 to -2.3 and a total exposure dose of ~43 e⁻/Å² averaging 30 frames.

Clamp loader, sliding clamp, and ADP•BeFₓ sample: This sample was imaged on a Titan Krios operated at 300 kV equipped with an GIF energy filter at x105000 magnification and a pixel size of 0.415 Å (bin=0.5), using a K3 Summit detector in superresolution counting mode. Movies were collected using SerialEM (Mastronarde, 2003). 4791 micrographs were collected at a target defocus range of -1.0 to -2.4 and a total exposure dose of 49-50 e⁻/Å² averaging 30 frames.

Clamp loader, sliding clamp, p/t-DNA, ADP•BeFₓ sample: This sample was imaged on a Talos Arctica operated at 200 kV equipped with a Gatan K2 Summit direct electron detector and a Gatan energy filter. Micrographs were collected at x45000 magnification and a pixel size of 0.435 Å (bin=0.5), using a K3 detector in super-resolution mode. Movies were collected using SerialEM (Mastronarde, 2003). 3,846 micrographs were collected at a target defocus range of -1.0 to -2.4 and a total exposure dose of 49-50 e⁻/Å² averaging 28 frames.

Clamp loader, sliding clamp, p/t-RNA, ADP•BeFₓ: This sample was imaged on a Glacios operated at 200 kV equipped with a Falcon4 (Thermo Fisher) direct
electron detector and a Selectris energy filter operated at 10 eV. The Falcon4 camera was operated in electron event representation (EER) mode (Guo et al., 2020). Micrographs were collected at x130000 magnification and a pixel size of 0.88. Movies were collected using SerialEM (Mastronarde, 2003). 3,132 micrographs were collected at a target defocus range of -1.1 to -2.3 and a total exposure dose of ~45 e/Å² averaging 1975 frames.

**Cryo-EM data processing**

**Clamp loader, sliding clamp, ATPγS:** Movie frames were aligned in IMOD with 2x binning, resulting in a pixel size of 0.87 Å per pixel. After frame alignment, all further data processing was completed within cryoSPARC (Punjani et al., 2017). The initial Contrast Transfer Function (CTF) estimation was done using CTFFind4 (Rohou & Grigorieff, 2015). Following CTF correction, one round of Blob picking (90-160 Å) was performed to complete reference free particle picking. Particles were then extracted with a box size of 256 pixels. To identify quality particles, we performed two rounds of 2D classification and selected classes with well-defined features, resulting in a stack of ~75,000 particles. This particle stack was used to train a Topaz particle picking model (Bepler et al., 2019, 2020). We implemented this Topaz particle picking model and identified 424,836 particles, which were extracted (box size 300 pixels). The particles were then subjected to two rounds of 2D classification and class selection. 2D classes with well-defined features were selected and then used to generate three *ab initio* models. The highest quality *ab initio* model appeared to be of a clamp
loader/sliding clamp complex, and its associated particle stack was selected for further processing (211,589 particles). We then 3D classified this particle stack. We performed homogeneous refinement on the final particle stack of 44,484 particles. The B-factor used for sharpening the 3D reconstruction was 622.7 Å² and the cryo-EM map had a resolution of 7.7 Å (FSC=0.143). All reference models used during data processing were lowpass-filtered to at least 30 Å. A data processing pipeline is provided in Figure 2.3 and data collection/3D volume reconstruction details are provided in Table 2.3.

Clamp loader, sliding clamp, ADP•BeF₆. Cryo-EM data processing was performed within cryoSPARC (Punjani et al., 2017). Cryo-EM movies were patch motion corrected followed by CTF correction using cryoSPARC’s implementation of Patch CTF. Blob picker was used for the initial particle picking (90-150 Å), which was followed by particle extraction (box size=256 pixels). These particles were then 2D classified and 2D classes with well-defined features were selected. These particles were then used as templates in the “Template Picker” tool and identified particles were extracted (box size=352). Another round of 2D classification followed by 2D subset selection was performed on the new particles. After 2D classification, the particle stack was used to generate 4 ab initio models. One of the ab initio classes was of a clamp loader/sliding clamp complex. This particle stack was then subjected to several rounds of 3D classification and homogeneous refinement to identify different conformations of the complex. A focus-mask was applied to the sliding clamp during the initial 3D
classification to identify any open and closed states. This step was followed by sequential non-masked 3D classification. Non-uniform refinement was used for the 3D refinement of the final map (Punjani et al., 2020). A data processing pipeline is provided in Figure 2.5 and data collection/3D volume reconstruction details are provided in Table 2.3.

**Clamp loader, sliding clamp, ADP•BeF₆, and p/t-DNA:** Cryo-EM data processing was completed within cryoSPARC (Punjani et al., 2017). Cryo-EM movies were patch motion corrected followed by CTF correction using cryoSPARC’s implementation of Patch CTF. Blob picker was used for the initial particle picking (90-150 Å), which was followed by particle extraction (box size=256 pixels). These particles were then 2D classified and 2D classes with well-defined features were selected. Particle picking was then performed using these particles as templates in the “Template Picker” tool and identified particles were then extracted (box size=300). Multiple rounds of 2D classification and 2D subset selection were performed to identify high-quality particles. After 2D classification, the particle stacks were used to generate 4 *ab initio* models. One of the *ab initio* models was of a clamp loader/sliding clamp complex. To identify the Open-DNAp/t, Closed-DNA1, and Closed-DNA2 states, 3D classification was first performed by applying a mask over the sliding clamp and A and B subunits of the *ab-initio* reconstruction. Subsequent 3D classification was performed without applying a focus-mask. To identify the Altered-Collar state, 3D classification was first performed by applying a mask over the collar of the *ab-initio* reconstruction.
Non-uniform refinement was used for the refinement of the final maps (Punjani et al., 2020). A data processing pipeline is provided in Figure 2.8 and data collection/3D volume reconstruction details are provided in Table 2.3.

**Clamp loader, sliding clamp, ADP•BeF₆, and p/t-RNA:** Cryo-EM data processing was completed within cryoSPARC (Punjani et al., 2017). Cryo-EM movies were patch motion corrected followed by CTF correction using cryoSPARC’s implementation of Patch CTF. Blob picker was used for the initial particle picking (90-150 Å), which was followed by particle (box size=260 pixels). These particles were then 2D classified and 2D classes with well-defined features were selected. Particle picking was then performed using these particles as templates in the “Template Picker” tool and identified particles were extracted (box size=260 pixels). These particles were then 2D classified, which was followed by 2D subset selection. After 2D class selection, the particles were extracted (box size=300 pixels, binning 2x). The resulting particle stack was used to generate 4 *ab initio* models. One of the *ab initio* models was of the clamp loader, sliding clamp, p/t-RNA complex. This particle stack was extracted (box size=300 pixels, binning=1x). The fully extracted particles were then 3D classified without a reference volume into 5 classes. No major differences were observed between the best three classes and they were pooled and refined by non-uniform refinement (Punjani et al., 2020). The map was sharpened with a B-factor of -92.3 Å² within cryoSPARC and has a resolution of 3.2 Å (FSC=0.143). A data processing pipeline is provided in Figure 2.7 and data collection/3D volume reconstruction details are provided in Table 2.3.
reconstruction details are provided in Table 2.3.

Table 2.3 Cryo-EM data collection, processing, and model statistics

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Microscope</th>
<th>Voltage (kV)</th>
<th>Cumulative Exposure (e-/Å²)</th>
<th>Detector</th>
<th>Pixel Size (Å)</th>
<th>Defocus range</th>
<th>Micrographs Collected</th>
<th>Symmetry</th>
<th>Initial-Binding Final Refined Particles (no.)</th>
<th>Applied B factor (Å²)</th>
<th>Map Resolution (Å, FSC 0.143)</th>
<th>Model-Map CC_mask</th>
<th>Bond lengths (Å), angles (°)</th>
<th>Ramachandran outliers, allowed, favored</th>
<th>Poor Rotamers (%), MolProbity score, Clashscore</th>
<th>EMD ID</th>
<th>PDB ID</th>
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<tbody>
<tr>
<td>ATP Binding</td>
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</table>
Model Building, refinement, and validation

We used crystal structures of the γ-complex bound to DNA-p/t (3GLF) (Simonetta et al., 2009) and the β-clamp (2POL) (Kong et al., 1992) as initial models. Each subunit was split into globular domains and then each domain was fit into the cryo-EM maps using UCSF ChimeraX (Pettersen et al., 2021). Molecular Dynamics flexible fitting (MDFF) as implemented in NAMD 2.14 was used to generate the model of the Initial-Binding complex from the clamp loader, sliding clamp, ATPγS dataset (Phillips et al., 2020; Trabuco et al., 2008). Input files were prepared via the MDFF plugin in VMD 1.9.4a55 (Humphrey et al., 1996). The rigid body fit model and cryo-EM map were used as the starting model and bias potential. The protein interactions were described with the CHARMM36 force field (Huang & Mackerell, 2013), and additional restraints added to maintain secondary structure, prevent cis peptide bonds, as well as maintain chirality. All other models were built using a combination of Coot for manual building (Emsley & Cowtan, 2004), Starmap for automated building (Lugmayr et al., 2022), and Phenix real-space refinement (Adams et al., 2010). After the initial rigid body fitting, the Semi-open, Fully-Open, Open-DNAp/t, Open-RNAp/t, Closed-DNA1, and Altered-Collar models were adjusted in Coot. Following manual adjustment, the models and maps were used as inputs for Starmap, for rosetta-driven molecular structure refinement. The models from the final iterations of Starmap, were inspected and manually adjusted in Coot. The Closed-DNA1 Starmap
output model was fit into the Closed-DNA2 map, the fitted model and map were then used as inputs for Starmap. The model from the final Starmap iteration for the Closed-DNA2 model was then manually adjusted in Coot. After all models were inspected and adjusted in Coot, they were refined using Phenix real-space refine. Errors in the models after refinement were manually fixed in Coot, and then a final round of real-space refinement was performed on each model. Real-space refinement statistics are listed in Table 2.3. Figures and analyses were made using Pymol (L., 2002), UCSF ChimeraX, and the PISA server (Oiki et al., 2007).

**ATPase assays**

0.3 µM γ-complex was incubated with 0.2 µM (Figure 2.2B) or 1 µM (Figure 2.6E, Figure 2.14E, Figure 2.12C) β-clamp, 0.4 µM (Figure 2.2B, Figure 2.6E, Figure 2.13E) or 1 µM (Figure 2.12C) p/t-junction and a mix of 5 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 1 mM ATP, 150 µM phosphoenolpyruvate, 50 µM NADH, 25 mM Tris (pH 7.5), 100 mM potassium glutamate, 5 mM magnesium chloride. The oligonucleotide sequences are listed in Table 2.1. The ATPase rates were measured at 25°C with a VICTOR Nivo multimode plate reader (Perkin Elmer) to detect NAD+. Rates were obtained from a linear fit of the data in GraphPad Prism.

**Fluorescence measurements**

To measure the affinity of different p/t-junction substrates to the clamp loader, sliding clamp, ADP•BeF₃ complex we used a fluorescence anisotropy-based
binding assay (Goedken et al., 2005). We used 5’ TAMRA-labeled template DNA which was annealed to either an 11 bp DNA or RNA primer, similar as previously described (Kelch et al., 2011). The oligonucleotide sequences are listed in Table 2.1. Anisotropy measurements were made with a FluoroMax 4 (Horiba Jobin Yvon Inc) at 25°C. Fluorescence was excited with a wavelength of 550nm (2 mm slit width) and emission was detected at a wavelength of 580 nm (7 mm slit width). Buffer conditions were 50 mM Tris (pH 7.5), 250 mM potassium glutamate, 2 mM DTT, 10% glycerol, and 1 mM magnesium chloride. 1 mM of ADP•BeF<sub>x</sub> was prepared in the sample as described above. Then 5 µM sliding clamp and 150 nM p/t-junction substrate was added. Finally, the clamp loader was titrated into the solution. Raw data were fit using Prism (GraphPad Software) with the equation:

\[ Y = B_{\text{max}} \frac{X^h}{(K_d^h + X^h)} + Z, \]

where \( X \) is the clamp loader concentration, \( Y \) is the anisotropy, \( h \) is the Hill slope, \( Z \) is the starting anisotropy, and \( B_{\text{max}} \) is the maximum anisotropy.

**Results**

**Structures of \textit{E. coli} clamp loader opening the \( \beta \)-clamp**

We reconstituted the clamp opening reaction using a purified recombinant sliding clamp (\( \beta \)) and clamp loader (\( \gamma \)-complex) (\textbf{Figure 2.2A}). The reconstituted clamp loader is active because it displays the expected response to ATP and p/t-DNA (\textbf{Figure 2.2B}). As seen in previous studies (Hingorani et al., 1999; Turner et al.,
1999a), β-clamp binding suppresses ATPase activity, but the presence of clamp and p/t-DNA synergistically activates ATPase activity.

Figure 2.2. Characterization and cryo-EM map validation of the bacterial clamp loader. **A)** Purified γ complex and β-clamp. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of the purified γ-complex and β-clamp proteins, and samples of the γ complex combined with the β-clamp before and after crosslinking with BS3 crosslinker. **B)** ATP activity profile of the bacterial clamp loader. Steady-state ATPase activity of the purified clamp loader complex in combination with the sliding clamp and p/t-DNA (mean ± S.D., n=3). **C-D)** Local resolution and FSC curves of the clamp loader cryo-EM maps. Local resolution (Fourier shell correlation (FSC)=0.5) of the reconstructions and a representative section of each density map with fitted model. The overall resolution of each map was determined by the FSC of each half-map using Gold-standard cutoff of 0.143 (blue line). For a clearer version of this figure see Landeck et al., 2024.
To reveal how the *E. coli* clamp loader opens its clamp, we used single particle cryo-electron microscopy (cryo-EM) to determine structures of various clamp opening intermediates (Figure 2.2C). In order to suppress the background ATPase activity in our cryo-EM samples, we replaced ATP with either the slowly hydrolysable ATP analog ATPγS or the non-hydrolysable analog ADP•BeF₆. From these two cryo-EM datasets, we obtained three distinct reconstructions of the sliding clamp/clamp loader complex.

**Clamp loader bound to closed clamp:** In the presence of ATPγS, we obtained an ~8 Å resolution reconstruction of the clamp loader bound to a closed clamp (Figure 2.3A; Figure 2.3). Despite the modest resolution of the reconstruction, we can unambiguously identify the location and orientation of all the protein domains of both the clamp loader and sliding clamp. We modeled these domains as rigid bodies, followed by Molecular Dynamics Flexible Fitting (Trabuco et al., 2008, 2009) to adjust the domains while preventing steric clashes (Figure 2.4A).

We observe that the γ-complex contacts the sliding clamp primarily through the A, D, and E subunits; the B and C subunits make minimal contact with the surface of the clamp (Figure 2.3A). Furthermore, the AAA+ modules of the B and C subunits make minimal contact with each other (Figure 2.4B). As inter-subunit contacts are necessary to hydrolyze ATP, this separation between subunits likely inhibits ATPase activity. Because the clamp is closed in this state, we attribute this conformation as the Initial-Binding complex.
Figure 2.3 Structures of the clamp loader/sliding clamp complex throughout the process of clamp opening. **A-C** The clamp loader/sliding clamp complex in the Initial-binding (A), Semi-Open (B), and Fully-Open (C) states. Left, cryo-EM maps in two views. Center, bottom view of the cryo-EM maps of bound sliding clamps. Right, a top view of the sliding clamp with residues that contact the clamp loader colored according to the contacting clamp loader subunit. The change in clamp/clamp loader interface area between states is quantified on the far right. There is no recognizable density for domain I of subunit II (indicated with a dashed circle) of the sliding clamp in the Semi-Open and Fully-Open states. **D** Conformational changes of the sliding clamp upon opening. Models of the Initial-Binding, Semi-Open, and the Fully-Open states are displayed as low contour surfaces. Sliding clamps were aligned on subunit II and displacements of domains within subunit I are shown as vectors. We quantify clamp opening using the distance between the centers of mass of domain II of subunit II and domain III of subunit I (dashed lines). **E** Conformational changes of the clamp loader between the Semi-Open and Fully-Open states. The Rossmann domains of the Semi-Open state (gray) and the Fully-Open state (color) are displayed as low contour surfaces. The structures are aligned on the Rossmann domain of subunit E, and the displacements of the centers of mass of the Rossmann domains of subunits A and B are shown as vectors. Subunits C, D, and E move less than 3 Å. The change in interface area between adjacent Rossmann domains is quantified to the right.
Figure 2.4 Schematic of the Cryo-EM processing workflow for the clamp loader, sliding clamp, ATPγS dataset. All data processing was performed using cryoSPARC. A) Representative aligned micrograph and particle-picking pipeline. (Left) Particles were first picked with cryoSPARC’s blob-picker tool. Particles were the extracted and 2D classified. Particles from the selected 2D classes were used as templates to train Topaz. Particles picked by Topaz were then extracted and used for downstream processing. (Right) Representative 2D classes following Topaz particle picking. B) Ab-initio reconstruction. Particles from the selected 2D classes were used to generate three Ab-initio models. One of the three models is of the clamp loader/sliding clamp complex (pink) C) 3D classification and reconstruction. Particles from the selected ab-initio class were 3D classified into three classes. Two of the 3D classes were combined and homogeneous refinement was used to generate the final 3D reconstruction (pink), which was used to build the Initial-Binding model. For a clearer version of this figure see Landeck et al., 2024.
Clamp loader bound to Semi-Open or Fully-Open clamp: In micrographs of the sample containing ADP•BeF$_x$, we observe two different conformations of the clamp loader/sliding clamp complex, both with the clamp open (Figure 2.5). However, the degree of opening is drastically different between these two states. In the first state, the clamp is barely opened and adopts a very tight spiral conformation (overall resolution = 3.9 Å; Figure 2.3B). In the second state, the sliding clamp is opened into a wide, planar conformation (overall resolution = 3.0 Å; Figure 2.3C). Therefore, we designate these two states as the Semi-Open and the Fully-Open states, respectively.
Figure 2.5. Schematic of the Cryo-EM processing workflow for the clamp loader, sliding clamp, ADP•BeF, dataset. All data processing was performed using cryoSPARC. A) A representative patch-motion corrected micrograph and particle-picking pipeline. Particles were first picked with cryoSPARC’s blob-picker tool. Identified particles were extracted and 2D classified. Particles from the selected 2D classes were used as templates for CryoSPARC’s template picker. Particles identified by the template picker were extracted and 2D classified. Representative 2D classes were selected following template picking. B) Ab-initio reconstruction. Particles from the selected 2D classes were used to generate four Ab-initio models. One of the four models resulted in a reconstruction of the clamp loader/sliding clamp complex (pink). C) 3D classification and reconstruction. A focus mask was applied to the sliding clamp of the ab-initio model, which was used to perform a focused 3D classification of the particles into five classes. One of the resulting classes was in a “semi-open” clamp loader/sliding clamp complex (pink), while three were of the open clamp loader/sliding clamp complex (green). Another focused 3D classification was performed on this “Semi-Open” class using a mask on the sliding clamp. One of the resulting classes (pink) was selected for further 3D classification. Non-uniform refinement and map sharpening was used on the final particle stack. The 3D reconstruction was used to build the Semi-Open model. The three open classes (green, right) were combined and further 3D classified. Non-uniform refinement and map sharpening was used on the final particle stack. The 3D reconstruction was used to build the Fully-Open model. For a clearer version of this figure see Landeck et al., 2024.
Unlike the Initial-Binding complex, in the Semi-Open state all five subunits of the clamp loader contact the sliding clamp (Figure 2.3B). These additional contacts between the clamp loader and sliding clamp help stabilize the initial opening of the sliding clamp in a spiral conformation. Interface contacts between the A and B AAA+ modules and the C and D AAA+ modules are strong, while contacts between the B and C AAA+ modules remain minimal.

As in the Semi-Open state, all five subunits of the clamp loader also make contact with the sliding clamp in the Fully-Open state. The primary conformational change between these two states is a dramatic increase in interfacial contacts between the B and C subunits. This change is accomplished by a single pivoting motion at a point between the B and C subunits (Figure 2.3E). Because the B and C subunits are contacting the sliding clamp, this pivoting motion imparts an equivalent pivoting motion within subunit I of the sliding clamp, resulting in the wide planar opening (Figure 2.3D). The first β-clamp subunit undergoes a substantial change (Cα RMSD ~3.0 Å), while the second β-clamp subunit is essentially unchanged (Cα RMSD ~0.7 Å). These motions pull the first subunit ‘upward’ into a flat “C” shape, rather than the spiral seen in structures of T4 phage and yeast clamp loading complexes (Gaubitz et al., 2022; Kelch et al., 2011; Schrecker et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022). A planar conformation of an open sliding clamp has been recently observed in the structure of the 9-1-1 clamp bound to the Rad24-RFC loader and resected DNA (Castaneda et al., 2022a; Zheng, Georgescu, Yao, Li, et al., 2022).
In both the Semi- and Fully-Open states, there is no significant density for Domain I of the second subunit of the β-clamp, which is at the site of ring opening (Figure 2.3B,C). This is the only domain not directly held by a clamp loader subunit. All of the other domains of the β-clamp are clearly observable, including Domain II directly adjacent to the missing domain indicating that the entirety of Domain I is too mobile to be clearly observed. These results are consistent with hydrogen-deuterium exchange data, which indicates that this domain of the *E. coli* β-clamp unfolds upon opening (Fang et al., 2011, 2014). The lack of density for Domain I makes it challenging to measure the true width of the β-clamp opening. Instead, we measure the distance between the centers of mass of Domain III of the first subunit and Domain II of the second (Figure 2.3D).

**DNA binding activates ATP hydrolysis and closes the clamp**

We next investigated how the *E. coli* clamp loader binds to p/t-junctions. Bacterial clamp loaders typically load onto RNA-primers at each Okazaki fragment *in vivo* (Kornberg, Arthur; Baker, 1992), but most *in vitro* measurements of structure or activity have been performed using DNA-primed p/t-junctions. Thus, we determined structures of the *E. coli* γ-complex and β-clamp bound to both RNA-primed and DNA-primed p/t-junctions. To prevent hydrolysis of the bound nucleotide, we used the non-hydrolyzable ATP mimic ADP•BeF₅ for both samples. We determined the structure of the γ-complex bound to an open β-clamp and an RNA-primed p/t-junction at 3.2 Å overall resolution (Figure 2.6C;
Figure 2.7). The dataset using the DNA-primed p/t-junction resulted in four conformations of the ternary complex after 3D classification (Figure 2.8). The first two are open states that differ in their collar domains (Open-DNAp/t and Altered-Collar), while the latter two are closed states that differ in their interactions between the loader and the clamp (Closed-DNA1 and Closed-DNA2).
Figure 2.6 Clamp loader/sliding clamp complex bound to p/t-DNA or p/t-RNA.

A) Cryo-EM map of the Open-DNAp/t complex. The clamp loader subunits are colored as before. The template strand is orange and the primer strand is yellow. The 3D reconstruction of the Open-DNAp/t complex has density for all six domains of the sliding clamp.

B) The p/t-junction bound complex adopts a helical conformation. Models of the Rossmann domains of the Fully-Open (gray) and Open-DNAp/t (color) states are displayed as low contour surfaces. The black line displayed on all models connects the center of masses of the Rossmann domains of the A and E subunits of the Fully-Open state. The models were aligned on the E subunit. The center of mass of the A subunit shifts ~10 Å after p/t-junction binding.

C) Cryo-EM map of the Open-RNAp/t complex.

D) The Open-DNAp/t (gray) and Open-RNAp/t (color) aligned on the collar of the A subunit. The DNA primer (gray) shifts registry, reaching higher into the central chamber compared to the RNA primer (yellow) which is accommodated by a 3.1 Å Cα shift in separation pin Y316, which stacks with the last base of the primer.

E) Steady-state ATPase activity of the clamp loader stimulated by p/t-RNA or p/t-DNA (Mean ± S.D., n=3).

F) The affinity of the clamp loader for p/t-RNA or p/t-DNA measured by anisotropy of TAMRA-labeled template DNA (Kd ± SEM, n=3).

Figure 2.7. Schematic of the Cryo-EM processing workflow for the clamp loader, sliding clamp, p/t-RNA, and ADP•BeFx dataset. All data processing was performed using CryoSPARC. A) A representative patch-motion corrected micrograph and particle-picking pipeline. Particles were first picked with cryoSPARC’s blob-picker tool. Identified particles were extracted and 2D classified. Particles from the selected 2D classes were used as templates for cryoSPARC’s template picker. Particles identified by the template picker were extracted and 2D classified. Representative 2D classes were selected following template picking. B) Ab-initio reconstruction. Particles from the selected 2D classes were used to generate four Ab-initio models. One of the four models resulted in a reconstruction of the clamp loader/sliding clamp/p/t-RNA complex (pink). C) 3D classification and reconstruction. The selected particles were 3D classified into four classes. Non-uniform refinement and map sharpening was used on the final particle stack. This 3D reconstruction was used to build the Open-RNAp/t model. For a clearer version of this figure see Landeck et al., 2024.
Figure 2.8. Schematic of the cryo-EM processing workflow for the clamp loader, sliding clamp, p/t-DNA, and ADP•BeF dataset. All data processing was performed using CryoSPARC. A) A representative patch-motion corrected micrograph and particle-picking pipeline. Particles were first picked with CryoSPARC’s blob-picker tool, extracted, and 2D classified. Particles from the selected 2D classes were used as templates for template picking. Identified particles were extracted and 2D classified. B) Ab-initio reconstruction. Particles from the selected 2D classes were used to generate four Ab-initio models. One of the four models consisted of the clamp loader/sliding clamp/p/t-DNA complex (blue) and one closed clamp loader/sliding clamp complex (tan). The three clamp loader/sliding clamp/p/t-DNA complexes were pooled and iteratively 3D classified. The final class had density for the clamp loader, p/t-DNA, and all six domains of the sliding clamp. The closed class was 3D classified resulting in five classes of the closed clamp loader/sliding clamp/DNAp/t complex (orange and red). After 3D classification, non-uniform refinement was performed to generate the final reconstructions of the Open-p/t-DNA, Closed-DNA1, and Closed-DNA2 complexes. In parallel, the particles from the ab-initio model were 3D classified into five classes while applying a focus mask to the collar and lid domains of the clamp loader/sliding clamp/DNAp/t complex (right). One of the classes had an altered collar (cyan). After 3D classification, non-uniform refinement was performed to generate the final reconstruction of the Altered-Collar class. For a clearer version of this figure see Landeck et al., 2024.
Open-DNAP/t (overall resolution 3.7 Å): We first discuss the next on-pathway state where p/t-DNA binds inside the clamp loader and clamp, causing both to constrict around DNA (Figure 2.6A). The primary motion is pivoting of the A and B subunits out-of-plane into a spiral. The AAA+ modules of the γ-complex and the sliding clamp adopt a helical arrangement that matches the helical symmetry of DNA (Figure 2.6B, Figure 2.9B), similar to other structures of clamp loaders bound to p/t-DNA (Gaubitz et al., 2022; Kelch et al., 2011; Simonetta et al., 2009). This constriction results in the A-gate closing by ~5 Å, measured by the centers of mass of domains A and E. The clamp then closes slightly, by 11 Å (Figure 2.9A). Clamp closure is primarily driven by relaxation of β-clamp subunit I into a conformation that is more like that of the closed form ($C_\alpha$-RMSD$_{FullyOpen_vs_Closed}$ ~3.3 Å versus $C_\alpha$-RMSD$_{DNA-Open_vs_Closed}$ ~1.8 Å).
Figure 2.9. Conformational change induced by p/t-junction binding. A) Conformational changes of the sliding clamp between states. Models of the sliding clamp of the Fully-Open and Open-DNAp/t states are displayed as low contour surfaces. The distance between domain III of subunit I and domain II of subunit II of the sliding clamp in each state is shown. The Fully-Open and Open-DNAp/t sliding clamps were aligned on subunit II and displacements of domains within subunit I are shown as vectors. B) Compatibility of the Rossmann domain with template DNA binding. Surfaces of the Rossmann domains of the B-D subunits of the Semi-Open, Fully-Open, and Open-DNAp/t models are shown. Models were aligned on the D subunit and the template strand from the Open-DNAp/t model is superimposed on the Semi-Open and Fully-Open models. C) Compatibility of template binding residues with template DNA binding. Surfaces of the Rossmann domains of the B-E subunits of the Semi-Open, Fully-Open, and Open-DNAp/t models are shown. Models were aligned on the E subunit and the template strand from the Open-DNAp/t model is superimposed on the Semi-Open and Fully-Open models. Key template strand gripping residues are highlighted in yellow. D) Per residue RMSD of the clamp loader/sliding clamp complex comparing the Open DNAp/t and Open-RNAp/t states. Structures were globally aligned and per-residue Cα RMSD was calculated. E) Alignment of template DNA binding residues with the template strand in different states. Surfaces are shown of the Rossmann domains of the B-E subunits of the Semi-Open, Fully-Open, and Open-DNAp/t models. Models were aligned on the E subunit and the template strand from the Open-DNAp/t model is superimposed on the Semi-Open and Fully-Open models. Residues that bind to the template strand are shown in yellow.
Open-RNAp/t (overall resolution 3.2 Å): We find that primer composition (RNA vs. DNA) has very little impact on the conformation of the complex. The $C_\alpha$ RMSD between the Open-RNAp/t structure and that of the Open-DNAp/t is only ~0.7 Å (Figure 2.9D). Both structures show that the duplex region of the p/t-junction adopts A-form geometry as expected (Simonetta et al., 2009). Because double-stranded DNA prefers B-form geometry in solution, it must be converted to A-form upon binding in the central chamber. On the other hand, RNA-DNA hybrids are preconfigured as A-form. Thus, the clamp loader does not need to overcome this energetic penalty for loading at the RNA-primed p/t-junction. The only difference between loading complexes is that the RNA-primed p/t-junction does not move up as high in the central chamber of the clamp loader as a result of flexibility in the “separation pin” (Figure 2.6D, Figure 2.9E).

We then wondered whether these subtle differences in p/t-junction binding manifest as differences in clamp-loading competency or p/t-junction binding affinity. To test the ability to load clamps, we measured steady-state ATPase rates, which report on the turnover of the clamp loader upon clamp loading. A p/t-junction containing an RNA primer is slightly more effective at activating the clamp loader ATPase activity, although this effect disappears in the presence of $\beta$-clamp (Figure 2.6E). To test whether the primer composition affects binding affinity, we performed an anisotropy assay with TAMRA-labeled p/t-junctions. Surprisingly, we find that the affinity for an RNA-primed p/t-junction is identical to
that of a DNA-primed junction (Figure 2.6F). We speculate that the difference in registry of the p/t-junction within the central chamber provides additional binding energy for the DNA-primed substrate that offsets the energetic penalty of converting the DNA duplex to A-form.

**Altered-Collar** (overall resolution 3.8 Å): A surprising class of DNA-primed particles exhibits an open clamp structure (Figure 2.10A), but the collar region is in a different conformation from all other γ-complex structures reported herein or elsewhere (Jeruzalmi, O'Donnell, et al., 2001; Leu & O'Donnell, 2001; Simonetta et al., 2009). The collar region of the B subunit shifts upward away from the AAA+ spiral. The collar regions of the C, D and E subunits are static, and the A subunit only moves slightly (Figure 2.10B). The movement of the collar opens a large pore between the A and B subunits (Figure 2.10C). A pore opens and closes at this site in the eukaryotic clamp loader RFC, although it manifests through a different mechanism (Gaubitz et al., 2022; Schrecker et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022).
Figure 2.10 Structure of the Altered-Collar conformation of the bacterial clamp loader. A) Cryo-EM map of the Altered-Collar state (bound to p/t-DNA and ATP-analog). B) Comparison of collar conformation across states. All structures were aligned on the collar of the D subunit. (Left) The Altered-Collar (color) is compared to the Open-DNAp/t structure (gray), and (Right) Open-p/tDNA is compared to the psi-bound (wheat, PDB: 3GLI) state. C) The interface between A and B subunits. The Altered-Collar conformational change results in a large opening between the A and B subunits of the clamp loader (yellow dashed contour). D) Modeling the psi peptide into the Altered-Collar. (Left) A solved co-crystal structure of the clamp loader (surface) bound to psi peptide (cartoon). (Right) The co-crystal structure was aligned to the Altered-Collar structure as in panel (B). This alignment was used to superimpose the psi peptide onto the Altered-Collar structure.
Moreover, this movement alters the center of the collar at the binding site for the ψ protein, an accessory factor that, together with the χ protein, acts as an adaptor linking the clamp loader to Single-Stranded Binding Protein (SSB) (Gulbis et al., 2004; Marceau et al., 2011; Newcomb et al., 2022; Simonetta et al., 2009). The Altered-Collar state shifts the binding site such that it is incompatible with the known binding pose of the ψ protein (Simonetta et al., 2009) (Figure 2.10D). We attribute this structure to an off- or parallel-pathway intermediate and discuss potential implications for the Altered-Collar conformational changes in the Discussion.

Closed-DNA1 & Closed-DNA2 (overall resolution 3.8 Å & 3.9 Å): The Closed-DNA1 and Closed-DNA2 structures reveal how the clamp closes and yields insight into sliding clamp release (Figure 2.11A&B). Both of these structures have a closed β-clamp, which is in the same conformation as the free clamp (Cα-RMSD 1.4 Å and 1.3 Å from PDB: 2POL). By comparing the Open-DNAp/t and Closed-DNA1 structures we infer that the clamp closes through two motions. The sliding clamp subunit bound to A, B, and C moves laterally, while the second sliding clamp subunit bound to C, D, and E pivots down and in towards the A-gate (Figure 2.11D). In spite of this, the conformation of the clamp loader is essentially identical across Open-DNAp/t, Closed-DNA1, and Closed-DNA2 (Figure 2.11C). The only difference between Closed-DNA1 and Closed-DNA2 lies within the contacts between the clamp loader and the sliding clamp (Figure 2.11E&F). In Closed-DNA1, all five subunits of the γ-complex contact the β-
clamp, although the D and E subunits lose substantial contact relative to the Open-DNAp/t state. The Closed-DNA2 state further loses contact between β-clamp and subunits C, D, and E of the γ-complex (Figure 2.11F). Between Closed-DNA1 and Closed-DNA2 the sliding clamp separates from the E subunit by ~4 Å. We propose that the Closed-DNA1 conformation precedes the Closed-DNA2 conformation in a trajectory towards clamp release. The disengagement of the D and E subunits from the clamp is similar to that seen in RFC and PCNA (Gaubitz et al., 2022).

In Closed-DNA1 and Closed-DNA2 structures, the clamp makes multiple direct contacts with DNA, primarily mediated by Gln15, Arg80 and Arg73 of clamp subunit I, and Gly23, Arg24, Arg80, and Gln149 of the second β-clamp subunit. Previous work showed Arg24 and Gln149 of the β-clamp are important for efficient loading (Georgescu et al., 2008). Similar residues in PCNA contact DNA and have been shown to aid in clamp loading by RFC (Gaubitz et al., 2022; McNally et al., 2010). Our findings support their hypothesis that these residues contribute directly to the clamp closure step.
Figure 2.11. Closing the sliding clamp. A-B) Cryo-EM maps of the Closed-DNA1 and Closed-DNA2 conformations of the clamp loader and sliding clamp complex. C) Per-residue RMSD of the clamp loader comparing the Open-DNAp/t state to the Closed-DNA1 state and the Closed-DNA1 state to the Closed-DNA2 state. Structures were globally aligned and per-residue Cα RMSD was calculated. D) Comparison of the sliding clamp in open and closed conformations with p/t-DNA. Structures were aligned on the E subunit of the clamp loader and are shown as low contour surfaces. Center of mass shifts in the sliding clamp domain III of subunit I and domain I of subunit II are shown in orange vectors. The position of the clamp in the Open-DNAp/t conformation is outlined in orange to help guide the eye. E) Interface area between the sliding clamp and clamp loader. Models of the sliding clamp are shown as surfaces, and residues that contact the clamp loader are highlighted with color according to the contacting clamp loader subunit. The region that changes the most is highlighted with a dashed oval. F) The change in interface area between the clamp loader and sliding clamp during clamp closure.
Insights into the stimulation of ATPase activity

All structures reported here contain ATP analogs in the three ATPase active sites, which allows us to observe the steps that align the catalytic machinery for ATP hydrolysis. The Initial-Binding state contains ATPγS, all other states contain ADP•BeFx. Prior to p/t-junction binding, the ATPase sites are not optimally positioned for ATP hydrolysis. In the Semi-Open state, there is a gap between the B and C subunits, which prevents the trans-acting arginine finger (Arg169 in γ, Arg158 in δ′) from interacting with ATP (Figure 2.3E). In the Fully-Open state, the arginine finger of the C subunit contacts the B subunit’s ATP. Furthermore, we find no evidence for a previously hypothesized DNA-dependent allosteric switch involving Lys100 and Arg98 of γ acting in cis (Kelch et al., 2011), as this residue does not significantly alter its conformation upon DNA binding (Figure 2.12A). Because DNA stimulates ATPase activity (Figure 2.2A), we propose that p/t-junction binding aligns other residues that are necessary for ATP hydrolysis. We find that upon p/t-junction binding and the transition from a planar to helical clamp, a trans-acting lysine (Lys141 in γ, Lys130 in δ′) pivots upward to contact Asp126 of the Walker B motif in the neighboring active site (Figure 2.12B).

We hypothesized that this trans-acting lysine plays a key role in stimulating ATPase activity in response to p/t-junction binding. To test this hypothesis, we made three variant clamp loader complexes in which this residue is mutated to alanine. The γ-K141A variant perturbs the active sites of the B and C subunits, while the δ′-K130A variant perturbs the D subunit’s active site. The γ-K141A/δ′-
K130A double-mutant perturbs all three active sites. We find that all three variants have modestly impaired ATPase activity (Figure 2.12C), and that addition of p/t-DNA boosts ATPase activity of all three variants in a manner similar to wild-type (Figure 2.12D). These observations do not support our hypothesis that this lysine is the conformational trigger for ATPase activity.
Figure 2.12. Examination of ATPase activation mechanism. A) No repositioning of the hypothesized “switch” residues. Overlay of the Fully-Open (gray) and Open-DNAp/t (color) models. In each panel, the models are aligned on the Rossmann domain of the ATP binding subunit. It was hypothesized that R98 and K100 act as allostERIC switches by controlling the conformation of the catalytic residue E127 in response to p/t binding. No such repositioning is observed. B) Repositioning of the hypothesized DNA-activating lysines. Overlay of the Fully-Open (gray) and Open-DNAp/t (color) models. In each panel, the models are aligned on the Rossmann domain of the ATP binding subunit of the interface. The trans-acting lysine and cis-aspartate 126 are shown as sticks. C) The steady-state ATP hydrolysis rate of the Wild-Type, δ'-K130A, γ-K141A, and γ-K141A/δ'-K130A clamp loaders. The steady-state ATP hydrolysis rates of the clamp loaders alone and in response to β-clamp (left). The steady-state ATP hydrolysis rate of the clamp loaders in response to p/t-DNA or p/t-DNA and β-clamp (right) (Mean ± S.D., n=3). D) Fold change of the ATP hydrolysis rate in response to p/t-DNA. The fold change ± standard deviation of the ATP hydrolysis rate of the clamp loader + p/t-DNA or clamp loader + sliding clamp and p/t-DNA over the clamp loader alone is shown (Fold changes calculated using ATPase rates from C, n=3).
Discussion:
Multi-step opening of the bacterial sliding clamp into a planar conformation

Our structures reveal how the *E. coli* clamp loader opens and places the β-clamp onto DNA. By arranging our structures into a series of steps, we produce a nearly complete mechanistic description of the clamp loading reaction in bacteria. We divide the clamp loading cycle into five different phases: i) clamp binding, ii) clamp opening: iii) p/t-junction binding, iv) clamp closure, and v) clamp loader recycling (Figure 2.13). The structures reported here illuminate four of the five phases of the clamp loading cycle. Future studies will focus on the clamp loader recycling phase, wherein the clamp loader exchanges ADP and P$_i$ for ATP, thus resetting the clamp loader. We acknowledge that these structures could be placed in a different sequence, but that mechanism proposed here best aligns with the available data (Ason et al., 2003; Goedken et al., 2004b; Hayner & Bloom, 2013; Hingorani et al., 1999; Kelch et al., 2011; LG et al., 2017; Paschall et al., 2011; Thompson et al., 2009; Turner et al., 1999a). Our structure of the Initial-Binding state shows the clamp binding phase of the cycle. Despite the low resolution of the structure, we unambiguously observe that the γ-complex initially binds to a closed β-clamp (Figure 2.3A; Figure 2.13 i). The existence of a closed binding complex was predicted based on rapid kinetics studies which showed that clamp binding occurs before clamp opening (S. Chen et al., 2009; Paschall et al., 2011; Thompson et al., 2009, 2012). The γ-complex binds the β-clamp using the A, D and E subunits, and with the A-gate of the γ-complex open. The open A-gate was predicted based on FRET experiments (Goedken et al.,
and highlights one difference from the eukaryotic clamp loader, which
binds to its clamp with a closed A-gate (Gaubitz et al., 2022).

The clamp opening phase (Figure 2.13 ii) begins with the intermediate Semi-
Open state and is followed by the Fully-Open state. This order of intermediates is
consistent with time-resolved FRET data (LG et al., 2017; Paschall et al., 2011).
The Semi-Open state, which was predicted by rapid kinetic studies (LG et al.,
2017), has all five AAA+ modules of the γ-complex gripping the β-clamp. The
AAA+ modules adopt a spiral matching the tight spiral of the clamp. The clamp
opening phase ends in the Fully-Open state, with the β-clamp opened into a
wide, planar arrangement (Figure 2.6B). This conformational change is driven by
a pivoting motion between the B and C subunits that flattens the spiral of both the
AAA+ modules and β-clamp as they open.

Our structures show that the clamp loader undergoes a dramatic
conformational change when opening the sliding clamp. Paradoxically, a previous
FRET study indicated that the distance between the A and E subunits does not
change during opening, and it was interpreted that the γ-complex does not alter
its conformation during clamp opening (Goedken et al., 2004b). An equilibrium
between the constricted Semi-Open and the splayed Fully-Open states could
explain the FRET data. The inter-probe distance estimated from the FRET data is
50-52 Å. The estimated inter-probe distances in our Semi- and Fully-Open states
are ~40 Å and ~55 Å, respectively. Taken together, the FRET data suggests that
the Fully-Open state accounts for ~75% of γ-complex bound to clamp in solution.
The β-clamp lacks density for an entire domain at the clamp opening in the Semi- and Fully-Open states, suggesting that the domain unfolds. Unfolding of this domain upon β-clamp opening was predicted from hydrogen-deuterium exchange data (Fang et al., 2011, 2014). This unfolding changes the size of the opening in the clamp, potentially affecting how DNA enters into the central chamber. Unfolded proteins are more extended, so this domain may partially block the gap through which DNA must enter. We speculate that the wide opening in the Fully-Open state allows for p/t-junction passage despite the potential steric block of the unfolded domain.

The third phase of the clamp loading cycle (p/t binding) is illuminated by our structures of γ-complex and β-clamp bound to p/t-junctions (Figure 2.13 iii). The p/t-bound state is nearly identical in the presence of either a DNA- or RNA-primer (Figure 2.6; Figure 2.9D), providing confidence in experiments using DNA-primed p/t-junctions. After p/t-junction binding, the clamp loader AAA+ domains tighten around the DNA helix, constricting the whole complex. This constricted spiral state follows the DNA backbone and is distinct from that observed in the Semi-Open state ($C_\alpha$-RMSD ~5.1 Å; Figure 2.9C).

Binding of a p/t-junction stimulates ATPase activity (Turner et al., 1999a), but it remains unclear how. We previously proposed that specific residues would trigger ATP hydrolysis after a conformational change upon p/t-junction binding. We first predicted that γ Lys100 and γ Arg98 within the AAA+ module controls ATPase activity in cis (Hedglin et al., 2013; Kelch, 2016; Kelch et al., 2011).
However, we observed that γ Lys100 or γ Arg98 do not have the predicted conformational change between the Fully-Open and Open-p/t structures (Figure 2.12A). On the other hand, trans-acting lysines (γ Lys141 and δ Lys130) directly contact the Walker B motif (Asp126) of the neighboring ATPase active site only after p/t-junction binding (Figure 2.12B). These lysine residues have been proposed to play a role in ATP hydrolysis based on bioinformatic (Neuwald, 2006) and deep mutational scanning analyses (Subramanian et al., 2021). We find that clamp loader variants with these residues mutated are still able to efficiently stimulate ATPase activity in response to p/t-junction binding (Figure 2.12D). This indicates that these lysines are not the DNA coupling element, as we hypothesized. However, the mutated variants have lowered overall ATPase activity (Figure 2.12C), suggesting that this trans-interaction plays a role in supporting the active site. We also could not identify the trigger for DNA-dependent ATPase stimulation from similar structures of the eukaryotic clamp loader (Gaubitz et al., 2022).

The clamp closure phase of the cycle is illuminated by our structures of the γ-complex bound to a β-clamp that is closed around p/t-DNA (Closed-DNA1 and Closed-DNA2) (Figure 2.13 iv). These structures show that clamp closure can occur with no significant structural change in the clamp loader. Nonetheless, there is a substantial loss of interaction of the D and E subunits with the sliding clamp. After clamp closure, the loader continues to disengage with the E, D, and C subunits. We speculate that this is on-pathway towards complete
Moreover, these structures indicate that clamp closure does not require ATP hydrolysis. Prior experiments showed that ATP hydrolysis precedes clamp closure (Hayner & Bloom, 2013). Our structures show that ATP hydrolysis is not strictly required for closure, but is more likely driving complete disengagement of the clamp loader. To reconcile our structures and the kinetic data, we note that all of the structures bound to p/t-junctions have their ATPase sites engaged, regardless of whether the p/t-junction contains an RNA or DNA primer, or whether the clamp is open or closed. Therefore, ATP hydrolysis presumably can manifest from any of these states, including prior to clamp closure.
Figure 2.13. Updated bacterial sliding clamp loading mechanism. (i) Clamp loaders first bind ATP, then the sliding clamp. (ii) Sliding clamp opening occurs in two steps: first, a spiral state with a partial opening of the sliding clamp, and then a wide open state with a planar conformation. Domain I of subunit II of the sliding clamp is unstructured in both. (iii) The clamp loader/sliding clamp complex binds a p/t-junction and adopts a helical conformation. (iv) Binding of the p/t-junction triggers ATP hydrolysis and the sliding clamp closes around the p/t-junction. (v) The sliding clamp dissociates from the closed sliding clamp and ADP, and the loaded sliding clamp can be used by DNA replication and repair factors.
Different opening mechanisms between bacterial and eukaryotic clamp loaders

The *E. coli* clamp loader has been studied for decades and has been the archetype for bacterial clamp loaders (Jeruzalmi et al., 2002; Kelch et al., 2012). Although eukaryotes have multiple clamp loaders (Kelch, 2016; Majka & Burgers, 2004), their primary clamp loader is RFC, analogous to the γ-complex. Recent structural studies have revealed RFC’s mechanism in unprecedented detail (Bowman et al., 2004a; Gaubitz et al., 2020, 2022; X. Liu et al., 2022; Schrecker et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022), which allows us to compare bacterial and eukaryotic clamp loaders.

The two clamp loaders bind to their sliding clamps differently during the clamp loading cycle, which may reflect differences within the sliding clamp itself (Figure 2.14A). Each sliding clamp subunit has a strong partner binding site within one domain, while the remaining domains contain weak binding sites (Figure 2.14B). These strong binding sites are used by other DNA replication and repair proteins. Because β-clamp and PCNA have different stoichiometries (dimer vs. trimer), β-clamp has two strong binding sites whereas PCNA has three. The different symmetries change the registry of the strong binding sites with the clamp loader subunits. For instance, β-clamp’s strong binding sites are aligned with the A and D subunits of the clamp loader. Accordingly, γ-complex initially binds the β-clamp primarily using its A, D and E subunits (Figure 2.14A). In contrast, PCNA’s strong binding sites are aligned with the A, C, and E subunits of RFC, and RFC first
binds to the clamp using its A and C subunits with contributions from the B subunit (Bowman et al., 2004a; Gaubitz et al., 2020, 2022).

In either case, clamp opening corresponds with complete engagement of the remaining clamp loader subunits with the sliding clamp. For the *E. coli* system, β-clamp opening occurs primarily by engaging the B and C clamp loader subunits, whereas PCNA opening is concomitant with engagement of the D and E RFC subunits. Both loaders interact with the clamp most extensively in an open state. However, binding of a p/t-junction further increases the contacts between γ-complex and β-clamp but causes RFC to lose interaction with PCNA. Upon clamp closure around p/t-junctions, both complexes lose substantial interactions between the sliding clamp and the E subunit. In general, the D subunit of γ-complex has the most extensive interaction with β-clamp, while the A subunit of RFC has the tightest interaction with PCNA. This observation is surprising because the A subunit of the γ-complex (the δ protein) shows the tightest affinity with the sliding clamp (Leu & O'Donnell, 2001). We hypothesize that the different stoichiometry of the bacterial and eukaryotic sliding clamps and ultimately the positioning of strong binding sites is a major factor underlying the different binding and opening mechanisms.

The bacterial and eukaryotic sliding clamps also open into distinct conformations by distinct motions. The γ-complex opens its clamp in two steps, both of which have one domain of the sliding clamp unfolded. The clamp is first broken open with a downward motion into a spiral Semi-Open conformation. A
large pivoting motion at a single interface between the B and C subunits transitions the Semi-Open conformation into the planar Fully-Open state (Figure 2.14C). In contrast, RFC uses multiple pivoting motions between nearly all subunits to open PCNA into a spiral conformation with no obvious intermediates and no unfolding (Gaubitz et al., 2022). The planar opening of the bacterial β-clamp is wider than that seen in the spiral PCNA (Figure 2.14D). In addition, the γ-complex’s lack of an A’ domain could allow for greater conformational flexibility leading to the wider A-gate opening seen in the E. coli clamp loader. We speculate that wider opening of the planar β-clamp and A-gate allows for efficient DNA passage into the central chamber despite the steric block of the unfolded β-clamp domain.

Our comparative study leads to a hypothesis for how the conformation of the open clamp alters ATPase activity. We hypothesize planar conformations inhibit ATPase activity because the active sites are misaligned. Conversely, a spiral configuration of AAA+ domains results in better alignment of the ATPase active sites (Gaubitz et al., 2022). In support of this hypothesis, addition of PCNA boosts RFC’s ATPase activity (Gomes & Burgers, 2001), while addition of β-clamp to γ-complex inhibits ATPase activity (Figure 2.14E & (Turner et al., 1999a)). Moreover, Rad24-RFC decreases in ATPase activity in the presence of its cognate clamp (Figure 2.14D,E & (Majka & Burgers, 2004)), which it opens in a planar fashion (Castaneda et al., 2022a; Zheng, Georgescu, Yao, O’Donnell, et al., 2022a).
Are the different clamp opening mechanisms primarily driven by the energetics of the clamp loader or by that of the sliding clamp? We speculate that the sliding clamp plays the predominant role in dictating the opening geometry, although almost undoubtedly both components play some role. Molecular Dynamics (MD) simulations have suggested that PCNA prefers a right-handed helical conformation with multiple pivot points (Kazmirski et al., 2005), whereas the *E. coli* clamp prefers to adopt a planar conformation with pivoting at the same site as when bound to the γ-complex (Jeruzalmi, Yurieva, et al., 2001). These same geometries are observed in structures of the open sliding clamps bound to their respective clamp loaders. Thus, the clamp loader does not appear to be imparting the conformation onto the sliding clamp. In further support of this hypothesis, the T4 bacteriophage sliding clamp (in the presence of p/t-DNA) is opened into a right-handed spiral by its cognate clamp loader (Kelch et al., 2011) and MD simulations show that the free T4 clamp prefers to adopt a right-handed spiral in the open form (Oakley, 2016). We also note that Rad24-RFC, which shares four out of five subunits with the RFC loader, opens the 9-1-1 clamp into a planar conformation (Castaneda et al., 2022a; Zheng et al., 2023; Zheng, Georgescu, Yao, O’Donnell, et al., 2022a). Accordingly, we predict that the free 9-1-1 clamp prefers planar opening, even without the presence of the Rad24-RFC loader. Moreover, addition of PCNA to Rad24-RFC results in a boost in ATPase activity (Figure 2.14E) (Majka & Burgers, 2004), presumably because PCNA prefers to open into a right-handed spiral and thus aligns the ATPase sites more
optimally.
Figure 2.14. Comparing bacterial and eukaryotic clamp loader mechanisms. A) Interface area between the *E. coli* (left) and *S. cerevisiae* (right) clamp loader and sliding clamp at comparable stages during clamp loading\(^2\). When multiple structures correspond to one state, we report the average value across all structures. B) Location of strong binding domains of the sliding clamps are highlighted with asterisks. C) Comparison of the clamp opening motions of the bacterial clamp loader and RFC. The colored shapes represent the Rossmann domains of each subunit and the gray rings represent the sliding clamp (viewed from above). Arrows indicate the motion of the Rossmann domains transitioning from the initial binding to fully open conformations. D) Structures of the open bacterial clamp loader, Rad24-RFC (PDB: 7ST9), and RFC (PDB: 7TI8) complexes bound to their cognate sliding clamps (displayed as low contour surfaces). The solid line approximates the plane of the corresponding closed sliding clamp. E) The ATPase activity of the bacterial clamp loader, Rad24-RFC\(^{9,1-1}\), and RFC\(^{2}\), either alone or with a sliding clamp. ATPase activities are relative to the rate of the clamp loader alone (Mean ± SEM, n=3)
A final distinction between bacterial and eukaryotic clamp loaders is that RFC partially unwinds the p/t-junction (Gaubitz et al., 2022), while the *E. coli* loader has no obvious base-melting activity. In our structures or elsewhere (Simonetta et al., 2009), we observe no unwinding of the p/t-junction, regardless if the primer is DNA or RNA. Moreover, our previous fluorescence data indicate that the *E. coli* clamp loader is unable to unwind a DNA p/t-junction (Gaubitz et al., 2022). RFC’s unwinding activity is thought to facilitate its role in loading at nicked DNA for performing long-patch Base Excision Repair (H. Li et al., 2022; X. Liu et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022). Because the *E. coli* clamp loader is not known to play a role in Base Excision Repair, presumably it does not require DNA unwinding activity. Nonetheless, the *E. coli* γ-complex is competent to load β-clamp onto nicked DNA (N. Yao et al., 2000). This activity remains mysterious, as our structure suggests that at least five nucleotides of ssDNA in the template strand are necessary to exit the central chamber of the loader. We note that the *E. coli* clamp loader has flexibility in its separation pin element (Figure 2.6D), which allows it to adopt two different heights of the primer 3’ end (Figure 2.9E). We propose that this flexibility may play a role in loading at nicked DNA. Future studies will illuminate this mechanism.

**Speculation on the Role of the Alternate Collar Conformation**

We observe a novel conformation of the collar region in the Altered-Collar class of particles that contains p/t-DNA. This conformational change is much larger than seen in previous structures, in which minor modification of the collar
resulted in modulation of the ψ protein binding site (Simonetta et al., 2009). The Altered-Collar state observed here severely disrupts the ψ protein binding site (Figure 2.10D), suggesting that this conformation is incompatible with ψ protein binding. It is possible that the Altered-Collar state functions as a means for disengaging the clamp loader’s linkage to SSB through the ψ protein.

We also observe that a pore opens between the AAA+ modules of the A and B subunits (Figure 2.10C). The position of this pore is similar to a pore that we recently discovered in the yeast RFC complex that is important for loading onto nicked DNA for repair (Gaubitz et al., 2022; X. Liu et al., 2022; Schrecker et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022). Because the E. coli clamp loader can readily load β-clamp onto nicked DNA (N. Yao et al., 2000), we speculate that this pore could function as a channel for the unwound primer strand of nicked DNA to exit the central chamber, similar to RFC. Future experiments will test these two non-mutually exclusive hypotheses.

Conclusions
Clamp loaders and sliding clamps are the most evolutionarily conserved components of the replication fork across life (N. Y. Yao & O’Donnell, 2016). Our comparative study of clamp loading in bacteria and eukaryotes reveals the intricate molecular choreography that underlies clamp loading over one billion years of evolution. We find numerous distinctions between bacterial and eukaryotic clamp loading, despite the universal conservation of clamp loading throughout life. Our work opens the door for potentially targeting the clamp
loading process of bacteria for development of novel antibiotics.

**Data Availability**

Cryo-EM maps and corresponding models have been deposited to the Electron Microscopy Data Bank (accession codes EMD-43096, EMD-43095, EMD-43098, EMD-43102, EMD-43094, EMD-43099, EMD-43100, and EMD-43101) and the PDB (accession codes 8VAN, 8VAM, 8VAP, 8VAT, 8VAL, 8VAQ, 8VAR, and 8VAS).

**ACKNOWLEDGMENTS**

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Chapter III: Inhibition of the E. coli clamp loader by N4 phage protein gene product 8
Preface
The work presented in this chapter was completed by myself and fellow graduate students in the Kelch lab. I prepared the figures and the written contents of this chapter. I developed the purification methods for gp8 and performed the ATPase assay presented in Figure 3.3A. The human Replication Factor C (clamp loader) and Proliferating Cell Nuclear Antigen (sliding clamp) were purified by Xingchen Liu and Joseph Magrino, respectively, and gifted to us to perform the assays presented in Figure 3.4. Krishna Ahn prepared the samples, performed the ATPase assays, and analyzed the data presented in Figures 3.4B and 3.4C.
Introduction:
Phages evolved multiple mechanisms to efficiently replicate inside their host cells. By inhibiting host growth, phage preserve resources for their own replication, and replicate more effectively. One previously identified example of this is phage lamba Kil protein, which inhibits *E. coli* cell division (Haeusser et al., 2014; Sergueev et al., 2002). Another method used by bacteriophage is to target the host RNA polymerase preventing host transcription (Tagami et al., 2014). Similarly, N4 coliphage express the protein gene product 8 (gp8) which inhibits host DNA replication (Schito, 1974; Yano & and Rothman-denes, 2011).

Yano and Rothman-Denes cloned an *E. coli* strain with inducible gp8 expression (Yano & and Rothman-denes, 2011). Gp8 expression blocked cellular replication, and the resulting cells had an altered elongated morphology. Fluorescent colocalization microscopy showed that fluorescent gp8 formed foci that colocalized with the tau subunit of the clamp loader. Because fluorescence microscopy has a resolution limit of ~250 nm, this result alone does not show that gp8 interacts with the clamp loader, only that gp8 colocalizes with the replication fork (Macdonald et al., 2015). To investigate gp8 binding partners, Yano et al. performed *in vivo* crosslinking followed by mass-spectroscopy. Several proteins crosslinked with gp8, and the major protein identified was the tau subunit of the clamp loader. Tau is the most abundant protein in the DNA polymerase III holoenzyme. Yano et al. also identified two mutations in the delta protein that suppress gp8’s growth inhibition. One resistant colony was identified from
gamma/tau mutagenesis, but this mutation was not identified. Thus, gp8 likely binds to the clamp loader \textit{in vivo}.

To obtain direct evidence that gp8 targets the clamp loader, Yano et al. next tested gp8 \textit{in vitro}. To test whether gp8 inhibits DNA synthesis \textit{in vitro}, they used a DNA synthesis assay in which the DNA polymerase III holoenzyme is reconstituted with primed ssDNA and radiolabeled nucleotides. DNA synthesis can then be assessed by the production of radiolabeled DNA (Glover & Mchenry, 1998). Gp8 inhibited DNA synthesis in a concentration dependent manner. Additionally, this assay was repeated such that the sliding clamp was preloaded onto primed DNA to test whether gp8 inhibited clamp loading or clamp usage. This experiment found that gp8 did not affect DNA synthesis when the sliding clamp was preloaded onto the DNA, showing that gp8 inhibits clamp loading but not its usage. Finally, to directly test if gp8 binds to the delta subunit, pull-down assays were performed between gp8 and delta or the gp8 resistant delta mutants. Gp8 and wild-type delta coelute, whereas gp8 did not coelute with either delta variant. Taken together, this study supports that gp8 inhibits clamp loading by targeting the A subunit (delta).

In the following section, I summarize my research on gp8. Taken together, Yano et al. showed that gp8 inhibits DNA replication by targeting the host clamp loader. The exact mechanism by which gp8 interacts with and inhibits the clamp loader is still unknown. Gp8 may inhibit how the clamp loader binds the sliding clamp and/or p/t-DNA, binds or hydrolyzes ATP, or releases the sliding clamp. I
was unable to identify how gp8 interacts with or inhibits the clamp loader. Surprisingly, I found that gp8 copurifies with DNA, which it holds with high affinity. Future work will be needed to understand how gp8 interacts with the E. coli clamp loader and to investigate its interactions with DNA.

**Materials and Methods**

*Expression plasmid construction:*

6x His-SUMO-gp8 pSTM3: Inserts with the coding region for Gp8 and flanking regions containing restriction digest sites for XhoI and BamHI, were purchased from Twist Bioscience (Twist Bioscience, South San Francisco, CA). The insertion site for gp8 is immediately downstream of an N-terminal 6x His-SUMO tag coded for in the pSTM3 vector. We treated the inserts and the pSTM3 backbone with the restriction enzymes BamHI and XhoI and then purified them by gel extraction using the QIAquick Gel Extraction kit (Qiagen). The gp8 coding region was ligated into the pSTM3 vector using T4 DNA ligase in T4 DNA ligase buffer (New England Biolabs). These mixtures were transformed into chemically competent DH5α *E. coli* (New England Biolabs) and plasmids were recovered by miniprep (Qiagen). Plasmids were checked for proper gene insertion by Sanger sequencing (Genewiz). The resulting plasmid codes for 6x His-SUMO-gp8.

*Protein Expression:*

The 6x His-SUMO-gp8 pSTM3 plasmid was transformed into BLR21(DE3) *E. coli* cells (Millipore), grown overnight at 37°C, and then transferred into 50 ml Luria Broth media supplemented with 100 µg/ml Kanamycin pre-warmed to
These cultures were grown for 4 hours while shaking at 37°C. Then, the 50 mL cultures were used to inoculate 1 L of Terrific Broth media pre-warmed to 37°C. The cultures were grown until they had an optical density at 600 nm of 0.6-0.8. The cultures when then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM final concentration. Following induction, the cultures were grown for 4 hours at 37°C. Cells were then harvested by centrifugation at 5,000 x g for 30 minutes. Cell pellets were flash frozen in liquid nitrogen and then stored at -80°C.

Protein purification:

The bacterial clamp loader and sliding clamp were prepared as described in Chapter II.

To purify the 6x His-SUMO-gp8, the frozen pellets were thawed on ice and then resuspended in 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole (Buffer Ni-A) and lysed with a cell homogenizer (Microfluidics Inc, Westwood, MA). Cell lysates were then clarified by centrifugation at 20,000 x g for 20 minutes. Lysates were then loaded onto Nickel NTA Agarose Beads (GoldBio) pre-equilibrated with Buffer Ni-A. The loaded resin column was washed with at least 10 column volumes (CV) of Buffer Ni-A. The protein was then eluted with 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM BME, and 250 mM imidazole (Buffer Ni-B). Fractions were then checked for protein by SDS-PAGE and pooled.
His-SUMO-gp8 was treated with ULP1 protease while dialyzing into Buffer Ni-A overnight at 4°C to remove the His-SUMO tag. The dialyzed protein was then passed over nickel NTA agarose beads to trap the 6x His-SUMO tag while the cleaved gp8 passed through the resin. The flowthrough was collected and dialyzed into 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM BME overnight at 4°C. Finally, the gp8 was concentrated, flash frozen, and stored at -80°C.

His-SUMO-gp8 wash treatments

His-SUMO-gp8 was expressed, and the lysate was prepared and loaded onto Nickel NTA Agarose (GoldBio) beads as described in the previous section. The nickel-resin was then separated into separate columns to allow for them to be treated independently. Each column was washed with at least 50 CV of one of the following buffers:
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni-A</td>
<td>20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole</td>
</tr>
<tr>
<td>2 M Salt Wash</td>
<td>20 mM Tris pH 8.0, 2000 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole</td>
</tr>
<tr>
<td>pH 11.0</td>
<td>20 mM Caps pH 11.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole</td>
</tr>
<tr>
<td>Potassium Glutamate</td>
<td>20 mM Tris pH 8.0, 500 mM KGlutamate, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole</td>
</tr>
<tr>
<td>DNase I Buffer</td>
<td>20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole, 1 U/ml DNase I</td>
</tr>
</tbody>
</table>

Table 3.1 Buffer composition used to wash the His-SUMO-gp8.

After washing the resins with the first buffer, the resins were washed with 10 CV of buffer Ni-A. The protein was then eluted with Ni-B buffer.

*Purification under denaturing condition and refolding:*

His-SUMO-gp8 was expressed and the lysate was prepared as previously described. Guanidine HCL salt was then added to the lysate to a final concentration of 6 M. Nickel NTA Agarose resin pre-equilibrated in buffer Ni-A was added to the lysate and incubated at 4°C for 1 hour while mixing. The resin was then pelleted by centrifugation at 500 rpm for 7 minutes and collected. The
resin was washed with 10 CV of Ni-A + 6 M guanidine HCl. Protein was then eluted in buffer Ni-B + 6 M Guanidine HCL.

**His-SUMO-gp8 Refolding:**

*Dialysis:* The eluted His-SUMO-gp8 was dialyzed into 1000x (v/v) Ni-A buffer overnight at 4° C.

*Rapid-Refolding:* The eluted His-SUMO-gp8 was added drop-by-drop into 1000x (v/v) ice cold Ni-A while constantly being mixed.

**Sample Imaging:**

*SDS-page gel:* To prepare the samples for SDS-Page analysis, 10 ul of the wash or elution from each sample were combined with 2 μl 5x SDS-Page Loading Buffer to a final concentration of 50 mM Tris-Cl pH 6.8, Bromophenol Blue 1.6%, 20 mM dithiothreitol, and 8% glycerol. The SDS-Page sample were then heated at 95° C for 5 minutes. The samples were loaded onto a 14% acrylamide gel and run at 200 V for ~1 hour. The SDS-Page gels were then stained with Coomassie Blue.

*Native gel:* Samples were prepared by combining 10 μl of either wash or elution sample and 2 μl 5x loading dye. The samples were then run on a 1.5% agarose gel. The gel was then fixed and stained with 5% ethanol and 7.5% acetic acid and Coomassie Blue for 1 hour at room temperature. The gel was then destained overnight at room temperature in 5% ethanol and 7.5% acetic acid.
**Urea Denaturing Gel:** Samples were prepared by combining 10 μl of either wash or elution sample and 2 μl 5x SDS-Page Loading Buffer. A 5% acrylamide urea denaturing gel was prepared by combining 4 ml of 5x TBE buffer, 2.5 ml of 40% (w/v) acrylamide/bisacrylamide, and 19.2 g of Urea (8 M final concentration), 40 μl TEMED, and 400 μl of 10% (w/v) APS and bring the solution to a final volume of 40 ml. The gel was pre-run with 1x TBE for 30 minutes. Samples were then loaded and run on the gel for 1 hour. After running, the gel was washed with 1x TBE for 15 minutes. The gel was stained with GelRed® in 1X TBE for 1 hour and then imaged under a UV light.

**Native gel and assessment of the gp8 band migration:**

A native gel of the His-SUMO-gp8 sample was prepared as previously described accept that the gel was 2.5% agarose supplemented with GelRed®. The sample was run with a TriDye™ Ultra Low Range DNA Ladder. The gel was imaged under UV light. Then the gel was treated with 5% ethanol and 7.5% acetic acid solution supplemented with Coomassie Blue to stain for protein for 1 hour at room temperature. Finally, the gel was destained in a 5% ethanol and 7.5% acetic acid solution overnight at room temperature. The image of the DNA migration of the gel was analyzed in ImageJ (Schneider et al., 2012). The distance from the well to the base of each distinct band was measured in pixels. The migration distance of each step in the ladder was plotted against the natural log of its length in base pairs. The line was then fit, length $y_{\text{length}} = mx_{\text{migration distance}} + b$,.
where \( m = -0.0045 \) and \( b = 6.9 \). The formula was then used to calculate the lengths of the distinct bands in the gp8 sample.

**DNA Protection Assay**

**Dnase I Treatment:** Samples of purified gp8 with the copurified DNA were diluted to 50 ng/μl of DNA in 10 mM Tris pH 8.0, 25 mM NaCl, 2.5 mM MgCl\(_2\), and 0.5 mM CaCl\(_2\). Then, Dnase I was added to a final concentration of 0.05 U/μl. Sample were heated at 37°C for 1 hour. Then, to inactivate the Dnase I, EDTA was added to a final concentration of 5 mM. An aliquot of the post-Dnase I treatment sample was taken for further analysis. Next, to digest any gp8 bound to the DNA, proteinase K was added to a final concentration of 100 μg/ml and the sample was incubated at 37°C overnight. Finally, the proteinase K was inactivated by heating the sample at 95°C for 10 minutes. To prepare a proteinase K alone treated sample, purified gp8 was diluted to 50 ng/μl of DNA in 10 mM Tris pH 8.0, 25 mM NaCl, 2.5 mM MgCl\(_2\), and 0.5 mM CaCl\(_2\) and proteinase K was added to a final concentration of 100 μg/ml and the sample was incubated at 37°C overnight the proteinase K was inactivated by heating the sample at 95°C for 10 minutes.

As a control the same treatments were perform on genomic bacterial DNA prepared by ethanol precipitation according to [https://lab.rockefeller.edu/chen/assets/file/DNAPrecipitation.pdf](https://lab.rockefeller.edu/chen/assets/file/DNAPrecipitation.pdf).
**Bacterial DNA preparation:** Briefly, DH5α *E. coli* cells were grown in 25 ml of Luria broth overnight at 37°C. They were then lysed with a homogenizer (Microfluidics Inc, Westwood, MA). The lysate was clarified by centrifugation at 20,000 rpm for 20 minutes and then the solution fraction was taken. 2.5 ml of 3M Na-Acetate was added to the lysate. Next, 50 ml of 100% ethanol was added that was pre-chilled to -20°C. The solution was then stored at -20°C for 1 hour. The solution was then transferred to 50 ml conicals and centrifuged at 4,000 rpm for 20 minutes. The ethanol was then decanted, and the pellet was washed with room temperature 70% ethanol. The pellet was air dried for 20 minutes at room temperature. Finally, the DNA was resuspended in 10 mM Tris pH 8.0 and 1 mM ETDA. The concentration was then quantified by measuring absorbance at 260 nm.

**Sample Analysis:** Samples of the bacterial DNA and gp8 treatments were prepared for analysis by agarose gel. 2 μl of 5x gel loading dye was added to 8 μl of each sample. The samples were then loaded onto a 2% agarose gel containing GelRed®. The gels were then visualized under UV fluorescence.

**Protein Visualization:** After the gels were visualized under UV light, the agarose gel was stained with as solution of Coomassie blue in 5% ethanol and 7.5% acetic acid to visualize the protein for 1 hour at room temperature. After staining, the gel was destained in a solution of 5% ethanol and 7.5% acetic acid overnight at room temperature.
ATPase assays

Boiled gp8 samples: Aliquots of purified gp8 were treated at 95°C for 15 minutes to denature the protein. The solutions were then centrifuged at 20,000 RFC for 10 minutes in an Eppendorf 5424 centrifuge. The supernatant was then collected to be used for testing.

Bacterial clamp loader plus gp8: 0.3 µM γ-complex was incubated with 0.2 µM β-clamp, 10 µM gp8, 0.4 µM p/t-junction, and a master mix of 5 U/ml Pyruvate kinase, 5 U/ml lactate dehydrogenase, 1 mM ATP, 150 µM phosphoenol pyruvate, 50 µM NADH, 25 mM Tris (pH 7.5), 100 mM potassium glutamate, 5 mM magnesium chloride. The oligonucleotide sequence for the 20-bp primer is 5’-GCAGACACTACGAGTACATA-3’ and the 30-bp template is 5’-TTTTTTTTTTATGTACTCGTAGTGTCTGC-3’, oligonucleotides were purchased from Genewiz. ATPase rate was measured at 25°C with a VICTOR Nivo multimode plate reader (Perkin Elmer) to detect NAD+. Rates were obtained from a linear fit of the data in GraphPad Prism.

Human RFC plus gp8: The assay is performed the same as with the bacterial clamp loader except that the protein concentrations were, 0.15 µM RFC and 1 µM PCNA, 10 µM Gp8. Additionally, the buffer conditions were the 5 U/ml Pyruvate kinase, 5 U/ml lactate dehydrogenase, 1 mM ATP, 150 µM phosphoenol pyruvate, 50 µM NADH, 25 mM Tris (pH 7.5), 100 mM potassium acetate, and 5 mM magnesium chloride.
Cryo-EM Studies

Sample preparation: We combined equimolar clamp loader and sliding clamp and gp8 to a final concentration of 3 µM, then ATPγS was added to a final concentration of 1 mM. We then treated the sample with 1 mM of bis(sulfo succinimidyl) suberate (BS3, Thermo Scientific Pierce) for 15 minutes at room temperature. To neutralize the cross-linking reaction, Tris-HCl was added to a final concentration of 25 mM. The sample was stored on ice until grid preparation.

Cryo-EM grid preparation: The Quantifoil R 0.6/1.0 grids were first washed with ethyl acetate. The grids were then glow discharged on a PELCO easiGlow for 60 s at 25 mA (Ted Pella) (negative polarity). 3.5 µL of sample was applied to a grid and the grid was then blotted on both sides with a blot force of 5, a blot time of 5 seconds, and wait time of 0 seconds. The grids were then vitrified by plunging into liquid ethane using a Vitrobot Mark IV (FEI) at 10°C and 95% humidity.

Cryo-EM data collection: The sample was imaged on the Talos Arctica operated at 200 kV equipped with an GIF energy filter at x45000 magnification and a pixel size of 0.435 Å (bin=0.5) using a K3 Summit direct electron detector (Gatan) in superresolution counting mode. The data were collected with a target defocus range of -1.1 to -2.3 and a total exposure of ~43 e⁻/Å² averaging 30 frames.

Cryo-EM data processing: Micrographs were aligned in IMOD with 2x binning, resulting in a pixel size of 0.87 Å per pixel. After micrograph alignment, all further
data processing was completed within cryoSPARC (Punjani et al., 2017). The initial Contrast Transfer Function (CTF) estimation was done using CTFFind4 (Rohou & Grigorieff, 2015). Following CTF correction, one round of Blob picking (90-160 Å) was performed to complete reference free particle picking. Particles were then extracted with a box size of 256 pixels. To identify quality particles, we performed two rounds of 2D classification and selected classes with well-defined features. The particles were then subjected to 2 rounds of 2D classification and class selection. 2D classes with well-defined features were selected and then used to generate three ab initio models. The highest quality ab-initio model appeared to be of a clamp loader, and its associated particle stack was selected for further processing (211,589 particles). We then subjected this particle stack to two rounds of 3-D classification followed by homogeneous refinement. Homogeneous refinement was implemented on the final particle stack. The map was sharpened with a B-factor of -210.7 Å$^2$ and the cryo-EM map had a resolution of 6.9 Å (FSC=0.143).

Model Building, refinement, and validation: We used the crystal structures of the apo-γ-complex (PDB:1JR3) (Jeruzalmi, O’Donnell, et al., 2001) as an initial model. Each subunit was split into globular domains and then each domain was fit into the cryo-EM maps using UCSF ChimeraX (Pettersen et al., 2021). The fit of the model was improved using Coot for manual building. The by residue cross-correlation was calculated using Coot (Afonine et al., 2018).

Model prediction:
To obtain a model prediction for the gp8 monomer, I submitted a fasta file of the gp8 sequence to the Cosmic2 server and ran Alphafold2 (Cianfrocco et al., 2017; Jumper et al., 2021). To generate a model prediction of gp8 bound to either delta or the sliding clamp, I submitted a fasta file with the sequence for gp8 and either the full-length delta subunit or the Rossmann domain (residues 1-150) to the Cosmic2 server and ran Alphafold-multimer (Evans et al., 2022). To predict models of gp8 bound to dsDNA, I uploaded the predicted gp8 monomer model, predicted from Alphafold, and a model of 20 base pairs of b-form dsDNA to the pyDockDNA Server (Rodríguez-Lumbreras et al., 2022). The models visualized and analyzed with a combination of PyMol and UCSF ChimeraX (Meng et al., 2023).

Results

Gp8 binds to genomic bacterial DNA

When purifying gp8 from BLR21 E. coli, it became apparent that gp8 was binding DNA. Eluted protein that was devoid of protein contaminants (as shown by SDS-PAGE) had a 260/280 absorbance ratio of >1.6, in contrast to pure protein that has a 260/280 ratio of ~0.6. I attempted to remove the DNA by immobilizing, washing, and eluting the protein over a ceramic-hydroxyapatite type (CHT) column as was done in Yano et al. (Yano & Rothman-denes, 2011) or a heparin column, but neither approach removed the DNA. To determine if the DNA could be removed from gp8 with an aggressive wash treatment, I treated
samples of his-SUMO-gp8 with various buffers (Table 3.1). To do this, I immobilized his-SUMO-gp8 from cell lysate on nickel resin. I then divided the resin into separate columns so that I could treat the immobilized protein with different buffer conditions. Next, I washed the resins with >50 CV of one of the buffer conditions from Table 3.1. The base condition of the wash buffer was 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole. One aspect of the buffer was altered to try to remove the DNA. The 2 M sodium chloride buffer, instead of 500 mM, was used to disrupt electrostatic interactions. The solution buffered with pH 11.0 Caps, instead of pH 8.0 Tris, shifted the pH of the buffer about the predicted pI of gp8 (pI ~ 9.6). The salt used was switched to potassium glutamate, because potassium glutamate is the most abundant cytoplasmic salt in bacteria and not sodium chloride (Bennett et al., 2009). Finally, Dnase I was added to the base buffer to digest unprotected DNA. Following the individual washes, the protein from each column was eluded with the base buffer plus 250 mM imidazole.

Following the individual washes, samples of the wash and elution were collected and analyzed by native gel, denaturing acrylamide gel, and SDS-PAGE. The SDS-PAGE analysis shows that each elution predominantly contains gp8 and no other proteins (Figure 3.1C). In the second lane, a sample of his-SUMO-gp8 was treated with ULP1 and purified under normal buffer conditions. The predominant band is ~10 kDa, as expected for gp8. The other samples, lanes 3-6, were run prior to ULP1 treatment and so the predominant band is ~23 kDa, as
expected for His-SUMO-gp8. Samples of gp8 run on native PAGE are smeared as opposed to running as distinct bands (Figure 3.1A). When the gp8 protein samples were stained for DNA using the ethidium analog GelRed® to visualize the DNA, all of the samples contained DNA migrated as smears, indicating that they are of varying sizes (Figure 3.1.B). The DNase I treated sample showed that a DNA smear was still present on the gel, indicating that this treatment did not remove the DNA, nor cleave it to a specific size (Figure 3.1.B, lanes 7-9). To determine if disrupting the structure of gp8 would remove the DNA, I attempted to purify gp8 with 6M guanidine. A Coomassie stained SDS-PAGE gel of the lysate and eluate of His-SUMO-gp8 shows that gp8 was successfully eluted off of the nickel resin after guanidine treatment and had no visible protein contaminants (Figure 3.1D). This sample is free of DNA, as determined by staining with the ethidium analog GelRed® in an agarose gel (Figure 3.1E). Thus, I was only able to isolate DNA-free gp8 protein by using an aggressive denaturation strategy.

Following unfolding, I attempted to refold the His-SUMO-gp8 to obtain active DNA-free protein. In the first method, the unfolded His-SUMO-gp8 eluate was dialyzed into 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5 mM β-mercaptoethanol (BME), and 20 mM imidazole overnight. This method slowly decreases the concentration of the eluate, providing time for gp8 to fold into its native conformation (Yamaguchi & Miyazaki, 2014). The second method that I attempted was rapid-refolding (Yamaguchi & Miyazaki, 2014). In this technique, I diluted his-SUMO-gp8 in 20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol (v/v), 5
mM β-mercaptoethanol (BME), and 20 mM imidazole drop-by-drop, immediately diluting the denaturant to refold the protein. Following both techniques, I planned to concentrate the protein by immobilizing it on a nickel-resin and eluting with a smaller volume. Unfortunately, the protein in the refold samples precipitated, suggesting that His-SUMO-gp8 was unable to refold or was unstable once refolded under these conditions.
Figure 3.1. Protein and DNA imaging of gp8 purified under different conditions. A. Protein migration under native conditions of purified gp8. Gp8 Agarose gel run under native conditions of His-SUMO-gp8 samples treated with different non-denaturing conditions during the wash step of the purification. Samples are labelled FT for flow-through, W for wash, or E for Elution. The agarose gel was stained with Coomassie Blue to visualize the protein migration. B. DNA migration under denaturing condition of purified gp8. Acrylamide gel run under denaturing conditions image for DNA of His-SUMO-gp8 samples washed with different buffer compositions. The gel was stained with GelRed® and imaged with UV light to visualize the DNA migration. C. SDS-Page gel visualizing the protein in the elutions from each of the different wash methods. His-SUMO-gp8 is ~25 kDa while gp8 is ~8 kDa. D. SDS-Page gel showing gp8 purified under denaturing condition (6M guanidine). Lane 1 shows the lysate, lane 2 shows the eluted protein. E. Agarose gel comparing samples of gp8 purified under denaturing conditions. The gel was stained with GelRed® and DNA was visualized under UV illumination.
When the migration of the gp8 sample is visualized under non-denaturing conditions periodic bands are present (Figure 3.1E, lane 2). The bands suggest that gp8 binds to DNA in a uniform manner. To determine the size of these bands, I separated the his-SUMO-gp8 sample on an agarose gel and quantified their sizes (Figure 3.2A). I measured the migration of the ladder and plotted the distance migrated against the band size, ln(bp), to obtain a standard curve which could be fit to calculate size (bps) based on the migration distance. The bands migrated as 200, 170, 130, and 95 base pairs, respectively. The distance between the bands is 35 ± 4 bps suggesting that there is uniform assembly.
Figure 3.2. Untreated gp8 agarose gel migration. A. *Purified gp8 migrates through an agarose gel forming distinct bands*. Visualization of gp8 run through an agarose gel stained with GelRed and imaged by UV-light. The locations of the distinct band are indicated next to the gel. B. *Linear fit of the ladder verse migration distance*. The natural logarithm of the bands known sizes were then plotted against the distance that they migrated (measured in ImageJ). The equation for the linear fit of they values is displayed as well as
**Gp8 DNA protection**

To determine whether gp8 could protect DNA from digestion by nucleases and the size of the DNA fragment that gp8 protects, I performed a DNA protection assay. The purified gp8 bound to DNA was treated with DNase I to degrade any unprotected DNA. To remove the gp8 from the DNA, the samples were treated with proteinase K. Treated samples were then analyzed by agarose gel staining for either DNA (using GelRed®) or for protein (using Coomasie). A schematic of the assay is depicted in Figure 3.3A.

We observed that gp8 protects DNA (Figure 3.3B). The untreated gp8 sample shows DNA staining between 75-700+ base pairs, with the banding pattern we observed previously. Treating the sample with only proteinase K caused the DNA to migrate faster through the gel as a smear. Interestingly, there is no visible DNA when gp8 is treated with Dnase I alone (Figure 3.3B, lane 4). It was expected that we would observe a band of DNA in this sample corresponding to gp8 bound DNA. I hypothesize that the nuclease degraded the exposed DNA and that gp8 prevents the dye from associating with the DNA. Finally, there is a distinct band between 25-35 base pairs when gp8 is treated with both Dnase I and proteinase K. As a control, I performed the same treatments on genomic *E. coli* DNA. The control DNA migrated the same in the
untreated and proteinase K treated samples and no DNA was visible in either of DNase I treated samples.

When the samples were analyzed under nondenaturing conditions and the protein was visualized, staining was not observed in samples treated with proteinase K (Figure 3.3C, lanes 2 and 4). However, staining was observed in the completely untreated sample (lane 1) and the sample treated with DNase I alone (lane 4) (Figure 3.3C).
Figure 3.3. Purified gp8 treated with nuclease. A. Schematic of the experimental design and the hypothetical results. B. DNA migration of the samples through an agarose gel. Samples were treated, run through and agarose gel, and the DNA was imaged by staining with GelRed and imaging under UV light. C. Agarose gel stained with Coomassie Blue to visualize protein migration in the prepared samples.
The effect of the purified gp8 on ATP hydrolysis

To understand if the purified gp8 inhibits the *E. coli* clamp loader, I tested the effect of gp8 on the ATPase activity of the *E. coli* clamp loader. The clamp loader hydrolyzes ATP at the completion of each clamp loading cycle, and so monitoring the clamp loader’s ATPase activity reports on successful clamp loading events. If gp8 were to inhibit any stage of clamp loading, it would result in a decrease in the rate of ATP hydrolysis under steady-state conditions. To measure the clamp loader’s steady-state ATPase activity, I used an enzyme-coupled ATPase assay which has previous been used to study ABC transporters, Origin Recognition Complex, and clamp loaders (Hughes et al., 2019; X. Liu et al., 2022; Schmidt & Bleichert, 2020). The assay links the hydrolysis of ATP to ADP with the oxidation of NADH to NAD$^+$ through a multistep enzymatic pathway (Kiianitsa et al., 2003). ATP is regenerated throughout the assay and so its concentration is held constant. The conversion of NADH to NAD$^+$ is monitored by measuring the absorbance at 340 nm over time. Because the hydrolysis of one ATP molecule is coupled with the oxidation of one NADH molecule, the change in absorbance at 340 nm can be used to measure ATP hydrolysis. This assay is limited in that it cannot determine which step in the clamp loading cycle gp8 inhibits since preventing any step in the cycle would decrease ATP hydrolysis.

The ATPase activity of the clamp loader samples that were not treated with gp8 had the anticipated ATPase profile (Figure 3.4A) (Turner et al., 1999b). The ATPase activity decreases with the addition of the sliding clamp, while the
addition of p/t-DNA stimulated the ATPase activity over that of the clamp loader alone. Finally, the greatest ATPase activity is observed with the clamp loader, sliding clamp, and p/t-DNA together.

Because gp8 inhibits clamp loading, we expected it to reduce or completely inhibit ATP hydrolysis. Surprisingly, we observed that gp8 stimulates the clamp loader’s ATPase activity (Figure 3.4A). The clamp loader treated with gp8 protein had a 5-fold increase in the ATPase activity compared to the clamp loader alone, and gp8 stimulated the ATPase activity of the complex of clamp loader and sliding clamp by ~15-fold. However, when the clamp loader, sliding clamp, and p/t-DNA were all present, gp8 failed to further stimulate ATP hydrolysis.

Because DNA copurifies with gp8 and p/t-DNA stimulates clamp loader ATPase activity, the ATPase stimulation observed by adding gp8 may have been caused by the DNA and not the gp8. To test this, we boiled the purified gp8 sample to denature the protein to see if the sample would still stimulate ATPase activity. The boiled gp8 sample had a similar stimulatory effect on the ATPase activity as the non-boiled sample (Figure 3.4B). To further test if the stimulatory effect of gp8 was caused by contaminating DNA, we treated the human clamp loader (hRFC) with gp8. Because the *E. coli* and human clamp loaders are so widely diverged in sequence and structure, it is highly unlikely that gp8 can bind to the human clamp loader. However, both the human and *E. coli* clamp loaders are stimulated to hydrolyze ATP by DNA binding (Gomes et al., 2001; Hingorani
et al., 1999). When hRFC+PCNA was treated with both untreated gp8 and boiled gp8 the ATPase activity was stimulated (Figure 3.4C). Thus, the stimulatory effect of the gp8 sample is likely a result of the contaminant DNA and not directly due to gp8 protein.

**Figure 3.4. Effect of treating clamp loaders with the purified gp8 sample.**

**A. Effect of gp8 on the ATPase activity of the E. coli clamp loader.** Steady-state ATPase activity of the purified E. coli clamp loader complex in combination with the sliding clamp, p/t-DNA and gp8 (Mean ± SEM, n=3).  **B. Effect of boiled gp8 on the ATPase activity of the E. coli clamp loader.** Steady-state ATPase activity of the purified clamp loader complex in combination with the sliding clamp and p/t-DNA and gp8 (Mean ± SEM, n=3).  **B. Effect of gp8 on the ATPase activity of the human clamp loader.** Steady-state ATPase activity of the purified human clamp loader (hRFC) in combination with the sliding clamp (PCNA), p/t-DNA and gp8 (Mean ± SEM, n=3).
Cryo-EM study of a clamp loader:sliding clamp:gp8:ATPγS sample

To determine the structure of gp8 bound to the clamp loader and sliding clamp, we collected a cryo-EM dataset of the three proteins and the slowly hydrolysable analog ATPγS. After particle picking, 2D-classification, and \textit{ab-initio} model reconstruction, I generated independent maps of the clamp loader and sliding clamp (Figure 3.5). I did not clearly identify any class of the clamp loader bound to gp8. Surprisingly, the 3D reconstruction of the sliding clamp from this dataset has additional density that is unaccounted for by clamp alone.

Processing this data resulted in a map of the clamp loader in the absence of both gp8 and sliding clamp. The local resolution of the reconstruction ranges from $\sim$4 Å, where secondary structure elements are identifiable, to >12 Å, where the density for entire domains is lacking (Figure 3.6A). The global resolution of the reconstruction is 6.9 Å based on the Gold-Standard FSC cutoff of 0.143 (Figure 3.6B). A model was made by rigid-body fitting individual domains from each subunit of the clamp loader from a previously solved model of the clamp loader (PDB:1jr3) into the map. Density for the collar domain of each subunit is clearly observed, which confirms that this reconstruction is of the fully assembled complex. The density for the lid and Rossmann domains is strongest for subunits A and E, while the density for those domains is significantly poorer in subunits B, C, and D. Overall, the worst density is for the Rossmann domain of the D subunit, which is hardly present (Figure 3.6). To assess the quality of the model-
to-map fit for each chain, I determined the by residue cross-correlation for each chain (Figure 3.6D). This analysis shows that the collar domain is best supported by the density, whereas the Rossmann domains of chains A-C have the least correlation. After interrogating the model fit into the map there is no apparent unassigned density, and so it does not appear that gp8 is bound to the clamp loader in this reconstruction.
Figure 3.5. Cryo-EM data processing Clamp loader, sliding clamp, Gp8, ATPγS sample. All data processing was performed using cryoSPARC. A representative aligned micrograph is shown. The identified particles were extracted, 2D classified, and then 2D classes of interest were selected. Selected particles were first used to generate three \textit{ab-initio} volumes. The particles used to generate the highest quality \textit{ab-initio} reconstruction of the clamp loader where then 3D classified into three classes. The two highest quality 3D classifies were identical and so they were pooled to generate a final 3D-reconstruction.
Figure 3.6. 3D Reconstructions from the Clamp Loader from the clamp loader:gp8:ATPγS dataset. A. Local resolution of the clamp loader cryo-EM map. Local resolution (Fourier shell correlation (FSC)=0.5) of the reconstructions. B. FSC curve of the clamp loader cryo-EM map. The overall resolution of the reconstruction was determined by the FSC of the half-maps using Gold-standard cutoff of 0.143 (blue line). C. Clamp loader model fit into the cryo-EM map. Different views of the model fit into the cryo-EM map of the clamp loader from this dataset. D. Accessment of the fit of the model into the cryo-EM map.
From this dataset, I observed 3D classes of the sliding clamp that I used to determine a 3D reconstruction with an overall resolution of \( \sim 4.7 \, \text{Å} \). It shows that the clamp is in a closed ring, which is unsurprising (Kong et al., 1992). While the resolution of the map does not allow for a model to be built \textit{de-novo}, the previously determined crystal structure of the \textit{E. coli} sliding clamp fits into the map (Figure 3.7). Surprisingly, there are two extraneous regions of density on one face of the sliding clamp. The low resolution of the map does not allow for this density to be confidently fit. To test whether the density could be explained gp8 binding, I determined maps of the sliding clamps from two additional cryo-EM datasets. In one dataset the sample is of the clamp loader, sliding clamp, and ADP•BeFx that was crosslinked and in the other the sample is not crosslinked and is of the clamp loader, sliding clamp, p/t-DNA and ADP•BeFx (both datasets were discussed in Chapter II, see Chapter II for a detailed method of their preparation and collection). Similar to my results with the gp8 dataset, I was not able to determine a high-resolution reconstruction of the sliding clamp from either of these datasets. However, the extraneous density does not match that from the gp8 containing dataset. Instead, the density that appears at low contour values is not connected to the sliding clamp (Figure 3.7).
**Gp8 structure prediction:**

To better understand the structure of gp8 and how it may interact with the clamp loader and DNA, I used different structure prediction algorithms to predict its structure and binding interactions (Evans et al., 2022; Jumper et al., 2021; Rodríguez-Lumbreras et al., 2022). The prediction for the structure of the gp8-DNA complex used the predicted gp8 structure as an input, thus decreasing the overall confidence we can have in this prediction. Despite these limitations, structural models are useful for predicting how gp8 may behave and interact with different molecules.

AlphaFold2 predicts that gp8 is a simple structure composed of one alpha helix adjacent to a 4-stranded beta-sheet (Figure 3.8A). All five predicted models of gp8 are similar, with the RMSD between the least similar models only 1.1 Å. The majority of the differences occur at the N- and C- termini, but the core structure is unchanged. Additionally, the average predicted local-distance difference test (pLDDT) value is 77, indicating that AlphaFold has high confidence in the predicted model (Figure 3.8B) (Tunyasuvunakool et al., 2021).

To understand how gp8 may interact with the clamp loader, I used AlphaFold-Multimer to predict how gp8 interacts with the A subunit of the clamp loader (Evans et al., 2022). Pull-down assays show that gp8 and the A subunit bind to each other, but the binding patch was not determined and so I prioritized this subunit (Yano & and Rothman-denes, 2011). Gp8 may bind to other subunits
of the clamp loader or at the interface between two subunits. Future experiments should be done to model how gp8 could bind to the other subunits. The modelled complex shows gp8 bound to the collar in the same position in all of the models (Figure 3.8C). This points to a model where gp8 binds to the A subunit and prevents the assembly of complex clamp loader. However, I find this unlikely as this would have a limited effect on clamp loaders that are already assembled in the cell. Instead, this is likely an artifact of using an isolated A subunit for the prediction.

Because the predicted model of gp8 bound to the full-length Rossmann domain is likely an artifact, I next used AlphaFold-Multimer to predict how gp8 would bind to the Rossmann domain of the A subunit (Figure 3.8D). I chose to focus on the Rossmann domain since one of the residues that confers gp8 resistance is in this domain and the Rossmann domain binds with the sliding clamp (Jeruzalmi, Yurieva, et al., 2001; Yano & and Rothman-denes, 2011). To model this complex, I used the sequence of gp8 and residues 1 through 150 of the A subunit. AlphaFold-Multimer accurately predicted the structure of the Rossmann domain (average RMSD of the predicted structure from the accepted structure is <1.0 Å). AlphaFold-Multimer predicts two separate gp8 binding sites on the A subunit Rossmann domain. One site (Models 3 & 4) can be discarded as an artifact, because gp8 would clash with the lid domain of the full-length protein. Models 1, 2, & 5 are more plausible because they predict an interaction between the A subunit and gp8 that mimics the interaction between the A subunit
and the sliding clamp. Thus, this structural model would predict that gp8 prevents clamp binding. However, the gp8 resistance mutant, L17A, is not located close enough to gp8 to directly interact with it in any of the binding locations, closest distance being 17 Å.

To predict how gp8 may bind to dsDNA, I used pyDockDNA, an \textit{ab-initio} docking approach for predicting protein DNA interactions. Using PDB coordinate files as inputs, PydockDNA scores the predicted models based on the electrostatics, desolvation energy, and van der Waals energy (Rodríguez-Lumbreras et al., 2022). As inputs, I used the highest rank Alphafold prediction of the gp8 monomer and a 20 bp B-form dsDNA fragment. PydockDNA did not converge on a single model for how gp8 binds to DNA which reduces my confidence in the prediction. The top five models predict three different locations for dsDNA to bind to gp8 (Figure 3.9A). Since gp8 protects a ~35 bp fragment of DNA, I suspect that it may form multimeric complex on DNA. To see if the different gp8:DNA binding predictions are compatible with a oligomeric complex, I superimposed the five gp8:DNA models on the dsDNA fragment (Figure 3.9A). The three gp8 DNA binding modes, rank 1, rank 2, 3, &5, and rank 4, do not clash with each other and follow the major groove of the DNA helix.

Since gp8 binds DNA with high affinity, I would expect this to be predicted by PydockDNA’s evaluation metric. To test this, I compared the scoring energy of gp8 to the scoring energy of three known DNA binding proteins, DNA transcription factor Ndt80 (PDB:1MNN), restriction enzyme CFR101 (PDB:
1CFR), and human uracil-DNA glycosylase (PDB: 1AKZ). The average scoring energy of the top five gp8:DNA predictions had similar predicted scoring energies to CFR10I and human uracil-DNA glycosylase (-75.6, -63.5, and -77.7, respectively). However, Ndt80 was predicted to have a higher affinity for DNA (scoring energy -133.0) than the other models. As a negative control, I used pydockDNA to predict how an apoferritin monomer, a protein that does not bind DNA, would bind to the 20 bp DNA fragment. The top five predictions had an average scoring energy of -45.1. Taken together, these results suggest that the gp8:DNA interaction has similar energy other known DNA binding proteins. However, there remains significant uncertainty in the predicted modes for DNA-binding.

I next aligned a structure of the clamp loader with the predicted models of gp8 bound to the A subunit, and gp8 bound to the sliding clamp to predict how a clamp loader:gp8:DNA complex may assemble. To do this, I used the apo-clamp loader crystal structure (1jr3) and the top-scoring model of gp8 bound to the Rossmann domain of the A subunit and the predicted models of gp8 bound to DNA. I first aligned the clamp loader and gp8:A subunit model on the A subunit. I then aligned the different gp8:DNA models on the gp8 molecule. These composite models would allow me to see how the DNA was positioned compared to the clamp loader. Since the gp8:DNA predictions rank 2,3,& 5 are in similar poses, I only performed this alignment using rank 2 to represent the entire group. Therefore, I made three composite models (Figure 3.9B-D). The composite
model using the highest ranked gp8:DNA prediction (rank 1, Figure 3.9A), positions the DNA roughly perpendicular to the central chamber of the clamp loader, where DNA normally binds (Figure 3.9B). The alignment using the gp8:DNA model rank 2 positions the dsDNA outside of the central chamber of the ATPase module, where it clashes into the A subunit of the clamp loader (Figure 3.9C). This interaction would not assemble into a tripartite complex. Finally, the last gp8:DNA model, Figure 3.9D, shows the DNA molecule directed towards the interior of the ATPase module (Figure 3.9D). Of the three composite models, two could form complexes without clashes (Figure 3.9B and Figure 3.9D). Given the ambiguity in the predicted protein-protein or protein-DNA interactions, these composite models have limited utility. However, we anticipate testing some of these predictions using site-directed mutagenesis.
Figure 3.8. Alpha and Alphafold Multimer Prediction of gp8 and gp8 in complex with the delta subunit. A. Alphafold Prediction of the structure of gp8. The five highest ranked Alphafold prediction aligned onto each other. B. Accuracy prediction of the Alphafold model. Top Alphafold models where each residue is colored by its predicted local difference difference test (pLDDT) value. Blue signifies lowest confidence and red signifies highest confidence (left). Graph of the pLDDT of the top ranked Alphafold output model (Right). C. Alphafold Multimer models of the gp8 and full length delta. The five top gp8:delta models aligned on the delta subunit. Residues that confer gp8 resistance, L17 and N281, are shown as yellow spheres. D. Alphafold multimer models of gp8 model to the Rossmann Domain of the delta subunit of the clamp loader. The top five models of gp8 bound to the Rossmann domain of the delta subunit. Residue L17 is shown as yellow spheres.
Figure 3.9. PydockDNA models of gp8 bound to dsDNA and composite gp8:clamp loader:DNA models. A. Top five PydockDNA models of gp8 bound to dsDNA aligned on the dsDNA. B-D. Composite models of the gp8:DNA:clamp loader complex. The pydockDNA models, top AlphaFold multimer model of gp8 bound to the Rossmann domain of the delta subunit, and apo-clamp loader structure (PDB: 1JR3) were used to generate these models. The apo-clamp loader and gp8:delta subunit model were aligned on the delta subunit. The pydockDNA and gp8:delta subunit model were aligned on gp8.
Discussion

Gp8 copurifies with host DNA

It remains unclear how gp8 inhibits the clamp loader. The DNA that copurified with gp8 made this question challenging to address since the DNA interacted with the clamp loader. The strong affinity of gp8 for DNA suggests that DNA may play a role in the mechanism of inhibition or a structural role in the protein’s folding.

Due to the experimental challenges imposed by gp8, more work needs to be performed to purify gp8 without DNA or to purify gp8 bound to a uniform DNA substrate. I have already shown that the DNA can be removed from gp8 under denaturing conditions, however, since it precipitated from solution after removing the denaturant, I presumed that it was unfolded which may not be the case. If gp8 was folded but “salted-out” of the solution in the absence of DNA the buffer condition used (500 mM sodium chloride) (Protein Precipitation Using Ammonium Sulfate BASIC THEORY, 1998). Protein refolding is challenging and often requires trial and error to identify conditions that are successful. Taking a systematic approach for identifying refolding conditions may decrease the time and attempts required to successfully refold the protein (Y. Wang et al., 2017). If gp8 requires DNA to successfully fold, a defined DNA substrate could be provided during refolding to obtain a soluble active sample. To test if the protein is folded, circular dichroism (CD) can be performed to identify secondary
structure, which is predicted for gp8 by AlphaFold. A final approach for purifying gp8 minimizing the bound DNA would be to translate it in vitro using a cell-free gene expression system. In this approach, host DNAs or RNAs would be eliminated and so gp8 would only have access to bind the coding DNA used in the system (Garenne et al., 2021).

Once gp8 is purified, it would be necessary to determine if the protein is active. Protein activity could be assessed using the ATPase assay, since active gp8 should inhibit clamp loading and the clamp loader’s ATPase activity, if Yano et al.’s findings are correct. Once a method to purify gp8 is determined, testing how gp8 interacts with the clamp loader can be done using the wealth of assay that have already been developed to study clamp loader activities.

What is the gp8 DNA assembly?

It remains unclear how gp8 binds to DNA with such high affinity and the significance of the DNA binding activity. The generated models of gp8 bound to DNA predict that gp8 binds to the major groove which could form an oligomer which could coat stretches of DNA. In the predicted conformation, a single gp8 monomer could only protect ~8-10 bps of DNA. However, the results from digesting the purified gp8 with nuclease shows that the purified gp8 protects a ~35 bp DNA fragment, suggesting that gp8 either forms an oligomeric complex on DNA or has an alternate conformation compared to the AlphaFold prediction which may help to explain the high affinity observed between gp8 and DNA. A
larger complex would benefit from avidity effects where there are multiple interactions between gp8 and DNA instead of just one. Thus, I hypothesize that gp8 forms an oligomeric complex on DNA. To test this, it would be beneficial to produce a large amount of the product gp8:DNA complex from the DNA protection assay. This sample could then be analyzed by either size exclusion chromatography or size exclusion chromatography-multi-angle light scattering (SEC-MALS). Both of these approaches would assess the molecular weight of the gp8:DNA complex. Both assays require a sample of 250 μl at ~1 mg/ml which may be challenging to obtain based on the efficiency of the purification and need to process the sample. However, knowing the molecular weight of this species may allow for the determination of the stoichiometry of the complex and modelling of its binding conformation.

*Is there a function role for DNA binding by gp8?*

I found that gp8 binds to DNA, but was unable to determine if this interaction plays a functional role. To investigate this, I computationally modelled gp8 bound DNA and created a composite model of gp8 bound to DNA and the clamp loader. These models did not come to a clear consensus prediction. Two of the composite models (Figure 3.9B, D) suggest that gp8 couples the clamp loader to DNA. If this interaction is strong enough to prevent the clamp loader from disengaging DNA, gp8 would stall the replication fork.
I did not address whether gp8 binds to DNA in a sequence specific manner or not. Sequence specific binding could enable gp8 to bind to the replication fork early in replication. The bacterial origin of replication has several conserved regions such as a DNA unwinding element and a DnaA recognition sequence. However, gp8 forms foci that colocalize with elements of the DNA polymerase III holoenzyme (Yano & Rothman-denes, 2011). Since activities at the unwinding element and DnaA recognition box precede replication fork formation, it is unlikely the gp8 targets these sequences (Messer, 2002). There are other prominent bacterial DNA sequences that would be beneficial for a virus to sequester. One of which is the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR) loci. These regions are between 24-48 bps long, which aligns with the length of the DNA segment that gp8 protects in the protection assay (Deveau et al., 2010). The sequence that gp8 targets may also be species specific. N4 phage are from a well-studied subspecies of phage with many members, yet only five other phages have been identified with sequences similar to gp8 (Ackermann, 1999). Gp8 may be poorly conserved because the sequence it targets is not conserved; thus it provides no advantage to other phages.

**Conclusion and Future Directions**

My studies did not identify how gp8 inhibits the *E. coli* clamp loader. Surprisingly, I found that gp8 binds exceptionally tightly to DNA. Several attempts were made to separate gp8 from co-purified DNA, however the only successful method was
to purify the protein under denaturing conditions, which also caused gp8 to precipitate. Initial results suggest that gp8 may inhibit the clamp loader by hyperactivating its ATPase activity, but further testing shows that this was a product of the contaminant DNA. I attempted to determine the structure of gp8 bound to the clamp loader and sliding clamp using cryo-EM. Unfortunately, this was unsuccessful. Finally, I used structure prediction algorithms to predict models of gp8 and how it may bind to the A subunit of the clamp loader and dsDNA, which could provide insight into how gp8 inhibits the clamp loader. To investigate the mechanism of gp8 further, it is necessary to purify gp8 such that it is free of DNA or so that the DNA has a consistent and known composition. Moreover, there are many interesting questions that can be addressed by understanding how gp8 binds to DNA so well. Determining the structure and requirements of the gp8 DNA complex may provide a tool to develop chimeric proteins with remarkably tight DNA affinity.
Chapter IV: Discussion
Preface
The following section summarizes what was learned from my thesis and the future directions that I see for this research. I prepared the content of this section.
**Thesis Summary:**

This thesis had two main goals to understand the structural mechanisms by which the *E. coli* clamp loader loads the sliding clamp onto DNA, and to understand how this clamp loader can be inhibited. Clamp loaders are essential factors in DNA replication in all domains of life (Kelch, 2016). They bind to and load sliding clamps onto primed DNA at the replication fork as well as load the sliding clamp onto DNA in DNA repair pathways (López de Saro & O’Donnell, 2001; Onrust et al., 1995). Sliding clamps then act as binding scaffolds for DNA polymerases, increasing both the speed and processivity of DNA polymerases by 100-fold and 5000-fold, respectively (Maki et al., 1988; N. Y. Yao et al., 2009).

The clamp loading processes requires ATP hydrolysis, not to power sliding clamp opening, but to coordinate the timing of events in the clamp loading process (Hingorani & O’Donnell, 1998). Clamp loaders from different domains of life have different architectures, which leads to the possibility that they may have different mechanisms of action (Bowman et al., 2004b; Jeruzalmi, O’Donnell, et al., 2001; Kelch et al., 2011). I sought to address how the different architectural elements of clamp loaders affect their mechanisms by examining the structure of the *E. coli* clamp loader throughout its opening cycle and comparing these to similar structures of the *S. cerevisiae* clamp loader. Clamp loading is an essential process, and so it is not surprising that it is targeted by bacteriophage which co-opts host machinery to replicate itself. The second goal of this thesis project was to understand how the *E. coli* clamp loader is inhibited by the viral protein gp8.
(Yano & and Rothman-denes, 2011). Since the clamp loading mechanism has multiple steps, there are several means by which the clamp loader could be inhibited by this protein. In the following sections, I summarize my research on the clamp loader mechanism, its inhibition by gp8, and I discuss future directions for this research.

**Summary: Mechanism of the *E. coli* clamp loader**

Prior to this thesis research, there were no structures of bacterial clamp loaders bound to its sliding clamp. Without these structures several questions about the clamp loader’s mechanisms, such as how it opens its sliding clamp and how p/t-DNA binding stimulates ATP hydrolysis by the complex remained. Fluorescence resonance energy transfer experiments (FRET) concluded that the *E. coli* clamp loader opens its sliding clamp without undergoing any major conformational changes (Goedken et al., 2004b). In contrast, structures of a eukaryotic clamp loaders show that it undergoes a dramatic conformational change upon opening its sliding clamp (Gaubitz et al., 2022). To understand how differences in the structures of bacterial and eukaryotic clamp loaders alter their mechanisms, I determined structures of the *E. coli* clamp loader throughout its clamp loading process.

These structures reveal how the clamp loader first binds and then opens the sliding clamp (the Initial-Binding, Semi-Open, and Fully-Open states). Taken together, these states show that the *E. coli* clamp loader opens its sliding clamp
with a crab-claw like mechanism where the primary conformational changes in
the clamp loader occur in the A and B subunits. This mechanism is distinct from
that of eukaryotic clamp loaders, since opening is primarily driven by a
conformation change at a single pivot point instead of multiple pivot point
throughout the ring. I wanted to understand how binding to p/t-DNA alters the
conformation of the complex, so I collected micrographs of the clamp loader and
sliding clamp bound to p/t DNA. This dataset resulted in four structures: an open
clamp loader:sliding clamp:p/t-DNA complex (Open-DNApt), two closed clamp
loader:sliding clamp:p/t-DNA complexes (Closed-DNA1 and Closed-DNA2), and
a clamp loader:sliding clamp:p/t-DNA complex with an alternate collar
conformation (Altered-Collar). I found that binding to p/t-DNA causes the clamp
loader:sliding clamp complex to transition from a planar to helical conformation.
Next, I found that following clamp closure the D and E subunits lose contact with
the sliding clamp, suggesting that release of the sliding clamp is ordered E, D, C,
B, A. It is unclear what the role of the Altered-Collar conformation would be in
loading the sliding clamp and so this state is not likely on-pathway for clamp
loading. The Altered-Collar Complex has a similar conformation to that of the
eukaryotic clamp loader when loading onto nicked DNA, and so I speculated that
the conformation may be relevant for DNA damage repair.

**Open Questions:** *E. coli* clamp loading mechanism

*How would the inclusion of tau, chi, and psi affect the clamp loading mechanism?*
The structures of the *E. coli* clamp loader used in chapter II were completed using a clamp loader with a stoichiometry of $\delta', \delta, \gamma_3$. However, in the replication fork the gamma complex is part of the DNA polymerase III holoenzyme and contains the tau, psi, and chi proteins. These auxiliary factors bind to different constituents of the replication fork, and so they are thought to be important for organizing the bacterial replication fork.

The collar domain of the clamp loader alters its conformation upon binding p/t-junctions. A crystal structure shows that when a peptide of the psi protein’s clamp loader binding motif is bound, it preconfigures the collar into the configuration of the p/t-junction bound complex (Simonetta et al., 2009). In the Fully-Open state, presented in Chapter II, the collar is not in the preconfigured p/t-junction bound conformation showing that the sliding clamp does not preconfigure the complex to bind p/t-junctions like psi does.

In Chapter II, I show that the clamp loader:sliding clamp complex first opens into a planar arrangement and, after binding p/t-junctions, transitions into a helical arrangement. Could this be an artifact of these structures being determined without psi bound? I am confident that this is not the case, as a similar cryo-EM study on the *E. coli* clamp loader:sliding clamp complex which included the psi peptide also observed the open clamp loader:sliding clamp complex in a planar conformation (Xu et al., 2023). The psi and chi heterodimer links the clamp loader to SSBs that are bound to the lagging strand (Glover & Mchenry, 1998). This linkage does not alter the rate that the clamp loader:sliding
clamp complex binds to and closes on p/t-junctions in-vitro (Newcomb et al., 2022). SSB increases the lifetime of the clamp loader sliding clamp p/t-junction complex (Newcomb et al., 2022). Perhaps, increasing the duration of this complex plays a role in coordinating the exchange of the sliding clamp from the clamp loader to the DNA polymerase.

Similar to the psi:chi heterodimer, tau proteins are associated with coordinating the replication fork. Different domains of the tau subunit interact with primase, helicase, and DNA polymerase and regulate their speed (Chintakayala et al., 2009; N. Y. Yao & O’Donnell, 2016). The clamp loader complex consisting exclusively of gamma subunits ($\gamma_3:\delta:\delta'$) is sufficient for loading the sliding clamp, and so tau does not play an essential role in the clamp loading mechanism (Onrust et al., 1995). To understand the role that tau plays in coordinating clamp loading at the replication fork further studies are necessary. The entire complex likely assembles into three subcomplexes; the clamp loader, DNA helicase and RNA primase, and the DNA polymerase III. Each of these subcomplexes can be individually purified and many have been studied by cryo-EM (Arias-Palomo, O’shea, et al., 2013; Makis & Kornberg, 1985; Moks & Marianss, 1987; Onrust et al., 1991). Reconstituting the entire complex has not been done and may reveal how these complexes interact and organize in the replication fork. The flexible nature of DNA and tau protein would likely lead to challenges in determining the assembly in a single reconstruction (Su et al., 2007). However, various techniques have been used to determine cryo-EM structures of highly flexible
particles, such as with HECT ubiquitin ligase and histone complexes (Dilorio & Kulczyk, 2023; Hunkeler et al., 2021; W. Li et al., 2023). Overcoming this challenge would enable us to learn about the organization of the replication fork and how the components interact with each other.

_How does ATP binding activate the clamp loader to bind the sliding clamp?_

It is not understood how ATP binding activates the clamp loader. Early research found that the delta subunit binds the sliding clamp on its own without ATP binding, but inclusion of delta prime subunit competes with sliding clamp to bind the delta subunit (Naktinis et al., 1995; Turner et al., 1999a). These results led to the hypothesis that in the apo state the Rossmann domain of the delta prime subunit interacts with the delta subunit occluding the sliding clamp.

Two independent studies tried to identify how ATP binding activates the clamp loader. One group truncated the Rossmann domain on the delta prime subunit and tested if the complex could still bind to the sliding clamp (Indiani & O'Donnell, 2003). If the delta prime domain physically blocked the sliding clamp binding domain, removing it should enable the clamp loader to bind the sliding clamp in the absence of ATP. However, the truncated complex was unable to bind to the sliding clamp (Indiani & O'Donnell, 2003). Moreover, FRET studies show that ATP binding does not induce a change in the distance between the A and E subunits (Goedken et al., 2004b; Indiani & O'Donnell, 2003). If a large conformational change between these subunits was induced by ATP binding, it
would have been expected that the distance between the two subunits would change and be detected in the FRET study.

Previous structural work determined a crystal structure of the clamp loader bound to ATPγS (Kazmirski et al., 2004). I discuss my concerns of this structure in greater detail in Appendix I. Briefly, there are prominent crystal contacts that may prevent the complex from being in its native state. Additionally, this structure cannot form a complex with a closed sliding clamp without undergoing multiple conformational changes. To address these concerns, I determined a cryo-EM structure of the *E. coli* clamp loader bound to ADP•BeF₆, since it is a non-hydrolyzable ATP analog whereas ATPγS is slowly hydrolyzable. This structure has strong density for the entire collar domain and the ATPase modules of the D and E subunits (Appendix I). However, the resolution of the Rossmann domain and lid of the A, B, and C subunits are poor, suggesting that these subunits are flexible.

I propose that ATP binding does not alter the conformation of the clamp loader. However, when the clamp loader interacts with the sliding clamp, ATP is necessary to stabilize the interfaces between the subunits and form a stable clamp loader:sliding clamp complex. This hypothesis would explain why the FRET studies did not detect a change upon ATP binding. In support of this hypothesis, rapid kinetics show that the association rate constant of the clamp loader and sliding clamp is the same in the presence and absence of ATP, and so the tighter affinity of the clamp loader for the sliding clamp in the ATP bound state
is primarily driven by an increased dissociation rate constant (Thompson et al., 2009).

Future studies are needed to test my hypotheses. My cryo-EM studies reported in this work do not conclusively show that the ATP bound clamp loader is dynamic. No attempts were made to optimize the cryo-EM sample and so screening different grid conditions or using different microscope settings may improve the quality of the reconstruction to resolve the entire AAA+ module. I attempted various cryo-EM techniques to resolve mobile subunits, I did not exhaust all options. Processing this dataset in cryoDRGN may reveal conformational heterogeneity in the complex that can be interpreted as movement (Zhong et al., 2021). Additionally, molecular dynamics simulations could be used to investigate the dynamics of the clamp loader. Modelling both the apo- and ATPγS bound structures would enable a comparison of how ATP alters the complex’s dynamics. Single-molecule FRET experiments could be performed to directly test whether ATP binding alters the flexibility of the ATPase module (Mazal & Haran, 2019). One limitation of this method is that it would be difficult to interrogate any changes other than the ones between the A and E subunits since subunits B-D are all the same.

Intermediate states in clamp opening?

In chapter II, I present my data on the clamp opening mechanism of the bacterial clamp loader. I show three states throughout clamp opening, Initial-Binding, Semi-Open, and Fully-Open. We propose a mechanism where the
clamp loader transitions through these states which is supported by prior biochemical data which predicted the Semi-Open state (LG et al., 2017). During my cryo-EM data processing, I did not only determine maps of the Semi-Open and Fully-Open states, but several other intermediate states throughout clamp opening.

By classifying the dataset of the clamp loader, sliding clamp, and ADP•BeF\(_x\) sample, I determined three other classes of intermediate open states, one that appears to be between the Initial-Binding state and Semi-Open state and two between the Semi-Open state and Fully-Open state. I predict that these states are ordered as shown in Figure 4.1A. To analyze the maps, I rigid-body fit either the Semi-Open or Fully-Open model into the map, I then adjusted individual domains to fit them into the density (Figure 4.1B). These models are not finished. The backbone and sidechains have not been adjusted to fit into the maps and refinement has not been performed on the models.

These additional states suggest that the clamp loader sliding clamp complex’s conformation is fluctuating prior to binding p/t-junctions. It is well known that proteins transition between many conformations in solution and several cryo-EM data processing algorithms have been developed to identify and analyze these states (Dsouza et al., 2023). After comparing the models, the C, D, and E subunits of the clamp loader do not undergo significant conformational changes when transitioning between the clamp opening states. The position of the Rossmann domain of the A and B subunits of the clamp loader and of the
sliding clamp subunit bound to clamp loader subunits A, B, and C change through opening. The additional conformations agree well with those that are predicted by atomic trajectories transitioning through the Initial-Binding, Semi-Open, and Fully-Open states. This supports that the trajectories are a probable model for clamp opening.

I observe that the states cluster around the Semi-Open and Fully-Open conformations and I do not observe a continuum of states between the Semi-Open and Fully-Open states. I proposed that the Semi-Open and Fully-Open states are local minima states and so conformations that deviate from these states are less energetically favorable and less common in the data. If this is true, then I would not expect states between the Semi-Open and Fully-Open states to be evenly populated. Instead, I would expect to predominantly observe states that closely resemble either the Semi-Open or Fully-Open state. This hypothesis needs further testing. Not all cryo-EM samples accurately portray the energy landscapes of their samples. To test this hypothesis, smFRET experiments could be performed monitoring the distance between the A and E subunits of the clamp loader or the opening of the sliding clamp.
In the Semi-Open and Fully-Open states of the clamp loader sliding clamp complex the density for one of the domains is not visible (Chapter II). I interpreted this as this domain being highly flexible upon clamp opening.

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) assays show that
the domain of the sliding clamp that lacks density in these states is more prone to unfolding that the other domains of the sliding clamp (Fang et al., 2011, 2014). While these data agree with each other both of these experiments are carried out on extended timescales and whether or not this domain becomes flexible during clamp loading has not been confirmed.

The increased flexibility of this domain of the sliding clamp may be necessary to enable the sliding clamp to closed around p/t junctions. The clamp loader does not undergo a significant conformational change between the Fully-Open, Open-p/t junction bound, or Closed-p/tDNA states and so, it does not appear that the clamp loader actively closes the sliding clamp through a conformational change in this system. Molecular dynamic stimulations show that a simulated open beta-clamp preferentially closes during a simulation (Oakley, 2016). However, the simulated open sliding clamp was not in the conformation observed in the Fully-Open state. Perhaps for the sliding clamp to close when bound to the clamp loader, Domain I of the sliding clamp subunit bound to clamp loader subunits C, D, and E must slightly unfold, enabling it to make contacts with domain III of the adjacent sliding clamp subunit. Once these contacts are established, the domain refolds which transitions the sliding clamp into a closed conformation.

Alternatively, the lack of density for this domain of the sliding clamp may be an artifact. In single-particle cryo-EM, particles can associate with the air-water interface, which may alter the particles conformation (Noble et al., 2018). If
this domain of the sliding clamp is interacting with the air-water interface, the air-water interface may promote unfolding of this domain. The 3D reconstructions of the complex have preferred orientation where either the A-gate of the clamp loader sliding clamp complex or back of the back of the complex are preferred, suggesting that this domain may be interacting with the air-water interface. I was able to determine reconstructions with density for this domain of the sliding clamp in the p/tDNA dataset, however the majority of the particles stacks in the open state lacked density for this domain (Figure 2.8). This was the only cryo-EM sample that was not crosslinked. In datasets that were crosslinked, I was unable to determine maps in the open state that had density for this domain. Potentially, when samples interacted with the air-water interface domain I gets distorted and the crosslinker formed crosslinks in this domain the prevent the domain from refolding. This domain has three lysine residues that could form crosslinks with the BS3 crosslinker used. Notably, the samples were only crosslinked for 15 minutes and the BS3 crosslinker was neutralized with Tris prior to grid preparation to try and prevent excessive crosslinking. Future studies should be performed to test whether or not domain I of the sliding clamp bound to clamp loader subunits C, D, and E in open complexes unfolds.

**ATP hydrolysis state of the clamp loader complexes**

Primer/template-junction binding triggers ATP hydrolysis across all clamp loaders. Different studies have tried to elucidate whether ATP hydrolysis occurs sequentially or as a single burst (Hingorani et al., 1999; Johnson & O'Donnell,
2003; J. Liu et al., 2017). A model has been proposed for bacterial clamp loaders, based on biochemical experiments, where sliding clamp binding triggers ATP hydrolysis in one ATPase pocket and p/t-junction binding triggers ATP hydrolysis in the remaining two (Williams et al., 2003). A similar model where sequential ATP hydrolysis is linked to specific clamp loading events is also supported biochemically in eukaryotic systems as well (Gomes et al., 2001).

Ideally, the structures presented in chapter II would inform on the ATP hydrolysis state of each ATP binding pocket. Unfortunately, they are not able to. In the Fully-Open and p/t-junction bound complexes, each of the ATP binding pockets are in a conformation which appears to be competent for ATP hydrolysis. Additionally, the density for the BeF₆ ion is too poor in all of my 3D reconstructions to reliably assess whether ADP•BeF₆ or ADP is bound in the pocket. This is not entirely unexpected as anions often do not have strong density in cryo-EM maps (J. Wang & Moore, 2017). Structures of Swi/Snf2 family ATPases (PDB 7ZKE: 3.6 Å/PDB 5Z3U 4.3 Å), Arp2/3 complex (PDB 8UZ1: 3.6 Å), cdc48-Npl4 complex (PDB 6OAB: 3.6 Å), and even a 2.6 Å structure of SSoMCM (PDB 8EAF) bound to ADP•BeF₆ have weak if any density supporting the BeF₆ ion (Chavali et al., 2024; M. Li et al., 2019; Meagher et al., 2022; Twomey et al., 2019; Woike et al., 2023). In each of my clamp loader models, I modelled ADP•BeF₆ into each ATP binding site. ADP•BeF₆ is a non-hydrolyzable ATP mimic, and so even if the complex would hydrolyze ATP at that intermediate state in clamp loading, I would expect to observe hydrolysis ADP•BeF₆ in my
maps based on the ATP analog used in this study. Additionally, in the anisotropy-based DNA binding assays presented in Chapter II, titrating in excess ADP decreases the anisotropy signal, presumably by simulating ATP hydrolysis and initiating complex disassembly (data not shown). Therefore, if the BeF$_x$ ion was released from each of the ATP binding pockets, I would expect the complex to have disassembled, and so each of the states must have at least partial ADP•BeF$_x$ occupancy.

The clamp loader does not form a stable complex with the sliding clamp unless it is bound to ATP and ATP hydrolysis triggers complex disassembly. Therefore, the Initial-Binding, Semi-Open, and Fully-Open states in vivo are either fully or partially bound to ATP. Binding to a p/t-junction triggers ATP hydrolysis and complex disassembly. If the ATP analog used in the study was hydrolysable, the open p/t-junction state may inform on whether hydrolysis is ordered or stochastic. If ATP hydrolysis occurs upon p/t-junction binding and is ordered, I would expect to observe density for either ATP or ADP in each of the nucleotide pockets. If ATP hydrolysis is stochastic, then each particle would not have the same nucleotide in each of its nucleotide binding pockets. As a result, I would expect the density for the gamma phosphate to be weak making it challenging to conclusively determine the hydrolysis state in each binding pocket. Classifying the particles by the hydrolysis state in each binding pocket would be challenging or impossible, unless the hydrolysis was paired with a larger
conformational change in the complex, due to the size of the mask that would need to be used.

_How does p/t-DNA trigger ATP hydrolysis in clamp loaders?_

A question that remains is how p/t-junction binding triggers ATP hydrolysis by clamp loaders. In both the bacterial and eukaryotic structures, the ATP binding pockets appear to be in a conformation competent for ATP hydrolysis after sliding clamp opening and prior to DNA binding (Gaubitz et al., 2022). This conformation is unexpected, as biochemical studies find that clamp opening does not require ATP hydrolysis and that p/t-junction binding stimulates ATP hydrolysis (Gomes and Burgers 2001; Onrust, Stukenberg, and O’Donnell 1991). Therefore, it was expected that the ATP binding pockets would not be in a hydrolysis competent state prior to p/t-junction binding.

There are several possibilities as to why a p/t-junction sensing element has not been identified. 1) The conformation observed in the open clamp loader:sliding clamp structures, prior to p/t-junction binding, are not representative of the *in vivo* conformation. 2) ATPase activation is caused by a change in the complex’s dynamics which cannot be observed in static structures. 3) The conformational change is present in the structure, but we have not identified it.

Both crystallography and cryo-EM occur in non-native conditions and so they can induce artifacts onto the resulting structures. In all the cryo-EM studies
of clamp loaders both reported here and elsewhere the samples were stored at 4°C prior to plunging, whereas they natively function at 30 or 37°C (Gaubitz et al., 2020, 2022; X. Liu et al., 2022; Zheng, Georgescu, Yao, Li, et al., 2022). The temperature of samples prior to plunging can alter the conformation of the sample (C.-Y. Chen et al., 2019). However, if clamp loaders were in a conformation more competent for hydrolysis at lower temperatures, it would be expected that they would have a faster rate of ATP hydrolysis at lower temperatures. While I do not know if this has been directly tested, the ATPase activity of related ATPases decrease with temperature (Das & Bajpai, 2023). Alternatively, if ADP release was the rate-limiting step, it would be expected that the post-hydrolysis state would be dominant at lower temperatures. Another concern is that the Fully-Open complex (Chapter II) and the open RFC:PCNA structures were both crosslinked (Gaubitz et al., 2022). The hypothesized DNA sensing element in bacteria, lys100, is available for crosslinking by BS3, and so potential its conformational has been altered by the crosslinker. Fortunately, there are no additional lysines in range for crosslinking in the structure and so this is not likely an issue. Additionally, mass-spectroscopy of the crosslinked yeast RFC PCNA sample was used to map the locations of the crosslinks and few crosslinks were observed near the ATPase pockets (Gaubitz et al., 2022). Moreover, it seems unlikely that in both the bacterial and eukaryotic samples, crosslinking would alter the structures such that they transitioned from a native state that is
incompetent for ATP hydrolysis, to a non-native state that appears competent for ATP hydrolysis.

Another possibility is that the open clamp loader:sliding clamp complex prior to p/t-junction binding forms an ATP hydrolysis capable conformation but on a slower timescale than when p/t-junction is present. Structural studies often make it seem that protein complexes are rigid, but they are dynamic in solution. Perhaps it should even have been expected that the p/t-junction bound and unbound states are in an ATP hydrolysis competent state, since they both have ATPase activity, just at different rates (Turner et al., 1999b). In support of this possibility, I note evidence from another system, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) which transports calcium ions from the cytosol into the sarcoplasmic reticulum in muscles and has calcium dependent ATPase activity (Inesi et al., 2006). Molecular dynamics (MD) simulations and FRET studies show that SERCA can adopt a conformation similar to the ATP hydrolysis competent conformation in the absence on Ca\(^{2+}\), but the transition occurs on a longer timescale (Espinoza-Fonseca & Thomas, 2011; Winters et al., 2008). Perhaps p/t-junction binding in clamp loaders is similar to Ca\(^{2+}\) binding in SERCA, it increases the rate that the clamp loader adopts the ATP hydrolysis competent conformation, but it not essential for it.

It was expected that by comparing the Fully-Open complex with the Open-p/tDNA or Open-p/tRNA complex that a conformational change that activates the clamp loader for ATP hydrolysis would be identified. However, in
this work and work on eukaryotic systems, both states are in an ATP hydrolysis competent conformation prior to p/t-junction binding. If a conformational change does occur, perhaps our ability to identify it is limited by the resolution of the data (Fully-Open = 3.0 Å and Open-RNAp/t = 3.2 Å). I do not think it is likely that the resolution limits are ability to observe the conformation change or that it is worthwhile to push the resolution of these structures without further evidence of it. Instead, I think future work using MD-simulations to understand the dynamics of both complexes will be useful to understand the role of p/t-junction binding in stimulating ATP hydrolysis.

*Interpretation of the p/t-junction sensing residues results*

In Chapter II, I tested whether specific residues in the clamp loader, gamma K141 and delta prime K130, trigger ATP hydrolysis when the complex binds to a p/t-junction. I found that mutating these residues to alanine resulted in a small reduction of the ATPase activity of the clamp loader and that p/t junction binding stimulated ATP hydrolysis under steady-state conditions. As a result, I concluded that the proposed residues are not responsible for sensing p/t-junction binding.

The effect of the lysine mutants on the clamp loader where not exhaustively studied. The enzyme-coupled ATPase assay used to assess the effect of the mutations on the clamp loader only reports changes in the rate-limiting step of the reaction, since the assay measures steady-state conditions.
Furthermore, the rate-limiting step in clamp loading is not ATP hydrolysis but either sliding clamp or ADP release (Hayner & Bloom, 2013; J. Liu et al., 2017). Therefore, the ATPase assay cannot measure the degree that the mutations alter the rate of ATP hydrolysis, since ATP hydrolysis occurs faster than sliding clamp or ADP release. Potentially the mutants hydrolyze ATP much slower than the wild-type clamp loader when the complex binds p/t-junctions, which would support that these residues are the p/t-junction sensing residues.

To ensure that the proposed residues do not trigger ATP hydrolysis in response to p/t-junction binding, it is necessary to measure the system under pre-steady state conditions. It is particularly important to measure the change in the rate of ATP hydrolysis and not the rate of another event in clamp loading. To measure the rate of ATP hydrolysis under pre-steady state conditions, an assay can be used that rapidly quenches ATP hydrolysis of radiolabeled ATP molecules using an acid quench. The formation of ADP can then be measured by separating the ATP from ADP by thin layer chromatography and the formation of ADP can then be quantified and plotted (Hingorani et al., 1999). If these experiments find that ADP formation is hindered in the mutants upon addition of p/t-junctions then the proposed residues play a role in coordinating ATP hydrolysis following p/t-junction binding.

*What drives sliding clamp release?*
The work presented in Chapter II of this thesis illustrates the mechanisms by which the *E. coli* clamp loader 1) binds to the sliding clamp, 2) opens the sliding clamp, 3) binds to p/tDNA, and 4) closes the sliding clamp, and provides a clue as to how the clamp loader initiates release of the sliding clamp. However, it is still unclear how the clamp loader releases the sliding clamp and how the clamp loader exchanges ADP for ATP post hydrolysis to restart the clamp loading cycle.

The final state of clamp loading determined in Chapter II was of the clamp loader bound to a closed sliding clamp and p/t-junction and so I did not see how ATP hydrolysis drives sliding clamp release. Presumably the structure of the clamp loader bound to ADP would be the most like the conformation at release. There is a crystal structure of the *E. coli* clamp loader bound to two ADP molecules bound to the B and D subunits (Kazmirski et al., 2004). This structure has high \( R_{\text{free}} \) and \( R_{\text{work}} \) values, 0.369 and 0.366, and so some care should be taken while interpreting it. Even still, at the time of sliding clamp release the clamp loader would have ADP bound in each of its active sites and so this structure may best represent the complex during ADP exchange. This suggests that ADP exchange in the B and D subunits occurs prior to exchange in the C subunit. A structure of the clamp loader in the post ATP hydrolysis state where all subunits are bound to ADP + P\(_i\) may be necessary to understand how hydrolysis initiates clamp release.
It maybe possible to determine the structure of the clamp loader in the post hydrolysis state using cryo-EM by adding ADP to the sample. If this results in a similar structure to the crystal structure, with partial ADP occupancies, it may be possible to determine the state of the clamp loader post hydrolysis by using ATP as the nucleotide and allowing for ATP hydrolysis to occur. This would likely result in a heterogeneous dataset with particles both pre- and post- hydrolysis. Care would need to be taken when classifying the particles to identify discrete states and enable proper interpretation of the structures.

*How is the E. coli sliding clamp unloaded from DNA?*

Two potential mechanisms have been proposed to perform clamp unloading in bacteria, either the gamma complex performs clamp unloading or the lone delta subunit unloads sliding clamp (Leu et al., 2000). Previous *in vitro* studies have shown that both the gamma complex and the lone delta subunit are able to remove the sliding clamp from dsDNA (Leu et al., 2000). Notably, the delta subunit is expressed in excess over the other constituents of the clamp loader and so there are a greater number of free delta subunits in the cytoplasm than clamp loader complexes (Leu et al., 2000). My results in Chapter II are ordered as a clamp loading reaction, but it is interesting to consider the events in the reversed order. To unload sliding clamps, hypothetically, the A subunit would first bind to a closed sliding clamp on dsDNA, then the remaining subunits would bind. The dsDNA would then clash with the collar of the complex. To relieve this clash, once the sliding clamp is opened, the complex may be pried off the
dsDNA. However, at this point it would seem like the complex is at a dead end. The clamp loader binds the sliding clamp with high affinity and only releases the sliding clamp after binding to p/t-junctions and ATP hydrolysis which occurs slowly without p/t-junctions. Thus, the clamp loader would sequester the sliding clamp from being loaded at subsequent Okazaki fragments, which is counter-productive to the role sliding clamp recycling. This model seems unlikely without a means for the clamp loader to quickly disengage the sliding clamp which might suggest that the free delta subunit is the unloader, or a third, unknown model is responsible for unloading.

*Implications for the bacterial clamp loader and sliding clamp in DNA repair*

DNA replication requires high fidelity, otherwise the resulting errors would result in deleterious mutations to the host. The error rate of the primary polymerase, α, of the DNA polymerase III holoenzyme is 1 in $10^5$ and the proof-reading subunit further improves its accuracy by about 2 orders of magnitude to 1 in $10^7$ (Maki & Kornberg, 1987). Following synthesis and proof-reading by the initial polymerase, mismatch repair proteins mutL, mutS, and mutU increase the overall fidelity to 1 error in $10^8$-$10^{10}$ nucleotides (Modrich, 1991). Following replication, DNA can become damaged by several mechanisms including reactive oxygen species, irradiation, UV light (Wozniak & Simmons, 2022). In eukaryotes, the clamp loader has been shown to play a role DNA repair by loading sliding clamp onto regions of damaged DNA. The primary clamp loader, RFC, can load the sliding clamp onto short-gapped or nicked DNA (X. Liu et al.,
Additionally, an alternative clamp loader complex, Rad17-RFC, loads sliding clamps onto 5’ recessed DNA (Castaneda et al., 2022b; Day et al., 2022). However, alternative clamp loader complexes have not been identified in prokaryotes despite facing the same challenges while maintaining their genomes.

UV irradiation produces cyclobutyl pyrimidine dimers, where two adjacent pyrimidines bind to each other, forming a DNA lesion that needs to be corrected. Pyrimidine dimers physically distort the helix resulting in the unwinding of a couple of bases (Lee et al., 2004). In E. coli, over expressing the sliding clamp while being exposed to high UV light resulted in the E. coli developing fewer mutations but decreased their survival rate (Tadmor et al., 1992). It is easy to imagine how alterations to the topology of DNA could alter the ability of the sliding clamp to slide along it and cause issues during DNA synthesis.

Researchers studying how E. coli cope with UV irradiation found that there is an early frame shift within the DnaN gene, which codes for the sliding clamp, producing a 26-kDa isoform of the sliding clamp, beta*, with 134 residues truncated from the N-terminal end, corresponding to Domain I (Skaliter, Paz-Elizur, et al., 1996a). To test if the overproduction of this isoform increases the UV resistance of E. coli, an inducible expression strain of beta* was generated and challenged with UV irradiation. Under inducing conditions, the beta* strain was up to 6-fold more resistant to UV light than under non-inducing conditions (Skaliter, Paz-Elizur, et al., 1996b). Experiments characterizing beta* in vitro
showed that it assembled into a trimer in native conditions and promotes DNA synthesis by the DNA polymerase III holoenzyme (Skaliter, Bergstein, et al., 1996).

Potentially, instead of having alternative clamp loaders, prokaryotes use this alternative sliding clamp to circumvent DNA damage. However, in lieu of an alternative clamp loader, the gamma complex must load this sliding clamp onto DNA. How does the bacterial clamp loader, which primarily loads a dimeric sliding clamp, load a trimeric form of the clamp? Is the same clamp opening mechanism used or does the clamp opening mechanism resemble that of eukaryotic clamp loaders more closely? Additionally, is this sliding clamp opened into a planar or helical arrangement prior to p/t-junction? In Chapter II, I raised questions about whether the clamp opening mechanism was driven by the clamp loader or the sliding clamp. Researching this isoform of the sliding clamp will allow us to address some of those questions.

I hypothesize that the clamp opening mechanism of the *E. coli* clamp loader will be altered when loading beta*. The domain that is truncated from beta*, domain I, is the most dynamic domain of the sliding clamp (Fang et al., 2011). On the subunit of the sliding clamp that is bound to clamp loader subunits C-E, Domain I is unfolded in the open state (Chapter II). On the subunit of the sliding clamp that is bound to clamp loader subunits A-C, the region between Domain I and Domain II undergoes the largest conformation change in the entire sliding clamp during clamp opening (Chapter II). In addition to lacking the most
dynamic domain, beta* is missing one of the natural dimerization interfaces in the complex (domain I to domain III). Instead, I expect Domain II to form an interface with Domain III. Moreover, instead of having two interfaces, the sliding clamp will have three. Taken together, this sliding clamp has several major differences between itself and the normal sliding clamp that should both alter its stability as a complex and the flexibility of each of the subunits. I hypothesize that beta* will be loaded onto p/t-junctions with a different mechanism than the normal sliding clamp and that this sliding clamp is less stable as a complex on DNA.

While beta* can be used by DNA polymerase to increase its processivity, this use may not be its primary function. Instead, this sliding clamp may specialize as a DNA repair replication factor. DNA polymerases and DNA ligase interact with domain III of the sliding clamp to increase their affinity for DNA (Naktinis & Turner, 1996; Pandey et al., 2016). Additionally, the sliding clamp can bind polymerase III and polymerase IV simultaneously and switch between the two. This activity supports a toolbelt model, where the sliding clamp switches between polymerases to synthesize and repair DNA. Beta* has three protein-protein binding domains which potentially allows it to bind with an additional binding partner, if the three polymerase do not clash with each other, or for it to bind with low affinity partners tighter since the additional domain may limit dissociation (Indiani et al., 2005). It will be interesting to study how the clamp loader loads beta* as well as investigate its binding dynamics with polymerases and other DNA repair proteins.
How does gp8 inhibit the *E. coli* clamp loader?

What are the implications of gp8 DNA binding on previous results?

Yano et al. did not report that they found that gp8 copurified with DNA (Yano & and Rothman-denes, 2011). In their purification they wash gp8 with 50 column volumes of a 1 M sodium chloride buffer and then purified the protein over a hydroxyapatite column (CHT Type). However, I washed gp8 with the same amount of 2 M sodium chloride buffer and purified gp8 over a CHT Type and DNA remained bound. Therefore, based on my results it remains possible that Yano et al. had DNA contamination in their preparation.

If gp8 was bound to DNA in Yano et al.’s gp8 preparation, how would this alter the interpretation of their findings? It does not alter my interpretation that the induction of gp8 in *E. coli* alters their growth and inhibits DNA synthesis. Yano et al. placed a fluorescent label on gp8 to visualize where it localizes in *E. coli*, finding that it formed foci that colocalized with replication proteins (Yano & and Rothman-denes, 2011). If gp8 has a higher affinity for either single stranded DNA, or primer template DNA I would anticipate this result. I would also expect these foci from a protein that forms a complex with DNA and additional proteins. The delta subunit of the clamp loader does not bind to DNA on its own and so the pull-down assays are likely observing interactions between the delta subunit and gp8 and not delta and DNA.
Finally, how would DNA contamination affect the results of the *in vitro* clamp loading/DNA synthesis assays? Yano et al. quantified the amount of new DNA synthesized by measuring the incorporation of radiolabeled nucleotides and then either visualized these products by polyacrylamide gel phosphor screen autoradiography or quantified it by scintillation. Samples treated with gp8 had less newly synthesized DNA than those that were not. This gel is only sensitive to radiolabeled synthesis products, and so it would not show contaminant DNA from gp8. It does not appear that contaminant DNA was used as a template for DNA synthesis. If so, I would have expected there to be more short off-target products in the lanes containing gp8. The lack of these products suggests that, if DNA is a contaminant in their gp8 preparation, it must either be blunt or only have short overhangs (<22 bp) to prevent it from being used as a template (N. Yao et al., 2000). The *E. coli* clamp loader sliding clamp complex still binds to blunt DNA constructs, and so contaminant DNA could act as a clamp loading sink, where it competes with the intended DNA substrate for clamp loader activity (Ason et al., 2003). If this were the case, then when the contaminant DNA is present in high enough concentrations, it would out compete the intended DNA substrate, and cause gp8 to appear like a clamp loading inhibitor. In these assays, Yano et al. require a ~125x higher concentration of gp8 than clamp loader to completely inhibit the DNA synthesis which aligns with the hypothesis that these results could be a product of contaminant DNA and are not a product of gp8 activity since the interaction is not particularly tight.
Taken together, I have some reservations on the published findings on gp8. I find that gp8 binds to DNA exceptionally well and is challenging to remove. This contaminant DNA can affect biochemical assays studying the clamp loader since the clamp loader may use it as a substrate. Regardless of the potential impact of DNA binding activity on some of the results, many of the results are unaffected. Gp8 binding to DNA does not alter the microscopy results showing that gp8 colocalizes with the replisome or the mutagenesis and pull-down assays showing that mutations to the clamp loader confer gp8 resistance.

**Future Directions for the development of clamp loader inhibitors**

One of the motivations in studying gp8 was to inform the design of bacterial clamp loader inhibitors. Antibiotic-resistant infection account for over 2.8 million infections per year in the United States, which is predicted to become a growing problem in the coming years (Centers for Disease Control, 2019). Therefore, it is necessary to design antibiotics that target new pathways. Clamp loaders are essential for host growth and therefore are a viable target. Understanding the mechanism of gp8 would reveal a means to inhibit the clamp loader and may provide the groundwork for structure-guided drug design.

Once gp8 can be purified such that it no longer is bound to DNA or is bound to a uniform DNA substrate it will be much easier to determine its mechanism. Previous work has outlined several approaches that measure various aspects of the clamp loader, from sliding clamp binding to clamp release
(Hayner & Bloom, 2013; LG et al., 2017; Turner et al., 1999b). These experiments can provide the framework for assessing how gp8 interacts with the clamp loader. Alternatively, if gp8 does not interact with the clamp loader and inhibits DNA synthesis through another means, it will need to be elucidated. The crosslinking data is not impacted by whether gp8 has DNA binding activity, and so repeating that assay and determining the identity of the other proteins that crosslink with gp8 would reveal potential candidates. Because they are all essential, any of the core DNA replication proteins in *E. coli* would be a viable antibiotic target.

My work from Chapter II shows that the bacterial and eukaryotic clamp loaders have distinct mechanisms which may enable the design of specific drugs against the bacterial clamp loader. Inhibition of the clamp loader could be achieved through preventing it from interacting with any of its binding partners, i.e. ATP, sliding clamp, or p/t DNA, or one of the steps in the clamp loading pathway. To target any of the activities, it would be ideal for the structure of the bacterial and eukaryotic protein to differ to reduce the potential of host-target affects. The ATP binding domains are all very similar both structurally and by sequence (Neuwald, 2006) (Figure 4.2A). The interactions between the A subunit and the sliding clamp differ between bacterial and eukaryotes (Figure 4.2B). Perhaps designing molecules that interfere with clamp loader sliding clamp binding will be successful. There are also differences in how the clamp loaders interact with their respective p/t-junctions (Figure 4.2C). Additionally, bacteria
bind to RNA primed templates *in vivo* whereas eukaryotes bind to DNA primed templates. Leveraging the difference between the bacterial and eukaryotic clamp loaders may allow for structure guided drug design efforts to be successful.

The high degree of similarities would make designing inhibitors against the bacterial clamp loader without additional guidance challenge. *In silico* techniques for designing protein binding partners are advancing and can be implemented against the existing structures of the clamp loader to design inhibitors (Cao et al., 2022). Additionally, large libraries of molecules are available for high throughput screening. These libraries have been successful at identifying inhibitors against *Staphylococcus aureus* and *Enterococcus faecium* (Ayon & Gutheil, 2019; Gargvanshi & Gutheil, 2022). With the rise antibiotic resistance variants there is an increasing need to develop new drugs and clamp loaders may be a viable target.
Figure 4.2. Comparison of potential antibiotic target sites between bacterial and eukaryotic clamp loaders. The *E. coli* clamp loader is colored by subunit, whereas the eukaryotic complex is color grey. A. Alignment of the Rossmann domains of the Fully-Open state and the open RFC:PCNA complex (PDB:7TI8) B. Alignment of the Fully-Open state and the open RFC:PCNA complex (PDB:7TI8) structure on the sliding clamp subunit bound to the A subunit of the clamp loader C. Alignment of the Open-p/tRNA state and the open RFC:PCNA:p/tDNA complex (PDB:7TI8) structure on the sliding clamp subunit bound to the A subunit of the clamp loader.
Concluding Remarks

This thesis investigates the mechanisms of the *E. coli* clamp loader. I determined eight structures of the *E. coli* clamp loader bound to its sliding clamp which reveal how the clamp loader binds and opens its sliding clamp, forms a complex with p/t-junctions, and closes the sliding clamp onto p/t-junctions. Future work should focus on understanding the mechanism behind ATP turnover and how binding ATP activates the complex. My work also studied how the clamp loader can be inhibited by the viral protein gp8. It was inconclusive and did not determine how gp8 inhibits the clamp loader, it provides the groundwork for future research.
Appendix I: Cryo-EM study of the *E. coli* clamp loader bound to \( \text{ADP}\cdot\text{BeF}_x \)
Preface
I prepared the figures and contents of this section. I prepared the cryo-EM sample and processed the data with the help of Krishna Ahn.
Introduction:

In Chapter II, we investigate how the clamp loader binds and loads a sliding clamp onto p/t-DNA after binding to ATP. However, we still do not know how binding ATP alters the conformation of the clamp loader. This knowledge is necessary to understand why ATP binding is necessary for the clamp loader to bind to the sliding clamp. We hypothesized that the ATP bound complex has a similar conformation to that of in the Initial-Binding Complex (Chapter II), where the ATPase module is assembled into a tight ring that matches that of the sliding clamp.

The first structure of the assembled *E. coli* clamp loader was a crystal structure solved in the apo state (Jeruzalmi, O'Donnell, et al., 2001). In this state, the Rossmann domains of the ATPase module were splayed out such that the interfaces between the A-B, B-C, and D-E subunits were not making extensive contacts with each other. Modelling the closed sliding clamp to its binding pocket on the A subunit shows that in this conformation the clamp loader would make significant clashes with the sliding clamp. Studies show that a conformational change occurs in the clamp loader following ATP binding (Hingorani & O'Donnell, 1998; Naktinis et al., 1995). In the Initial-Binding state of the clamp loader (Chapter II), the clamp loader is bound to the closed sliding clamp with the A, D, and E subunits making strong contacts with the sliding clamp. These results present two possibilities: either ATP binding promotes the clamp loader to assemble into a conformation that templates the closed sliding clamp like the one
observed in the Initial-binding state, or after ATP binding, the clamp loader undergoes conformational changes upon making contact with the sliding clamp that allow the A, D, and E subunit to bind to the sliding clamp.

To investigate how ATP binding alters the conformation of the clamp loader, we sought to determine a cryo-EM structure of the clamp loader bound to ADP•BeF₆. A crystal structure of the clamp loader bound to the slowly hydrolysable analog ATPγS had previously been solved to 3.45 Å (PDB: 1XXH) (Kazmirski et al., 2004). We have two concerns over this structure. The *E. coli* clamp loader is capable of hydrolyzing ATPγS (Turner et al., 1999a), and so the crystal is likely composed of both pre- and post- hydrolyzed molecules. Additionally, there are extensive contacts between the ATPase modules in the crystal lattice, which may prevent the clamp loader from adopting its native conformation even if hydrolysis is not a significant concern. Therefore, we hypothesize that the cryo-EM structure determined with ADP•BeF₆ will be in a different conformation than the crystal structure of the ATPγS bound conformation.

We determined a 3D reconstruction of the clamp loader bound to ADP•BeF₆. The reconstruction has a global resolution of 4.5 Å, however there is significant variability in the resolution of the map. No attempts have been made to optimize this sample, either by changing the buffer/freezing conditions or collecting additional data. Additional experiments will likely improve the overall resolution of this map.
Materials and Methods

Protein Expression and Purification

The protein used in this section was expressed and purified with the same methods described in Chapter II.

Clamp loader and ADP•BeF₅ sample: We made a solution of 1.0 mg/ml (4.7 μM) of clamp loader in 25 mM Hepes pH 7.5, 200 mM NaCl, 4 mM MgCl₂, and 1 mM TCEP. 1 mM of ADP•BeF₅ was prepared in the sample according to Kelch et al. 2011 (Kelch et al., 2011). The sample was then crosslinked with BS3 according to the procedure described in Chapter II.

Cryo-EM grid preparation: All grids were first washed with ethyl acetate. The UltrAufoil R2/2 grids were then glow discharged on a PELCO easiGlow for 30 s at 20 mA (Ted Pella) (negative polarity). 3.5 μL of sample was applied to a grid and the grid was then blotted on both sides with a blot force of 5, a blot time of 5 s, and a wait time of 0 s. The grids were then vitrified by plunging into liquid ethane using a Vitrobot Mark IV (FEI) at 10°C and 95% humidity.

Cryo-EM data collection: The grids were imaged on a Talos Arctica operated at 200 kV equipped with an GIF energy filter at x45000 magnification. The frames were collected with a pixel size of 0.435 Å using a K3 Summit direct electron detector (Gatan) operated in superresolution counting mode. The data were collected with a target defocus range of -1.1 μm to -2.3 μm and a total exposure of ~43 e⁻/Å² averaging 30 frames.
Cryo-EM data processing: All data processing was completed using cryoSPARC (Punjani et al., 2017). Micrographs were aligned in using Patch Motion Collection with 2x binning, resulting in a pixel size of 0.87 Å per pixel. The contrast transfer function was determined using Patch CTF. Following CTF correction, one round of Blob picking (70-160 Å) was performed to complete reference free particle picking. Particles were then extracted with a box size of 256 pixels and 4x binning. To identify quality particles, we performed two rounds of 2D classification and selected classes with well-defined features, resulting in a stack of ~577,000 particles. The resulting particle stack was used to generate two ab-initio models. The highest quality ab-initio model appeared to be of the clamp loader and its associated particle stack was selected for further processing (458,000 particles). We then subjected this particle stack to two rounds of 3D classification followed by homogeneous refinement. Homogeneous refinement was implemented on the final particle stack of 102,460 particles. The B-factor used was 133.0 Å² and the cryo-EM map had a resolution of 4.5 Å (FSC=0.143). All reference models used during data processing were downfiltered to at least 30 Å.

Results:

Cryo-EM data process and 3D-reconstruction validation

We determined a map of the clamp loader bound to ADP•BeF₆, global resolution of 4.5 Å, in a single conformation (Figure A1.1, Figure A1.2B). The local resolution of the map is highly variable ranging from <4 Å to >12 Å (Figure A1.2A,C). The map clearly shows the secondary structure of the collar domain
for each subunit (Figure A1.3C). The ATPase module for the D and E subunits are clearly resolved in the map, and the model for these subunits is well supported by the density. There is sufficient density to identify an ADP molecule is bound in the ATP binding domain between the D and E subunit (Figure A1.3B). The BeF$_x$ molecule is not observed. This may be due to the limited overall resolution of the map or because BeF$_x$ molecules have a strong negative charge which are often not resolved in cryo-EM (Bartesaghi et al., 2014). The density for the ATPase modules consistently decreases rotating clockwise around the complex starting at the E subunit. The lid and ATPase modules for the C subunit can be rigid-body-fit into the density, but secondary structure is not defined. The lid and Rossmann domains for the A and B subunit have the lowest resolution in the map. These domains were fit into the available density, their position remains somewhat uncertain.

One significant factor that contributes to the overall resolution of a cryo-EM map is how well the particles align to each other. The resolution of flexible regions of samples suffer since these regions do not align to each other well (Serna, 2019). Perhaps, the ATPase modules of the A and B subunits are highly flexible which is decreasing the quality of these regions. To try and improve this region of the map I tried several approaches. First, I applied a focus mask to this region to focus the alignment of the particles on this area. I also tried processing the particle stack using Three-Dimensional Flexible Refinement (3DFlex) in cryoSPARC (Punjani & Fleet, 2023). 3DFlex employs a motion-based neural
network model to the 3D density for highly flexible domains. Unfortunately, neither masked refinement nor 3DFlex improved the resolution of this region of the map. Perhaps, this region of the complex was too flexible for either of these methods to properly align the particles. The mask that I applied to the refinement covers both the A and B subunits. However, if the domains of the A and B subunits move independently from each other the mask would not improve the alignment. Applying a mask to each subunit independently would circumvent this problem. Unfortunately, masking is unlikely to be effective on regions of a complex <100 kDa for two reasons, 1) The more features that a particle has, the easier it is for alignment algorithms to align them. 2) Larger particles have a better Signal-to-Noise Ratio (SNR) than smaller particles (Herzik et al., 2019). Each ATPase domain is ~25 kDa and so these regions cannot be masked independently, and the original mask was already small, focusing on ~60 kDa.

Perhaps, this region of the complex has unfolded. If so, the region would become so flexible each particle would likely have its own unique confirmation and generating a 3D volume would be impossible. If the lack of density in this region is due to protein unfolding, is this an actual behavior of the clamp loader, or an artifact of the experiment? There are a few reasons why unfolding the A subunit of the clamp loader after ATP binding would be beneficial for clamp loading. Presumably, the A subunit of the clamp loader makes the first contact with the sliding clamp, since is the only subunit that binds to the sliding clamp alone (Naktinis et al., 1995). By unfolding, the Rossmann domain of the A
subunit, which binds to the sliding clamp, would extend away from the rest of the complex and have a much larger search radius to find and bind to a sliding clamp. This conformation would also relieve any clashes between the sliding clamp and other clamp loader subunits, such as the ones that would occur between the clamp loader in the apo conformation and the closed sliding clamp. Since the clamp loader is localized to the replication fork by tau proteins, in an active replication fork, the clamp loader is locally constrained which would limit its ability to contact a sliding clamp. Unfolding presents a major problem, which is, after binding to the sliding clamp it must refold. Further experiments would need to be performed to verify that the A subunit unfolds after ATP binding. Hydrogen deuterium exchange mass spectroscopy could be performed on the clamp loader with and without ATP to test how ATP binding alters the complex’s flexibility. If the A or B subunit unfolds after binding to ATP, it would be expected that exchange would occur in these subunits faster when ATP is present than in its absence. Alternatively, the unfolding of these region could be an artifact of the assay. An estimated 90% of particles contact the air-water interface during grid preparation, an interaction that can damage the particles and lead to protein unfolding (Floris et al., 2019; Noble et al., 2018).
Figure A1.1. Schematic of the cryo-EM processing workflow for the clamp loader and ADP•BeF\textsubscript{x} dataset. All data processing was performed using cryoSPARC. 

A. A representative patch-motion corrected micrograph and particle-picking pipeline. Particles were first picked with cryoSPARC’s blob-picker tool. Identified particles were extracted and 2D classified. Particles from the selected 2D classes were used as templates for cryoSPARC’s template picker. Particles identified by the template picker were extracted and 2D classified. Representative 2D classes were selected following template picking.

B. Ab-initio reconstruction. Particles from the selected 2D classes were used to generate two ab-initio models. One of the models resulted in a reconstruction of the clamp loader.

C. 3D classification and reconstruction. The particle stack from the ab-initio model was 3D classified to separate out discrete classes and improve the final model. 

D. Homogeneous refinement of the 3D classes. Each 3D class after the second round of 3D classification was refined. The resolution calculated by the FSC of each half-map using Gold-standard cutoff of 0.143 for each map is shown as well as the number of particles.

Figure A1.2. 3D reconstruction of the clamp loader ADP•BeF\textsubscript{x} complex. A. 3D reconstruction of the complex. The map is shown at two contours levels, 0.06 and 0.1. The individual subunits of the clamp loader are colored. B. FSC curve of the clamp loader cryo-EM map. The overall resolution of each map was determined by the FSC of each half-map using Gold-standard cutoff of 0.143 (black line). C. Local resolution of the clamp loader cryo-EM map. Local resolution (Fourier shell correlation (FSC)=0.5).
Figure A1.3. Fit of the clamp loader ADP•BeFx model into the 3D reconstruction. The model of the clamp loader ADP•BeFx complex is shown with a cartoon representation. The map that the model was built into is shown at different contours for different regions of the map. A. The Rossmann domain of the E subunit (contour = 0.1). B. The ADP•BeFx molecule bound at the D:E subunit interface (contour = 0.1). C. The collar domain of the complex (contour = 0.1). D. The collar and lid domains of the A and B subunits (contour = 0.1). E. The Rossmann domain of the A subunit (contour = 0.04). F. The Rossmann domain of the C subunit (contour = 0.07). G. Model-to-map cross-correlation of each chain of the complex. The vertical dotted lines show the boundaries between the different domains of the complex.
Conformational changes between the ADP•BeF₆ state and previously solved E. coli clamp loader structures

Here I compare the models of the clamp loader bound to ADP•BeF₆ (determined in this work), to the apo- (PDB:1jr3), ATPγS bound (PDB:1xxh), and the Initial-Binding complex (Chapter II). The collar domains of each structure are very similar where the greatest difference is between the ADP•BeF₆ bound clamp loader and the Initial-Binding complex, where the RMSD is only 2.6 Å. Additionally, the E subunit undergoes the least conformational change between the states where the greatest difference in RMSD between the ADP•BeF₆ is again with the Initial-Binding complex, where the RMSD is 2.4 Å. It is not surprising that the collar domain and the E subunit undergo the least conformational changes as the collar serves as the oligomeric domain and the E subunit has previously been call the “stator,” and has been predicted to serve as a backboard for the rest of the complex to push against when opening the sliding clamp (Indiani, 2003).

While the collar and E subunit did not undergo large conformational changes, differences are present between the position of the ATP modules of subunits A-D (Figure A1.4). I had hypothesized that binding to ADP•BeF₆, would stabilize interactions between the Rossmann domains and produce a conformation similar to the Initial-Binding complex. However, the results do not support this hypothesis. The Rossmann domains of the ADP•BeF₆ bound clamp loader are the most splayed out, especially those of the A and B subunits. The density of subunits A and B are the weakest and so the position of these subunits
cannot be confidently assigned. The lack of strong density suggests that these subunits are flexible which is not consistent with the hypothesis that ATP binding stabilizes the conformation of the clamp loader. In the apo, ATPγS bound, and ADP•BeF₉₆ bound complex the ATPase domains are in an overall planar conformation in contrast to the semi-helical conformation seen in the Initial-Binding state. These structures show that binding to the sliding clamp is concomitant with a conformational change in the clamp loader.

This work starts to help us understand the conformational changes that occur after ATP binding. Unfortunately, the quality of the map leaves several questions remaining about how ATP binding affects the conformation of the clamp loader and the actual state that was solved in this work. While ADP is observed in the D subunit’s ATP binding pocket, the resolution of the map does not allow us to determine whether ADP•BeF₉₆ is bound to either the B or C subunits. Interestingly, in the ATPγS bound structure, ATPγS is only observed in the B and D subunit’s ATP binding pockets. This result is supported by isothermal titration calorimetry (ITC) data and nitrocellulose membrane binding assays, which find that the clamp loader alone only binds to two ATP molecules (Hingorani & O'Donnell, 1998; Kazmirski et al., 2004). Perhaps, in this state ADP•BeF₉₆ is only bound in either the B or C subunit and one subunit is empty. In the ATPγS bound structure the ATPγS, molecules are modelled into the D and B binding pockets. Perhaps, the B and D subunits first bind ATP then, after sliding clamp binding, the C subunits binds to the third ATP molecule which would be an
interesting mechanism of coordinated ATP binding events. Future work will need to be conducted to optimize the cryo-EM sample to better understand how binding to ATP affects the conformation of the sliding clamp.
Figure A1.4. Conformational differences between the ADP•BeF₇, bound clamp loader and previously determined structures. Models of the apo-clamp loader (PDB: 1jr3), clamp loader ADP•BeF₇ complex, clamp loader ATPγS complex (PDB: 1xxh), and the clamp loader from the Initial-Binding State (Chapter II) are displayed as low contour surfaces. All clamp loaders were aligned on the E subunit. A. Conformational changes of the clamp loader between the apo- and ADP•BeF₇. The Rossmann domains of the apo-state (gray) and the ADP•BeF₇ state (color) are displayed as low contour surfaces. The black arrows show the displacement of the centers of mass of the Rossmann domains of subunits A-E as vectors. B. Conformational changes of the clamp loader between the ATPγS and ADP•BeF₇ bound complexes. The Rossmann domains of the ATPγS state (gray) and the ADP•BeF₇ state (color) are displayed as low contour surfaces. The black arrows show the displacement of the centers of mass of the Rossmann domains of subunits A-E as vectors. C. Conformational changes of the clamp loader between the ADP•BeF₇ bound complex to the Initial-Binding complex. The Rossmann domains of the ADP•BeF₇ state (color) and the Initial-Binding complex (gray) are displayed as low contour surfaces. The black arrows show the displacement of the centers of mass of the Rossmann domains of subunits A-E as vectors.
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