DNA replication by *Escherichia coli* DNA polymerase III is regulated by the *umuD* gene products

A dissertation presented

by

Michelle C. Silva

to

The Department of Chemistry and Chemical Biology

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In the field of

Chemistry

Northeastern University

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry and Chemical Biology in the College of Science at Northeastern University

July 19, 2013
DNA polymerase III (DNA pol III) efficiently replicates the *Escherichia coli* genome, but it cannot bypass DNA damage. Instead, translesion synthesis (TLS) DNA polymerases are employed to replicate past damaged DNA; however, the exchange of replicative for TLS polymerases is not understood. The *umuD* gene products, which are up-regulated during the SOS response, were previously shown to bind to the α, β, ε subunits of DNA pol III. Full-length UmuD inhibits DNA replication and prevents mutagenic TLS, while the cleaved form UmuD’ facilitates mutagenesis. In this work, we investigate the interactions between the α subunit of DNA pol III, the single-stranded DNA binding protein SSB, and UmuD and we find that these interactions regulate DNA replication in *E. coli*.

We show that the α subunit possesses two UmuD binding sites: at the N-terminus (residues 1-280) and the C-terminal domain (residues 956-975) of α. The C-terminal site favors UmuD over UmuD’. We also find that UmuD, but not UmuD’, disrupts the α-β complex. The C-terminal binding site is also adjacent to the single-stranded DNA (ssDNA) binding site of α. We have used single molecule DNA stretching experiments to demonstrate that UmuD specifically inhibits binding of α to ssDNA. We predict using molecular modeling that UmuD residue D91 is involved in the interaction between UmuD and α, and demonstrate that mutation of these residues decreases the affinity of α for UmuD. We propose that the interaction between α and UmuD contributes to the transition between replicative and TLS polymerases by removing α from the β clamp and from ssDNA.
E. coli SSB binds to and protects ssDNA present during various DNA processing mechanisms such as DNA repair, recombination, and replication. Here, we investigate the role of SSB during DNA replication by DNA pol III. It has been previously shown that when SSB is bound to ssDNA at the replication fork, replication by the polymerase core, which consists of the α, ε, and θ subunits, is inhibited. In order for replication to occur in the presence of SSB the χ and ψ subunits of DNA polymerase III are required. To date, the mechanism of this inhibition is poorly understood. We demonstrate that SSB inhibits DNA polymerase III by directly binding to the C-terminal domain of the α polymerase subunit. Interestingly, instead of binding the disordered tail of SSB like other proteins including the χ subunit, the α subunit binds the globular N-terminal domain of SSB that is responsible for binding ssDNA. Using single molecule force spectroscopy, we also show that the α subunit stabilizes the SSB/ssDNA interaction. In the absence of the α subunit, SSB stabilizes ssDNA below 20 pN and fully dissociates above 20 pN. However, in the presence of the α subunit, SSB does not dissociate above 20 pN and the energy required to dissociate SSB from ssDNA is increased by a factor of two. We show here that SSB inhibits replication by α both through direct interactions between the two proteins as well as through SSB binding to DNA; the overall effect of these interactions is that α stabilizes SSB binding to DNA, which contributes to the inhibition.

We also show that SSB binds both full-length UmuD and the cleavage product UmuD’, with a slightly higher affinity for UmuD’ versus full-length UmuD. Like with other SSB binding partners, binding was localized to the C-terminal tail, as a decrease in binding to UmuD was observed with the SSB-113 variant, which harbors the C-terminal tail mutation P177S. But unlike other SSB protein binding partners, UmuD and especially UmuD’ also bind the globular
N-terminal domain due to our observation that the isolated SSB-OB domain binds both UmuD and UmuD’. *In vivo*, elevated levels of UmuD and UmuC but not UmuD’ complemented the temperature sensitive phenotype of the *ssb-113* allele, suggesting that the interactions between UmuD and SSB and between UmuD’ and SSB have different physiological consequences for the cell. Overall, we show that a network of protein-protein and protein-DNA interactions modulates DNA replication under normal cellular conditions and in response to DNA replication stress.
I would like to thank my advisor Dr. Penny J. Beuning for allowing me to work in her lab all these years. I am especially grateful to her for giving me the opportunity to manage the chemical biology lab class. This opportunity inspired me to maybe consider a career in academia one day.

I would like to thank my thesis committee members: Dr. Mary Jo Ondrechen, Dr. David Budil, and Dr. Carla Mattos. Thank you for accepting my invitation to be on my committee and for advising me through the many thesis committee meetings we have had over the past couple of years.

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LIST OF ABBREVIATIONS

~ Approximately
° Degrees
µL Microliter
µM Micromolar
A Alanine
A Adenosine
Å Angstrom
Ala Alanine
Asn Asparagine
Asp Aspartic Acid
ATP Adenosine triphosphate
BMH Bis-maleimidohexane
BSA Bovine serum albumin
C Cytidine
CaCl₂ Calcium Chloride
CD Circular Dichroism
CV Column volume
Cys Cystiene
D Aspartic Acid
DNA Deoxyribonucleic acid
DNA pol I DNA polymerase I
DNA pol III DNA polymerase III
DNA pol IV DNA polymerase IV
DNA pol V DNA polymerase V
DNA pol β DNA polymerase β
dsDNA Double-stranded DNA
DTT Dithiothreitol
E. coli Escherichia coli
EDTA Ethylenediaminetetraacetic acid
EPR Electron paramagnetic resonance
F Phenylalanine
FRET Fluorescence resonance energy transfer
G Glycine
G Guanosine
Gly Glycine
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr Hour
I Isoleucine
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$K_a$</td>
<td>Equilibrium association constant</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>nt/s</td>
<td>Nucleotides per second</td>
</tr>
<tr>
<td>OB</td>
<td>Oligonucleotide/oligosaccharide binding</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PHP</td>
<td>Polymerase and histidinol phosphatase</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>pN</td>
<td>Piconewtons</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-stranded DNA binding protein</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>T</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Ts</td>
<td>Temperature Sensitive</td>
</tr>
<tr>
<td>TLS</td>
<td>Trans-lesion Synthesis</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
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CHAPTER 1: Overview of DNA Replication in *Escherichia coli*

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1.1 *E. coli* DNA Polymerase III

DNA replication requires the coordination of many different proteins to accomplish the goal of simultaneous replication of the two antiparallel stands of DNA. This process is tightly regulated so that DNA is replicated in a timely and accurate manner. Moreover, DNA replication is highly
processive, which allows efficient replication of over three million base pairs in every *E. coli* cell cycle.

In *E. coli*, DNA polymerase III (DNA pol III) is responsible for the majority of DNA replication (Figure 1.1). Each cell expresses approximately ten copies of DNA pol III core (see below) (1). Along with auxiliary proteins, DNA pol III semi-discontinuously replicates DNA at a speed of approximately 1 kilobase (kb) per second (2), making less than one error in approximately $10^5$ nucleotide additions (1, 3). In the presence of proofreading and mismatch repair, the error frequency is approximately $10^{-10}$ per base pair (1, 4). At each replication fork, the DNA pol III core acts as an asymmetric dimer (5-9): one monomer acts in the continuous replication of the leading strand and another acts in the discontinuous replication of the lagging strand (Figure 1.1). Even though only two polymerase cores are needed to replicate DNA, it has been shown that the replisome may contain three DNA polymerase cores. The third polymerase core may function on the lagging strand or serve as a spare polymerase, able to replace either polymerase when needed (10).
Figure 1.1 DNA polymerase III holoenzyme (dimer form) at a replication fork.

The two polymerase cores (green), which are tethered to the β clamps (light blue), contain the three subunits α, ε, and θ. The γ complex (blue), assembled with two τ subunits, couples the polymerization of both the leading strand and the lagging strand. The single-stranded lagging strand is threaded through DnaB helicase (orange) and is coated with SSB (gray). Also shown is the primase (light green) that synthesizes the RNA primers (red) on the lagging strand. The ψ and χ subunits are not shown but would connect the γ complex to SSB.

*E. coli* DNA pol III consists of ten subunits (2, 6, 9, 11) that can be classified into three subassemblies: the core, the clamp and the clamp loader complex (9, 12) (Table 1.1). The core is composed of three subunits: α, ε, and θ. The α subunit contains the polymerase activity and is responsible for synthesizing DNA. The processivity of the isolated α subunit, defined as the
number of nucleotides incorporated into the nascent DNA per association event, is low (1-10 nucleotides) compared to >50 kb for the holoenzyme (1, 13-17). The $\varepsilon$ subunit is a 3′ to 5′ exonuclease that is responsible for the proofreading capability of the core. In the absence of $\varepsilon$, the frequency of mutations due to misincorporations during replication increases by approximately forty-fold (18). When coupled, the exonuclease and polymerase activities of the $\alpha$ and $\varepsilon$ subunits increase significantly (18). The third component of the core, $\theta$, is not required for high processivity (19).

The $\beta$ clamp, also known as the processivity clamp, is the major contributor to the processivity of DNA pol III (1). The $\beta$ clamp encircles DNA and tethers the $\alpha$ subunit to its DNA substrate (Figure 1.1). The $\beta$ clamp is loaded onto DNA by a complex known as the clamp loader, which is composed of six subunits: $\tau$, $\gamma$, $\delta$, $\delta'$, $\chi$, $\psi$ (Table 1.1) (20). The $\tau$ subunits coordinate replication on both strands by coupling the polymerase cores to the clamp loader complex (9, 21). When $\tau$ is coupled to the core, processivity increases approximately six-fold (1). The $\gamma$ subunit is an ATPase. Along with $\delta$ and $\delta'$, $\gamma$ is responsible for loading the clamp onto DNA (2, 11). The two other subunits, $\psi$ and $\chi$, bind single stranded DNA binding protein (SSB) and help regulate replication on the lagging strand (22, 23). The $\psi$ subunit also has a role in clamp loading (24).
Table 1.1. Components of DNA polymerase III.

<table>
<thead>
<tr>
<th></th>
<th>Mass (kDa)</th>
<th>Gene</th>
<th>Function</th>
<th>References</th>
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<tr>
<td><strong>Polymerase Core</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>130</td>
<td>dnaE</td>
<td>polymerase</td>
<td>(25-28)</td>
</tr>
<tr>
<td>ε</td>
<td>27.5</td>
<td>dnaQ (mutD)</td>
<td>3'-5' exonuclease</td>
<td>(18, 29-36)</td>
</tr>
<tr>
<td>θ</td>
<td>10</td>
<td>holE</td>
<td>stabilizes the core</td>
<td>(33, 36-39)</td>
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<tr>
<td><strong>Clamp Loader</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>τ</td>
<td>71</td>
<td>dnaX</td>
<td>coordinates replication</td>
<td>(9, 21, 42-51)</td>
</tr>
<tr>
<td>γ</td>
<td>47.5</td>
<td>dnaX</td>
<td>ATPase</td>
<td>(47, 52-54)</td>
</tr>
<tr>
<td>δ</td>
<td>35</td>
<td>holA</td>
<td>“wrench”; opens clamp</td>
<td>(52, 55)</td>
</tr>
<tr>
<td>δ’</td>
<td>33</td>
<td>holB</td>
<td>mediator between γ &amp; δ</td>
<td>(52, 56)</td>
</tr>
<tr>
<td>χ</td>
<td>15</td>
<td>holC</td>
<td>binds SSB</td>
<td>(22, 23, 57, 58)</td>
</tr>
<tr>
<td>ψ</td>
<td>12</td>
<td>holD</td>
<td>bridges γ complex &amp; χ</td>
<td>(22, 57)</td>
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<tr>
<td><strong>Clamp</strong></td>
<td></td>
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<tr>
<td>β</td>
<td>40.6</td>
<td>dnaN</td>
<td>clamp</td>
<td>(11, 28, 40, 59-63)</td>
</tr>
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1.2 The Polymerase Core

The DNA pol III core includes the polymerase and proofreading exonuclease activity of the replisome. Alone, the core can replicate DNA at a rate of approximately 20 nucleotides per second (nt/s) with a processivity of 11 nucleotides, values much lower than the entire replisome (2, 11). The following is an in-depth description of each of the subunits of the core.

1.2.1 The Polymerase Subunit, α
The α subunit, encoded by the dnaE gene and a member of the polymerase C family, is a protein of approximately 130 kDa, containing several distinct domains. Although this polymerase has been studied for decades, the crystal structure of the α subunit of *E. coli* was only solved recently (25). Like other polymerases, the structure resembles a right hand with three characteristic domains: the palm, the fingers, and the thumb domains (64-66). The palm domain contains the active site of the polymerase consisting of three aspartic acid residues: Asp401, Asp403, and Asp555 (67). This domain is similar to the palm domains of polymerases in the X family, especially to that of DNA pol β (25). Modeling DNA onto the *E. coli* DNA pol III α structure using the human DNA pol β co-crystal structure with DNA (68) as the modeling template, showed that the DNA strand collides with a short α-helix in the palm domain in a sterically unfavorable interaction (25). This suggests that although DNA pol III and pol β have similar active sites, there may be differences in how they bind DNA.

The finger domain includes four sub-domains: the index finger, the middle finger, the ring finger, and the little finger. These sub-domains are responsible for binding the incoming nucleotide. The thumb domain guides the newly formed DNA duplex as it leaves the active site. The Polymerase and Histidinol Phosphatase (PHP) domain is located in the “wrist” position, relative to the hand of the polymerase domain. The exact role of this domain is unknown but because of the domain’s sequence similarity to histidinol phosphatases, it was proposed to possess pyrophosphatase activity for the pyrophosphate produced during replication (69). Based on the crystal structure of DNA pol III α, this domain is unlikely to harbor such activity (25). However, it has been demonstrated that the *Thermus thermophilus* DNA pol III α subunit contains a Zn^{2+}-dependent 3’ to 5’ exonuclease activity (70).
The C-terminal domain is not present in the crystal structure of *E. coli* DNA pol III α. This domain, which is located C-terminal to the tip of the little finger domain, includes an oligonucleotide/oligosaccharide binding (OB) fold (25, 26) and the binding sites for both the τ subunit (49, 71) and the β clamp (28, 50). The recently-solved structure of DNA pol III α from *Thermus aquaticus* (26) includes this C-terminal domain, consisting of an OB fold. The OB fold domain consists of five β-strands arranged in a β barrel, similar to that of other OB folds (72). The OB fold domain of α has been shown to bind single-stranded DNA (ssDNA) specifically (73). Compared to other ssDNA binding proteins, the OB fold domain of the α subunit is somewhat unusual in that it does not actively melt DNA, but rather binds to ssDNA that is pre-formed, in this case by force-induced melting (73). Along with the rest of the polymerase, this function provides insight into the regulation of DNA pol III α, discussed below.

A co-crystal structure of *T. aquaticus* DNA pol III α bound to primer-template DNA and an incoming deoxynucleoside 5′-triphosphate has been determined with a resolution of 4.6 Å (27). When compared to the structure of *T. aquaticus* DNA pol III α without DNA, it is possible to see significant movements of the thumb, finger, and β-binding domains. These movements position the protein on the DNA, allowing for the interaction with the DNA backbone at the minor groove. This structure also indicates that the DNA and incoming nucleotide bind in a similar fashion to that of DNA pol β. The C-terminal domain undergoes an approximately 30° rotation putting the OB fold in position to bind the single stranded template DNA. The internal β-binding motif also seems to be correctly positioned in the structure with DNA in order to bind to the hydrophobic pocket on the β clamp (27, 28).
The structure of a ternary complex of PolC, the replicative polymerase of the gram-positive bacterium *Geobacillus kaustophilus*, with primed DNA and an incoming dideoxynucleoside substrate has been solved to 2.4 Å resolution (74). Unlike the DNA pol III α polymerase, PolC contains an intrinsic 3′ to 5′ exonuclease domain as an insertion within the PHP domain. Instead of being located in the C-terminal domain, the OB fold of PolC is located N-terminal to the palm domain and is positioned so it could bind the single-stranded template strand approximately 15-20 nucleotides away from the polymerase active site. The thumb domain contains β-strands that bind DNA in the minor groove, possibly allowing for the detection of mismatched base pairs after incorporation (74). Flexibility in the palm domain suggests a large conformational change upon DNA binding (74, 75).

### 1.2.2 The ε Subunit

The ε subunit, a 27.5-kDa protein encoded by the *dnaQ* (also known as *mutD*) gene, is responsible for the 3′ to 5′ exonuclease activity of the polymerase core (1) and forms a tight complex with DNA pol III α. Whereas in *E. coli* the polymerase and exonuclease reside on two different polypeptides, in other cases, the exonuclease activity and the polymerase activity are part of the same polypeptide, as in gram positive PolC and in DNA pol I (64, 66, 74), another eubacterial polymerase. Such an interaction between α and ε enhances the overall activity of each protein. In fact, it has been shown that α has greater polymerase activity in complex with ε than alone (18). The exonuclease activity of the ε subunit is also substantially stimulated within the complex (18).
Although the structure of the N-terminal domain of \( \varepsilon \) has been determined by X-ray crystallography (29) and by NMR (76), no structures for the full-length \( \varepsilon \) subunit have been determined due to the difficulty in obtaining large amounts of pure protein. The structures include the 186 N-terminal residues of \( \varepsilon \) responsible for its exonuclease activity. The 57 residues that are not present in the structure include the C-terminal domain, which contains a flexible linker that has been shown to bind to the \( \alpha \) subunit (30-33). The \( \varepsilon \) subunit binds \( \alpha \) in the PHP domain located in the \( \alpha \) N-terminal domain (77).

1.2.3 The \( \theta \) Subunit

The third subunit of the polymerase core is \( \theta \), the 10-kDa product of the \textit{holE} gene (1). The \( \theta \) subunit binds to \( \varepsilon \) close to the \( \varepsilon \) active site (37, 38), although it is unlikely to play a direct role in exonuclease activity (36). Extensive hydrophobic surfaces define the interactions between \( \theta \) and \( \varepsilon \) (36, 39, 78). The \( \theta \) subunit has not been shown to bind directly to \( \alpha \) (38, 79), but it may have a stabilizing effect on the \( \alpha: \varepsilon \) complex. Such a function is supported by the results of a series of yeast two-hybrid experiments indicating that the interaction between \( \alpha \) and \( \varepsilon \) is strengthened in the presence of \( \theta \) (80). The \( \theta \) subunit also seems to enhance the exonuclease activity of the \( \varepsilon \) subunit by stabilizing \( \varepsilon \) (38, 81). Deletion of \( \theta \) results in a slight increase in the spontaneous mutation frequency of \textit{E. coli} (80). In biochemical experiments, the exonuclease activity of \( \varepsilon \) I170T/V215A double mutant was not substantially stimulated in the presence of either \( \alpha \) or \( \theta \) (82). Upon addition of both \( \alpha \) and \( \theta \), however, activity of the \( \varepsilon \) I170T/V215A variant was stimulated (82).
1.3 The Clamp Loader Complex and the β Clamp Subunit

The clamp loader complex consists of at least two τ subunits, up to three γ subunits, and one each of the δ, δ’, χ, and ψ subunits (2, 9, 11). As mentioned above, DNA pol III can be assembled as a trimer (10). In this case, three τ subunits are present in the absence of γ subunits. Although not required for the clamp loading process, the τ subunits play a critical role in managing replication at the fork (9, 11). The γ subunits, closely related to τ, bind ATP, and facilitate loading the β clamp onto DNA (11, 40). The δ and δ’ subunits are directly involved with the loading of the clamp, δ as the “wrench” and δ’ as the mediator between γ and δ (40, 55).

1.3.1 The γ Complex: δ':γ1:γ2:γ3:δ; Loading the Clamp

The dnaX gene encodes both τ and γ. The τ subunit is the full-length product of the gene and γ is produced due to a -1 frameshift, which causes a stop codon to be inserted prematurely, forming the shorter product, γ (47, 83, 84). This frameshift is caused by two factors: a heptanucleotide sequence that induces frameshifts and a downstream RNA stem-loop structure (Figure 1.2A). Therefore, γ consists of only the first three of the five τ domains (Figure 1.2B). These three domains contain the ATPase site and so both τ and γ are ATPases. In the crystal structure of the γ complex, containing γ3δδ’ (52), it is possible to distinguish these domains. Domains I and II
contain the nucleotide binding site in which ATP binds at the interface between the subunits and Domain III forms a circular collar with the other subunits of the γ complex.

**Figure 1.2 The τ and γ subunits of DNA pol III.**

(A) The segment of the dnaX mRNA, responsible for coding the γ and τ subunits. The two factors that cause the -1 frameshift are a heptanucleotide sequence (bold) and a downstream stem-loop. As a result, the γ subunit consists of the first 430 residues of the dnaX gene product shared with the τ subunit, followed by a glutamic acid residue and a stop codon. (B) A side-by-side comparison of the domains of the τ and γ subunits. The γ subunit, the shorter protein, contains only the first three domains of the entire gene product. These domains include the ATPase active site and the collar domain. Domains IV and V of the τ subunit contain the binding sites for both the pol III α subunit and DnaB.

A crystal structure of the first 243 residues of γ (Domains I and II) solved with and without nucleotides (53), shows a conformational change upon nucleotide binding, suggesting a mechanism for binding to the clamp and ATP hydrolysis (Figure 1.3). In the crystal structure of the γ complex (52), the γ subunits, together with δ and δ’, are arranged in a heptameric complex.
resembling an opened ring in the order $\delta':\gamma_1:\gamma_2:\gamma_3:\delta$ (Figure 1.3). At each $\gamma$ interface there is an ATP binding site (52). Such a configuration suggests that upon ATP binding, the $\gamma$ complex undergoes a conformational change from a closed state (without ATP) to an open state (with ATP) (53). The $\delta$ subunit is then free to interact with the $\beta$ clamp.

Figure 1.3 The process of loading the $\beta$ clamp onto the primer-template DNA duplex. ATP binds the $\gamma$ complex, causing a conformational change from a closed state to an open state, which allows the N-terminal domain of the $\delta$ subunit (dark blue) to bind to the $\beta$ clamp. This interaction disrupts the dimer interface of the $\beta$ clamp, creating an opening for the primer-template DNA duplex to enter. The bound ATP is then hydrolyzed, causing the $\gamma$ complex to relax back to its closed state. The $\delta$ subunit then releases the $\beta$ clamp, re-establishing the dimer interface.

The $\delta'$ subunit, the 33-kDa member of the $\gamma$ complex encoded by the *holB* gene, acts as a mediator between $\gamma$ and $\delta$ (1). Although sequence and structure alignments of $\delta'$ and $\gamma$ suggest that these two subunits are homologous, $\delta'$ does not have a functional nucleotide binding domain,
as shown in the crystal structure of the δ′ subunit (56). This crystal structure shows that δ′, like γ, consists of three consecutive domains organized in a C-shaped architecture. The first domain consists of a β sheet with five parallel strands surrounded by six α-helices similar to the nucleotide binding domain of RecA (56, 85). The δ′ subunit contains a zinc-binding module whose function is unknown but because it is found on what resembles a phosphate binding loop, it may help couple DNA binding with ATP hydrolysis by the clamp loader (56).

The δ subunit, a 35-kDa product of the holA gene, is considered the “wrench” of the clamp loader complex because its binding to the β clamp causes a spring-like conformational change (55). This allows the dimeric ring of the β clamp to transition from its default closed state, where both dimeric interfaces are intact, to an open state, where only one dimeric interface exists (Figure 1.3) (55, 86-88). This spring-like mechanism facilitates loading of the β clamp onto the primer-template DNA duplex (2, 9, 11, 55). The “wrench”-like interaction between δ and the β clamp is shown in a crystal structure involving the N-terminal 140 residues of δ and a variant form of the β clamp that cannot dimerize (55). Attempts to crystallize δ with the full-length β clamp dimer have been unsuccessful (55). In fact, δ binds to the monomer form approximately fifty-fold more tightly than to the dimer. The binding interface between these two subunits is contained in a hydrophobic tip of δ and a hydrophobic pocket on the surface of the β clamp that is also known for binding other components of the replisome (55, 63, 89) and DNA polymerases (90-93). When the structure of the monomer form of the β clamp without δ was compared to that with δ, a distortion of the curvature of the β clamp that results in a ~15 Å opening can be seen (55). Such an opening is enough to allow ssDNA into the center of the clamp (40, 55).
The crystal structures of the clamp loader subunits suggest a multistep process for loading the β clamp onto a primed DNA strand (9, 11, 40), powered by the binding of two ATP molecules (54) (Figure 1.3). When ATP binds to the closed ring-shaped γ complex, a conformational change takes place disrupting the interaction between δ and δ′. The δ subunit is then free to bind to the hydrophobic pocket of the β clamp dimer. This binding event distorts the β dimer interface allowing DNA to enter the clamp, creating an opened ring-like structure consisting of the opened γ complex and clamp. Site-directed mutagenesis (94), electron microscopy (95), and X-ray crystallography (41) experiments have shown that DNA can bind to the center chamber of the complex. Once ATP is hydrolyzed, the dimer interface of the β clamp is restored, allowing the γ complex to relax back to its closed state. This causes the clamp loader to release its hold on the β clamp, the rate-limiting step of the clamp loading process (96). The β clamp and primer-template DNA duplex are then competent for polymerase loading (97).

The recent crystal structure that shows the clamp loader complex coupled to DNA (41) supports the notched screw-cap model for clamp loading as described above. The structure shows that the complex forms a right-handed spiral-like structure and is loaded onto dsDNA like a cap, allowing ssDNA to exit through a slit formed by the complex. In this structure, the γ complex does not recognize DNA via both strands of the primer:template complex as previously thought (95, 98). Rather, recognition occurs on the phosphate backbone of the template strand alone, and at just the 3′ nucleotide of the primer strand (41). This allows for both DNA and RNA primers to be recognized, the mechanism of which was previously unclear (41, 99).
1.3.2 The $\chi$ and $\psi$ Subunits

The $\chi$ and $\psi$ subunits, products of the *holC* and *holD* genes, respectively, are subunits of the clamp loader complex (2, 9, 11, 100). These two subunits function during replication on the lagging strand (22, 23). Because DNA polymerases replicate DNA only in the 5′ to 3′ direction, the lagging strand must be replicated in a direction opposite to the movement of the replication fork. This is accomplished by replicating DNA in ~1 kb fragments, called Okazaki fragments (101). As a result, an abundance of ssDNA is present and coated with SSB (Figure 1.1). The $\chi$ and $\psi$ subunits help to coordinate replication on the lagging strand (22, 23). The $\psi$ subunit acts as a mediator between the $\gamma$ complex and $\chi$ by binding the collar domains (Domain III) of the $\tau$ and $\gamma$ subunits (41, 57). The $\chi$ subunit binds to the C-terminal domain of SSB (23), thereby coupling it to the replisome and allowing the clamp loader complex to be in close proximity to the primer-template DNA on the lagging strand (58). Together, the $\chi$ and $\psi$ subunits constitute a tightly held complex that increases the affinity of $\tau$ and $\gamma$ for $\delta$ and $\delta'$ (57). The $\psi$ subunit also serves to increase affinity of the clamp loader for the $\beta$ clamp in the presence of ATP$\gamma$S (24).

1.3.3 The $\beta$ Clamp Subunit

Once it is loaded onto the primer-template DNA duplex, the $\beta$ clamp (*dnaN*) has two specific roles. It tethers the polymerase to the DNA and contributes to the mobility of the polymerase on the DNA strand. The $\beta$ clamp, a ring-shaped homodimer (Figure 1.4) (61, 62), is the major contributor to processivity (1), by allowing the polymerase to maintain close contact with the DNA. In order to facilitate processive DNA synthesis, the clamp must remain bound to the DNA
with or without the polymerase present, which has been shown (59, 102). Other studies show that the clamp can remain on a circular plasmid two to three times longer than the time it takes for cells to divide (60). With the use of single molecule fluorescence spectroscopy, it was found that the diffusion constant for the β clamp is at least three orders of magnitude lower than for diffusion through water. The β clamp seems to be held at the 3′ end of the primer in the presence of SSB (103). The relatively slow motion of the clamp may be due to the attractive interactions of positively charged residues on the inside of the clamp that come in contact with the negatively charged phosphate backbone of the DNA (103, 104).

![Figure 1.4 Residue substitutions in the β clamp that are implicated in interactions with UmuD (left), UmuD′ (middle) and α subunit of Pol III (right) (105).](image)

Positions in green are important to the interaction between the β clamp and all three proteins listed above. Positions in purple exhibit only a modest effect. Substitutions that result in an increase or decrease in the affinity of UmuD and UmuD′ for the β clamp by formaldehyde or glutaraldehyde cross-linking are shown in red. Residue Lys74 shown in grey (left) cross-links to UmuD using formaldehyde. The hydrophobic channel is shown in brown (residues Leu177, Pro242, Val247, Val360, Met362) (55) (106), while the rim interaction residue Leu98 is shown in black. Structures were generated using VMD (107) and coordinates for β (2POL) from the PDB (61).
Binding experiments of the clamp with DNA pol III α and other components suggest that the same hydrophobic region on the surface of β to which δ binds is also responsible for binding DNA pol III α and other DNA polymerases (55, 63, 90-93, 105, 108, 109). It has also been suggested that the DNA pol III α C-terminus binds to the β clamp (see below) and binds τ. These observations suggest that the polymerases, δ, and τ compete with one another for binding to the β clamp, creating a mechanism for polymerase loading and switching on the β clamp (46, 50, 63, 110).

An internal binding site (α residues 920-924), rather than the 20 C-terminal residues, was shown to be responsible for the interaction between the β clamp and α (28). Replacement of all residues in this internal binding site eliminated binding to the β clamp, but binding between the τ subunit and α was not affected. When an analogous set of mutations was made in the C-terminal binding site of α, the β clamp still showed affinity for the α subunit (28). In fact, α participated in processive replication even when the entire C-terminal binding site was removed. The absence of the C-terminal peptide, however, specifically effected the interaction of τ with α. These findings suggest that the clamp does not bind to the C-terminus of α, but instead to an internal site. This discrepancy might be due to the use of an α variant with a relatively large C-terminal truncation in the previous study (28, 50), rather than site-directed mutant variants or more modest deletions (28).

There is also evidence that two different polymerases can simultaneously bind to the clamp. The β subunit, a homodimer, has two hydrophobic pockets per functional protein, thus allowing it to
bind two DNA polymerases (11, 105, 111, 112). Such a situation allows replication to alternate between the two DNA polymerases without the need for dissociation from the β clamp. This “toolbelt” hypothesis allows for high processivity, even under conditions where multiple polymerases are used. The ability of the clamp to bind both DNA pol III and pol IV (a Y family polymerase) was investigated using fluorescence resonance energy transfer (FRET). Proximity of the pol III α subunit and pol IV was detected in a β-clamp-dependent manner, providing experimental evidence that two different DNA polymerase molecules can simultaneously bind to the β clamp (112). The crystal structure of β with the C-terminal little finger domain of pol IV (106) showed that the pol IV polymerase domain is angled off to the side providing enough room for another polymerase, such as DNA pol III, to bind. Modeling the full length structure of Dpo4 (113) (a Pol IV homolog from Sulfolobus solfataricus), containing a primer-template duplex and incoming nucleotide, onto that of the little finger structure of E. coli pol IV with the β clamp, showed that when bound in this position, the polymerase likely does not have access to the DNA strand (106). The little finger domain can likely undergo a conformational change (106), positioning the polymerase onto the DNA substrate.

Another model for polymerase switching is “dynamic processivity” (114). This model involves polymerase replacement without affecting apparent overall processivity. Such a scheme was observed during bacteriophage T4 DNA replication (114). The addition of a catalytically inactive variant D408N of gp43, the T4 DNA polymerase, to an active replication fork, arrested replication while still retaining wild-type-like affinity for DNA and the clamp (gp45). This observation suggested that the active polymerase is quickly (<1 min) replaced by the inactive
variant (114). This dynamic processivity of polymerases implies that multiple replicative polymerases may be required to replicate normal, undamaged DNA.

1.3.4 The τ Subunit

Although similar to the γ subunit, the role of the τ subunit is distinct from the remainder of the γ complex. As a central component of the DNA pol III replisome, τ coordinates replication on both strands by connecting the subassemblies of the core and β clamp as τ binds to the polymerase (48) and the helicase DnaB (45, 48), and is part of the clamp loader complex (57). Numerous distinct roles have been assigned to τ, as summarized in a review (9). The roles of the τ subunit in replication are to: 1) coordinate replication on both strands; 2) bind to DnaB; 3) prevent premature removal of the β clamp; and 4) function in the processivity switch.

When DNA polymerase III' (core + τ) was first isolated (21), it was observed that two polymerases were coupled by two τ subunits, suggesting that τ may be a key factor in coordinating replication (Figure 1.1). This hypothesis was tested by varying the concentration of τ (42). At low concentrations of τ, shorter DNA fragments were observed upon agarose gel electrophoresis analysis of replication reaction samples (42). These results suggest that the two polymerase cores must be coupled through τ in order to effectively replicate both the lagging and leading strands.

DNA pol III α binds τ through the α C-terminal domain (28, 50) with an equilibrium dissociation constant ($K_D$) of 4 nM (48). The α binding site on τ was determined to be at the τ C-
terminal domain (45), which is not part of γ (Figure 1.2B). Along with determining the NMR solution structure for this domain (51), combinatorial binding studies were conducted to determine which residues bind to α (49). It was concluded that the 18 C-terminal residues of τ are required for binding to α (49).

Four different reconstituted clamp loader complexes, γ3δδ′χψ, τ1γ2δδ′χψ, τ2γ1δδ′χψ, τ3δδ′χψ, have similar rates of loading the β clamp onto DNA (10). The complex containing τ3 may coordinate three DNA polymerases at the replication fork. A “triple-polymerase” model has been proposed in which two of the pol III cores function on the lagging strand to synthesize Okazaki fragments. Alternatively, one pol III core is utilized on the lagging strand with the third pol III core held “in reserve” off of the DNA (10). The latter model suggests a possible switching mechanism between high and low fidelity DNA polymerases (10).

The affinity of the τ subunit for DnaB (45) also seems to affect the rate at which the replication fork proceeds (8, 43). DnaB, a hexameric helicase, unwinds DNA ahead of the fork while encircling only the lagging strand (Figure 1.1) (8). Without the replisome, the helicase unwinds DNA at a rate of approximately 35 nt/s, similar to that in the presence of the complex without τ. When τ is added, the rate increases to at least 400 nt/s, approaching that of DNA pol III (43, 45).

The τ subunit may also indirectly prevent the premature removal of the β clamp by the γ complex, thereby maintaining processivity. The length of DNA produced in the absence of τ is directly proportional to the concentration of β and inversely proportional to the concentration of the γ clamp loader complex (44). This suggests that the clamp removal function is inhibited
during normal replication in the presence of the γ complex, allowing the β clamp to stay on the DNA.

Discontinuous replication of the lagging strand requires that DNA pol III α must constantly dissociate from and re-associate with different β clamps. Such a cycle is known as the processivity switch and is thought to involve τ (46). It has been shown that the polymerase core/τ complex must complete the newly formed strand, leaving only a nick, before the switch can be activated (46). In such a scenario, τ loses affinity for α when primed DNA is present. Then when replication is completed, τ gains affinity for α, releasing α from the clamp (46). The ssDNA binding function of α also appears to play a role in this process (110).

1.4 The Single-Stranded DNA Binding Protein, SSB

E. coli SSB plays a significant role in DNA replication, recombination, and repair by binding ssDNA present during these DNA processing events, preventing ssDNA from forming secondary structures and protecting the ssDNA from chemical and nucleolytic attacks (115-117). SSB is a homo-tetramer with a D$_2$ axis of symmetry (118). Each monomer contains an N-terminal globular domain and a disordered C-terminal tail (119).

The N-terminal globular domain of SSB contains an oligonucleotide/oligosaccharide-binding (OB) fold that binds ssDNA via stacking interactions with one phenylalanine (F61) and two
tryptophan (W41 and W55) residues of SSB (120-125). ssDNA binds SSB by wrapping around the homo-tetramer in two binding modes: (SSB)$_{35}$ and (SSB)$_{65}$ (126). In the (SSB)$_{35}$ binding mode, ssDNA only wraps around two monomers of the homo-tetramer, favoring high cooperativity. On the other hand, the (SSB)$_{65}$ binding mode wraps around all four monomers and favors limited cooperativity (126).

In addition to binding and protecting ssDNA, SSB also serves as a hub for at least 15 proteins, such as proteins in homologous recombination, DNA pol II and pol V in damage tolerance, and the χ subunit of DNA pol III in replication, involved in numerous DNA processing pathways (127). Most of these proteins have been found to bind the C-terminal tail suggesting that SSB uses this tail to facilitate interactions with other proteins involved in DNA maintenance (128). The temperature sensitive allele ssb-113 is associated with the mutation P177S (129) and impairs DNA replication at temperatures higher than 30°C (130, 131). The P177S mutation has been shown to disrupt the interaction with proteins that bind this C-terminal tail (127).

SSB, as well as protecting ssDNA present at the replication fork, also has been observed to directly impact replication activity of DNA pol III. SSB increases processivity of the polymerase when all the subunits of DNA pol III are present. In contrast, when only the core subunits are present, SSB inhibits replication (13). This inhibition is alleviated when the χ subunit of the clamp loader complex is present due to a direct interaction between χ and the C-terminal tail of SSB (22, 132-134).
1.5 DNA Damage disrupts DNA Replication

DNA pol III function requires finely tuned interactions of multiple proteins for efficient and accurate DNA replication. When this complex encounters non-canonical DNA structures, including DNA damage, it is not equipped to replicate them. DNA damage is ubiquitous, arising from numerous exogenous and endogenous sources. For example, it is estimated that 10,000 abasic sites are formed per human cell per day (135). The outcome of the replisome encountering DNA damage in the template may depend on whether the damage is encountered during leading strand or lagging strand synthesis, but typically the rate of progress of replication is decreased (136, 137). A lesion in the leading strand has been observed to slow progression of the replication fork, but was not observed to interfere with lagging strand replication (138). On the other hand, a lesion in the lagging strand does not block overall progression of the replication fork. Instead, the lagging strand DNA polymerase appears to re-initiate downstream of the lesion at the next Okazaki fragment, leaving a gap (138-141). Indeed, it has been shown that replication can restart downstream of obstacles, even in the case of leading strand synthesis (142).

1.6 Specialized DNA Polymerases facilitate DNA Damage Tolerance

In *E. coli* and some other bacteria, DNA damage and other stresses lead to induction of the SOS response (135). Stalling of DNA replication at damaged sites results in the accumulation of single stranded DNA (ssDNA). RecA polymerizes on the ssDNA, forming a nucleoprotein
filament that serves as the inducing signal for the SOS response. As the result, the expression of at least 57 genes is induced (143, 144). SOS-regulated genes code for proteins involved in regulation of cell division, nonmutagenic repair of chemically modified DNA or in damage tolerance mechanisms, which can be mutagenic or error prone (Figure 1.5) (135, 143, 145, 146).

**Figure 1.5 Regulation of SOS induced genes after DNA damage.**

The SOS response is induced by the formation of a RecA nucleoprotein filament on single stranded DNA (RecA*). This stimulates the auto-proteolysis of the LexA repressor which leads to the induction of at least 57 genes. Among these genes are Y family polymerases pol IV (DinB) and pol V (UmuD'C). UmuD2 undergoes RecA* facilitated cleavage of its N-terminal 24 amino acids to yield UmuD'2, the form that is active in translesion synthesis. Lon and ClpXP proteases play a role in regulating the levels of UmuD2, UmuD'2, and UmuC in the cell. ClpXP also specifically targets UmuD' in UmuDD' heterodimers (not shown).
Three of the five known *E. coli* DNA polymerases are under SOS inducible regulation (145, 147). These SOS inducible DNA polymerases are pol II (*polB*), pol IV (*dinB*), and pol V (*umuDC; UmuD'C*). The latter two belong to the Y family of DNA polymerases, which are characterized by their ability to perform translesion synthesis (TLS) on damaged DNA templates, as well as their relatively low fidelity on undamaged DNA (148). Y family DNA polymerases also lack intrinsic 3'-to-5' exonucleolytic proofreading and exhibit low processivity (145, 148, 149).

Although high-resolution structures of *E. coli* Y family polymerases have not yet been experimentally determined, the structures of Y family polymerases from *Sulfolobus solfataricus* and *Sulfolobus acidocaldaricus* homologs provide insights into their function (113, 150, 151). While there is no obvious sequence homology between replicative and Y family DNA polymerases, the crystal structures of the latter reveal a similar right-hand structure of the catalytic domain consisting of thumb, palm, and finger domains, common to other DNA polymerases. Another domain, the little finger domain, is present in the Y family polymerases, providing additional DNA binding contacts in the major groove (113, 148). This domain may be responsible for both substrate specificity and processivity (152). Moreover, the O helix that is responsible for high fidelity in the replicative polymerases (153-155) is not present in Y family polymerases, suggesting a structural basis for the low fidelity of Y family DNA polymerases while replicating undamaged DNA. The specialized ability of Y family polymerases to replicate damaged DNA has been attributed to their loose, flexible active sites that accommodate aberrant DNA structures (113, 148, 151). In addition, Y family polymerases have fewer contacts with their DNA substrates than replicative DNA polymerases (113, 151). Although the crystal
structures show that the catalytic domains of Y family polymerases have similar overall folds, these polymerases exert different efficiencies and fidelity in bypassing various DNA lesions (148, 156).

1.6.1 Regulation of Y Family DNA Polymerases

Expression of the *umuDC* and *dinB* gene products is negatively regulated by the LexA repressor as part of the SOS transcriptional response. LexA binds to a sequence in the operator region of the genes (135, 157, 158). Derepression of the *umuDC* and *dinB* operons occurs when the RecA protein binds to single-stranded regions of DNA that develop at replication forks that are stalled by DNA damage (159). The RecA/ssDNA nucleoprotein filament serves as a coprotease to facilitate cleavage of the LexA repressor (Figure 1.5). As the cellular concentration of LexA diminishes, the genes whose expression is normally repressed by LexA are transcribed (135).

The *umuDC* genes are among the most tightly regulated SOS genes; the equilibrium dissociation constant \(K_D\) is 0.2 nM for LexA binding to the “SOS-box” in the promoter region (157). In comparison, the \(K_D\) values for LexA binding to the “SOS-boxes” of the *recA* and *lexA* genes are estimated to be 2 nM and 20 nM, respectively (160). Immunoblotting assays have shown the cellular steady-state levels of UmuD to be ~180 copies per uninduced cell and ~2400 copies per cell under SOS induction (161). A single protein in a compartment with the volume of a typical *E. coli* cell is present at a concentration of ~1 nM. The level of UmuC is approximately 12-fold lower than UmuD with about 15 molecules per cell in the absence of induction and ~200 molecules of UmuC per cell under SOS induced conditions (161). There are ~250 molecules of
DinB per cell in the absence of induction and ~2500 molecules of DinB per cell after treatment with the DNA damaging agent mitomycin C (162). It should be noted that the \textit{dinB} gene is also present on the \textit{E. coli} F$'$ episome, and expression levels of DinB from the episome under uninduced and induced conditions are approximately three-fold higher than from the chromosome (162). Expression of the \textit{umuDC} genes initially produces UmuD (139 amino acids) which undergoes a RecA/ssDNA-stimulated autodigestion reaction after induction resulting in UmuD'$'$ (115 amino acids) (Figure 1.5 and 1.6) (163, 164). UmuD is the predominant species for the first approximately 20-40 min after SOS induction, after which UmuD'$'$ is the predominant species (165). UmuD proteins exist in solution as UmuD$_2$ and UmuD'$'$$_2$ homodimers as well as the UmuD-UmuD'$'$ heterodimer, which is more stable than either of the homodimers (166-169). The $K_d$ for UmuD$_2$ dimerization is estimated to be in the low-pM range, so UmuD is likely to be present in the cell as a dimer under most conditions (170, 171).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Model of full-length UmuD (172) and crystal (169) and NMR (168) structures of UmuD'$'$.}
\end{figure}

UmuD model shown in the \textit{trans}, elbows down conformation (left). Crystal structure (middle) and NMR structure (right) of UmuD'$'$ in \textit{trans} conformation. The N-terminal arms of UmuD'$'$ are cleaved between Cys24 and Gly25. Residues 1-24 are shown in magenta; residues 25 to 40 are shown in blue. Active site Ser60 and Lys97 are highlighted in red and green, respectively.
SOS-induced mutagenesis is also regulated at the post-translational level. UmuD is functionally inactive for facilitating TLS until it undergoes RecA/ssDNA-mediated cleavage to generate UmuD’ (164, 173, 174). Full-length UmuD also inhibits -1 frameshift mutagenesis by DinB (175). Efficient cleavage of UmuD in vitro and in vivo was observed at elevated levels of activated RecA, suggesting that TLS likely occurs when cells are under more severe environmental stress (161, 173). The removal of the UmuD N-terminal 24 amino acids through the cleavage of the Cys24-Gly25 bond occurs via an intermolecular pathway, that is, one protomer of the dimer acts as an enzyme, while the other is the substrate (176). The crystal structure of UmuD’ revealed that the active site, consisting of conserved serine (Ser60) and lysine (Lys97) residues, is found at the end of a cleft within the C-terminal globular domain of the protein and these residues are poised for cleavage (Figure 1.6) (169). In the NMR structure of UmuD’ Ser60 and Lys97 are further apart and not correctly oriented for catalysis. It has been suggested that the crystal structure of UmuD mimics the effect of the RecA/ssDNA nucleoprotein filament as it serves to realign these residues, thereby activating UmuD for self-cleavage (168, 177).

The two different forms of UmuD provide a temporal switch between accurate and mutagenic phases of the cellular response to DNA damage (165, 178, 179). The combination of uncleaved UmuD and UmuC specifically decreases the rate of DNA replication and increases resistance of cells to killing by UV radiation (165, 180). Uncleaved UmuD’C improves DNA damage survival by allowing time for error-free repair mechanisms to act before the combination of cleaved UmuD’ and UmuC (UmuD’C, pol V) initiates potentially error-prone TLS (165). The combination of UmuC and noncleavable UmuD (S60A) significantly delayed recovery of cell
growth after UV radiation (165, 179). Therefore, a model for a umuDC-dependent DNA damage checkpoint in *E. coli* was proposed wherein a delay in DNA synthesis provides time for error-free nucleotide excision repair to remove DNA lesions. TLS is then enabled by the presence of UmuD' (181-183). This model suggests that the different umuD gene products, in combination with UmuC, are involved in distinct survival pathways after UV damage.

Increasing UmuD'2C protein complex concentration was found to antagonize RecA-mediated recombination of a UV-damaged gene (184); this effect was also observed *in vitro* (185). In the proposed model, high concentrations of the UmuD'2C proteins induce replisome switching from recombination to SOS mutagenesis. Indeed, the UmuD'2C complex has been shown to bind directly to the RecA/ssDNA filament and could disrupt the DNA pairing activity of RecA (186, 187). Notably, in the presence of homologous DNA sequences, homologous recombination repair is more prevalent than TLS in responding to DNA damage (188).

The mutagenic potential of Y family polymerases may be further regulated by preferential formation of heterodimers between UmuD and UmuD', thereby depleting the cell of mutagenically active UmuD' homodimers (166). UmuD' is degraded by the ATP-dependent protease ClpXP while in a heterodimeric complex with UmuD (189, 190). Formation of UmuDD' heterodimers in preference to mutagenically active UmuD' homodimers therefore specifically targets UmuD' for proteolysis. UmuD also targets its UmuD homodimer partner for proteolytic degradation by ClpXP (191). The ATP-dependent serine protease Lon is responsible for the degradation of both UmuD and UmuC proteins *in vivo* (192). Targeted proteolysis of the
*umuD* and *umuC* gene products is one mechanism for returning protein levels to their uninduced state.

1.6.2 Structural dynamics of *UmuD* and *UmuD’*

The *umuD* gene products interact with multiple replication factors such as polymerases *UmuC* (as *UmuD’2C*, pol V), *DinB* (pol IV) and components of the pol III holoenzyme. The latter include the polymerase subunit α, proofreading subunit ε, and the processivity clamp β (161, 170, 172, 175, 193, 194). These interactions are due in part to the relative flexibility of full-length *UmuD* and its *UmuD’* cleavage product as shown biochemically and by X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and circular dichroism (CD) (168-170). The cleaved form *UmuD’* contains disordered N-terminal arms that expose the C-terminal globular domain to solvent upon cleavage, while in full-length *UmuD*, the arms are more stably bound to the globular domain (168, 169). Therefore, *UmuD* and *UmuD’* make specific contacts that facilitate a variety of protein-protein interactions (168, 170, 193, 195).

The crystal structure of *UmuD’* reveals extended N-terminal arms (residues 25-39) and a globular C-terminal body (residues 40 to 139) that contains the catalytic dyad Ser60 and Lys97 (169). Although two dimer interfaces (designated as molecular and filament) were observed in the crystal structure, NMR and cross-linking experiments support the conclusion that the so-called filament dimer interface is the form present in solution (Figure 1.6) (168, 169, 195-200). However, there is evidence that the filament structure may be biologically relevant (196). To date, there is no high resolution structure of the *UmuD2* homodimer. Cross-linking studies of a
series of single-cysteine derivatives of UmuD are consistent with the UmuD2 homodimer interface resembling the interface of the UmuD′2 homodimer, involving contacts between the C-termini of the monomers and intermolecular interactions between Asn41 and Leu44 of α helix 1 (168, 171, 200). NMR experiments also suggest that the UmuDD′ heterodimer most closely resembles the UmuD2 homodimer (168). Dimerization appears to be important for biological activity, as UmuD′ variants that resulted in decreased UV-induced mutagenesis also have severe deficiencies in their abilities to form homodimers *in vivo* (197, 201, 202).

Four models of the UmuD homodimer have been generated from NMR, Electron Paramagnetic Resonance (EPR), and cross-linking studies, and by homology to LexA (168, 172, 203, 204). One model shows UmuD with the N-terminal arms in *trans* with the elbows down, where the N-terminal arm of one monomer folds down across the C-terminal body of the adjacent monomer and crosses the catalytic site (Figure 1.6). Each UmuD monomer cleaves the N-terminal arm of its partner at Cys24-Gly25 (172). A *trans*, elbows up model positions the arms along the outer edge of the globular domains. Two *cis* versions with elbows up and elbows down suggest that each N-terminal arm could bind over its own globular domain (172). The N-terminal region (residues 1-14) is likely to be in a random extended conformation. Cross-linking and chemical modification experiments suggest that the *trans*, elbows down conformation of the N-terminal domain is the most prevalent in solution (172, 203). However, given the dynamic nature of UmuD, all four conformations may be physiologically relevant (170, 172).

Circular dichroism (CD) spectroscopy simulating physiological conditions detected random coil conformations for both UmuD dimers (170), rather than the β-sheet-rich structure determined by
X-ray crystallography and NMR spectroscopy (168, 169, 195). At higher salt concentration both UmuD and UmuD’ dimers have more typical β-sheet appearance. Thus, the umuD gene products belong to the group of intrinsically disordered proteins (IDPs) (205). Like their IDP counterparts, UmuD dimers are capable of making a remarkable number of specific protein-protein contacts.

It has been shown that UmuD and UmuD’ interact with the α, β, and ε subunits of DNA polymerase III (193). UmuD interacts less strongly with the α subunit in vitro than cleaved UmuD’ (193). Moreover, uncleaved UmuD interacts more strongly with the β clamp than cleaved UmuD’ as observed by affinity chromatography (193). Different interactions of UmuD and UmuD’ with the β clamp suggest that these interactions regulate how the umuD gene products access the replication fork. Overexpression of the α subunit or the β clamp inhibits cleavage of UmuD to UmuD’ in vivo, further supporting the specific interactions between the pairs of proteins (193). It has also been found that overexpression of the β clamp reduces UV-induced mutagenesis (206).

1.6.3 UmuD-Beta clamp interactions

To date, interactions between the umuD gene products and the β clamp have been studied in much more detail than other interactions involving the umuD gene products. Proteins that interact with the β clamp, with the exception of UmuD and UmuD’, contain the eubacterial clamp-binding motif (QL[S/D]LF) (207). UmuD contains a $^{14}$TFPLF$^{18}$ sequence within its N-terminal arm (207). Although the motif lies in a region of UmuD that is important for its interaction with the β clamp (194), the interaction does not depend on the sequence identity of
the motif (172). That is, a UmuD variant containing mutations in this motif binds to the β clamp with similar affinity as that of wild-type UmuD (see below) (172).

UmuD and UmuD’ affinity chromatography and in vitro cross-linking studies confirm that the β clamp has a higher affinity for UmuD than UmuD’ (194). However, it has been shown that both the N-terminal arms and C-terminal globular domains of UmuD are important for interaction with the β clamp (194). UmuD lacking its N-terminal nine residues is proficient for interactions with the β clamp, while UmuD lacking the N-terminal 19 residues results in reduced formaldehyde cross-linking to β (194). The UmuD variant UmuD-3A (T14A L17A F18A), a non-cleavable variant with mutations of the most conserved residues of the TFPLF motif, possesses some of the biological functions of the cleaved form UmuD’. Although the $K_d$ values for interaction of the β clamp with UmuD (5.5 +/- 0.8 μM) and UmuD-3A (6.1 +/- 0.5 μM) are similar, their interactions with the β clamp may be different, as observed by intrinsic tryptophan fluorescence of the β clamp (172). Tryptophan fluorescence is a relatively sensitive probe of the microenvironment. The single tryptophan of the β clamp is located on a flexible loop between domains I and II of β, and thus is a sensitive reporter of conformational changes (61, 172). The β clamp tryptophan fluorescence differed dramatically upon UmuD vs. UmuD-3A binding, suggesting they have different binding modes (172).

UmuD, UmuD’, the α catalytic subunit, UmuC, DinB and clamp loader all interact with the β clamp around the β clamp hydrophobic pocket, approximately defined by residues Leu177, Pro242, Val247, Val360, Met362 (Figure 1.4) (52, 90, 93, 105, 106). UmuD, UmuD’ and the α subunit interact with overlapping regions of β, suggesting that there may be competition for
binding (Figure 1.4) (193). This implies that UmuD plays a regulatory role following the SOS response, interacting with components of Pol III, interfering with α binding to β, slowing replication and allowing time for error-free repair mechanisms to act (165, 193). It is also possible that α and UmuD or UmuD′ bind the homodimeric β clamp simultaneously. A model has been proposed in which cleavage of UmuD to form UmuD′ reduces binding to the β clamp, thereby releasing the DNA damage checkpoint and enabling translesion synthesis (208).

1.7 DNA Polymerase Switching

Replicative DNA polymerases are unable to copy damaged DNA under most circumstances (209, 210). In the polymerase-switching model (111, 112, 211, 212), a lesion blocks the progress of the replicative DNA polymerase at the replication fork, and translesion polymerases act to allow replication to proceed (Figure 1.7). Since the SOS inducible polymerases are characterized by low processivity and low-fidelity replication, specialized polymerases must subsequently be replaced by replicative polymerases to restore efficient and accurate DNA replication.
Figure 1.7 Polymerase switching in response to DNA damage.

Replicative DNA polymerases (green) are generally unable to copy damaged DNA. A polymerase switch occurs allowing a TLS polymerase (orange) access to DNA. The TLS polymerase synthesizes DNA opposite the lesion and far enough beyond it that the replicative polymerase can resume synthesis without disruption due to the lesion. In eukaryotes, there is evidence that two different TLS polymerases act in a stepwise fashion to insert nucleotides opposite the lesion and then extend the primer beyond the lesion (213).

1.7.1 Replisome Dynamics

Elevated levels of UmuD and UmuC inhibit DNA replication, a phenomenon suggested to represent a primitive DNA damage checkpoint (165, 180). DinB (pol IV) also interferes with replication fork progression both in vitro and in vivo, and its overexpression inhibits cell growth (212, 214). Neither DinB catalytic activity nor its canonical β-clamp binding motif are required
for inhibition of replication (214). Both pol II and DinB inhibit replication fork progression by altering the speed of the DnaB helicase (215). In this case, the β-clamp binding motif is required for DinB to occupy the replisome and slow the helicase (215). This decrease in helicase velocity allows time for accurate DNA repair processes to take place while maintaining the overall structure of the replication fork (215). Taken together, these observations suggest a high degree of plasticity in the replisome as well as parallels between the physiological effects of pol IV and pol V on the replication fork.

The β clamp binds to, and increases the processivity of, all five known E. coli DNA polymerases (1, 108, 216) and is important for the lesion-bypass activity of UmuD’C (90, 91, 93). Both UmuD and UmuD’ interact with the β clamp and the α and ε subunits of pol III (193). In general, the β clamp-binding proteins possess a canonical peptide motif that binds to the hydrophobic channel on the β clamp (109, 207), suggesting that they compete for the same binding site. Extensive analysis of DNA polymerase binding to site-directed mutants of the β clamp show that the different polymerases have partly, but not completely, overlapping sites of interaction on the β clamp (217-219). Based on the co-crystal structure of the little finger domain of DinB with the β clamp, a physical basis for recruitment of Y family polymerases to the replisome was proposed (106). Two interfaces between the DinB little finger domain and the β clamp were identified. One is a peptide-protein interaction involving the extended C-terminal tail of the DinB little finger domain and hydrophobic channel, or “cleft”, on the surface of the β clamp (106). A second interaction was observed between surface loops of the DinB little finger (residues 303-305) and the outer rim of the β clamp at the dimer interface (residue 98) (106). Modeling of the entire S. solfataricus DinB-ortholog Dpo4 protein onto the structure of the DinB little finger
domain showed that, when bound in this position, DinB would likely not have access to a linear DNA duplex (106). Therefore, DinB could have two modes of binding to the β clamp: a ‘locked-down’ conformation with no access to DNA, and a ‘tethered’ complex attached to the β clamp via the C-terminal canonical β clamp binding motif, which would allow interactions with DNA (106). By analogy, mutations that disrupt the proposed β clamp outer rim interaction of UmuC result in increased UV-induced mutagenesis, presumably because the ‘lock’ is released, giving UmuC more access to DNA (90). Recent crystal structures of Dpo4 show that it also adopts multiple conformations whether in the apo form, bound to DNA or bound to the sliding clamp (220). Specifically, the little finger rotates around the linker region and adopts different conformations depending on the presence of different binding partners of Dpo4. While the Dpo4 little finger domain makes additional contacts with the clamp beyond the canonical interaction motif (PIP-box), unlike DinB the Dpo4 little finger domain lacks interactions with the outer rim of the clamp.

1.7.2 Polymerase Switching: Tool-Belt and Active Exchange Models

The multiple conformations and flexibility of polymerase binding to the β clamp support the ‘tool-belt’ model of polymerase switching (90, 93, 106, 112, 211), in which several polymerases simultaneously attach to the clamp. Depending on the DNA substrate, either a replicative or a translesion polymerase is given access to the DNA. The proposed triple polymerase replisome provides another possible mechanism for efficient switching between high- and low-fidelity polymerases (10). The observed tilt angle of the β clamp on DNA may also facilitate polymerase switching (104). It was recently reported that only one of the two possible hydrophobic channels
that are sites of polymerase interaction on the β clamp is required for switching between pol III and DinB (221). This result challenges the “tool-belt” model (221). Other studies suggest an active exchange model in which one polymerase readily displaces another at the replication fork (114).

DNA replication assays reveal the exchange of the replicative pol III and TLS pol V on DNA in the presence of DNA damage (111, 222). The pol III holoenzyme replicates up to the nucleotide position preceding the damaged base, but not beyond it. Through contacts between their minor groove recognition domain and the DNA, replicative DNA polymerases sense distortions in the DNA within the last four to five nucleotides replicated (223-225). In agreement with these structural studies of other replicative polymerases, it has been experimentally shown that pol III requires the primer to be four to five nucleotides beyond the lesion for resumption of efficient replication (111). If the primer is not extended sufficiently beyond the lesion and pol III has access to the DNA, the proofreading subunit ε will degrade the primer (111). In the presence of DNA damage, the SOS response is induced and RecA coats ssDNA. Pol V binds to RecA and the β clamp and inserts nucleotides opposite the lesion. The less constrained active site of pol V permits insertion and extension of the 3' terminus of the primer beyond the lesion. Pol V synthesizes a ‘TLS patch’ ranging from 1 to 60 nucleotides, averaging 20 nucleotides in a single binding event (111, 222). Once pol V synthesizes a TLS patch of at least four nucleotides, the pol III holoenzyme is able to efficiently resume DNA replication even in the presence of a RecA/ssDNA nucleoprotein filament (111).
There is growing genetic evidence that multiple polymerases act during replication and in lesion bypass (226-230). It was hypothesized that SOS inducible polymerases II, IV, and V alter the fidelity of DNA replication by interfering with the cellular functions of pol I and III. This was tested by exploiting the observation that each polymerase leaves a unique mutagenic ‘fingerprint’ when copying DNA (226). The spectrum of mutations in the rpoB gene arising in various ΔmutL strains was measured (226). Deletion of mutL provides a genetic background deficient in postreplicative mismatch repair to allow determination of actual polymerase-specific misincorporation events, rather than simply assaying mutations that escape repair. Furthermore, polymerase expression levels were modulated using strains with mutations in recA and lexA (226). Analysis of the rpoB genes in strains with deletions of different combinations of polymerases revealed changes in the spectrum of mutations. This result was interpreted as evidence that E. coli DNA polymerases compete for access to genomic DNA, thereby promoting or suppressing mutagenesis (226). Specifically, multiple spontaneous transversion mutations are due to pol V or to the combined action of pol IV and pol V (226). Moreover, the chromosomally encoded levels of pol V are the limiting factor for the production of the mutations and even modest low-level expression causes an increase in transversion at specific hot spots. Facile switching between the five DNA polymerases of E. coli during genome replication suggests that spontaneous mutations arise due to the interactions of multiple polymerases (226).

Additional evidence for switching between pol III and pol V on UV-damaged DNA came from a study revealing that strains harboring variants of E. coli pol V with mutations of F10 or Y11 (the steric gate residue) display hypersensitivity to UV light and dominant negative genetics (230). The steric gate residue has been shown to prevent incorporation of ribonucleotides by sterically
occluding the 2'-OH moiety in the Y family polymerases (231, 232). The dominant negative character of *umuDC Y11A* is suppressed by disruption of *dnaQ*, the gene encoding the ε proofreading subunit (Table 1.1). It seems likely that pol V Y11A is recruited to damaged DNA and prevents other DNA polymerases from accessing the replication fork. The steric gate variants of pol V (Y11A) and pol IV (F13V) conferred on a wild-type *E. coli* strain extreme sensitivity to both nitrofurazone and UV radiation (230, 233). Considering that pol IV copies *N*²-furfuryl-dG lesions efficiently and accurately (233), this finding suggest that the pol V steric gate variant prevents another Y family polymerase from accessing damaged DNA.

1.7.3 Gap-filling Model

Several studies have suggested that lesion bypass does not occur at the replication fork, but at gaps that are left by the replication machinery (141). This model is supported by the observation that the rate of replication recovery after DNA damage is independent of the presence of pol IV or pol V (234, 235). Moreover, as discussed above, lesions in the lagging strand do not slow the overall rate of replication, but lead to re-initiation downstream of the lesion, leaving a gap (138-140). Finally, it has been observed that β clamps can be left behind when the polymerase dissociates from DNA (102), possibly as an aid to recruitment of other replication factors, including Y family polymerases. The observation that Rev1, a Y family polymerase in yeast, is upregulated in the G₂-M transition of the cell cycle, rather than in S phase when most DNA replication occurs, is also consistent with this model (236). Ultimately, the two models of coordinated polymerase switching and gap filling are not mutually exclusive. The specific
mechanism a particular cell utilizes likely depends on the type of lesion encountered and the conditions in the cell at that time.

In this work, we investigate the interactions between the α subunit of DNA pol III, the single-stranded DNA binding protein SSB, and UmuD. Overall, we show that a network of protein-protein and protein-DNA interactions modulate DNA replication under normal cellular conditions and in response to DNA replication stress.

1.8 REFERENCES


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CHAPTER 2: Selective disruption of the DNA polymerase III α-β complex by the umuD gene products

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Michelle C. Silva designed bulk biochemical experiments, purified proteins, performed experiments, analyzed the data, and wrote the manuscript; Philip Nevin performed FRET studies;
2.1 INTRODUCTION

DNA polymerase III (DNA pol III) is the main DNA polymerase in *Escherichia coli* and is responsible for replicating the entire genome. It contains ten subunits that allow for accurate and processive replication to occur (1-4). These ten subunits are organized into three subassemblies: the polymerase core; the β processivity clamp; and the clamp loader complex (5). The polymerase core consists of the α polymerase subunit, the ε proofreading subunit and the θ subunit whereas the clamp loader complex loads the processivity clamp onto primer:template DNA (6) and coordinates continuous replication on both leading and lagging strands of DNA (7-9). Once the β clamp is positioned, the clamp loader complex regulates the loading of the polymerase core onto the β clamp (9). This ensemble of proteins allows for continuous replication to occur on the leading strand and discontinuous replication to occur on the lagging strand (10), allowing for efficient replication of DNA.

A crystal structure has been solved of a truncated form of the *E. coli* α subunit (residues 1-917) (Figure 2.1A) (11). With a resolution of 2.3 Å, it is possible to distinguish the characteristic domains of DNA polymerases including the palm domain containing the conserved aspartic acid residues of the active site, and the fingers and thumb domains (12, 13). The N-terminal domain,
which was thought to possess pyrophosphatase activity (14), is also present in the crystal structure. This domain contains the binding site of the ε proofreading subunit (15). The C-terminal domain, not included in the crystal structure, contains the oligonucleotide/oligosaccharide binding (OB) fold (11, 16) responsible for binding single-stranded DNA (17), the binding site for the τ subunit of the clamp loader complex (9, 18-20) and the binding site for the β processivity clamp (Figure 2.1A) (21, 22). It was originally suggested through the use of truncations of α that the C-terminus is responsible for binding to the β clamp (22); however, site-directed mutagenesis experiments revealed that residues 920-924 are primarily responsible for binding to the β clamp and that the C-terminus plays a minor role (21). Because of these findings, we refer to residues 920-924 as the β binding site on α.

Figure 2.1 Models of α and UmuD.

(A) The x-ray crystal structure of α 1-917 (PDB code 2hnh (11)) (left) shows the major domains of the polymerase, emphasizing the active site residues and the binding site for ε (15). A homology model of full-length α (23) (right) depicts the C-terminal domain that is not present in the crystal structure containing the β (21) and τ (9) binding sites as well as the OB domain (11, 17). (B) The homology model of UmuD (24) and the x-ray crystal structure of UmuD′ (25)
showing one monomer in green and the other in purple. The first 24 N-terminal residues missing from the UmuD’ structure (yellow) are cleaved as part of a primitive DNA damage checkpoint by the active site (orange). The single cysteine residues at C24 is shown (red) in full-length UmuD.

The α subunit, together with the other subunits of DNA pol III, is capable of efficiently replicating undamaged DNA. But when it encounters DNA damage, replication by the polymerase is disrupted (26). DNA pol III is capable of inserting nucleotides opposite some DNA lesions, but usually cannot extend beyond the unusual primer terminus that it generates. Thus, it can become trapped in a futile cycle of insertion and exonucleolytic proofreading at sites of DNA damage (27). On the leading strand, the blockage of DNA synthesis is disruptive, causing the inhibition of replication fork progression (28, 29). On the lagging strand however, progression of the replication fork is less affected because the polymerase can reassemble on the next primer terminus downstream (28). In order to withstand DNA damage, the SOS response is employed by the cell (30).

The initial molecular trigger for the SOS response is an abundance of ssDNA caused by the stalled DNA polymerase (31). RecA coats the ssDNA forming a nucleoprotein filament that facilitates the cleavage of the LexA repressor and up-regulates the expression of at least 57 SOS genes (32). The products of these genes are involved in DNA damage tolerance mechanisms, such as recombination, DNA repair and translesion synthesis (TLS), which is the process involved in replicating damaged DNA. TLS is carried out by potentially mutagenic Y family DNA polymerases that can replace a stalled replicative DNA polymerase (33-37).
Y family DNA polymerases have the specialized ability to bypass DNA damage by inserting nucleotides opposite the lesion (33-40). Of the five DNA polymerases present in *E. coli*, DNA pol IV (DinB) and DNA pol V (UmuD’2C) are members of this family (41-43). DNA pol V is composed of the UmuD’2 dimer, which is the RecA/ssDNA-facilitated cleavage product of UmuD2, and UmuC, which possesses polymerase activity (44, 45). During the first 20-40 min after the activation of the SOS response, UmuD2 is the predominant species (46, 47). UmuD2 together with UmuC serves to affect a primitive DNA damage checkpoint, inhibiting replication to allow time for accurate DNA repair processes to act (46, 48, 49). After approximately 40 min, UmuD’2 becomes the predominant species, releasing the primitive DNA damage checkpoint (46, 50, 51). UmuD’2 and UmuC form DNA pol V (44, 45, 52), which is the form active in TLS.

UmuD2 is a very tight dimer (*Kd* <10 pM) (53) and is expected to be dimeric in all experiments reported here; therefore we use UmuD to refer to homodimeric UmuD and variants. Each UmuD monomer consists of two domains: the N-terminal arms and a C-terminal globular domain. One model of UmuD places the N-terminal arms folded down onto the globular domain, positioning the cleavage site (between C24 and G25) at the active site (S60 and K97) (an “arms down” conformation) (Figure 2.1B) (24, 54). But because of the dynamic nature of arms, UmuD can exist in a conformation where the arms are unbound (an “arms up” conformation) exposing the globular domain (24, 53, 55).

The *umuD* gene products have also been shown to interact with other proteins such as DNA pol IV (*Kd* 0.62 µM) (56), and components of DNA pol III including the α polymerase, the ε proofreading subunit and the β processivity clamp (57). UmuD and UmuD’ differentially interact with components of DNA pol III (57). Specifically, affinity chromatography experiments
suggested that UmuD binds more strongly to the β processivity clamp than to α (57), supporting
the role of UmuD in inhibiting DNA synthesis as part of a primitive DNA damage checkpoint
(50), however the α-UmuD interaction has not been characterized at the molecular level. It has
also been suggested that upon binding to the β clamp or to DinB, the structure of UmuD
becomes less disordered (53). These interactions suggest that both UmuD and UmuD′ play a role
in replication fork management.

The process by which exchange of the α polymerase subunit and a TLS polymerase occurs is not
fully understood. There are likely a number of factors that facilitate such exchange; for example,
it has been shown that the presence of DinB inhibits DNA pol III replication (58). In this work,
we characterized the interactions of α with the umuD gene products. We determined the binding
affinity between UmuD and α. We also determined that there are two UmuD binding sites on α,
one of which favors UmuD over UmuD′. The UmuD-specific site overlaps with the β binding
site on α and consequently, we found that UmuD, but not UmuD′, specifically disrupts the α-β
interaction. Therefore, we find that UmuD plays a key role in regulating polymerase access to
the β clamp.

2.2 MATERIALS AND METHODS

2.2.1 Proteins and Plasmids
UmuD and UmuD′ were expressed from pSG5 and pSG4 plasmids, respectively, as previously described (59). UmuD3A (UmuD containing the triple mutation T14A, L17A, and F18A), UmuD-S60A, and UmuD′-S60A variants were previously described (24). Plasmids that express His-tagged wild-type α and the truncations α1-280, α1-917, α1-956 and α1-975 were provided by Dr. Meindert Lamers and Prof. John Kuriyan, UC Berkeley (11). The plasmids encoding the truncations α1-835 and α917-1160 were cloned as described previously (17). Wild-type α and truncations were expressed in Tuner(DE3) (Novagen) competent cells and purified using the established protocol and stored at -20 °C in a buffer containing 50 mM Hepes (pH 7.5), 100 mM NaCl, and 50% glycerol (protein storage buffer) (11). The β processivity clamp was purified according to published protocols (60). LexA was purified from pJWL228 (provided by Dr. Ronaldo Mohana-Borges, Instituto de Biofísica Carlos Chagas Filho-UFRJ, Brazil) as described (61).

To improve the stability of the α protein, various conditions were tested including different buffer conditions and varying temperatures. The most stable proteins were those stored at -20 °C in a buffer containing 50% glycerol, at 25 µM or lower concentrations. The high concentration of glycerol was necessary to keep the proteins from freezing. It was noted that the stability of α was reduced when the protein was frozen and thawed before use. In solutions with lower glycerol concentrations, aggregation was observed. We found that the high concentration of glycerol interfered with many of the experiments described here; therefore, before each experiment, the buffer was exchanged to the relevant buffer for each assay. This was accomplished by using Zeba Desalt Spin Columns (Thermo Scientific) with the buffer exchange protocol provided.
2.2.2 Tryptophan Fluorescence Assay

The intrinsic tryptophan fluorescence of α in the presence of increasing amounts of UmuD was used to determine the equilibrium constant for α binding to UmuD. UmuD has no tryptophan residues and α has eight tryptophans. To determine the fluorescence of α alone, 60 µL of 5 µM α in 50 mM Hepes (pH 7.5), 100 mM NaCl was excited at 278 nm, and emission was monitored from 300 to 500 nm. To determine the binding constant between α and UmuD, the sample of α was then titrated with UmuD. After each addition, the sample was excited at 278 nm and emission was monitored from 300 to 500 nm, with both excitation and emission slits set to 5 nm. To analyze the change in fluorescence of α, the maximum of each peak was determined and corrected for the dilution caused by adding UmuD. To correct for any fluorescence contribution from UmuD alone, fluorescence emission was monitored from UmuD in reaction buffer at the same concentrations used to titrate α. To produce a binding curve, the fraction of fluorescence from α quenched by the addition of UmuD (Q) was plotted versus the ratio of UmuD to α. The curve was fit to the following equation using GraphPad Prism (La Jolla, CA):

\[
Q = B_{max} \left[ 0.5 \left( 1 + \frac{[UmuD]}{[\alpha]} + \frac{K_d}{P - \frac{P}{T}K_d} \right) - \sqrt{0.25 \left( 1 + \frac{[UmuD]}{[\alpha]} + \frac{K_d}{P - \frac{P}{T}K_d} \right)^2 - \frac{[UmuD]}{[\alpha]}} \right]
\]

(Equation 2.1)
where P is the initial concentration of α and T is the concentration of the UmuD stock used in the titration. The binding constant at pH 10 for α and UmuD was obtained in alkaline cleavage buffer (see below).

2.2.3 Thermal-Shift Assays

Reactions were assembled in 96-well PCR plates (Applied Biosystems) in which each sample (16 μL total volume) consisted of thermal-shift assay buffer (50 mM Hepes (pH 7.5), 100 mM NaCl), and 25x Sypro Orange (Invitrogen) excited at 490 nm. In experiments in which the melting transitions of UmuD were observed in the presence of α, each sample contained 45 μM UmuD (monomer concentration) and 1 μM α. Samples were incubated for 2 h at room temperature before detection. In experiments in which the stability of the truncations of α was compared with that of wild-type α, each construct of α was added to a final concentration of 5 μM without incubation before detection. In order to increase the thermal stability of the α proteins for ease of detection, 30% glycerol was added to each sample. Once the plate was sealed with optical adhesive film (Applied Biosystems), an iCycler iQ5 Real-Time PCR (Bio-Rad) was used to increase the temperature from 25 °C to 75 °C with an increment of 0.1 °C and a 10 sec dwell time per temperature increment. The fluorescence intensities emitted at 575 nm and detected by the built-in CCD camera were plotted versus temperature. The melting temperature (T_m) was calculated as described (62, 63). The assays were repeated several times with similar observations and consistent melting temperatures.

2.2.4 UmuD in vitro Cleavage Assays
RecA/ssDNA nucleoprotein filament-facilitated UmuD cleavage reactions were carried out as described in LG Buffer (59). Samples containing α, UmuD and buffer were incubated for 2 h at room temperature before the addition of RecA/ssDNA, after which reactions were carried out at either 37 °C or 30 °C for 45 min. The reaction temperature was adjusted to 30 °C wherever possible because of the relatively low melting temperature of α (approximately 38 °C). To rule out any non-specific interactions, BSA was used instead of α, or, alternatively, LexA cleavage was assayed in the presence of α. Alkaline cleavage of UmuD was also carried out as previously described (59). The alkaline cleavage buffer (100 mM Glycine (pH 10), 10 mM CaCl₂, 50 mM NaCl, 10 mM dithiothreitol (DTT), 0.25 mg/mL BSA) was added after a 30 min incubation at room temperature, and the reactions were carried out at either 37 °C or 30 °C for 48 hours. Reactions of cleavage assays were analyzed by using 18% SDS-PAGE.

2.2.5 Cross-Linking of UmuD using Bis-maleimidohexane (BMH)

Bis-maleimidohexane (BMH, Pierce) cross-linking (24, 64) was carried out as described by incubating 10 µM UmuD-S60A in the presence of 10 µM wild-type α, α917-1160, or α1-280 in 10 mM Sodium Phosphate (pH 6.8), 100 mM NaCl and 1 mM BMH. The reactions were incubated for 5 min at room temperature, after which the reactions were quenched with 50 mM DTT. Cross-linked dimers of UmuD-S60A were resolved from monomers by 4-20% SDS-PAGE (Bio Rad). An immunoblot was used to identify those bands containing UmuD-S60A using rabbit anti-UmuD as the primary antibody as described (24, 59).

2.2.6 Fluorescence Resonance Energy Transfer
Protein labeling was carried out using Alexa Fluor 488 C₅-maleimide (Invitrogen) for the β clamp and Alexa Fluor 647 C₂-maleimide (Invitrogen) for the α subunit, in a reaction buffer containing 50 mM Hepes (pH 7.5) and 200 mM NaCl. The β clamp (100-150 μM) and α (20-50 μM) were each labeled by adding 5-10 molar excess of the respective Alexa Fluor maleimide at room temperature, followed by overnight incubation at 4 °C in the dark. Unreacted reagent was separated from labeled protein on a Sephadex G-50 (GE Healthcare) column at 4 °C using a buffer containing 25 mM Hepes (pH 7.5) and 200 mM NaCl. Fractions were analyzed by SDS-PAGE and the gels were analyzed on a Storm 860 phosphorimager (Molecular Dynamics) by monitoring fluorescence after irradiating the gels at 450 nm. The degree of labeling was determined as described by the manufacturer and was on average 3-4 fluorophores per α subunit and 1-2 fluorophores per β monomer.

The interaction between the α subunit and the β clamp was monitored by FRET. A reaction mixture containing 350 nM donor-labeled β subunit (βₐ₄₈₈) and 1 μM acceptor-labeled α subunit (αₐ₆₄₇) in 70 μL FRET buffer (25 mM Hepes (pH 7.5), 25 mM NaCl, 1 mM DTT, and 0.5 mM EDTA) was incubated for 15 min on ice, followed by analysis at room temperature with a Cary Eclipse spectrofluorimeter (Varian). Samples were excited at 495 nm and emission spectra were recorded from 500 to 700 nm, with slits for both excitation and emission set to 5 nm. The effects of UmuD, UmuD’ and UmuD-S60A were investigated by adding up to 40 μM UmuD or variants to each sample prior to analysis. FRET efficiency (E) was calculated by finding the ratio between the emission intensity of the donor in the presence of the acceptor (Fd’) to the emission intensity of the donor alone (Fd), which is then subtracted from one (Equation 2.2).
\[ E = 1 - \frac{F_d'}{F_d} \]

(Equation 2.2)

### 2.3 RESULTS

#### 2.3.1 α binds UmuD via two UmuD Binding Sites on α

In order to develop a more complete picture of the interactions of UmuD with subunits of the replisome, we probed the interaction between α and the umuD gene products. Because α has eight tryptophans and UmuD has none, we were able to use the intrinsic tryptophan fluorescence of α to determine the equilibrium binding constant for the interaction between α and UmuD. By analyzing the extent of α fluorescence quenching observed with the addition of UmuD, the \( K_d \) was determined to be 1.1 ± 0.6 µM (Figure 2.2). This binding constant is similar to that for other protein interactions with UmuD (UmuD and the β clamp: 5.5 ± 0.8 µM \(^{24}\); UmuD and DinB: 0.62 µM \(^{56}\)). To localize the binding site of UmuD on α, a number of truncations of α were constructed and dissociation constants for their interactions with UmuD were determined (Figure 2.3A). The truncation α1-975 has a binding constant (3.1 ± 1.0 µM) similar to that of full-length α indicating that the binding site is located within this region. When the truncation α1-956 was analyzed, a significant decrease in affinity was observed (\( K_d = 14.7 ± 1.9 \) µM), suggesting that
there is a UmuD binding site between residues 956-975 positioned in the C-terminal domain (Figure 2.1A). This region is also near the β-binding site (residues 920-924) (21).

Figure 2.2 UmuD binds wild-type α with a $K_d = 1.1$ μM.

The curve representing the fraction of α tryptophan fluorescence quenched by various concentrations of UmuD was fit to Equation 2.1, which produced a $K_d$ value of $1.1 \pm 0.6$ μM at pH 7.5. The error bars represent the standard deviation from five independent experiments.
Figure 2.3 UmuD binds α at two distinct binding sites.
(A) $K_d$ values for UmuD binding to various α truncations. The large difference between the $K_d$ values for the truncations α1-975 and α1-956 indicates that one binding site is between residues 956-975. The reduced but still significant binding constants for the truncations α1-956, α1-917, α1-835, and α1-280 suggests another UmuD binding site is present in the N-terminal domain α1-280. The domains of α are labeled above the schematic of the protein; ε, β, and τ indicate the location of the respective binding site for each protein, HhH indicates the helix-hairpin-helix domain involved in double-stranded DNA binding, OB indicates the OB-fold domain involved in single-stranded DNA binding. (B) Melting curves for the various α truncations used show comparable stability to that of full-length α.
The $K_d$ values for truncations $\alpha1$-917, $\alpha1$-835, $\alpha1$-280 are all similar to that of truncation $\alpha1$-956. That binding was still detected with these truncations suggests that there is a second UmuD binding site within $\alpha$. N-terminal truncations smaller than 280 residues were expressed poorly and so could not be analyzed. As a result, this second binding site could not be localized any further than to residues 1-280, the PHP domain (Figure 2.1A). The $K_d$ value for UmuD binding to the truncation $\alpha917$-1160, which contains the proposed C-terminal UmuD binding site ($\alpha$ residues 956-975), was determined to be $13.9 \pm 5.1 \mu$M which is somewhat similar to that for truncation $\alpha1$-280 (7.3 $\mu$M) but greater than that of full-length $\alpha$ (1.1 $\mu$M). The increased affinity for UmuD seen with full-length $\alpha$ as opposed to that of the truncations containing only one binding site may be due to the simultaneous binding of two UmuD dimers to $\alpha$, as the two UmuD binding regions are spatially somewhat distant (Figure 2.1A).

A thermal-shift assay was used to determine if any of the truncations assayed here were unstable. This assay involves the use of an environmentally-sensitive fluorescent dye where the fluorescence intensity is proportional to the hydrophobicity of its environment. When a folded protein is present, the dye is in an aqueous environment and so the fluorescence of the dye is quenched. As the temperature reaches the melting point of the protein, the dye binds to the exposed hydrophobic interior of the unfolding protein, increasing the fluorescence intensity of the dye. The temperature at the midpoint of the transition between the folded and unfolded states is known as the melting temperature, or $T_m$. Because the $T_m$ is related to the stability of the protein, a protein with a higher $T_m$ is more stable than a protein with a lower $T_m$. All $\alpha$ truncations were found to be about as stable as full-length $\alpha$ (Figure 2.3B); while the truncations $\alpha1$-280 and $\alpha917$-1160 were even more stable than full-length $\alpha$. 
2.3.2 α Inhibits RecA/ssDNA-Facilitated Cleavage in vitro

It was previously shown that overexpression of α inhibited UmuD cleavage \textit{in vivo} (57). We wanted to determine whether this phenomenon could be observed \textit{in vitro} and therefore could be used to further characterize the UmuD binding site on α. Assays of RecA/ssDNA-facilitated UmuD cleavage were conducted in the presence of 10 to 25 µM α, which resulted in approximately 50% less cleavage of UmuD at 37 °C than in assays without α (Figure 2.4A). Because the melting point of α is approximately 38 °C at pH 7.5 (Figure 2.4B), the assay was also conducted at 30 °C to minimize denaturation of α present in the reaction. This resulted in even less UmuD cleavage, approximately 60% less cleavage than with UmuD alone. In order to determine whether the inhibition observed was specific for both UmuD and α, two controls were employed: (1) UmuD cleavage in the presence of BSA; and (2) LexA cleavage in the presence of α, as LexA also undergoes RecA/ssDNA-facilitated self-cleavage (61). In both cases, there was minimal change in cleavage efficiency (Figure 2.4D), indicating that the inhibition of UmuD cleavage by α is specific.
Figure 2.4 The presence of α inhibits UmuD cleavage.

After each cleavage reaction, UmuD and UmuD’ were resolved by 18% SDS-PAGE. Representative data are shown in A and C. (A) RecA/ssDNA nucleoprotein filament-facilitated UmuD cleavage assays were performed in the presence of 10 and 25 μM α wild type and at 37 °C and 30 °C. (B) To determine stability, the melting temperature was obtained for full-length α at pH 7.5 (25 μM α, 50 mM Hepes (pH 7.5)) and at pH 10 (25 μM α in alkaline cleavage buffer). (C) Cleavage assays were conducted under alkaline conditions in the absence of RecA/ssDNA. (D) A comparison of the cleavage assay performed under different conditions: RecA/ssDNA facilitated UmuD cleavage in the presence of α (red); UmuD cleavage under alkaline conditions in the presence of α (purple); RecA/ssDNA facilitated UmuD cleavage in the presence of BSA (blue); RecA/ssDNA facilitated LexA cleavage in the presence of α (green). Percentage of cleavage was calculated by comparing the density of cleaved product to the total amount of protein present. Each point represents the average of at least three experiments and the error bars...
Inhibition of UmuD cleavage in the RecA/ssDNA-facilitated cleavage assay may be due to the competitive binding of RecA/ssDNA and α for UmuD. When bound to α, UmuD cannot bind to the RecA/ssDNA filament and so cleavage cannot occur under these conditions. It is also possible that in addition to competitive binding, α may directly affect the ability of UmuD to cleave itself. In order to assess the possibility that α inhibited UmuD cleavage directly, rather than via competition with the RecA/ssDNA filament, cleavage assays were conducted under alkaline conditions (pH 10) (59) without the RecA/ssDNA nucleoprotein filament (Figure 2.4C). It was previously shown that UmuD can undergo autocleavage, albeit slowly, in the absence of RecA/ssDNA under alkaline conditions, as the elevated pH apparently facilitates deprotonation of the S60 nucleophile on UmuD (59, 65). Under these conditions, no change in cleavage efficiency was observed, except at the highest concentrations of α and at 30 °C. At pH 10, α was determined to be stable with a melting temperature of approximately 36 °C (Figure 2.4B). However, we found that at pH 10, the $K_d$ was higher than at pH 7.5, indicating a decrease in affinity of α for UmuD (Figure 2.4E). Consequently, the observation that α did not substantially change the cleavage efficiency of UmuD under alkaline conditions may be attributed to the decreased affinity at pH 10. As a result, we conclude that in the case of RecA/ssDNA-facilitated cleavage, cleavage is inhibited likely due to competition for binding to UmuD between α and the RecA/ssDNA filament.

represent the standard deviation from three independent experiments. (E) The $K_d$ at pH 10 was determined to be 21.7 ± 11.7 µM.
To confirm the binding sites between α and UmuD, the RecA/ssDNA-facilitated cleavage assay was conducted in the presence of two α truncations: the α1-280 truncation containing the N-terminal binding site (Figure 2.5A) and the α917-1160 truncation containing the C-terminal binding site (Figure 2.5B). As the concentration of α1-280 was increased from 0 µM to 40 µM, inhibition of cleavage was observed (Figure 2.5A). Approximately four times more α1-280 than wild-type α is required to observe the same extent of cleavage inhibition, consistent with the decreased affinity of α1-280 for UmuD. In contrast, the presence of 0 to 5 µM α917-1160 modestly increased UmuD cleavage efficiency (Figure 2.5B), which suggests that UmuD binds the C-terminal binding site in a conformation that may facilitate cleavage.

**Figure 2.5** The α truncation 1-280 inhibits UmuD cleavage.
RecA/ssDNA nucleoprotein filament-facilitated UmuD cleavage was carried out at 30 °C in the presence of (A) the α1-280 truncation and (B) the α917-1160 truncation. SDS-PAGE analysis showed that approximately four times as much α1-280 is needed to produce the same extent of cleavage inhibition as wild-type α. On the other hand, the presence of α917-1160 increased cleavage efficiency. Percentage of cleavage, shown below each lane, was calculated by comparing the density of cleaved product to the total amount of UmuD present.

2.3.3 The Interaction between α and UmuD is Conformation Dependent

A thermal-shift assay was used to determine whether the presence of α had an effect on the conformational dynamics of UmuD. Previously, it had been shown that UmuD exhibits two distinct melting transitions: one at approximately 28 °C and another at approximately 60 °C (63). The absence of the first transition in the case of UmuD' suggests that the lower-temperature transition represents the dissociation of the N-terminal arms of UmuD from the globular domain (55, 63). In order to observe the effect of α on these two melting transitions, the thermal-shift assay was conducted in the presence of α (Figure 2.6A). By comparing the melting curve of UmuD alone with the melting curve of UmuD with α, it is clear that upon addition of α, the first melting transition is diminished. This suggests that α binds UmuD in a conformation-dependent manner. Two scenarios are possible (Figure 2.6C) when α binds to UmuD: (1) the arms are confined in such a way that they cannot dissociate from the body (“arms down”) or (2) the arms are not bound to the body and therefore do not undergo the transition to the unbound conformation (“arms up”). The second melting transition is essentially unchanged in the presence of α.
Figure 2.6 Binding to α alters the conformation of UmuD.

(A) Melting curves of 45 μM UmuD (solid line), 45 μM UmuD with 1 μM α (dotted line), and 45 μM UmuD' (dashed line). The solid line shows the melting transitions for UmuD at 32 °C and 60 °C. The disappearance of the first melting transition suggests that the interaction between α and UmuD influences the conformation of the arms. (B) Percent UmuD-S60A cross-linked by using BMH in the presence of either full-length α, α1-280 or α917-1160. (C) Cartoon showing two possible conformations of the UmuD N-terminal arms. The star indicates the position of the C24 residues, which are cross-linked by BMH.

To determine whether the C-terminal binding site binds UmuD in an “arms up” or “arms down” conformation (Figure 2.6C), UmuD was cross-linked using the homobifunctional thiol-specific reagent bis-maleimidoehexane (BMH) in the presence or absence of α (Figure 2.6B). The UmuD dimer has two cysteine residues, one on each arm, which are represented by a star in Figure 2.6C. In order to cross-link with BMH, the two cysteine residues need to be in close proximity (within ~13 Å) to each other. If the arms are bound to the body in the “arms down”
conformation, the two Cys residues are too far apart to be cross-linked; the arms will only be efficiently cross-linked if they are unbound from the body. As a result, this cross-linking assay is ideal for looking at the conformational dynamics of the arms. In order to prevent contamination with the UmuD’ cleavage product, the UmuD-S60A non-cleavable variant was used. It was observed that in the presence of the α truncation α917-1160, the amount of cross-linked UmuD-S60A dimer compared to the total UmuD-S60A present in the reaction was significantly reduced compared to when UmuD was cross-linked alone (Figure 2.6B). This suggests that the C-terminus of α binds UmuD in an “arms down” conformation. The presence of the α truncation α1-280 showed a similar but less dramatic reduction of cross-linking efficiency (Figure 2.6B).

2.3.4 The C-terminal Binding Site Favors Full-length UmuD

To determine if the interaction between α and UmuD is selective for UmuD or UmuD’, binding constants were determined for the interaction of UmuD variants with full-length α, and the truncations α1-280 and α917-1160 (Table 2.1). When wild-type UmuD is purified, there is typically some amount of contaminating UmuD’. Moreover, cleavage can occur upon incubation or interaction with other proteins, so it is nearly always a complicating factor with wild-type UmuD. Furthermore, mixtures of UmuD and UmuD’ form UmuDD’ heterodimers (66); therefore, UmuD with some UmuD’ present will be a mixture of several dimeric species. Consequently, to determine the binding constant between full-length α and UmuD uncontaminated with UmuD’, the non-cleavable UmuD variant UmuD-S60A was used. The binding constant between UmuD-S60A and full-length α was determined to be 10.6 ± 2.9 µM.
With UmuD', the binding constant was 10.9 ± 1.6 µM, suggesting that full-length α does not favor UmuD' or UmuD.

Table 2.1 Equilibrium dissociation constants $K_d$ (µM) for binding of α truncations to UmuD variants.

<table>
<thead>
<tr>
<th>UmuD variants</th>
<th>Full-length α</th>
<th>α1-280</th>
<th>α917-1160</th>
</tr>
</thead>
<tbody>
<tr>
<td>UmuD</td>
<td>1.1 ± 0.6</td>
<td>7.3 ± 1.4</td>
<td>13.9 ± 5.1</td>
</tr>
<tr>
<td>UmuD-S60A</td>
<td>10.6 ± 2.9</td>
<td>10.3 ± 4.3</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>UmuD'</td>
<td>10.9 ± 1.6</td>
<td>8.6 ± 1.0</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>UmuD3A</td>
<td>8.7 ± 1.5</td>
<td>9.3 ± 3.2</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>UmuD'-S60A/UmuD-S60A</td>
<td>4.6 ± 0.9</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

Binding affinity was also analyzed between the α truncation α917-1160, containing the proposed C-terminal binding site (residues 956-975), and the UmuD variants UmuD-S60A and UmuD'. As shown in Table 1, α917-1160 binds significantly more strongly to UmuD-S60A (0.7 ± 0.3 µM) than to UmuD' (3.8 ± 0.9 µM), suggesting that the C-terminal binding site selectively favors full-length UmuD over the cleavage product, UmuD'. On the other hand, the α truncation α1-280, containing the proposed N-terminal binding site, does not distinguish between full-length UmuD and UmuD', exhibiting similar binding constants with UmuD-S60A (10.3 ± 4.3 µM) and UmuD' (8.6 ± 1.0 µM) (Table 2.1).

UmuD3A is a full-length UmuD variant that exhibits properties of UmuD' (24). UmuD3A contains three mutations (T14A, L17A, F18A) located in the N-terminal arms of the protein, which disrupt packing of the arms against the C-terminal globular domain and cause UmuD3A to adopt a more UmuD'-like conformation (24, 55, 63). As expected, UmuD3A has similar affinity for the α variants as UmuD' (Table 2.1). Because both UmuD' and UmuD3A have a more
exposed globular domain than wild-type UmuD, it seems that the α C-terminus preferentially binds UmuD when the arms are bound to the C-terminal globular domain of UmuD, which is consistent with our findings in the cross-linking experiments with BMH (Figure 2.6B).

The distinguishably different binding constants for wild-type UmuD compared to those of UmuD-S60A and UmuD' are consistent with our observation that preparations of wild-type UmuD may contain some UmuD'. To probe this further, we assembled a mixture of 1:1 UmuD-S60A:UmuD'-S60A (Table 2.1) to somewhat resemble the conditions of wild-type UmuD. Because it is known that a monomer of UmuD can cleave the arm of its partner (67), we used the active site mutation S60A in both UmuD and UmuD' to prevent any further cleavage. Under these conditions, the $K_d$ was determined to be 4.6 ± 0.9 µM, which is intermediate between the $K_d$s determined with UmuD compared to with UmuD' or UmuD-S60A.

2.3.5 UmuD Disrupts the DNA Pol III α-β Complex

Our observation that UmuD binds α at a site (residues 956-975) that is near the β-binding site on α (residues 920-924) prompted us to investigate the effect of the umuD gene products on the DNA pol III α-β complex. The β clamp and the α subunit were labeled with donor and acceptor fluorophores, respectively, as shown in Figure 2.7C, and FRET was monitored in the presence or absence of UmuD, UmuD' or UmuD-S60A (Figure 2.7A and Figure 2.7B). As expected, FRET was observed when the donor on the β clamp was excited in the presence of acceptor-labeled α subunit (Figure 2.7A), indicating that the two proteins interact with each other. Addition of UmuD to the reaction resulted in higher emission intensity from the donor, indicating a decrease
in FRET efficiency and consistent with disruption of the α-β complex. The effect was more apparent with the addition of UmuD-S60A, the full-length non-cleavable variant of UmuD. This suggests that UmuD is able to disrupt the interaction between the α subunit and the β clamp. In comparison, no difference in FRET efficiency was observed in the presence of UmuD′ (Figure 2.7A and 2.7B), suggesting that UmuD′ is not able to disrupt the α-β complex. These findings are consistent with our observation that the binding site for UmuD on the C-terminal domain of α, near where β also binds to α, binds full-length UmuD (UmuD-S60A) with higher affinity than it binds UmuD′. Taken together, these observations indicate that specifically full-length UmuD is capable of disrupting the α-β complex (Figure 2.7C).

**Figure 2.7** Fluorescence resonance energy transfer analysis shows that UmuD disrupted the interaction between the α polymerase, labeled with Alexa Fluor 647 C₂-maleimide, and the β processivity clamp, labeled with Alexa Fluor 488 C₅-maleimide.
(A) FRET was reduced in the presence of UmuD and UmuD-S60A but not in the presence of UmuD'. FRET between β and α was reduced in the presence of UmuD-S60A in a concentration-dependent manner, from 1-40 μM UmuD-S60A (not shown). (B) Bar graph showing FRET efficiency at each condition; representative of five trials. The error bars represent the standard deviation of at least three trials. (C) Schematic of FRET experiment showing that the FRET observed upon interaction of β and α is eliminated with the addition of full-length UmuD, but not UmuD'.

2.4 DISCUSSION

The disruption of replication fork progression caused by DNA damage necessitates TLS polymerases having access to the primer terminus at the site of the damage in order to carry out replication of damaged DNA. Two models have been proposed to explain how replicative and TLS polymerases exchange: the “toolbelt” model and the dynamic processivity model. It has been shown that the α subunit and pol IV can interact with the β clamp simultaneously (68), allowing replication to alternate between the two DNA polymerases without the need for them to dissociate from the β clamp (the “toolbelt” model). In such a model, when a DNA lesion is encountered, α stalls and a Y family polymerase takes over replication (27). Once the DNA lesion has been bypassed, α once again resumes replication. On the other hand, the dynamic processivity model (69) suggests that the replicating polymerase is constantly being exchanged with other polymerases without affecting overall processivity. DNA pol IV (DinB), a TLS polymerase, has been shown to inhibit DNA pol III replication, which may facilitate dissociation of the pol III α subunit and allow TLS to occur (58).

It has been shown that UmuDC inhibits DNA replication (46, 49), allowing time for accurate DNA repair processes to occur (46). There is also evidence that the umuD gene products play a
direct role in regulating access to the replication fork by interacting with the α, β, and ε subunits of DNA pol III (57). The interaction between UmuD and the β clamp (24) is believed to contribute to a primitive DNA damage checkpoint (57). In this report, we investigate the interaction between the α subunit and the umuD gene products, UmuD and UmuD'. Our observations suggest that another contribution to the DNA damage checkpoint is likely to be the disruption of the α-β complex by UmuD.

In this work, we identified two UmuD binding sites on α (Figure 2.3A): one in the N-terminal domain (residues 1-280) and one in the C-terminal domain (residues 956-975). It has been shown that UmuD and UmuD' differentially bind the α and β subunits, where UmuD' interacts more strongly with α than with β and UmuD interacts more strongly with β than with α (57). A comparison of the binding constant between UmuD and β (5.5 ± 0.8 µM) (24) with the binding constant for UmuD-S60A and wild-type α (10.6 ± 2.9 µM) (Table 2.1), supports the previous suggestion, based on affinity chromatography with cell extracts, that UmuD binds more tightly to the β clamp. On the other hand, the interaction between α and the umuD gene products is complicated by our observation of two UmuD binding sites on α and because the C-terminal binding site favors binding to full-length UmuD. Notably, the $K_{d}$s we determined for a variety of UmuD and α constructs (ranging from 0.7-14 µM, Table 2.1) are similar to those obtained between UmuD and β and between UmuD and DinB (24, 56). The cellular concentration of UmuD and UmuD' ranges from 0.25-1 μM depending on whether cells are SOS induced (70), which suggests that UmuD and α can interact in vivo.
The C-terminal domain of α appears to be a hub for protein and DNA interactions. This apparent regulatory domain contains the binding site for the β clamp (residues 920-924) (21), and a binding site for τ (residues 1120-1160) (9, 19), a subunit of the clamp loader complex (Figure 1A). We observed that the C-terminal domain of α (residues 917-1160) favors binding to full-length UmuD (Table 2.1), and that full-length UmuD is able to disrupt the binding of α to β. This domain is also believed to contain an OB fold, which has been shown to bind preferentially to ssDNA and acts as a sensor detecting ssDNA upstream from the primer terminus (17, 71). On the other hand, less is known about the N-terminal domain of α. Apart from binding UmuD, as we have demonstrated here, this domain has also been shown to bind the ε subunit (15). Unfortunately, it is difficult to probe possible direct effects of UmuD on the α-ε interaction because of the difficulty of acquiring purified, soluble ε.

The umuD gene products have distinct and temporally separated roles in the DNA damage response, first by taking part in a primitive DNA damage checkpoint by inhibiting DNA replication and then by activating TLS (46). We observed that FRET between α and β was reduced with the addition of full-length UmuD, suggesting that the interaction between α and β is disrupted specifically by UmuD (Figure 2.7C). FRET efficiency was not affected by the addition of UmuD’. This selective disruption of the α-β complex by full-length UmuD together with the preferential binding of full-length UmuD by the C-terminal domain of α suggests a specific role for full-length UmuD in polymerase management. Because UmuD is the predominant species at the beginning of the SOS response, the displacement of α from the β clamp most likely occurs before the appearance of DNA pol V (UmuD’C).
How can α differentiate between UmuD and UmuD’ at the molecular level? The primary difference between UmuD and UmuD’ is the absence of the N-terminal 24 amino acid residues in UmuD’ suggesting that the N-terminal arms are involved in the interaction between α and UmuD. As shown here, several lines of evidence, including thermal melting and cross-linking analysis, support a model in which α binds UmuD in an “arms down” conformation. Similarly, the interaction between UmuD and the β clamp has been shown to involve both the N-terminal arms and the C-terminal globular domain of UmuD (50); furthermore, the β clamp can bind to a UmuD variant that contains an artificial disulfide bridge (C24-F94C) locking the arms to the globular domain (50). Taken together, previous and current work suggests that both α and the β clamp bind UmuD in a similar fashion in which the N-terminal arms are bound to the C-terminal globular domain. Collectively, our findings suggest that the already extensive role of UmuD in responses to DNA damage includes releasing α from the β clamp.

2.5 REFERENCES


Chapter 3: Polymerase manager protein UmuD directly regulates *E. coli* DNA polymerase III α binding to ssDNA

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3.1 INTRODUCTION

DNA polymerase III (DNA pol III) holoenzyme is a ten-subunit protein complex that efficiently and accurately replicates the entire genome of Escherichia coli (1, 2). It is composed of three subassemblies: the polymerase core, the β processivity clamp, and the clamp loader complex. Polymerase and proofreading activities are conducted by the core subassembly which consists of the polymerase subunit α, the proofreading subunit ε, and the θ subunit, which has a role in stabilizing the core (3, 4). The β processivity clamp encircles the DNA and provides a platform for the polymerase core to bind, providing α with access to the primer-template and facilitating processive replication. The clamp loader complex, which consists of the γ, δ, δ’, τ, χ, and ψ subunits, loads the β clamp onto the DNA (5) with τ tethering the polymerase core to the replisome (6), and coordinating simultaneous replication of the leading and lagging strands of the replication fork (6, 7).
Although DNA pol III efficiently replicates undamaged DNA, replication is disrupted upon encountering damaged bases (8-11). Formation of a RecA filament on accumulated ssDNA triggers the SOS response (12), resulting in the upregulation of genes encoding numerous proteins involved in DNA damage repair and tolerance (13). These proteins include the potentially mutagenic Y-family polymerases DNA pol IV (DinB) and DNA pol V (UmuD′₂C) (14-16). Replication of damaged DNA can proceed once DNA pol III α is replaced with one of these Y-family polymerases, which can replicate damaged DNA in a process known as translesion synthesis (TLS) (17-20). DNA pol V is composed of two subunits, the cleaved form of UmuD and the UmuC polymerase. DNA polymerase manager protein UmuD regulates the cellular response to DNA damage in part, along with UmuC, by decreasing the rate of replication, thereby allowing time for non-mutagenic DNA repair processes to occur (21-23). UmuD undergoes a RecA/ssDNA-facilitated auto-cleavage of 24 amino acids of its N-terminal “arms” to form UmuD’. UmuD forms a tight dimer (UmuD₂), which is the predominant form for the first 20–40 minutes of the SOS response after which the cleaved form UmuD’ is the predominant species (22, 24). Although UmuD and UmuD’ are expected to be dimeric under all the conditions studied here (25) for simplicity we will refer to these dimeric forms as UmuD rather than UmuD₂ and UmuD’ rather than UmuD’₂, respectively. UmuD also regulates mutagenesis in the cell through its interaction with the Y-family DNA polymerase DinB, by inhibiting DinB-dependent -1 frameshift mutagenesis (14, 26, 27).

UmuD interacts with several components of DNA pol III, including the polymerase subunit α, the β clamp, and the proofreading subunit ε (28). Recent ensemble biochemical experiments have shown that there are two UmuD binding sites on the α subunit, one in the N-terminal domain and
one in the C-terminal domain (29) (Figure 3.1). The C-terminal binding site (residues 956-975), which has higher affinity for full-length UmuD relative to the cleaved form UmuD′ (29), is adjacent to the β clamp binding site (residues 920-924) (30), which tethers the polymerase to its DNA template. UmuD, but not UmuD′, releases α from the β clamp, which may inhibit DNA replication and facilitate polymerase exchange (29). The C-terminal UmuD binding site of α is also adjacent to the OB fold (residues 975-1160), through which α binds ssDNA (31), suggesting that UmuD may be competing with ssDNA for binding to α. We hypothesized that one way UmuD contributes to a DNA damage checkpoint is by disrupting the interaction between α and ssDNA, thereby inhibiting replication. To test this hypothesis, we have used single molecule DNA stretching to quantify α binding to ssDNA in the presence of wild-type UmuD and several UmuD variants designed from a computational docking analysis of the complex. We find that wild-type UmuD competitively inhibits α binding to ssDNA through UmuD-α interactions, while a single amino acid substitution, D91K, in UmuD disrupts this inhibition.

Figure 3.1 Diagram of DNA pol III α, with domain labels within the boxes and known interaction sites above the boxes (sequence numbering shown below). The two UmuD binding sites, one in the N-terminal domain and one in the C-terminal domain of α, are shown in yellow (29). The CTD binding site (residues 956-975) is adjacent to the β clamp...
binding site (residues 920-924, shown in blue) (30) and recent biochemical experiments show that UmuD displaces α from the β clamp (29). This UmuD binding site is also adjacent to the OB fold (red), where ssDNA binds α (31), an observation that led us to hypothesize that UmuD also inhibits α binding to ssDNA.

3.2 MATERIALS AND METHODS

3.2.1 Proteins and plasmids

Wild-type UmuD was expressed from the pSG5 plasmid in BL21 (DE3) (Novagen) as previously described (32, 33). UmuD D91 and G92 were changed to lysine by site-directed mutagenesis of pSG5 using the QuikChange Kit (Agilent) and confirmed by sequencing the resulting plasmids (Macrogen USA). Wild-type UmuD and all variants were purified as previously described (32).

Wild-type DNA pol III α was expressed from the pET28a-α plasmid in Tuner competent cells (Novagen) and purified using both a Nickel His-trap column (GE Healthcare) and a heparin column (GE Healthcare) as previously described (29). Fractions collected after the heparin column were diluted 6-fold with buffer HA (50 mM HEPES, pH 7.5; 1 M NaCl; 2 mM beta-mercaptoethanol; 20% glycerol) and loaded onto a hydroxyapatite column (BioRad Bioscale Mini CHT Type 1, 5-mL, 40 μm cartridge) to concentrate the protein; protein concentrator devices were avoided because they significantly reduced the stability and activity of DNA pol III α. After washing with 10 column volumes (cv) of buffer HA, buffer HB (100 mM sodium phosphate, pH 6.5; 1 M NaCl; 2 mM beta-mercaptoethanol; 20% glycerol) was used to elute the
protein from the column isocratically in 2 cv. Fractions containing DNA pol III α were dialyzed against protein storage buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; and 50% glycerol) and stored at -20 °C.

3.2.2 Single molecule DNA stretching

In DNA stretching experiments with optical tweezers, a single λ DNA molecule is captured between two polystyrene beads inside a flow cell. One bead is fixed on a micropipette tip, and the other is held in a dual-beam optical trap (34). As the fixed bead is extended at 100 nm/s, the tethered DNA molecule exerts a force on the trapped bead, which is measured by deflection of the trapping laser beams. The force on the DNA molecule is measured as a function of DNA extension. As the DNA is stretched, the dsDNA helix undergoes a force-induced melting transition into ssDNA (Figure 3.2A). The bases anneal upon DNA release, exhibiting minimal hysteresis, or mismatch between the extension and release curves. After the DNA is stretched and released in buffer only (10 mM HEPES, 100 mM Na⁺, pH 7.5), the solution in the flow cell is exchanged to include protein. Subsequent force-extension curves are obtained in the presence of α, UmuD, or both proteins.
Figure 3.2 DNA pol III α binding to ssDNA characterized with single molecule force measurements.

(A) Typical extension (solid black) and return (dashed black) of a single DNA molecule. The molecule undergoes a force-induced melting transition from dsDNA (red, Equation 3.2) to ssDNA (blue, Equation 3.3) at 62.6 ± 0.5 pN. (B) In the absence of protein (black), the DNA molecule anneals immediately upon release, exhibiting minimal hysteresis, or mismatch between the stretch (solid) and release (dashed) curves. The force extension curves in the presence of 500 nM UmuD are the same within uncertainty as those of DNA only (purple), which shows that UmuD does not measurably bind DNA. In the presence of 250 nM α (green), pausing at a fixed DNA extension (dashed arrow) after the melting transition exposes ssDNA to α for 30 minutes, as previously described (31). Upon DNA release, α remains bound to the ssDNA (open circles), prohibiting the two strands from annealing. The DNA molecule is therefore a combination of dsDNA and protein-bound ssDNA, and fitting the release curve (open circles) to Equation 3.1 (solid line) yields the fraction of ssDNA bound to α ($f_{ss} = 0.58 ± 0.02$).

Force-extension curves in the presence of 250 nM α alone exhibit significant hysteresis, since protein bound to the exposed ssDNA prohibits the two strands from annealing upon DNA release. All single molecule experiments were performed by waiting at fixed extension for 30 min prior to DNA release, which has been established as a quantitative method of characterizing α binding to ssDNA (31). The fraction of ssDNA bound $f_{ss}$ may be described in terms of observed length $b$ (31):
\[ b(F_{ss}) = b_{ds}(1 - f_{ss}) + b_{ss}f_{ss} \]
(Equation 3.1)

where \( b_{ds} \) as a function of force \( F \) is described by the Worm-Like Chain (WLC) model:

\[
b_{ds}(F) = B_{ds} \left[ 1 - \frac{1}{2} \left( \frac{k_B T}{P_{ds} F} \right)^{\frac{1}{2}} + \frac{F}{S_{ds}} \right]
\]
(Equation 3.2)

with persistence length \( P_{ds} \), end-to-end or contour length \( B_{ds} \), and stretch modulus \( S_{ds} \). The Freely-Jointed Chain (FJC) model describes the polymer elasticity of ssDNA:

\[
b_{ss}(F) = B_{ss} \left[ \coth \left( \frac{2P_{ss} F}{k_B T} \right) - \frac{1}{2} \frac{k_B T}{P_{ss} F} \right] \left[ 1 + \frac{F}{S_{ss}} \right]
\]
(Equation 3.3)

The WLC and FJC polymer models shown in Figure 3.2 have typical parameter values (\( B_{ds} = 0.34 \text{ nm/bp, } P_{ds} = 48 \text{ nm, and } S_{ds} = 1200 \text{ pN in Equation 3.2, } B_{ss} = 0.55 \text{ nm/bp, } P_{ss} = 0.75 \text{ nm, and } S_{ss} = 720 \text{ pN in Equation 3.3})). As previously described, the fits were confined to forces below 40 pN (31) to eliminate effects from changes in the force-extension curve of ssDNA due to protein binding.
These experiments were repeated in the presence of both \( \alpha \) and UmuD, and the fraction of \( \alpha \)-bound ssDNA \( f_{ss} \) as a function of UmuD concentration \( c_s \) was fit to a simple competitive DNA binding isotherm:

\[
f_{ss} = \left(1 - \frac{c_s}{K_d^{app}}\right) f_{sat}
\]

(Equation 3.4)

where \( K_d^{app} \) is the apparent equilibrium dissociation constant between UmuD and \( \alpha \) in the presence of ssDNA, and \( f_{sat} \) is saturated \( \alpha \)-ssDNA binding. A minor correction to added UmuD concentration \( c \) accounts for UmuD bound to \( \alpha \) in solution, so effective UmuD solution concentration \( c_s \) is (35):

\[
c_s = \frac{c}{1 + K_a c_\alpha}
\]

(Equation 3.5)

where \( K_a = 9.1 \times 10^{-5} \text{ M}^{-1} \) is the equilibrium association constant between UmuD and \( \alpha \) in bulk solution (29) and \( \alpha \) concentration \( c_\alpha \) is 250 nM.

3.2.3 Protein-protein docking

Protein-protein docking models were used to predict residues involved in the binding interaction between \( \alpha \) and UmuD. The structures used for these docking models were a homology model of
UmuD (33) and a homology model of full-length DNA pol III α (36). Protein complexes where predicted by docking DNA pol III α with UmuD using ClusPro 2.0 (37-40), GRAMM-X (41), and PatchDock (42). The top 10 results from each method were analyzed and compared. Local docking was performed using the RosettaDock server (43).

3.2.4 Thermal stability assay

A thermal stability assay was used to determine the melting temperature of UmuD variants relative to wild-type, as previously described (44). To determine whether mutations at positions D91 and G92 disrupt the stability of UmuD, melting temperatures of these variants were compared to those of wild-type. Samples containing 20 µM of each variant in 50 mM HEPES, pH 7.5, 100 mM NaCl and 25x Sypro Orange (Invitrogen) were exposed to temperatures from 25 °C to 80 °C while monitoring the fluorescence emission intensity at 575 nm. Melting temperatures $T_m$ were determined by taking the first derivative of the melting curves, as previously described (29).

3.2.5 RecA/ssDNA facilitated cleavage assay

Reactions were assembled as previously described (33) and incubated at 37 °C for 45 minutes. After incubation, the cleaved product UmuD′ was separated from full-length UmuD using 18% SDS-PAGE. Bands were then analyzed using the image analysis software ImageQuant TL (Amersham Biosciences). Control reactions in the absence of RecA, ssDNA and ATPγS were carried out to determine the amount of UmuD′ present due to spontaneous cleavage.
3.2.6 Tryptophan fluorescence assay

The equilibrium dissociation constant \( K_d \) between DNA pol III \( \alpha \) and the UmuD variants were determined with a Varian Cary Eclipse Fluorescence Spectrophotometer, as previously described (29). DNA pol III \( \alpha \) (5 \( \mu \)M in 50mM HEPES, pH 7.5 and 100 mM NaCl) was titrated with varying volumes of 200 - 400 \( \mu \)M UmuD variants. Tryptophan fluorescence quenching was used to quantitate binding constants, as previously described (29).

3.3 RESULTS

3.3.1 UmuD inhibits \( \alpha \) binding to ssDNA

In DNA stretching experiments, a single double-stranded \( \lambda \)-DNA molecule was captured between two polystyrene beads, one held in an optical trap and the other fixed on a micropipette tip. As the distance between the beads increases, measurements of the force on the DNA molecule yield a force-extension curve (Figure 3.2A, solid black line). At a constant force of 62.6 \( \pm \) 0.5 pN, the dsDNA molecule undergoes a force-induced melting transition to ssDNA. This overstretching transition has been established as force-induced melting in the presence of DNA binding proteins such as \( \alpha \), which is the case for the experiments presented in this work (34). When the tension on the DNA molecule is released (Figure 3.2A, dashed black line), the ssDNA generated by force anneals immediately into dsDNA, and the curve exhibits minimal
hysteresis, or mismatch between DNA extension and release curves. The molecule is a well-characterized combination of dsDNA and ssDNA along the force-induced melting transition (45), so waiting at constant extension for a fixed time exposes ssDNA to proteins in solution. The protein-bound ssDNA exhibits a change in observed length upon DNA release, which is a direct measurement of protein binding to ssDNA. This single molecule technique has been established as a quantitative method of characterizing α-ssDNA binding (31). As expected, constant extension experiments in the presence of 250 nM α exhibit large hysteresis, indicating significant ssDNA binding (Figure 3.2B). Fits to Equation 3.1 yield the fraction of ssDNA bound by α, which agrees with previous results obtained by this method (31).

Introducing the DNA damage response protein UmuD disrupts the binding of α to ssDNA (Figure 3.3A). Constant extension experiments generate ssDNA for 30 minutes in the presence of both α and UmuD, and the fraction of ssDNA bound by α decreases with UmuD concentration. Control experiments demonstrate that UmuD does not bind DNA, since force extension curves in the presence of UmuD are the same within uncertainty as those of DNA only (Figure 3.2B, purple). Therefore the interaction between UmuD and α inhibits α binding to ssDNA. A simple DNA binding isotherm (Equation 3.4) fit to the fraction of α bound as a function of effective UmuD concentration in solution yields the apparent equilibrium dissociation constant $K_d^{\text{app}} = 340 \pm 103$ nM between UmuD and α in the presence of ssDNA (Figure 3.3B).
The fraction of ssDNA bound by α decreases with increasing UmuD concentration.

(A) Force extension (solid black line) and release (open circles) curves for DNA in the absence (black) and presence of α and UmuD (open circles). Open circles are data points, and solid color lines are fits to Equation 3.1, which yield the fraction of ssDNA bound to α at each UmuD concentration. Fractions shown in these representative curves are 0.58 ± 0.02 in the absence of UmuD (green), 0.44 ± 0.02 at 200 nM UmuD (blue), 0.27 ± 0.02 at 500 nM UmuD (red), 0.09 ± 0.01 at 1 μM UmuD (brown), and 0.06 ± 0.01 at 3 μM UmuD (purple). (B) Fraction of ssDNA bound by α as a function of effective UmuD concentration in solution (Equation 3.5). Error bars represent standard error (N ≥ 3) for all points except 2.5 μM, which represents propagated error. The solid line is a $\chi^2$ fit to a simple DNA binding isotherm (Equation 3.4) that yields apparent equilibrium dissociation constant $K_{d}^{app} = 340 ± 103$ nM between UmuD and α in the presence of ssDNA, and a saturated α-ssDNA binding fraction ($f_{sat} = 0.51 ± 0.05$) consistent with previous single molecule results (31).

3.3.2 Specific UmuD variants disrupt the UmuD-α interaction

To predict potential α binding sites on UmuD, we used three global protein-protein docking methods. All three methods predicted an ensemble of complexes with UmuD binding near the N-terminal domain and C-terminal domain of DNA pol III α, consistent with the two previously characterized UmuD binding sites (29). At the C-terminal domain, a number of docking models suggested the formation of a salt bridge (Figure 3.4A) between the arginine residue of DNA pol III α at position 1068 (Figure 3.4B) and the aspartic acid residue of UmuD at position 91 (Figure
As a result, UmuD residues D91, along with its adjacent neutral residue G92, were each mutated to lysine in order to disrupt this salt bridge (Figure 3.4C). We did not construct corresponding mutants in DNA pol III α at position 1068 because such a mutant would likely disrupt DNA binding as well.

Figure 3.4 Docking model predicts residues involved in the interaction between DNA pol III α and UmuD.
(A) The docking model predicts a salt bridge between the C-terminal domain of DNA pol III α (pink) and UmuD (yellow) at α residue R1068 (green) and UmuD residue D91 (blue). UmuD residue G92 (red) may also be involved in this interaction. (B) The homology model of α (36) showing arginine residue R1068 (green). The C-terminal domain (residues 917-1160) containing the binding sites for the β clamp, UmuD, and ssDNA is shown in pink. (C) The homology model of full-length UmuD (33) showing the residues D91 (blue) and G92 (red) predicted to bind to DNA pol III α by protein-protein docking studies. The arms of UmuD are shown here in a “trans” conformation where the arm of one monomer (both arms shown in purple) is bound to the globular domain of the other monomer (both globular domains shown in yellow). The active site residues S60 and K97 (cyan) cleave the N-terminal arms (residues 1-24) to form UmuD'.

To verify that the mutations did not destabilize UmuD, the melting temperatures of each UmuD variant were determined (Figure 3.5A). It should be noted that UmuD has two melting transitions, which have been assigned to the dissociation of the arms from the globular domain (see drawing in Figure 3.5A) and the melting of the globular domain, respectively (46). Both melting transitions of the UmuD variants are comparable to those of wild-type UmuD, indicating that the variants are properly folded. To test the effect of each mutation on the cleavage activity of UmuD, a RecA/ssDNA-facilitated UmuD cleavage assay was also performed. All variants were able to cleave efficiently to form the cleavage product UmuD' (Figure 3.5B), so these mutations do not alter UmuD enzymatic activity. Because UmuD can undergo spontaneous cleavage, control reactions where RecA/ssDNA was not added to the UmuD variants were also carried out. The amount of UmuD' present in both the control reactions and the reactions with RecA/ssDNA were quantified separately to distinguish the amount of UmuD' produced only in the RecA/ssDNA facilitated reaction (Figure 3.5B). Thus, the UmuD variants constructed show similar stability and similar RecA-ssDNA-facilitated cleavage efficiency as wild-type UmuD.
Figure 3.5 UmuD variants are structurally stable and enzymatically active.

(A) Melting temperatures of all UmuD variants. The N-terminal “arms” dissociate from the globular domain (drawing above) in the first transition (dashed), and the globular domain melts in the second transition (solid). (B) Relative amount of UmuD′ produced by each variant in the RecA/ssDNA-facilitated self-cleavage reaction (45 min at 37 °C, drawing above), in the absence (dashed) and presence (solid) of RecA/ssDNA. Thermal stability and self-cleavage activity of all variants is similar to wild-type UmuD, suggesting that the mutations had minimal impact on protein stability and function. Error bars represent standard error (N ≥ 3).

As shown in Figure 3.6, in the absence of DNA the UmuD variant D91K disrupted the DNA pol III α-UmuD interaction by a 15-fold increase in $K_d$ (18 ± 1.7 µM, compared to 1.1 ± 0.6 µM for wild-type UmuD) while no change was seen with the adjacent UmuD variant G92K ($K_d = 1.3 ± 0.5$ µM). When both positions were changed to lysine, D91K-G92K, a similar effect of decreased binding to α was observed ($K_d = 33 ± 9.6$ µM), confirming this position as a binding site for DNA pol III α. Although it is initially surprising that an adjacent residue mutation does not also disrupt UmuD-α interactions, unlike D91, we predict that a side chain at 92 would be angled away from the surface of UmuD (Figure 3.4A), so it is less likely to participate directly in the interaction.
Figure 3.6 Binding curves between pol III α and UmuD variants measured by tryptophan fluorescence quenching.

(A) The equilibrium dissociation constant $K_d$ is $1.1 \pm 0.6 \ \mu M$. (B) Binding to α is compromised by the D91K ($K_d = 18.1 \pm 1.7 \ \mu M$) mutation relative to wild-type UmuD (29). (C) The G92K variant ($K_d = 1.3 \pm 0.5 \ \mu M$) binds α with nearly the same affinity as wild-type UmuD. (D) In contrast, the binding affinity of the D91K-G92K variant ($K_d = 32.5 \pm 9.6 \ \mu M$) is significantly weaker than that of the D91K variant. This suggests that both the D91 and G92 residues are involved in the interaction of UmuD and α. Error bars indicate standard error ($N \geq 3$).
3.3.3 Specific variants disrupt UmuD inhibition of α binding to ssDNA

We used single molecule DNA stretching experiments to test whether the three UmuD variants retain the ability to disrupt binding between α and ssDNA. We previously showed using this single molecule method that α possesses two distinct DNA binding activities: the (HhH)$_2$ domain binds dsDNA whereas the C-terminal domain containing the OB-fold domain binds ssDNA (31). This method allows us to probe specifically the binding of α to ssDNA without the potential complications of DNA secondary structure or dsDNA binding. The biochemical results show that the D91K mutation compromises the ability of UmuD to bind α, and single molecule experiments demonstrate that the fraction of ssDNA bound by α in the presence of 1 μM UmuD D91K is only slightly smaller than that of α alone (Figure 3.7A). UmuD G92K has an affinity for α similar to that of wild-type UmuD, and DNA stretching shows that this variant retains most of its ability to inhibit α binding to ssDNA (Figure 3.7B). However, the double mutation D91K G92K yields a UmuD variant whose binding to α is dramatically weakened, and is completely unable to disrupt α-ssDNA binding (Figure 3.7C). UmuD residue D91 is therefore required for UmuD to bind α and disrupt its binding to ssDNA. Residue G92 also participates in the interactions that are responsible for UmuD inhibition of α-ssDNA binding. Collectively, these results show that these direct interactions between UmuD and α are responsible for the ability of UmuD to inhibit α binding to ssDNA.
Figure 3.7 UmuD variants have compromised ability to disrupt α binding to ssDNA.

(A-C) Force extension (solid black line) and release (open circles) curves for DNA in the absence (black) and presence of protein (open circles). Open circles are data points, and solid color lines are fits to Equation 3.1, which yield the fraction of ssDNA bound by α. (A) The fraction of α-bound ssDNA in the presence of 1 μM UmuD D91K (blue, 0.54 ± 0.02) is similar to that of α alone (green). The D91K variant lost the ability to inhibit α-ssDNA binding relative to wild-type UmuD (brown), which indicates that the D91 residue is required for the interaction between α and UmuD that disrupts α binding to ssDNA. (B) The G92K variant (red, 0.23 ± 0.02) partially retains its ability to disrupt the interaction between α and ssDNA. (C) The D91K-G92K mutation (purple) fully abolishes the ability of UmuD to disrupt α-ssDNA binding, and fraction of ssDNA bound to α is the same without the UmuD variant (0.58 ± 0.02). (D) Fraction of ssDNA bound to α in the presence of 1 μM UmuD variants. These single molecule results indicate that UmuD residue D91 is essential for the interaction between UmuD and α that disrupts α binding to ssDNA, but G92 is also involved in the interaction.
3.4 DISCUSSION

In this work, we used a single-molecule method to demonstrate that UmuD inhibits the binding of α to ssDNA through its interaction with α. The apparent equilibrium dissociation constant \( K_{d}^{\text{app}} \) between UmuD and α is 340 ± 103 nM in the presence of ssDNA, which is similar to a previous measurement of the equilibrium dissociation constant between α and UmuD (\( K_{d} = 1.1 \pm 0.6 \mu\text{M} \)) determined by using a tryptophan fluorescence binding assay in the absence of DNA. The reasonable agreement between apparent binding affinity and direct binding affinity implies that direct UmuD interactions with α are responsible for ssDNA binding inhibition. In addition, the measured apparent binding affinity between α and UmuD is within the range of cellular UmuD concentrations, suggesting that this interaction plays an important role in regulating α activity in vivo (47).

In addition to directly demonstrating that UmuD inhibits α binding to ssDNA, we used protein-protein docking models to identify potential UmuD-α interaction sites. The resulting models predicted that binding between UmuD and α involves UmuD residues D91 and G92 and α residue R1068. Biochemical experiments confirmed that that corresponding UmuD variants exhibit a compromised ability to bind α, despite the fact that the variants were demonstrated to be thermally stable and active for cleavage. Single-molecule experiments showed that the D91K variant completely fails to disrupt the α-ssDNA interaction, while the G92K variant only partially inhibits the ability of α to bind ssDNA. These results demonstrate that a direct α-UmuD interaction at these residues is responsible for UmuD inhibition of α-ssDNA binding. Another UmuD variant at position 91, D91A, has been shown to disrupt the UmuD-DinB interaction (26),
suggesting the existence of a general binding site for polymerases on UmuD. In addition, peptide mapping experiments show that this region of UmuD provides a binding site for the protease ClpXP, which plays a role in modulating mutagenesis by degrading UmuD and UmuD′ as well as DinB (48, 49). Furthermore, the UmuD G92D mutation is poorly cleavable, but when this mutation was constructed in the context of UmuD′, cells harboring this variant were mutable to a similar extent as cells harboring wild-type UmuD (50, 51), suggesting that the G92D mutation does not alter the ability of UmuD′ to facilitate UmuC-dependent mutagenesis. On the other hand, the UmuD G92C variant was as proficient for cleavage as wild-type UmuD (52). Therefore, this region of UmuD seems to be an important site for a number of protein interactions.

The C-terminal domain of α facilitates numerous interactions necessary for efficient replication, as it interacts with the β clamp (30), ssDNA (31), and the τ subunit of the clamp loader (53, 54). The interaction between α and the β clamp is essential for high processivity. However, since replication on the lagging strand is carried out in a series of Okazaki fragments that are synthesized in a discontinuous manner, the polymerase on the lagging strand must be recycled for each Okazaki fragment. Although the exact mechanism of the processivity switch is unknown, the OB fold of α has been implicated as a sensor for ssDNA such that when synthesis of an Okazaki fragment is completed, the affinity of α for the β clamp is decreased and α is released from the clamp and from DNA (55-57). Thus, the interaction between α and ssDNA is proposed to act as a processivity switch. In this work, we demonstrated that SOS-induced levels of UmuD inhibit binding of α to ssDNA. UmuD also inhibits binding of α to the β clamp, and these two mechanisms likely work together to facilitate polymerase exchange.
UmuD, together with UmuC, specifically decrease the rate of DNA replication and therefore were proposed to participate in a primitive DNA damage checkpoint (21-23). We previously showed that UmuD, but not the cleaved form UmuD’, disrupts the binding of α to the β clamp, which provides a possible molecular explanation for the role of UmuD in a primitive checkpoint (29). The question then arises of the role of UmuD disruption of ssDNA binding by α. It has been shown that DNA damage on the lagging strand does not disrupt DNA replication, whereas DNA damage on the leading strand may severely disrupt replication or may cause leading strand replication to occur discontinuously as the polymerase can re-initiate synthesis downstream of the damage (8, 9, 11). Polymerases that encounter DNA damage can also become stalled in a futile cycle of insertion and excision of nucleotides (58) as they fail to copy the damaged DNA. Our observations suggest that UmuD would then rescue the stalled polymerase by preventing α from binding to ssDNA or to the β clamp, thereby allowing DNA repair proteins or translesion DNA polymerases access to the damaged DNA.

Taken together, our findings suggest that UmuD specifically disrupts the interaction between α and ssDNA, inhibiting access to the ssDNA template by the replicative polymerase as part of the primitive DNA damage checkpoint to allow DNA repair. UmuD may also prevent binding of α to ssDNA to facilitate polymerase exchange, allowing an error-prone TLS polymerase to copy damaged DNA so that DNA replication may proceed. Our results demonstrate a new mechanism by which UmuD may regulate the cellular response to DNA damage.
3.5 REFERENCES


Chapter 4: Replication by *E. coli* DNA pol III α is inhibited by direct binding to the OB domain of single stranded DNA binding protein

Michelle C. Silva designed bulk biochemical experiments, purified proteins, performed experiments, analyzed the data, and wrote the manuscript; Kiran Prant designed single molecule experiments, analyzed the data, and wrote the manuscript; Jana Sefcikova designed primer-extension assay, purified proteins, and wrote the manuscript; Susie Nimipattana performed single molecule experiments and analyzed data; Mark C. Williams and Penny J. Beuning designed experiments and wrote the manuscript.

4.1 INTRODUCTION

During replication, genome integrity must be efficiently maintained by DNA polymerases. In *Escherichia coli* (*E. coli*), processive replication of undamaged DNA is accomplished by the DNA polymerase III (DNA pol III) holoenzyme. It consists of ten subunits which are further organized into three subassemblies: the core, the β processivity clamp, and the clamp loader complex (1, 2). Polymerase activity is contained in the core subassembly which consists of the α
polymerase subunit, the ε proofreading subunit, and the θ subunit. The high processivity of the holoenzyme is conferred by the β processivity clamp which encircles DNA and tethers the core to its DNA substrate. This clamp is loaded onto DNA with the help of a clamp loader complex consisting of six different subunits: τ, γ, δ, δ′, χ, and ψ. The γ subunit along with δ and δ′ load the β processivity clamp on to DNA with concomitant ATP hydrolysis (1, 2). The τ subunit coordinates replication on both the leading and lagging strands by coupling the core to the clamp loader complex (3) while the χ/ψ complex helps regulate replication on the lagging strand (4). Due to the discontinuous nature of replication on the lagging strand, a significant amount of ssDNA may be present at one time which is coated with single-stranded DNA binding protein (SSB) preventing ssDNA from forming secondary structures and protecting the ssDNA from chemical and nucleolytic attack (5-7).

SSB is a homo-tetramer with a D₂ axis of symmetry (8). Each 178 amino acid-long monomer consists of an N-terminal globular domain and a disordered C-terminal tail (9). The N-terminal globular domain of SSB possesses an oligonucleotide/oligosaccharide-binding (OB) domain that binds ssDNA via stacking interactions with one phenylalanine (F61) and two tryptophan (W41 and W55) residues of SSB (10-15). Changing residue H55 to either tyrosine or lysine has been shown to disrupt the tetramer interface (16). SSB binds ssDNA by wrapping the DNA around the homo-tetramer in two binding modes: (SSB)₃₅ and (SSB)₆₅ where the integers 35 and 65 represent the number of nucleotides contacting the protein (17). In the (SSB)₃₅ binding mode, ssDNA only wraps around two monomers of the homo-tetramer, promoting high cooperativity. On the other hand, the (SSB)₆₅ binding mode favors limited cooperativity and ssDNA wraps around all four monomers (17).
In addition to binding and protecting ssDNA, SSB also serves as a hub for at least 15 proteins involved in numerous DNA processing pathways, such as proteins involved in homologous recombination, DNA pol IV (18) and pol V (19) in damage tolerance, and the χ subunit of DNA pol III in replication (20). Most of these proteins have been found to bind the C-terminal tail suggesting that SSB uses this tail to facilitate interactions with other proteins involved in DNA maintenance (21). The temperature sensitive allele ssb-113, encoding the mutation P177S (22), impairs DNA replication at temperatures higher than 30 °C (23, 24). The P177S mutation has been shown to decrease the interaction with proteins that bind this C-terminal tail (20).

SSB, in addition to protecting ssDNA present at the replication fork, also has been observed to directly impact replication activity of DNA pol III. SSB increases processivity of the polymerase when all of the subunits of DNA pol III are present. In contrast, when only the core subunits α, ε, θ are present, SSB inhibits replication (25). This inhibition is alleviated when the χ subunit of the clamp loader complex is present due to a direct interaction between χ and the C-terminal tail of SSB (4, 26-28).

Although much work has been focused on aspects that suppress inhibition of the α polymerase subunit by SSB, factors contributing to the inhibition have not been probed in detail. In this report, we demonstrate that the OB-fold domain of SSB directly binds to the C-terminal domain of the α polymerase subunit of DNA pol III. We also demonstrate that the α subunit stabilizes SSB on DNA. Taken together, our findings suggest that the inhibition of DNA pol III by SSB is due to the direct interaction between SSB and the α subunit and indirectly due to SSB binding ssDNA.
4.2 MATERIALS AND METHODS

4.2.1 Proteins and Plasmids

His-tagged DNA pol III α subunit was expressed from pET28a which was provided by Dr. Meindert Lamers and Dr. John Kuriyan (UC Berkeley) and purified using the protocol previously described (29). The α truncations were constructed as previously described (30). The plasmid encoding wild-type SSB, pEAW134, was provided by Dr. Mark Sutton at University at Buffalo and expressed in BL21-DE3 competent cells (Novagen). Cells were lysed using lysozyme (Sigma) and purified using the established protocol (31) which includes precipitation with polymin P and ammonium sulfate, followed by an ssDNA cellulose column. Purified SSB was stored at -20 °C in a buffer containing 50% glycerol to prevent freezing. High amounts of glycerol can affect the outcome of various experiments, especially primer extension assays. Consequently, before every experiment, the buffer was exchanged using Zeba spin columns (Thermo Scientific) to a buffer appropriate for that assay. The SSB variants F61A, D91N, and P177S (P177S the mutation encoded by the ssb-113 temperature sensitive allele; this SSB variant is referred to as SSB-113) were created by site-directed mutagenesis using a QuikChange kit (Agilent). The plasmid encoding the SSB-OB truncation (residues 1-113), pSSBOB, was developed from pEAW134 by inserting stop codons in all reading frames after the 113th residue.
The disordered C-terminal tail of SSB contains mostly acidic residues (32). Consequently, when this C-terminal tail is removed as with our SSB-OB truncation, the pI changes from 6 for full-length SSB to 9 for SSB-OB (33). Therefore, we developed the following method to purify SSB-OB. Cell pellets from 2 L of cell culture were thawed overnight at 4 °C in SA buffer (50 mM Tris, pH 8.3; 1 mM EDTA; 20% glycerol; 0.1 M NaCl; 1 mM dithiothreitol; ¼ protease inhibitor tablet (Roche)) and then cells were lysed using lysozyme (300 µg/mL; Sigma), DNaseI (1 µg/mL Roche), and a freeze-thaw cycle. Lysate was separated from cell debris by centrifugation at 12,000 x g and 4 °C for 1 hr and sterile-filtered using a 0.45 µm membrane filter (GE Healthcare).

The lysate was loaded onto a 5 mL HiTrap SP FF column (GE Healthcare) which was equilibrated with SA buffer. The column was washed with five column volumes of SA and then eluted with a gradient of 0-100% SB buffer (50 mM Tris, pH 8.3; 1 mM EDTA; 20% glycerol; 1 M NaCl; 1 mM dithiothreitol) over 15 column volumes. Since SSB-OB was not retained on the column, the flow-through was collected and applied to the column a second time. This allowed for the separation of impurities that are retained on the HiTrap SP FF column.

The column load and wash were collected and loaded onto 2 x 5 mL HiTrap DEAE FF columns (GE Healthcare) (10 mL total column volume) equilibrated with SA buffer. The column was washed with 10 column volumes and then eluted with a gradient of 0-100% SB buffer over 15 column volumes. This column was repeated a second time. Once again, SSB-OB was not retained on the column and so the DEAE column load and wash were combined for further purification.
Ammonium sulfate was added to a final concentration of 1 M. The mixture was then loaded onto 2 x 5 mL HiTrap phenyl-sepharose FF columns (GE Healthcare) (10 mL total column volume) pre-equilibrated with PSA buffer (50 mM Tris, pH 8.3; 1 mM EDTA; 20% glycerol; 0.1 M NaCl; 1 mM dithiothreitol; 1 M ammonium sulfate). The phenyl-sepharose column was washed with 10 column volumes of PSA. SSB-OB eluted approximately halfway through a gradient of 0-100% SA buffer over 15 column volumes. The fractions containing SSB-OB were pooled and the protein was concentrated using Vivaspin 6 centrifugal concentrators (5,000 MWCO; Vivascience) to less than 2 mL. At this point, SSB-OB was approximately 75% pure.

This partially purified, concentrated SSB was then injected onto a 300-mL Superdex 75 size exclusion column (GE Healthcare) using SA buffer. Fractions containing SSB-OB were pooled, concentrated using Vivaspin 6 centrifugal concentrators (5,000 MWCO) and stored in SSB storage buffer (20 mM Tris, pH 8.3; 1 mM EDTA; 50% glycerol; 0.5 M NaCl; 1 mM dithiothreitol) in the same manner as full-length SSB (31). We have found that SSB-OB is insoluble in buffer that does not contain a reducing agent. After the size exclusion column, SSB-OB was found to be approximately 90% pure, producing a total yield of 13 mg as quantified by Bradford assay (Bio-Rad).

4.2.2 Primer-Extension Assay

The 30-nucleotide primer and 100-nucleotide template strands used for the primer-extension assay (Eurofins MWG Operon) were purified using denaturing polyacrylamide gel electrophoresis followed by labeling of the primer with $^{32}$P at the 5′ end as previously described.
The sequences are as follows: primer, 5’ GCATATGATAGTACAGCTGCAGCCGGACGC 3’; and template, 5’ GGATAACAATTTCACACAGGAAACAGCTATGACCATTGGTTACTCAGATCAGGCCTGCGAAGACCTGGGCGTCCGGCTGCAGCTGTACTATCATATGC 3’. The DNA substrate was prepared by combining equal amounts (500 nM) of each strand in annealing buffer (20 mM HEPES, pH 7.5; 5 mM Mg(OAc)$_2$). DNA was annealed by first denaturing at 95 °C for 2 min, incubating at 50 °C for 1 hr and then allowing to cool to 37 °C.

DNA pol III α polymerase activity on a $^{32}$P end labeled primer-template DNA substrate was assayed in the presence of wild-type SSB and SSB variants as previously described (34). Reactions were assembled with 50 nM α, 0-500 nM SSB (tetramer concentration) and 100 nM $^{32}$P primer-template in primer extension buffer (30 mM HEPES, pH 7.5; 20 mM NaCl; 10 mM MgSO$_4$; 4% glycerol; 100 µg/mL BSA; 1 mM dithiothreitol). Replication was initiated with 100 µM dNTPs at 37 °C and then quenched with stop buffer (50 mM EDTA; 85% formamide; 0.025% bromophenol blue; 0.025% xylene cyanol) at selected time points (0, 5, 20 and 60 minutes). All reactions were analyzed on a denaturing 12% polyacrylamide gel and imaged using a phosphor screen and a Storm 860 Phosphorimager (GE).

**4.2.3 Tryptophan Fluorescence Quenching Assay**

Binding constants were determined by observing the change in intrinsic fluorescence of tryptophan residues of the α subunit in the presence of varying concentrations of SSB as previously described (30). Varying amounts of wild-type SSB and SSB variants (100-200 µM
monomer stock concentrations) were added to 5 µM α subunit in W buffer (50 mM HEPES, pH 7.5 and 100 mM NaCl). Because SSB also contains tryptophan residues, fluorescence contributed by SSB alone was subtracted. SSB/ssDNA binding was detected by titrating varying amounts of 50 µM 24-mer ssDNA (Eurofins MWG Operon) into 5 µM SSB variants (monomer concentration) in W buffer.

4.2.4 Single Molecule Force Spectroscopy using Optical Tweezers

To label the 3’ and 5’ ends of the same strand, lambda DNA (Roche, 48,500 bp) with two single-stranded 12 nt 5’ overhangs was digested with ApaI (Fermentas, Fast Digest restriction enzyme) for 15 minutes at 37 °C to create 3’ and 5’ overhangs on the same strand. The reaction was terminated by heat inactivation for 20 minutes at 65 °C. In the next step, the nucleotides, biotin-14-dATP, biotin-14-dCTP (Invitrogen), dTTP and dGTP (Fermentas) were incorporated into 5’-overhang in the presence of Klenow exo- DNA polymerase for 45 minutes at 37 °C. The reaction was terminated by heat inactivation for 15 minutes at 75 °C and was purified using ethanol precipitation. Biotinylated oligonucleotide (cTcTcTcTctctttctttctctttgccc, where T =Biotin-dT, a gift from Dr. Eriks Rozners, Binghamton University) was annealed to the 3’ overhang to the purified DNA (100:1 oligonucleotide:DNA) by heating the reaction mixture to 65 °C for 10 minutes, followed by slow cooling to room temperature. Then, the ligation reaction was carried out at room temperature for 60 minutes with T4 DNA ligase (Fermentas, Fast Ligase) and the DNA was purified using ethanol purification. The resulting DNA construct is 38,500 bp long with biotin at 3’- and 5’-ends of the same strand.
After successfully labeling both ends of the same strand with biotin, the DNA is captured by attaching to two streptavidin beads (Bangs Laboratories) where one is trapped in an optical laser trap and the other is attached to a glass micropipette by suction (Figure 4.1A and the inset of Figure 4.1C). The force-extension measurements were carried out by measuring the force on trapped beads as a function of distance (nm/nt) controlled by moving the pipette a known distance away from the laser trap to stretch the DNA between the two beads (Figure 4.1B). When DNA was stretched to ~60 pN, a constant force transition was observed in which DNA was converted from ssDNA to dsDNA (Figure 4.1B). However, when this process was reversed, the DNA strands anneal and double-stranded DNA is recovered. In order to obtain ssDNA between the two beads, we used the enzymatic activity of T7 polymerase. T7 DNA polymerization and exonucleolysis activities can be modulated by controlling the force exerted on the DNA (Figure 4.1B). Exerting a force above 40 pN increases the exonucleolysis activity of T7 DNA polymerase which is unaffected by the presence of dNTPs (36). In our experiments, we were able to detect T7 exonuclease activity in real time (Figure 4.1C), which converts dsDNA to ssDNA. The newly formed ssDNA was then stretched in the presence and absence of SSB. To investigate the binding affinity of SSB to ssDNA in the presence of DNA pol III α, the α subunit was added along with SSB.
Figure 4.1 5’ and 3’ biotin-labeled ssDNA was constructed using exonucleolysis by T7 DNA polymerase.  

(A) Schematic diagram of DNA stretching using optical tweezers. 38,500 bp long λDNA was prepared as described and attached between two beads, one held on the tip of a glass pipette and the other in an optical trap. (37) (B) Force-extension curves for dsDNA (black solid and dotted lines) and ssDNA (blue solid and dotted lines). The arrows show the direction of polymerization and exonucleolysis. (C) When dsDNA is stretched in the presence of T7 DNA polymerase and held at ~50 pN (red solid line), dsDNA (black solid and dotted lines) is converted to ssDNA (blue solid and dotted lines) by exonucleolysis.
4.3 RESULTS

4.3.1 DNA pol III α interacts with the OB fold of SSB

It has been previously determined that SSB inhibits replication by the core subunits of DNA pol III (25). Under our conditions, in which a 5′ $^{32}$P-labeled primer-template was extended by the α subunit of DNA pol III in the presence of wild-type SSB, we observed that inhibition increases with increasing concentration of SSB (Figure 4.2), further supporting previous work (25). Although studies have been conducted to determine how this inhibition is overcome (4, 26-28), the mechanism of inhibition has not been probed in detail.

![Diagram](https://via.placeholder.com/150)

**Figure 4.2 SSB inhibits primer extension by DNA pol III α.**

(A) A 5′ $^{32}$P-end labeled primer is extended by DNA pol III α in the presence of wild-type SSB. Polyacrylamide gels were used to resolve the extended primer. (B) As the SSB concentration is increased, bands representing full or partial extension of the DNA primer become less intense indicating inhibition of DNA replication by SSB.
The observations that SSB inhibits the α subunit, but stimulates the activity of other DNA polymerases such as DNA pols II and IV (18, 38), suggest that there may be a direct interaction between α and SSB. To test this, a tryptophan fluorescence quenching assay was used to detect binding. When increasing amounts of wild-type SSB were added to the α subunit, a fluorescence quenching effect was observed, confirming that wild-type SSB binds to the α subunit (Figure 4.3). The equilibrium dissociation binding constant $K_D$ was determined to be $1.8 \pm 0.4 \mu M$ (Figure 4.3), suggesting that SSB has a higher affinity for the α subunit than for the χ/ψ complex whose binding constant $K_D$ was previously determined to be $8.9 \pm 0.7 \mu M$ (28).

![Figure 4.3 SSB binds DNA pol III α with a dissociation binding constant, $K_D$, of $1.8 \pm 0.4 \mu M$.]
To localize the $\alpha$ binding site on SSB, the SSB-113 variant, which contains the C-terminal tail mutation P177S (22), was employed (Figure 4.4). This mutation, which is also associated with the ssb-113 temperature sensitive allele (22), has been shown to disrupt the interaction between SSB and its binding partners whose binding sites have been located, including the $\chi$ subunit of DNA pol III (39-41). The binding constant between the $\alpha$ subunit and SSB-113 was determined to be $0.18 \pm 0.1 \mu M$ (Figure 4.5A), a 10-fold increase in binding affinity when compared to wild-type SSB. This suggests that unlike other SSB binding partners, $\alpha$ does not bind the C-terminal tail of SSB. To determine whether the globular OB-fold domain of SSB binds $\alpha$, a truncation of SSB that contains only the OB fold (resides 1-113; SSB-OB) was constructed (Figure 4.4). The binding constant $K_D$ for this truncation was determined to be $2.8 \pm 0.2 \mu M$ (Figure 4.5A), suggesting that $\alpha$ binds this domain. To our knowledge, no other protein has yet been shown to bind directly to this domain of SSB.

To further localize the binding site in this domain, two SSB variants were constructed; SSB F61A and SSB D91N (Figure 4.4). It is known that the residue F61 interacts with ssDNA by stacking with the nucleotide bases of ssDNA. Changing this position to an alanine has been shown to disrupt this interaction (10). The binding constant between SSB F61A and the $\alpha$ subunit was determined to be $2.2 \pm 1.5 \mu M$ (Figure 4.5A), similar to that of wild-type SSB suggesting that SSB binds $\alpha$ and ssDNA at distinct sites of SSB.

The residue D91 was selected as a possible binding site because it lies on the surface of the N-terminal globular domain. As shown in Figure 4.4, each of the monomers are concave in shape with the C-terminal tail extending from one end, the ssDNA binding residues located in the
pocket, and the D91 residue positioned at the other end. To test whether D91 is involved in binding α, the SSB D91N variant was constructed. The D91N mutation did not affect the ability of SSB to bind ssDNA (Figure 4.5B). The binding constant between SSB D91N and the α subunit was determined to be 10.9 ± 2.3 µM (Figure 4.5A), an approximately five-fold decrease in binding. We also constructed this mutation in SSB-OB. The truncated SSB with this mutation, SSB-OB D91N, also showed a slight decrease in binding (4.0 ± 0.7 µM) when compared to otherwise wild-type SSB-OB (Figure 4.5A). This confirms that the α subunit binds the N-terminal globular OB fold of SSB.

Figure 4.4 The structure (9) of the SSB homo-tetramer with the SSB variants used in this work indicated.

The phenylalanine residue at position 61 (blue) is one of the residues responsible for binding to ssDNA. Changing the residue to an alanine resulted in decreased binding affinity between SSB and DNA (10). P177S (orange) located in the unstructured and unresolved C-terminal peptide is the mutation associated with the ssb-113 temperature sensitive allele (22) and has been shown to disrupt the interaction with various proteins including the χ subunit of DNA pol III (4). D91N (purple) disrupts binding to DNA pol III α.
Figure 4.5 SSB D91N disrupts the interaction with DNA pol III α but not with ssDNA.  
(A) $K_D$ values were determined for the interaction between DNA pol III α and SSB variants. A 5-fold decrease in binding was observed with the SSB D91N variant and a 10-fold increase in binding with SSB-113, suggesting that DNA pol III α binds the OB fold of SSB and not the C-terminal peptide. (B) The affinity of SSB D91N (♦) for ssDNA is similar to that of wild-type SSB (▲).

4.3.2 SSB interacts with the C-terminal domain of DNA pol III α

To localize the binding site for SSB on the α polymerase subunit, we used a series of truncations of α that contain different domains. The first truncation used contained only the N-terminal PHP domain, residues 1-280. No binding was detected with α1-280 (data not shown). A truncation consisting of residues 1-835 includes the PHP domain and the polymerase domain. The binding constant $K_D$ between this α1-835 truncation and SSB was determined to be $36 \pm 18 \mu$M (Figure 4.6), a significant 18-fold decrease in binding, suggesting that SSB only very weakly binds the
N-terminal 835 residues of the α polymerase subunit. In order to assure that the α subunit binds SSB protein in the active conformation, activity of both alpha subunit variants was assayed for primer extension activity. Both α-835 and α-917 are active, with α-917 as active as wild-type α, but α-835 significantly less active (data not shown).

The remaining α residues 835-1160 compose the C-terminal “regulatory hub” of α (30, 42-45). To further localize the binding site in this domain, two additional truncations were used, α residues 1-917 and α residues 917-1160. The truncation α1-917, along with the PHP and polymerase domains, contains the two helix-hairpin-helix motifs shown to bind dsDNA (44). The binding constant $K_D$ for α1-917 binding to SSB was 2.5 ± 1.5 μM, comparable to that of full-length α. The C-terminal truncation, α917-1160 contains the OB-fold domain shown to bind ssDNA (44), as well as binding sites for the β processivity clamp (42, 43), the τ subunit of the clamp loader complex (45-48), and the SOS response protein UmuD$_2$ (30). The equilibrium dissociation binding constant for α917-1160 binding to SSB was 0.54 ±0.43 μM (Figure 4.6). Taken together, these observations confirm that the C-terminal residues 835-1160 of the α polymerase subunit of DNA pol III binds to SSB. Localizing this interaction further shows that SSB binds α at the C-terminal domain “regulatory hub” of α.
**Figure 4.6** SSB binds the C-terminal domain of DNA pol III α subunit.

The large difference between the binding constants for the truncation α1-835 and full-length α indicate that the binding site is between residues 835-1160. The domains of α are labeled in blue above the schematic of the protein. The previously determined binding sites for UmuD₂ (30), and the ε (49), β (42, 43), and τ (45-48) subunits of DNA pol III are labeled in black.

### 4.3.3 SSB inhibits DNA pol III α when bound to ssDNA

DNA pol III α activity was assayed in the presence of numerous SSB variants. As seen in Figure 4.2, approximately 150 nM wild-type SSB is needed to significantly inhibit α polymerase activity. SSB-113 and SSB-OB, whose binding affinities to α are similar to that of wild-type SSB, showed a similar inhibitory effect consistent with their similar binding affinities (Figures 4.7A and 4.7C). As with wild-type SSB, significant inhibition of α by SSB-113 at 150 nM is observed and only slightly less inhibition of α by SSB-OB is observed. With the SSB variants that disrupt binding to the α subunit, SSB D91N and the truncation SSB-OB D91N, a significant amount of inhibition is observed only at higher SSB concentrations of 500 nM and 250 nM, respectively, which again is consistent with their binding affinities for α (Figures 4.7B and
4.7D). On the other hand, the variant SSB F61A with a similar affinity for α to that of wild-type SSB, showed less inhibition of α (Figure 4.7E). Significant inhibition was only seen with 500 nM SSB F61A. The F61A mutation has been shown to disrupt the interaction between SSB and ssDNA but not with α (10) (Figure 4.5), suggesting that in addition to the direct SSB-α interaction, the interaction between SSB and ssDNA also contributes to inhibition of α subunit polymerase activity.
Figure 4.7 SSB F61A and SSB D91N only minimally inhibit replication by DNA pol III α.

Primer extension by DNA pol III in the presence of (A) SSB-113, (B) SSB D91N, (C) SSB-OB, (D) SSB-OB D91N, and (E) SSB F61A. The extent of inhibition of DNA pol III α polymerase activity by SSB-113, SSB D91N, SSB-OB, SSB-OB D91N is consistent with the observed binding constants. On the other hand, SSB F61A is not as proficient at inhibiting DNA pol III α as other variants even though its affinity for DNA pol III α is similar to that of WT SSB. This suggests that both direct binding to DNA pol III α and binding to DNA contribute to the ability of SSB to inhibit DNA polymerase activity of DNA pol III α.
4.3.4 DNA pol III α stabilizes SSB on ssDNA

To determine if the DNA pol III α subunit affects the interaction between SSB and ssDNA, single-molecule force spectroscopy, a technique involving the stretching of ssDNA with optical tweezers (37, 50-52), was used with and without SSB and the α subunit. Single-stranded DNA was generated by using a DNA molecule with biotin on either end of the same strand and using the exonucleolytic activity of T7 DNA polymerase to remove one strand of DNA (Figure 4.1). A greater force is needed to stretch ssDNA in the presence of SSB than with ssDNA alone (Figure 4.8). The change in force is directly proportional to the binding affinity of SSB to ssDNA (37, 50).

The ssDNA stretching force increases by 2-3 pN in the presence of 200 nM SSB in 50 mM Na$^+$ (Figure 4.8), indicating that SSB binds to ssDNA at low force. Once the stretching force exerted on the ssDNA surpasses 15 pN, SSB starts to dissociate from ssDNA and is completely dissociated around 20 pN (Figure 4.8A and 4.8B), consistent with previous observations (53). At this point, the stretching curve of ssDNA in the presence of SSB overlaps with that of ssDNA alone. In contrast, in the presence of 275 nM α subunit, SSB is not completely dissociated from the ssDNA at 20 pN and more force is required to dissociate SSB (Figure 4.8C). This suggests that the affinity of SSB for ssDNA increases in the presence of DNA pol III α as the binding of pol III α alone to ssDNA does not result in any change in ssDNA stretching force within experimental uncertainty (Figure 4.8B).
Furthermore, we have calculated the free energy (ΔG) required to disrupt the SSB-ssDNA interaction in the absence and presence of DNA pol III α (Figure 4.8C). In the presence of proteins that bind ssDNA, the DNA stretching force increases and therefore the free energy to disrupt these interactions can be estimated as the area in between the red and blue solid curves for starting points at length per nucleotide = 0.35 and ending points at force of 40 pN for both curves (Figure 4.8C). In the presence of DNA pol III α, the free energy to disrupt the ssDNA-SSB interaction, is 0.23 ± 0.04 k_BT/nt, as compared to 0.10 ± 0.02 k_BT/nt for the ssDNA-SSB interaction only, which shows that the ssDNA-SSB complex is significantly more stable in the presence of DNA pol III α.

Figure 4.8 DNA pol III α stabilizes the ssDNA/SSB interaction.

(A) Force-extension curves for ssDNA (blue solid line) and ssDNA/SSB complex (red solid line). (B) Force-extension curves for ssDNA (blue solid line) and ssDNA in the presence of α (red solid line). (C) Force-extension curves for ssDNA (blue solid line) and ssDNA/SSB complex (red solid and dotted lines, which represent the stretch and release, respectively) in the presence of DNA pol III α. All experiments were carried out in 10 mM Hepes, pH 7.5; 45 mM NaCl (50 mM Na^+).
4.4 DISCUSSION

In this work, we probed the inhibition of DNA pol III α by SSB. We determined that the N-terminal globular domain of SSB binds the C-terminal domain of α. By doing so, the α subunit stabilizes the interaction between SSB and ssDNA. We also observed that along with binding to the α subunit, SSB needs to be bound to ssDNA for inhibition to occur. Taken together, these observations provide a better understanding into how replication by DNA pol III is regulated in *E. coli*.

Other *E. coli* polymerases have been shown to bind SSB including the Y family polymerases DNA pol IV and DNA pol V (18, 19). In both cases, SSB increases the efficiency of these polymerases. With DNA pol IV, in order to efficiently replicate a template strand coated with SSB, the polymerase needs to interact with SSB (18). A similar observation, an increase in processivity, was seen when the RB69 DNA polymerase was fused with its cognate SSB forming a chimeric protein (54). The interaction between DNA pol V and SSB has been shown to increase access to the primer termini (19). The major difference between these interactions and the α-SSB interaction is that these polymerases bind the C-terminal tail (18, 19) while the α subunit binds the N-terminal globular domain of SSB.

As demonstrated in previous works and further probed here, the effect of SSB on DNA pol III is more complex. Even though SSB increases the processivity of the assembled holoenzyme, SSB
also inhibits replication when only the α polymerase subunit is present. The mutation associated with the temperature sensitive allele ssb-113 (23), P177S (SSB-113) has been shown to disrupt the interaction between SSB and other proteins (20). Interestingly, the opposite is seen with the α subunit, as P177S actually increases the affinity of SSB for the α subunit. It has been previously suggested that the C-terminal tail of SSB binds to the DNA-binding pocket in the OB fold when no DNA is present (55). If so and if the P177S mutation also disrupts this interaction, the N-terminal globular domain of the SSB-113 variant may be more exposed than that of wild-type SSB. Because α binds the N-terminal globular domain, this would provide α better access to SSB, and thus an increase in affinity is observed with the SSB-113 variant.

The χ subunit of DNA pol III has been shown to suppress the inhibition of DNA pol III caused by SSB, by binding to the C-terminal tail of SSB (4, 26-28). As with other proteins, the SSB-113 variant disrupts the interaction with χ. When the polymerase activity of the α subunit was tested in the presence of the SSB-113 variant, more inhibition was observed than with wild-type SSB. This suggests that the disruption in DNA replication observed with the ssb-113 allele is a direct result of the inability of the χ subunit to alleviate DNA pol III inhibition and the increased affinity for the α subunit.

Taken together, our findings suggest that the inhibition of α replication by SSB is directly due to the interaction between α and SSB and indirectly due to SSB binding to ssDNA. Moreover, the presence of α stabilizes SSB on ssDNA, further enhancing the inhibition. Thus, DNA replication in E. coli is regulated in multiple layers by interactions between α, SSB, and ssDNA.
4.5 REFERENCES


Chapter 5: The *E. coli* single stranded DNA binding protein SSB binds the UmuD$_2$ polymerase manager protein

Michelle C. Silva designed bulk biochemical experiments, purified proteins, performed experiments, analyzed the data, and wrote the manuscript; Arianna DiBenedetto, Celeste Dang, Christine Alves, and Monyrath Chan performed experiments and analyzed the data; Penny J. Beuning designed experiments and wrote the manuscript.

5.1 INTRODUCTION

The SOS-induced *umuD* gene products, full-length UmuD and the auto-cleavage product UmuD', contribute to the overall regulation of DNA replication in *Escherichia coli*. Full-length UmuD$_2$ is a very tight homodimer ($K_D < 10$ pM) (1) and so is expected to be in its dimer form under all conditions reported here. For the sake of clarity, we will refer to the dimer UmuD$_2$ simply as UmuD throughout this report. Each UmuD monomer consists of two domains; a dynamic N-terminal arms domain containing the cleavage site (between residues C24 and G25) and a globular C-terminal domain containing the active site (S60 and K97). Due to the dynamic nature of the arms, they can exist in various conformations: in an “arms down” conformation
where the cleavage site is positioned in the active site, and an “arms up” conformation where the arms are unbound exposing the globular domain (1-4).

During the first 40 minutes after the SOS response is initiated, full-length UmuD is the predominant form (5, 6). It is believed that during this time, UmuD participates in a primitive DNA damage checkpoint by inhibiting DNA replication and allowing time for accurate DNA repair processes to act (6-9). By binding to the α polymerase and β processivity clamp subunits of DNA polymerase III (DNA pol III), UmuD inhibits interactions between α and β and α and ssDNA, likely contributing to the primitive DNA damage checkpoint (3, 7, 10, 11).

After these initial 40 minutes, the RecA-ssDNA nucleoprotein filament-facilitated auto-cleavage product, UmuD’ becomes the predominant form resulting in the activation of translesion synthesis (TLS) (6, 12, 13). TLS is the potentially mutagenic process in which DNA lesions are bypassed by a group of specialized DNA polymerases known as the Y-family polymerases (14-21). In E. coli, two of the five DNA polymerases are part of this specialized group. They include DNA pol IV (DinB) and DNA pol V (UmuD’2C) both of which are regulated by the umuD gene products (22-24). It has been shown that along with RecA, UmuD prevents DNA pol IV from creating -1 frameshift mutations (25). DNA pol V contains two subunits, UmuD’ and the UmuC polymerase subunit (22, 23). UmuC is specifically activated for TLS in the presence of UmuD’ and RecA that has been activated by binding to ATP and ssDNA (26).

E. coli SSB has been shown to be a key player in numerous DNA processes such as DNA replication, recombination, and repair due to its ability to bind single-stranded DNA (ssDNA)
present at the replication fork (27-29). It is a homotetramer where each monomer contains an N-terminal globular domain and an inherently disordered C-terminal tail (30, 31). The N-terminal globular domain consists of an oligonucleotide binding (OB) fold containing the tryptophan and phenylalanine residues responsible for binding ssDNA (32-36). Recently it has been shown that this domain is also responsible for binding the α polymerase subunit of DNA pol III (Chapter 4). The C-terminal tail has been shown to serve as a hub for numerous proteins including the Y family polymerases DNA pol IV and DNA pol V (37-39), as the SSB-113 variant (P177S), associated with the ssb-113 temperature sensitive allele, has been shown to disrupt the interaction between these proteins and SSB (38-42).

It has been recently shown that SSB increases accessibility of DNA pol V to the primer-termini through a direct interaction between the C-terminal tail of SSB and the polymerase subunit of DNA pol V, UmuC (38). In this work, we probed the interactions between SSB and the UmuC partner proteins full-length UmuD and the cleaved form UmuD'. We find that these interactions contribute to the regulation of the interface between DNA replication under normal cellular conditions and under stress.
5.2 MATERIALS AND METHODS

5.2.1 Proteins, Strains and Plasmids

Strains and plasmids used are listed in Table 5.1. Full-length UmuD (pSG5), the cleaved product UmuD’ (pSG4) and the variant UmuD S60A were overexpressed in BL21-DE3 (Novagen) and purified as previously described (3, 43). The variant UmuD S60A contains a mutation in the active site that greatly reduces cleavage efficiency (3). Dr. Mark Sutton from the University at Buffalo provided the plasmid pEAW134 that encodes wild-type SSB. The SSB variants SSB-113 (P177S), SSB D91N, SSB-OB, and SSB-OB D91N were constructed as previously described (Chapter 4). Wild-type SSB and full-length SSB variants were expressed in BL21-DE3 (Novagen) and purified using the established protocol (44). The SSB truncations (SSB-OB and SSB-OB D91N) were purified as described in Chapter 4. Purified SSB protein was stored in a buffer containing 50% Glycerol and stored at -20 °C.

The plasmids used for assays of temperature sensitivity contain the operon expressing the genes umuDC (pGY9739), umuD’C (pGY9738), umuD (pGYDΔC), and umuD’ (pGYD’ΔC) and encode resistance to spectinomycin (60 µg/mL). The pGYC1 plasmid, encoding only umuC, was constructed from the pGY9739 plasmid by inserting XhoI restriction sites before and after the umuD gene, followed by digestion with XhoI and ligation. The restriction site were inserted by site-directed mutagenesis using a Quickchange kit (Agilent Technologies) and the primers Cxho (CCACGTCGTTAAGCTCGAGCGCTGATGTTTGC) and DCxho (CAGTATAACTCGAGGCAGATTATTATGTTTG) (Operon). The strain PAM33 (provided by
the Coli Genetic Stock Center at Yale University), which contains the temperature sensitive allele ssb-113, were grown in standard Luria broth media at 30 °C unless otherwise specified. Competent cells of this strain were prepared using the CaCl₂ method (45).

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<td>pGYD'ΔC</td>
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5.2.2 Tryptophan Fluorescence Assay

All equilibrium dissociation constants (K_D) were determined by observing the intrinsic fluorescence of tryptophan residues as previously described (11). Before each experiment, the SSB buffer was exchanged to Trp Assay buffer (50 mM HEPES, pH 7.5; 100 mM NaCl) using zebaspin desalting spin columns (Thermal Scientific) to remove excess glycerol. Varying volumes of 200-400 µM full-length UmuD or the cleaved product UmuD’ (monomer concentration) were titrated directly into 5 µM wild-type SSB or SSB variants (monomer concentration) in Trp Assay buffer. Samples were excited at 278 nm and the emission was
recorded between 300 and 400 nm. Peak maxima were used to calculate the fraction quenched (Q), which was then plotted versus protein concentration. All binding curves produced by this method were fitted as previously described (11).

5.2.3 Quantitative Transformation Assay

To determine the effect of elevated levels of the umuDC gene products on the temperature sensitivity of the ssb-113 allele phenotype, transformation assays were used. Approximately 0.2 µg of each respective plasmid was added to 40 µL of PAM33 competent cells. To induce intake, cells underwent a 10 minute incubation on ice, a 5 minute heat shock at 37 °C, and an additional 10 minute incubation on ice, after which the cells were allowed to recover for 2 hours at 30 °C in 400 µL of Luria broth with gentle shaking. Half of each transformation sample (200 µL each) was plated on each of two Luria broth agar plates supplemented with spectinomycin (60 µg/mL) which were then incubated at 30 °C and 37 °C, respectively. After 48 hours, cell colonies were counted and the ratio between the non-permissive (37 °C) versus the permissive (30 °C) temperatures were recorded. The assay was repeated at least three times.

5.2.4 Bis-maleimidohexane (BMH) Cross-linking

Bis-maleimidohexane (BMH) is used as a cross-linking agent to analyze the dynamic N-terminal arms region of UmuD (43). Reactions contained 10 µM UmuD S60A (monomer concentration), 40 µM SSB or SSB variant (monomer concentration) with and without 50 µM ssDNA (25-mer; Operon) in BMH buffer (10 mM Sodium Phosphate, pH 6.8; 100 mM NaCl). To initiate cross-
linking, 1 mM BMH was added and reactions were incubated at room temperature for 5 minutes, after which 50 mM DTT was added to quench the reactions. Covalently cross-linked UmuD dimers were separated from unreacted UmuD using 18% SDS-PAGE. An immunoblot was employed to detect UmuD-containing bands using rabbit anti-UmuD as the primary antibody as previously described (3, 43). The variant UmuD S60A was used to eliminate the possibility of UmuD’ contamination.

5.3 RESULTS

5.3.1 SSB binds the umuD gene products

A previous study has shown that the presence of SSB increases accessibility of the 3’ primer terminus at the site of DNA damage for the TLS polymerase DNA pol V (38). This study also showed by immunoprecipitation that the polymerase subunit of DNA pol V, UmuC, directly binds to wild-type SSB (38). This study led us to hypothesize that SSB binds to the other subunit of DNA pol V, UmuD’ and its precursor, full-length UmuD, which has been shown to regulate replication by binding to proteins essential to DNA replication (3, 9, 11, 49, 50).

To determine if the umuD gene products bind to SSB, a tryptophan fluorescence assay was employed. This assay involves observing the intrinsic fluorescence of the tryptophan residues of SSB in the presence of varying concentrations of full-length UmuD or UmuD’, which have no
tryptophan residues. As shown in Figure 5.1, fluorescence quenching was observed with increasing concentrations of UmuD or UmuD’. This indicates that SSB does indeed bind both proteins. The equilibrium dissociation constants ($K_D$) for the SSB-UmuD and SSB-UmuD’ interactions were determined to be $14 \pm 1.7 \, \mu\text{M}$ and $6.6 \pm 0.7 \, \mu\text{M}$, respectively, suggesting that UmuD’ has slightly higher affinity for SSB than full-length UmuD.

![Figure 5.1](image)

**Figure 5.1 Wild-type SSB binds the umuD gene products.**
The curves represent the fraction of SSB tryptophan fluorescence quenched (Q) versus the ratio of UmuD to SSB concentration fitted as previously described (11). The UmuD’ binding curve (red) shows a slightly greater affinity for wild type SSB than that of the binding curve for full-length UmuD (blue) ($K_D^{\text{UmuD}} = 14 \pm 1.7 \, \mu\text{M}$; $K_D^{\text{UmuD}'} = 6.6 \pm 0.7 \, \mu\text{M}$). Binding was detected in Trp Assay buffer. The error bars represent the standard deviation of four experiments.
5.3.2 The variant SSB-113 disrupts the interaction with UmuD

Like other SSB binding partners, the polymerase UmuC was found to bind the disordered C-terminal tail of SSB due to the fact that the SSB-113 variant disrupts this interaction (38). To determine if UmuD or UmuD’ bind at the same binding site, the variant SSB-113 was utilized. The dissociation constants for the interaction between UmuD and SSB-113 and between UmuD’ and SSB-113 were found to be 50 ± 13 µM and 34 ± 7.5 µM, respectively, a 4-5-fold decrease in binding affinity (Figure 5.2). This suggests that, like other proteins that bind SSB, both UmuD and UmuD’ bind the C-terminal tail of SSB.

Since mutation of the C-terminal tail of SSB was not sufficient to eliminate binding, binding was also probed using the SSB truncation, SSB-OB. This truncation contains the first 113 residues containing the OB fold responsible for binding ssDNA and lacks the C-terminal tail known for binding other proteins. Surprisingly, only a 2-fold decrease in affinity was observed relative to wild-type SSB ($K_D^{UmuD} = 27 \pm 5.7 \mu M$; $K_D^{UmuD’} = 16 \pm 3.7 \mu M$) (Figure 5.2). If the C-terminal tail were the only binding site, a greater decrease in affinity would be expected with SSB-OB. This suggests that both the N-terminal globular domain and the C-terminal tail of SSB are involved in binding to full-length UmuD and UmuD’.

To date, the only other protein shown to interact with this N-terminal globular domain of SSB has been the α polymerase subunit of DNA pol III (Chapter 4). This α-SSB interaction was disrupted by substituting Asp91 with asparagine (SSB D91N) (Chapter 4). To test if UmuD or UmuD’ interacted with SSB in the same manner, binding was probed with the SSB D91N.
variant. A disruption in binding was observed with UmuD' but not with full-length UmuD
($K_D^{\text{UmuD}} = 11 \pm 1.3 \mu\text{M}; K_D^{\text{UmuD'}} = 22 \pm 5.6 \mu\text{M}$) (Figure 5.2). This suggests the N-terminal
globular domain may preferentially bind the cleaved UmuD' over full-length UmuD. The same
mutation in the context of SSB-OB eliminates discrimination between UmuD and UmuD'
(Figure 5.2).

![Figure 5.2 Equilibrium dissociation constant $K_D$ between UmuD or UmuD' and SSB variants measured by tryptophan fluorescence quenching.](image)

The variant SSB-113 (P177S) disrupts the binding between UmuD and UmuD' ($K_D^{\text{UmuD}} = 50 \pm 13 \mu\text{M}; K_D^{\text{UmuD'}} = 34 \pm 7.5 \mu\text{M}$) suggesting that UmuD and UmuD' bind the C-terminal tail of
SSB. The truncation of the N-terminal globular domain of SSB, SSB-OB shows only a slight
disruption in binding ($K_D^{\text{UmuD}} = 27 \pm 5.7 \mu\text{M}; K_D^{\text{UmuD'}} = 16 \pm 3.7 \mu\text{M}$). A mutation in this
domain SSB D91N shows a disruption in binding for only UmuD' ($K_D^{\text{UmuD}} = 11 \pm 1.3 \mu\text{M};
K_D^{\text{UmuD'}} = 22 \pm 5.6 \mu\text{M}$) when compared with wild-type SSB and UmuD' ($K_D^{\text{UmuD}} = 14 \pm 1.7
\mu\text{M}; K_D^{\text{UmuD'}} = 6.6 \pm 0.7 \mu\text{M}$). The D91 mutation in the context of SSB-OB eliminates
discrimination between UmuD and UmuD'. Error bars represent dissociation constant standard
error of at least three trials.
5.3.3 Full-length UmuD but not UmuD' complements the phenotype of the ssb-113 allele

The ssb-113 allele is an intensely studied ssb allele that confers a temperature sensitive phenotype for growth at 37 °C on strains that harbor it (40, 42). The PAM33 strain containing the ssb-113 allele was transformed with plasmids expressing the umuDC genes in order to test whether elevated levels of UmuD, UmuD', or UmuC suppress the phenotype. As seen in Figure 5.3A and Figure 5.3B, elevated levels of UmuD and UmuC (expressed from the plasmid pGY9739) suppress the temperature sensitive phenotype. In contrast, elevated levels of UmuD' and UmuC (expressed from pGY9738) only partially suppress the temperature sensitivity of the ssb-113 strain (Figure 5.3A and 5.3B). To further probe the suppression of temperature sensitivity, PAM33 was transformed with plasmids expressing umuD, umuD', and umuC individually. With these plasmids, growth at 37 °C was seen with umuD and umuC but not with umuD' (Figure 5.3A and 5.3B). Consequently, these observations show that UmuD and UmuC but not UmuD' suppress the temperature sensitivity of the ssb-113 strain. This suggests that the interactions between UmuD and SSB and between UmuD' and SSB play different roles in vivo.
Figure 5.3 Overproduction of UmuD but not UmuD' suppresses the temperature sensitive conditional lethality phenotype of the ssb-113 allele.

pGB2 = empty vector; pGY9739 = UmuDC; pGY9738 = UmuD'C; pGYDΔC = UmuD alone; pGYD'ΔC = UmuD' alone; pGYC1 = UmuC alone. (A) The PAM33 strain containing the ssb-113 allele was transformed with different plasmids. Equal amounts of each transformation reaction were plated on two to three plates (Luria broth-agar-spec), one for each temperature tested (30 °C, 37 °C, 42 °C). Plates were incubated at the appropriate temperature for 48 hours. (B) The ratio of cell growth 37 °C /30 °C is shown. Error bars represent the standard deviation of at least four independent trials.

5.3.4 SSB does not disrupt the dynamics of the N-terminal arms of UmuD

The N-terminal arms of UmuD are only weakly bound to the globular domain (4). The dynamic nature of the UmuD arms when UmuD is bound to other proteins can be probed with the thiol-specific, homobifunctional cross-linking agent BMH (11). Because each UmuD monomer contains only one cysteine residue at residue position 24, two covalently linked UmuD
monomers result from this cross-linking reaction. In order for cross-linking to occur, the two UmuD cysteine residues, one from each monomer, must be no more than 13 Å away from each other, which occurs only when the N-terminal arms of UmuD are unbound from the C-terminal globular domain. Hence this technique is useful to determine if the dynamic nature of the N-terminal arms of UmuD is altered in the presence of SSB.

The cross-linking efficiency of the variant UmuD S60A in the presence of SSB was compared to the cross-linking efficiency of UmuD S60A alone. As shown in Figure 5.4Ai, the presence of wild-type SSB, as well as the variants SSB-113 and SSB-OB, did not significantly change the cross-linking efficiency of UmuD. The addition of ssDNA had no effect as well (Figure 5.4Aii). The reaction was repeated multiple times resulting in no statistically significant change in cross-linking efficiency (Figure 5.4B). These results suggest that the interaction between SSB and UmuD does not change the dynamic nature of the N-terminal arms.

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Figure 5.4 The interaction between SSB and UmuD does not affect the dynamic nature of the N-terminal arms of UmuD.

(A) Percent UmuD-S60A cross-linked by bis-maleimido-heptane (BMH) in the presence of wild-type SSB, SSB-113, and SSB-OB (i) without ssDNA and (ii) with SSB. (B) Bar graph showing the average % cross-linked UmuD of three independent trials where error bars represent standard deviation.

5.4 DISCUSSION

In this work, we show that SSB binds both full-length UmuD and the cleavage product UmuD’, and that SSB has a slight increase in affinity for UmuD’ versus full-length UmuD. As with other binding partners, binding was localized to the C-terminal tail due to the decrease in binding with the SSB-113 variant. But unlike other protein binding partners, UmuD and especially UmuD’ also bind the globular N-terminal domain as binding is observed with the SSB-OB truncation. In vivo, elevated levels of UmuD and UmuC but not UmuD’ suppress the temperature sensitive phenotype of the ssb-113 allele, suggesting that the interactions between UmuD and SSB and between UmuD’ and SSB have different cellular consequences.

Due to the fact that UmuD is a key player in managing proteins involved in DNA replication and translesion synthesis, we hypothesized that UmuD would bind SSB, a protein that is also involved these DNA processes. As previously documented, full-length UmuD participates in a primitive DNA damage checkpoint (6, 7, 9). Furthermore, UmuD disrupts the interaction between the α and β subunits of DNA pol III by binding the two subunits (3, 11, 21), as well as disrupts the interaction between α and ssDNA (10). In addition, SSB has also been shown to
interact with various polymerases in *E. coli*, including the DNA pol III α subunit (Chapter 4), DNA pol IV (39) and the UmuC subunit of DNA pol V (38).

Of the approximately 15 proteins to date that have been shown to bind SSB, most have been shown to interact with the C-terminal tail (37). Only one protein, the DNA pol III α has been shown to interact with the N-terminal globular domain (Chapter 4). As we have shown here, the interactions between SSB and UmuD or UmuD' involve both the globular domain and the C-terminal peptide of SSB. There are two possible explanations for binding to the C-terminal tail. Because this region binds various proteins involved with different DNA processes, it has been suggested that this region serves as a protein hub providing access to DNA primer termini for these proteins. This increase in accessibility was shown with the interaction between SSB and UmuC (38) and so it seems possible that the interaction between SSB and UmuD/D' would serve the same purpose. The other possibility is that SSB may be required to directly interact to both subunits of DNA pol V (UmuD'C) to efficiently replicate DNA when the template is coated with SSB, similar to the increase in replication efficiency seen when DNA pol IV (DinB) binds SSB (39). On the other hand, the interaction with the N-terminal globular domain of SSB suggests that interaction of UmuD or UmuD' with SSB may affect the interaction between DNA pol III α and SSB.

As previously mentioned, the SSB P177S mutation associated with the *ssb-113* allele has a temperature sensitive phenotype due to the disruption of DNA replication (41). It has also been suggested that this disruption is due to the increased affinity of SSB-113 for DNA pol III α when compared with wild-type SSB, which in turn causes the inhibition of replication (Chapter 4). If
this is the case, the fact that UmuD suppresses the temperature sensitivity suggests that UmuD may disrupt the interaction between DNA pol III α and SSB. If so, it would be interesting to see if UmuD has an effect on the interaction of SSB with ssDNA as well. This would further solidify the role of UmuD as a manager protein in DNA replication.

5.5 REFERENCES


Chapter 6: Conclusions and Future Considerations

In 1985, it was reported that elevated levels of UmuDC inhibit DNA replication (1). Thus it was concluded that UmuD participates in a temporal primitive DNA damage checkpoint (Figure 6.1). Since then, it was determined that UmuD and UmuD’ bind the α, β, and ε subunits of DNA pol III suggesting a role in regulation of DNA replication (2). By far, the most extensively investigated interaction of these has been the interaction between UmuD and the β clamp (3-6). In contrast, the interaction with the ε subunit remains to be fully characterized. In this work, we report that UmuD prevents the α subunit from binding the β clamp and single-stranded DNA through the direct interaction between UmuD and the α subunit. We have also shown that the single-stranded DNA binding protein (SSB) binds UmuD and the α subunit. Overall, these observations suggest that UmuD participates in the primitive DNA checkpoint by removing the α subunit from the replication fork, thus disrupting replication, and by regulating other proteins associated with replication through protein-protein interactions.
Figure 6.1 The *umuD* gene products regulate *E. coli* DNA replication in the presence of DNA damage.

(A) UmuD (purple) disrupts the interactions of DNA pol III α (green) with the β processivity clamp (blue) and ssDNA, releasing the polymerase from the primer-termini. DNA damage is represented by a red X. (B) Full-length UmuD (purple) undergoes self-cleavage to the cleaved form, UmuD’. This reaction is facilitated by the RecA/ssDNA filament (blue). (C) UmuD’ (purple) activates UmuC (orange) for translesion synthesis (TLS); together, they make up DNA pol V. The interaction between UmuD and SSB also contributes to the regulation of DNA replication (not shown).
UmuD participates in a temporal switch by undergoing RecA/ssDNA facilitated self-cleavage to UmuD', the cleaved form (7) (Figure 6.1B). This takes approximately 20-40 minutes, after which UmuD' is the predominant product of the *umuD* gene. Previous work has shown that UmuD' activates UmuC for translesion synthesis (8, 9) (Figure 6.1C). When full-length UmuD is the predominant product, we now know that full-length UmuD but not UmuD' prevents the α subunit from binding the β clamp and ssDNA (Figure 6.1A). This suggests a timeline for UmuD regulation of DNA replication. When expression of the *umuD* gene is first upregulated due to the SOS response, the role of UmuD is to release the stalled replication fork, after which UmuD'C or DNA pol V bypasses the DNA damage. Thus the *umuD* gene products are important for DNA regulation under cellular stress. Although much progress has been made, more questions still remain:

**6.1 Does UmuD affect the processivity of DNA pol III?**

Because UmuD disrupts the interaction between the α subunit and the β clamp, which is the major contributor to processivity of DNA pol III (10), the next step would be to ascertain if UmuD has any effect on processivity. Processivity is referred to as the number of nucleotides incorporated onto the growing DNA strand per binding event. The α subunit alone exerts low processivity, approximately 1-10 nucleotides (10). When the β clamp is added, processivity levels can increase to near the levels for the entire DNA pol III holoenzyme (>50 kb) (10-12). Because wild-type UmuD also disrupts the α/ssDNA interaction, in order to test processivity, it
would be necessary to create a UmuD variant that does not affect the ability of the α subunit to bind ssDNA.

6.2 Do these proteins bind as a complex?

Now that we have shown that UmuD binds numerous proteins associated with DNA replication, the next question is whether these interactions occur independently or in complex with each other. For instance, when UmuD disrupts the interaction between the α subunit and the β clamp, is UmuD binding to only the α subunit, only the β clamp, or both? In Chapter 3, we showed that the UmuD variants D91K and D91A disrupt the interaction between the α subunit and UmuD. Do these variants also disrupt the interaction between the β clamp and UmuD? If these UmuD variants still interact with the β clamp, the contributions of the UmuD-α versus the UmuD-β interactions to prevent the binding of α to the β clamp can be tested. Methods such as gel filtration or pull-downs can be used to determine if the α subunit, the β clamp, and UmuD bind as a complex.
6.3 Does UmuD also have a role in regulating DNA repair?

As previously mentioned UmuD participates in a primitive DNA damage checkpoint by pausing replication allowing time for other non-mutagenic DNA repair mechanisms to occur (1, 13). Does this mean that UmuD could contribute to DNA repair mechanisms such as homologous recombination? For instance, it may be possible that the presence of UmuD increases the accessibility of proteins involved in repair to the replication fork, thus increasing efficiency. UmuD is already associated with RecA which is responsible for strand exchange in homologous recombination. The auto-cleavage of full-length UmuD to UmuD' is facilitated by the RecA/ssDNA filament (6, 7). RecA is also needed to activate pol V (UmuD'C) for TLS and pol V inhibits RecA-mediated homologous recombination (14, 15). UmuD may be the key to understanding the transition between repair and translesion synthesis.

Even though there has not yet been a high resolution structure solved for full-length UmuD, new findings regarding how UmuD regulates DNA replication and mutagenesis have been discovered within the past few years, including our work presented here. Taken together, these observations further establish the role of UmuD as a manager protein.
6.3 REFERENCES


