DESIGN AND CHARACTERIZATION OF LEXA DIMER INTERFACE MUTANTS

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Abstract

Two key proteins, LexA and RecA, are involved in regulation of the SOS expression system in bacteria. LexA and RecA act as the transcriptional repressor and inducer of the SOS operon, respectively. LexA downregulates the expression of at least 43 unlinked genes and activated RecA interacts with the repressor LexA and therefore, LexA undergoes self-cleavage. The ability of the LexA protein to dimerize is critical for its ability to repress SOS-regulated genes *in vivo*, as the N-terminal domain (NTD) alone has a lower DNA-binding affinity without the C-terminal domain (CTD) and the components for the dimerization of LexA are located in the CTD. Two antiparallel β-strands (termed β-11) in the CTD at the dimer interface of LexA are involved in the dimerization. LexA interacts with the active form of RecA *in vivo* during the SOS response. It was determined experimentally that monomeric and non-cleavable LexA binds more tightly to RecA and is resistant to self-cleavage. Therefore, we reasoned that if we can produce such LexA mutants we would be able to stabilize the LexA and active RecA complex for crystallization. Therefore, in this experiment, we attempted to make a non-cleavable and predominantly monomeric LexA that interacts intimately with RecA. We produced four single mutations at the dimer interface of the non-cleavable and NTD-truncated mutant of LexA (Δ_{68}LexAK156A) in order to weaken the interactions at the interface. The predominant forms of LexA mutants and the affinities of interaction between the mutant LexA proteins and RecA were examined. Δ_{68}LexAK156AR197P mutant was found as predominantly monomeric at a concentration of 33.3 μM both by gel filtration chromatography and dynamic light scattering (DLS) experiments. It also bound RecA more tightly than wild-type LexA. Another mutant, Δ_{68}LexAK156AI196Y, was also found as predominantly monomeric at a concentration of 33.3 μM by DLS. Both these proteins were subjected to crystallization with wild-type RecA protein. We were able to produce some predominantly monomeric LexA with good binding affinity for RecA; however, we were unsuccessful in co-crystallization.

Acknowledgement

First of all I want to acknowledge my supervisor Dr. Yu Luo for his inspiration and support to carry on my research project by providing interesting ideas, thoughts, opportunities and funding. His ingenious direction in this research was helpful for me to complete the work. He made me involved in several research areas in his lab and provided all the technical and intellectual supports. I want to thank all the lab members I met throughout my research period for their help and inspiration. I want to thank Mr. Jason Maley for the technical support in the Saskatchewan Structural Science Centre (SSSC). I am thankful to Canadian Istitute of Health research (CIHR) who provided the fund for my research project.

Dedication

This paper is dedicated to my uncle and aunt, Dr. Uma Aryal and Mrs. Nazmun Naher, who made my life easier in a foreign country.

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List of Abbreviations

ADP	Adenosine diphosphate
AMP-PNP	5'-adenylylimidodiphosphate
AMP-PCP	Adenosine 5'-(β,γ-methylene) triphosphate
ATP	Adenosine triphosphate
CFU	Colony forming units
d	Deoxy prefix
DNA	Deoxyribonucleic acid
ds	Double-stranded prefix
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
His tag	Hexahistidine tag
IPTG	Isopropyl-thio-β-D-galactoside
K_d	Dimer dissociation constant
LB	Luria Bertani
MES	N-(morpholino)ethanesulfonic acid
Mr	Molecular mass
MW	Molecular weight
NTP	Nucleoside triphosphate
OD	Optical density
PCR	Polymerase chain reaction

PDB	Protein Data Bank
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RMSD	Root-mean-square deviation
SDS	Sodium dodecyl sulphate
Ser/Lys	Serine/Lysine
ss	Single-stranded prefix
TAE	Tris-acetate-EDTA
Tris	Tris(hydroxymethyl)aminomethane
wt	Wild type

Chapter One: **Introduction**

Damage to the bacterial DNA by external agents such as ultra violet (UV) radiation can lead to ultimate cell death. Therefore, different DNA repair mechanisms have been developed by bacterial species to encounter the danger. The SOS response is such a coordinated cellular response to bacterial DNA damage. The expression of more than 40 genes in the SOS network is induced by DNA damage. Two key proteins, LexA and RecA, are involved in regulation of the expression system of the SOS operon (Courcelle *et al*., 2001a; Fernandez *et al*., 2000; Janion, *et al*., 2002; Walker, 1985; Witkin, 1991). LexA and RecA act as the transcriptional repressor and inducer of the SOS system, respectively. Under normal cellular conditions, the LexA protein binds to the operator region of SOS genes, called 'the SOS box', and downregulates the expression of at least 43 unlinked genes, including the *lexA* gene itself (Courcelle *et. al.*, 2001a; Fernandez *et al*., 2000). Damage during DNA replication produces single-stranded DNA (ssDNA) at the lesion site, which initiates the SOS response mechanism. RecA, a bacterial recombinase protein, wraps around the ssDNA forming a nucleoprotein filament and becomes active in the presence of ATP. The active RecA nucleoprotein filament interacts with the repressor LexA protein. As a result of this interaction LexA is proteolytically cleaved in a RecA-dependent fashion. This action is defined as the co-protease activity of RecA (Horrii *et al*., 1981; Kowalczykowski *et al*; 1994; Little *et al*., 1980). As a result, the concentration of LexA in the cell drops approximately tenfold (~2 μM to ~0.2 μM) and SOS genes are derepressed (Sassanfar and Roberts, 1990). DNA polymerase V, an error prone bacterial replication machine, forms due to *umuCD* gene expression in the SOS operon. This DNA polymerase V repairs the lesion site by an error-prone DNA replication procedure. After the damage is repaired, the induction signal disappears and the cell returns to the normal conditions. The amount of LexA in the cell also returns to normal.

The *E. coli* LexA repressor is a 22.7 kD protein and contains 202 residues (Little and Mount, 1982; Walker, 1985). It has two domains, an N-terminal DNA-binding domain (NTD, 1-69 residues) and a C-terminal dimerization and catalytic domain (CTD, 75-202 residues) (Hurstle et al., 1986; Luo *et al*., 2001). A short hinge region joins the two domains. LexA usually remains in the dimeric form under normal cellular conditions. Again, the two-fold symmetry of the SOS box suggests that LexA binds DNA as a dimer. The ability of LexA

protein to dimerize is critical for its ability to repress SOS-regulated genes *in vivo*, as the NTD alone has a lower affinity for DNA. Two monomers of LexA bind to the imperfect palindrome sequence of the DNA with their NTDs, which harbour a helix-turn-helix DNA-binding motif (Fogh *et al*., 1994; Knegtel *et al*., 1995). The two C-terminal domains (CTDs) from the two DNA-bound LexAs interact to form a dimer (Schnarr *et al*., 1988; Schnarr *et al*., 1985). The cleavage site, Ala84-Gly85, is also found in the C-terminal domain. Cleavage at this site separates the two domains and the LexA NTDs are split from the DNA-binding sequence. The SOS genes are thus derepressed and their expression occurs.

LexA slowly undergoes auto-cleavage in solution (Little, 1984). Active RecA stimulates the latent capacity of LexA for auto-digestion (Little, 1991). Two residues of the CTD of LexA, Ser119 and Lys156, serve as the catalytic residues during the cleavage (Ser/Lys dyad). This dyad cleaves the peptide bond between Ala84 and Gly85. All the LexAs from different bacterial species and its homologues (UmuD and λ repressor, for example) contain these conserved residues. The Ser/Lys dyad model is supported by mutational studies of these residues (Lin and Little, 1989; Lin and Little, 1988; Slilaty and Little, 1987). Replacing either the Ser119 or Lys156 residues with Ala (S119A or K156A mutations) produces non-cleavable LexA. RecA does not directly participate in the chemistry of the cleavage reaction. Therefore, it is called 'the co-protease'.

RecA is bacterial recombinase protein that has at least three known functions: homologous recombination, co-protease activity and translesion synthesis. Almost all forms of organisms contain proteins from the recombinase family. During replication or DNA damage, RecA monomers polymerize around the ssDNA in the 5′-3′ direction, forming a nucleoprotein filament with ~6 (6.16) monomers per turn in the presence of ATP or its analogs (Chen *et al*., 2008). ATP binding facilitates an allosteric conformational transition in RecA, which causes the RecA self-polymer to be stretched approximately 50-60% to make it a 93.96 Å pitch per turn. The RecA-bound DNA is also underwound to 18.5 base pairs (bp) per turn (Chen *et al*., 2008; DiCapua *et al*., 1992; DiCapua *et al*., 1990; Nishinaka *et al*., 1997; Yu *et al*., 2001). This filament provides binding sites for other molecules (three DNA strands or proteins such as LexA). The dimer dissociation constant of LexA (K_{dimer} or K_d) in solution was reported to be in the picomolar range determining by experiments involving pressure-dependent changes in the

LexA intrinsic tryptophan (residue number 201, buried in the dimer interface) (Mohana-Borges *et al*., 2000), but was not confirmed by other experiments. If true, this indicates that the LexA interacts with the RecA-ssDNA complex as dimer rather than monomer; though historical findings suggest that the LexA monomer binds RecA tighter than the dimer (Schnarr *et al*., 1988). However, the interactions of LexA with the RecA filament are hard to predict until a crystal structure of the complex is solved.

Highly ordered arrangements of molecules, called crystals, are abundant in nature. Various kinds of small molecules and macromolecules form crystals spontaneously. Likewise, protein molecules also can be prompted to form crystals when placed in appropriate conditions. Crystals are composed of a repeating series of unit cells where proteins align themselves in a consistent orientation. The lattice thus formed during crystallization is held together by non-covalent interactions (Rhodes, 1993). Protein crystals serve as a tool to solve three-dimensional structures via X-ray diffraction using a method called X-ray crystallography. RecA forms a self-polymer in a helical arrangement with or without DNA molecules. The two conformations of the RecA protein (compressed and extended) are quite different with regard to the helical pitch of the filaments. The active conformation has a helical pitch of ~94 Å (Chen *et al*., 2008; DiCapua *et al*., 1990; Flory *et al*., 1984; Morimatsu *et al*., 2000; VanLoock *et al*., 2003b**;** Yu *et al*., 2001) and inactive conformation has a pitch of ~82 Å (DiCapua *et al*., 1990; Flory *et al*., 1984; Vanlook *et al*., 2003b; Yu *et al*., 2001). The active conformation of RecA has always been difficult to crystallize. Chen *et al*. (2008) instead solved the crystal structure by physically joining the RecA subunits. Therefore, we reasoned that a protein that binds intimately to RecA in its active conformation may be able to stabilize that active conformation during crystal packing. LexA protein interacts with the active form of RecA. Due to this interaction, we presumed that LexA can be used as a tool to stabilize the active RecA conformation during crystallization. This will also help in understanding the structural details of the co-protease activity of RecA. The difficulty in crystallizing the RecA-LexA complex is that active RecA mediates the autocleavage of wild-type LexA. Again, the LexA monomer binds more tightly to RecA than the dimer (Schnarr *et al*., 1988). However, in a co-crystallization trial of RecA and LexA, the protein concentration has to be around 1 mM. Again, the dimer dissociation constant (K_d) of LexA is in the picomolar range (Mohana-Borges *et al*., 2000). Because of these traits,

LexA should predominantly exist as a dimer in co-crystallization experiments. Therefore, an attempt was previously undertaken in our lab to make a LexA mutant that is predominantly monomeric in solution, can tightly bind RecA and is resistant to auto-cleavage (Moya, 2006). Though it was possible to produce monomeric LexA mutants, the co-crystallization of LexA and RecA was unsuccessful. A crystal structure of the LexA dimer shows that interactions between the antiparallel β-11 strands at the dimer interfaces are favorable for dimer stabilization (Luo *et al*., 2001). Therefore, single mutations in the terminal residues of β-11 may produce monomeric LexA. In addition, the K156A mutation can make it non-cleavable. In this study, single mutations were introduced at three consecutive positions (residues 195 to 197) in the β-11 strands at the dimer interface of LexA in order to increase the K_d value to produce monomeric LexA. Their binding ability with RecA was also observed. Finally, the predominantly monomeric LexA forms found in the experiments were subjected to crystallization with RecA.

Chapter Two: **Literature review**

2.1 LexA is a repressor protein

Control of gene expression can be maintained at several points in living systems. Transcription initiation is a major control point of gene expression regulation. Recognition of the promoters by RNA polymerase can be inhibited or stimulated during transcription initiation. Many different kinds of regulatory molecules have been reported to enhance or reduce the stability of the transcription complex in prokaryotes (Browning and Busby, 2004). A bacterial protein, LexA, acts as this type of transcriptional regulator in bacterial cells. Under normal cellular conditions, LexA protein binds to the operator region of the SOS genes, called 'the SOS box', and downregulates the expression of at least 43 unlinked genes, including the *lexA* gene itself (Courcell *et al*., 2001a). SOS genes are expressed when the cell is threatened by any external agents and its DNA is damaged. With very few exceptions, LexA acts as a repressor protein of these genes found in a diverse range of bacteria.

2.2 SOS transcriptional response

The regulation of gene expression in response to different environmental conditions is necessary for the survival of the cell, as well as maintaining the structural and functional integrity of the genome. The SOS response is a coordinated cellular response to DNA damage and is found in most bacterial species. The Ames test, which is based on SOS mutagenesis, provided evidence about the relationship between bacterial DNA damage and the SOS response (Ames, 1973). The SOS response was first elaborately described in *Escherichia coli* (Walker, 1984). However, this phenomenon was first proposed and named by Miroslav Radman in 1974 (Radman, 1974). The SOS system is a programmed DNA repair regulatory network, which results in DNA mutagenesis and genetic exchange or recombination (Matic *et al*., 1995). This presumably helps the bacteria evolution, especially under stress (Radman *et al*., 1999). Stalled DNA replication and damage can occur through metabolic intermediates in the well-fed (Cox *et al*., 2000) or starved cells (Taddei *et al*., 1995). Exogenous treatments or agents that elicit DNA damage, or even the DNA replication fork can induce the SOS response in bacteria (Erill and Barbe, 2007; Kelley, 2006).

The interplay between two key regulatory proteins controls the SOS system. One of those proteins is the LexA repressor protein, which downregulates its own and several other proteins' expression during normal cell growth (Courcelle, *et al*., 2001a; Fernandez *et al*., 2000). The second is the inducer protein, RecA. RecA is a recombinase protein, which leads homologous DNA recombination in bacteria. The RecA protein becomes active by binding to single-stranded DNA in response to DNA damage or during homologous recombination and forms a nucleoprotein filament in the presence of ATP (Chen *et al*., 2008; Cox, 2007b). The ssDNA-RecA-ATP filament interacts with LexA and activates the self-cleavage activity of the LexA, which is also termed as 'auto-cleavage' (Little, 1991). RecA does not directly take part in the catalysis reaction and this is why RecA is also called the 'co-protease'. Self-cleavage occurs between residue Ala84 and Gly85 of the C-terminal domain (CTD) of LexA and while this occurs LexA dissociates from the SOS boxes. This causes induction of the expression of SOS genes. The co-protease activity of the RecA protein disappears after the DNA damage is repaired and allows functional LexA to re-accumulate and bind to the target DNA sites to prevent further expression of the SOS genes (Erill and Barbe, 2007).

2.2.1 Mechanism of the SOS mutagenesis

The basal level of LexA in the cell is high enough to repress most of the SOS genes and *lexA* itself. Upon cleavage by the RecA protein, LexA cannot bind to the operator; therefore, several genes from the SOS regulon are expressed to perform error-prone DNA replication to repair the damage. After the damage repair, the number of LexA molecules increases and the SOS genes are again repressed. A cascade of three major repair processes takes place during the SOS response: excision, recombination and mutagenic repair. The DNA lesions are removed in the first step by nucleotide excision or base excision. Recombination, greatly aided by RecA induction, occurs next and the RecA concentration increases to at least 100,000 monomers in the cell. This recombinational repair lasts for about 30 minutes. However, not all the lesions are removed by recombinational repair. Therefore, mutagenic repair takes place. Finally, after completion of the damage repair, the SOS signals disappear and the SOS genes are again blocked by the LexA repressors. Cell division then resumes normally. Figure 2.1 shows different steps during this mutagenic DNA repair system.

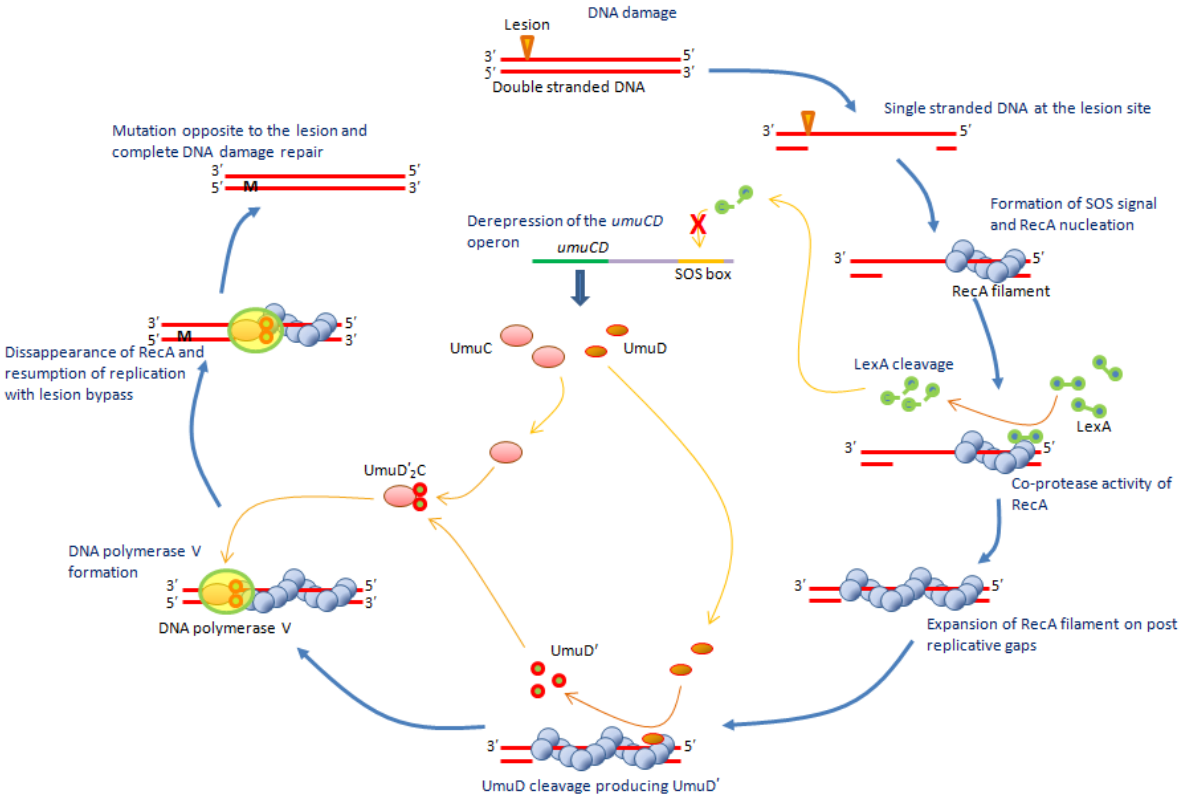


Figure 2.1 SOS mutagenic DNA repair.

The SOS mutagenesis is an error-prone DNA replication process that allows the bypass of DNA lesions induced by UV or other external agents via a special kind of DNA repair machinery (Walker, 1985). Transcriptional derepression of the SOS genes occurs at the initiation of this process, which includes cleavage of the LexA repressors by activated RecA nucleofilament at the *umuCD* operon.

Two proteins, UmuD and UmuC, are expressed as a result of the induction of the *umuCD* operon. These proteins associate together and produce a heterodimer called $UmuD_2UmuC$, which is responsible for the initiation of the DNA damage cell cycle check point (Opperman *et al*., 1999). Again, the activated RecA mediates the cleavage of UmuD (Figure 2.1) (removal of N-terminal 24 amino acids) and produces UmuD′ to generate either an UmuD/UmuD′ heterodimer or an $UmuD'_2$ homodimer (Burckhardt *et al*., 1988; Nohmi *et al*., 1988; Shinagawa *et al*., 1988). The $UmuD'_2UmuC$ complex along with activated RecA

constitutes a functional DNA polymerase, known as DNA polymerase V (UmuD′$_2$C-RecA-ATP) (Figure 2.1) (Jiang *et al*., 2009; Reuven *et al*., 1999; Tang *et al*., 1999). DNA polymerase V is responsible for bypassing the DNA lesions (translesion synthesis) that cannot be replicated by DNA polymerase III (Figure 2.1) (Tang *et al*., 2000; Tang *et al*., 1998). When the lesions are repaired by base inclusions and there is no longer any ssDNA left, RecA loses its active conformation and cannot cleave UmuD anymore. Translesion synthesis is inhibited by the remaining UmuD′-UmuD heterodimers (Battista *et al*., 1990). They are eventually removed from the cell by the ATP-dependent protease, ClpXP, which shuts down the SOS mutagenesis process (Gonzalez *et al*., 2000).

2.2.2 The SOS regulon

The components and mode of regulation of the SOS response exhibit diversity among the different bacterial phyla (Mazón *et al*., 2004a). All *Probacteria* subclasses have a *lexA*-like gene except for *Epsilonprobacteria* (Erill and Barbe, 2007). A *lex*A-like gene is present in all Gram-positive bacteria (Erill and Barbe, 2007), Cyanobacteria (Mazón *et al*., 2004b) and Green nonsulfur bacteria (Fernandez *et al*., 2002). Genomic studies on the LexA or SOS regulons from four different bacteria, *E. coli* (Courcell *et al*., 2001a; Fernandez *et al*., 2000), *Bacillus subtilis* (Au *et al*., 2005; Goranov *et al*., 2006), *Pseudomonas aeruginosa* (Cirz *et al*., 2006) and *Staphylococcus aureus* (Cirz *et al*., 2007), showed that the regulon is made up of at least 15 genes, including *recA*, *lexA* and at least one error-prone DNA polymerase (Butala *et al*., 2009). About 63 genes of the regulon are controlled by the lexA-like gene dinR (damage inducible gene) (Raymond and Guillen, 1991) in *Bacillus subtilis* (Au *et al*., 2005) and remarkably only seven of them are homologous in *E. coli.* This illustrates the diversity of the SOS network.

LexA participates as a transcriptional repressor in all these cases, blocking the access of RNA polymerase to the promoters. However, in few cases, LexA plays an opposite role. The LexA paralog in *Rhodobacter sphaeroides* (Tapias *et al*., 2002) and the *Synechocystic sp*. Cyanobacterium (Gutekunst *et al*., 2005) activates transcription. Interestingly, in several bacterial species of *Pseudomonas* and *Xanthomonas*, there are two copies of LexA, LexA1 and LexA2. Thus the *lexA* gene is duplicated in these cases (Abella *et al*., 2007). LexA1 takes part

in the conventional SOS response and LexA2 binds to a different SOS box of DNA and regulates the expression of two error-prone DNA polymerases (Butala *et al*., 2009).

2.3 Overall structure of LexA

E. coli LexA is a 202 amino acid protein encoded by the *lexA* gene. It folds into two structurally defined domains: a carboxy-terminal domain (CTD) and an amino-terminal domain (NTD), linked by a flexible hinge region (Figure 2.2A) (Luo *et al*., 2001; Oertel-Burchheit *et al*., 1993). The CTD helps the protein to dimerize (Luo *et al*., 2001; Schnarr *et al*., 1988) and the NTD helps the LexA protein to bind to DNA with its helix-turn-helix motif.

The crystal structure of the full length LexA mutant (PDB ID: 1jhh and 1jhf, Luo *et al*., 2001) and the three dimensional structure of the LexA-NTD by NMR spectroscopy were reported (PDB ID: 1lea) (Fogh *et al*., 1994). The crystal structure of *E. coli* LexA is presented in the Figure 2.2A. LexA is a member of the 'winged helix' family of DNA-binding proteins (Brennan, 1993; Dumoulin *et al*., 1996; Holm *et al*., 1994; Madan and Teichmann, 2003). The DNA-binding domain, the NTD, is composed of three α-helices and two β-sheets. Residues 6-21 (a1), 28-35 (a2) and 41-52 (a3) produce three α-helices and two anti-parallel β sheets (b1 and b2) follow the helices (Figure 2.2A). a2 and a3 form the helix-turn-helix (winged helix) DNA-binding motif (Fogh *et al*., 1994). The NTD is composed of the first 69 residues followed by a hinge region (domain linker) to connect it to the CTD. The two domains make contact through 470 $Å^2$ of buried surface in between them (Luo *et al*., 2001).

The domain linker or hinge is extended from residues Gln70 to Glu74 (light green, Figure 2.2B), which is a hydrophilic region and not a simple linker. Its sequence showed the importance of the formation of specific LexA-DNA contacts (Butala *et al*., 2007). Different crystal structures of the LexA mutants (S119A and G85D, PDB ID: 1jhh and 1jhf) showed that this connector region is solvent-exposed (Luo *et al*., 2001). These regions of each subunit maintain the positions of the two NTDs in a LexA dimer and do not allow any additional base pairs at an operator between the two DNA-binding motifs (Oertel-Burchheit *et al*., 1993).

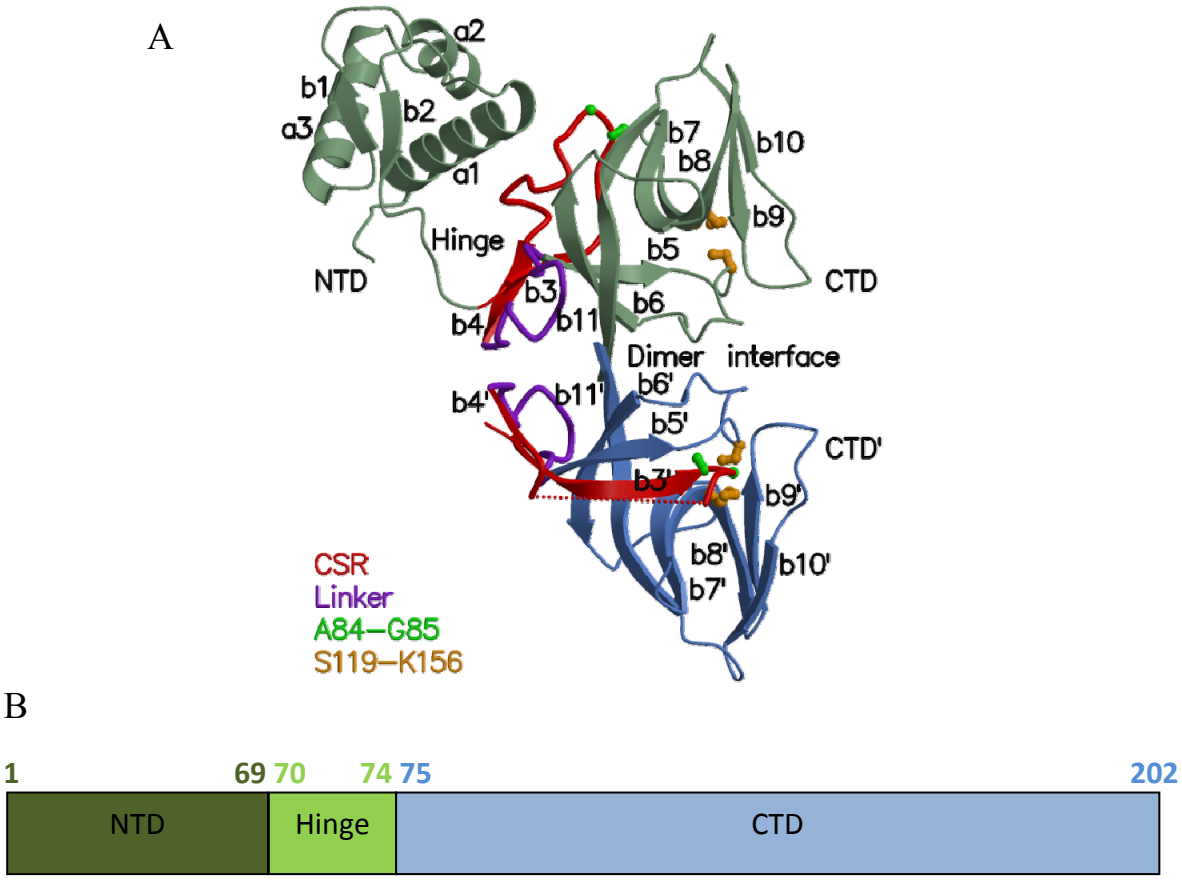


Figure 2.2 Crystal structure and a cartoon diagram of the domains of *E. coli* LexA.

A) Crystal structure of *E. coli* LexA dimer (Luo *et al*., 2001). Two different subunits are shown in different colors (top: dark green, bottom: blue). One of the N-terminal domains (NTD) was disordered during crystal packing and was omitted from the figure (bottom monomer). NTD is composed of three α helices (a1-a3) and two β strands (b1 and b2). C-terminal domain (CTD) is entirely composed of β strands (b3-b11). The catalytic core is composed of b5-b11. The cleavage site region (CSR) [composed of b3 and b4 with intervening loop (red)], linkers (purple) [between the b4 (CSR) and b5 (catalytic core)], cleavage site residues (light green) and catalytic residues (yellow) are shown in different colors. The hinge region is shown by an arrow in the top monomer. Two CTDs are interacting in part with the two anti-parallel β strands (b11). The figure was prepared from LexA S119A mutant (PDB ID: 1jhh) and the S119 residue was reconstructed by the authors (Luo *et al*., 2001) from the G85D mutant (PDB ID: 1jhf) B) Cartoon diagram showing the domains of a LexA monomer. The NTD, hinge and CTD are shown in dark green, light green and cyan, respectively. Residue numbers showing domain ranges are given on top of each domain and hinge, and the font colors are consistent with the domain colors. Reprinted with the permission from Elsevier (Cell) (Luo *et al*., 2001).

The C-terminal domain (CTD) of LexA is entirely composed of β strands (b3-b11, Figure 2.2A) and contains the determinants of oligomerization and self-cleavage (Luo *et al*., 2001). b5-b11 composed the catalytic core which contains the Ser119 and Lys156 (Ser/Lys dyad), the key catalytic residues in LexA (yellow, Figure 2.2A). They act as the serine nucleophile and general base, respectively (Slilaty and Little, 1987). The bond between residues Ala84 and Gly85 is cleaved by catalysis of the Ser/Lys dyad. Ala84 and Gly85 are located in a loop in the CTD, called the cleavage site region (CSR), in between the b3 and b4. The CSR is connected to the catalytic core (b5-b11) with a linker which helps the movement of CSR to switch between two different conformations (purple, Figure 2.2A; Figure 2.3). The loop is away from the catalytic site in one conformation (Figure 2.3A-B), whereas it is buried between the Ser/Lys dyad in another conformation (Figure 2.3C-D) (Luo *et al*., 2001). The LexA structure switches between these two conformations, called cleavable (C) and non-cleavable (NC) conformations. The CSR sweeps ~20 Å from the NC form to the C form to present the cleavage site to the catalytic site in.

2.4 The homologues of LexA

Several proteins have been reported as homologues of LexA. These are called LexA superfamily proteins. The lytic repressors from the lambdoid phages and the UmuD protein from bacteria are some well known homologues of LexA. UmuD is expressed from the derepression of *umuCD* operon upon LexA cleavage during the SOS response. *E*. *coli* UmuD is a mutagenic factor which is involved in an error-prone DNA synthesis machinery to bypass the DNA lesions (Rajagopalan *et al*., 1992; Shinagawa *et al*., 1988). The catalytic Ser/Lys dyad found in LexA and other repressors is conserved in the UmuD protein. However, the cleavage site in UmuD (Cys-Gly) is different from that of LexA (Ala-Gly). UmuD has a Cys residue instead of Ala. In spite of the difference, it is not surprising that UmuD can also be cleaved by the interaction with the activated RecA protein (auto-cleavage) and can follow the same catalytic mechanism as the LexA cleavage (Shinagawa *et al*., 1988). UmuD cleavage involves the removal of first 24 residues from the N-terminal and produces a truncated protein called UmuD′. UmuD′ generates the functional DNA polymerase V ($UmuD'_2C$-RecA-ATP) along

with UmuC and RecA, which helps to fill up the single stranded gaps in the DNA lesion sites (Jiang *et al*., 2009; Reuven *et al*., 1999; Tang *et al*., 1999).

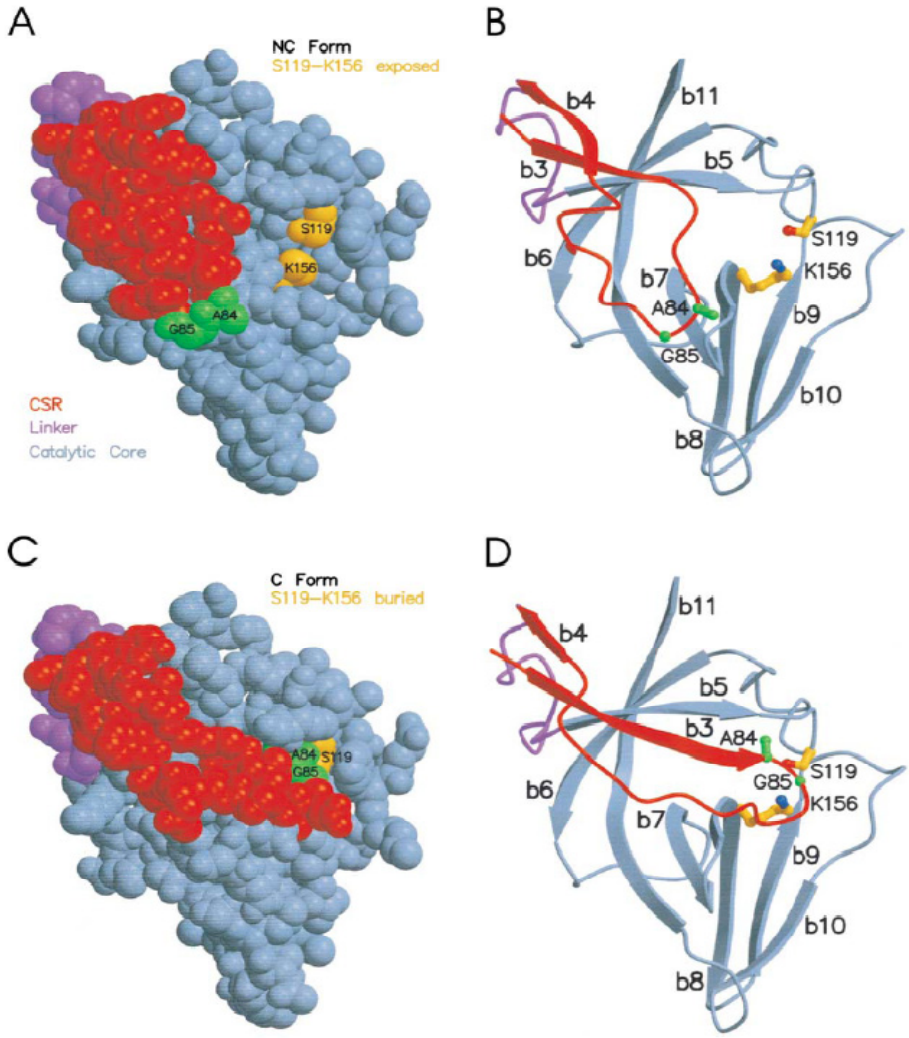


Figure 2.3 Two different conformations of LexA CTD.

Left side figures are space filling and right side figures are ribbon representations of the LexA CTD. A and B are in non-cleavable conformations where the cleavage site, Ala84-Gly85, (red polypeptide chain) is ~20 Å away from the catalytic site, Ser119/Lys156. While in the cleavable conformations (C and D), the cleavage site is completely buried in between the catalytic site. These figures were prepared from LexA S119A mutant (PDB ID: 1jhh) and the S119 residue was reconstructed by the authors (Luo *et al*., 2001) from the G85D mutant (PDB ID: 1jhf). Reprinted with the permission from Elsevier (Cell) (Luo *et al*., 2001).

During the lysogenic phase of the lambdoid phage infection, the phage DNA is integrated into the host chromosome and the expression of the phage proteins is repressed by the phage repressors. Escape of the prophage from the host (lytic phase) requires the inactivation of the repressor protein. In order to permanently inactivate the repressor, prophage exploits the part of the SOS pathway of the host. This includes the interaction of the activated RecA filament which stimulates the intrinsic autoproteolytic activity of the phage's repressor protein. Cl repressors from different phages (λ, φ80, p22, 434 etc.) have been shown to possess this kind mechanism (Eguchi *et al*., 1988; Roberts *et al*., 1978; Sauer *et al*., 1982).

Amino acid sequence alignment of the LexA repressors, SOS response proteins and cl phages repressors is presented in the Figure 2.4. The cleavage site (Ala/Cys-Gly) and catalytic residues (Ser/Lys) are conserved in all of the LexA superfamily proteins.

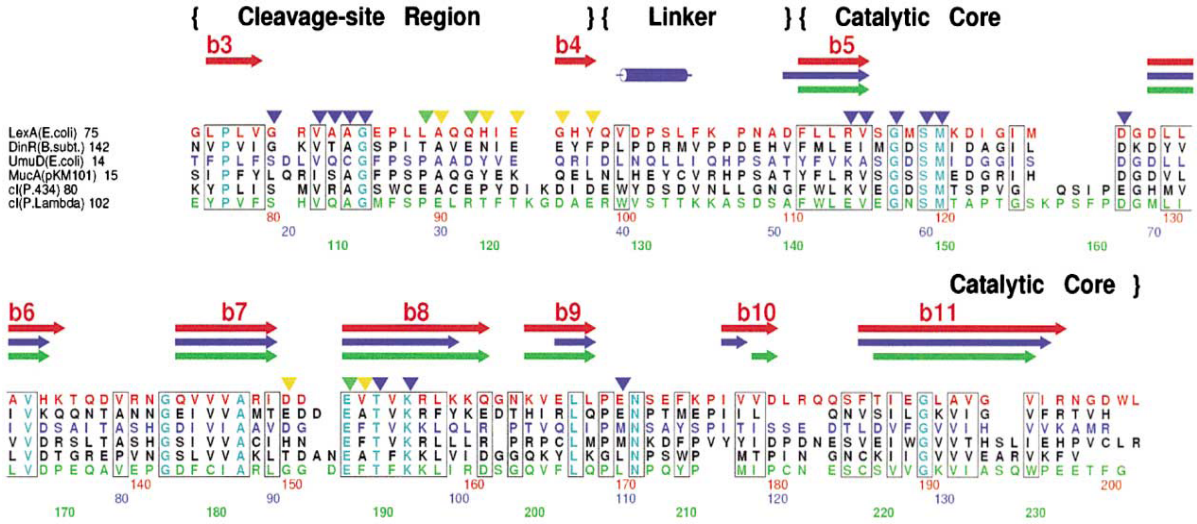


Figure 2.4 Sequence alignment of the CTDs of proteins from LexA superfamily.

Sequence of the C-terminal domains of LexA repressors (1st and 2nd), SOS response proteins (3rd and 4th) and phage repressors (5th and 6th) are aligned. The labels (left side) give the name of the proteins, name of the organisms in parenthesis and the starting sequence number. β-strands, residue sequences and numbering of LexA repressor, UmuD′ and λ Cl repressors are colored in red, blue and green respectively. The secondary structure diagrams of the three repressors are shown on top of the alignment and the colors are consistent with the residue colors. The conserved residues including the cleavage sites (A/C-G), and the catalytic residues Ser and Lys are marked with blue colors (fonts and triangles). Reprinted with the permission from Elsevier (Cell) (Luo *et al*., 2001).

2.5 Dimerization of LexA: the functional repressor formation

The number of LexA molecules in non-induced *E. coli* is approximately 1300, of which 20% are free and non-DNA-bound (Sassanfar and Roberts, 1990). The level of LexA falls 10-fold upon induction of the SOS response and when RecA becomes activated (Dri and Moreau, 1994; Sessanfar and Roberts, 1990). There is a tryptophan residue at position 201 of LexA and it is buried within the dimer interface. Because of this, pressure-dependent changes in the intrinsic tryptophan of LexA can be measured. The results from these experiments revealed that the LexA proteins are predominantly dimeric in solution and the level of monomeric LexA is minute (Mohana-Borges *et al*., 2000). Therefore, it can be understood that LexA interacts or recognizes its targets as a dimer. The target is the operator DNA sequence, which is composed of two symmetrically inverted binding elements. Each of those elements binds to a single subunit of LexA (Thliveris *et al*., 1991). This binding significantly increases the LexA dimer stability by about 1000-fold (Mohana-Borges *et al*., 2000). Dimerization of LexA maximizes SOS gene repression and brings about a high degree of cooperativity.

The determinants for the dimerization of LexA are found entirely in the CTD (Luo *et al*., 2001). The dimerization interface is composed of residues from the two loops (Gln99 to Asp110 and Ser116 to Gly128) and a portion of the β-11 strand of the CTD (Figure 2.2A). Residues 186 to 198 compose the two antiparallel β-11 strands of the CTD. It was observed from the crystal structure of LexA (PDB ID: 1jhh) that residues 195 to 198 (Val195-Ile196-Arg197-Gln198) of the β-11 strands participate in the interaction at the dimer interface. A total of approximately 1380 $Å^2$ of area is buried at the interface (Luo *et al*., 2001). The mechanism of LexA-DNA binding and the LexA dimerization were presumed to be as follows: two LexA monomers first separately sit on an operator with the individual NTD and then the two CTDs come close and interact with each other to form a dimer (Schnarr *et al*., 1988; Schnarr *et al*., 1985). This results in DNA bending as well. From the *in vitro* studies, the formation and dissociation of LexA dimers were observed as a slow process (Giese *et al*., 2008) that takes minutes rather than seconds. However, when LexA is inactivated by self-cleavage, the cleavage fragments dissociate faster from the heterodimer than the intact LexA dimers.

2.6 LexA operator and the specific DNA binding

LexA can access binding sequences in any part of the bacterial genome (Wade *et al*., 2005) in spite of chromosomal folding into the nucleoid (Dame, 2005). The consensus DNA sequence of LexA binding is called 'the SOS box', which is located in the operator region of the SOS genes. This box is found in most of the SOS genes. The DNA sequence in the SOS box is usually taCTGT(at)$_4$ACAGta and displays an imperfect palindromic structure. (Lewes *et al*., 1994). *E. coli* has this type of consensus sequence [CTGT(N)$_4$ACAG] (Walker, 1984). This consensus sequence is conserved in many other Gram-negative bacteria, whereas the consensus sequence is GAAC(N)$_4$GTTC in Gram-positive bacteria (Erill *et al*., 2007) and it is actually termed a Cheo box (Cheo *et al*., 1991). There are 30 LexA boxes that have been found in *E. coli*; however, LexA can bind to another 19 targets that lack the conventional site (Wade et al., 2005). The LexA repressor binds selectively to the CTGT sequence, particularly to the central TG bases.

Specific binding of the protein to the DNA targets requires energetically favorable interactions between the residues in the binding site and specific base pairs. This may be coupled with conformational changes in both the protein and the DNA (Lim *et al*., 2002; Tiebel *et al*., 1999; Wisedchaisri *et al*., 2004; Zhang *et al*., 1987). Many transcriptional regulators switch between the DNA binding and non-binding conformations. The crystal or NMR structure of the LexA-DNA complex has not yet been determined (Dumoulin, 1996). Therefore, the specific contacts of *E. coli* LexA NTD with DNA have been studied by several biochemical and biophysical methods (Dumoulin *et al*., 1993; Hurstel *et al*., 1988; Lloubes *et al*., 1991; Oertel *et al*., 1992; Oertel *et al*., 1990; Ottleben *et al*., 1991; Thliveris *et al*., 1991).

The binding of a half site of the LexA operator, that is with just one subunit of LexA, has 1000-fold lower affinity from the dimer (Kim and Little, 1992). Therefore, the interaction between the two subunits of LexA with a dyad symmetry is required for the tight binding to the consensus sequence. This stabilizes the interaction with both halves of the DNA (Kühner *et al*., 2004; Mohana-Borges *et al*., 2000).

It was revealed by a model that the free conformation of LexA found in the crystal structure (Luo *et al*., 2001) would not allow the NTD of both subunits to dock with both halves of a symmetric operator (Chattopadhyaya and Pal, 2004). A study using molecular dynamic

simulations of the LexA dimer binding to the operator DNA sequence created by CHARMM biomolecular simulation program (Brooks *et al*., 2004) showed that a reorientation of the NTD of LexA with respect to the CTD is essential for the specific operator binding (Butala *et al*., 2009). This was also confirmed by *in vitro* experiments exploiting cysteine crosslinking (Butala *et al*., 2007). Another study with a homolog from *Bacillus subtilis* indicated that either substantial DNA bending or a conformational change in LexA must occur for specific operator binding (Stayrook *et al*., 2008).

2.7 Overview of RecA protein and its functions

Bacterial RecA protein belongs to the universal recombinase superfamily found in almost every form of life. It is part of the group of ATPase proteins homologous to archaeal RadA (Sandler *et al*., 1996) and eukaryal Rad51 (Shinohara *et al*., 1992) and DMC1 (Bishop *et al*., 1992). The existence of RecA was first reported by Clark and Margulies in 1965 while describing a class of mutations in *E. coli* that completely lacked genetic recombination during bacterial conjugation (Clark and Margulies, 1965). As a result of their experiment and a series of other experiments over the last 40 years on bacterial, archaeal and eukaryotic recombinases, the RecA family was found to be the principal catalyst of homologous recombination (Radding, 1981; Seitz *et al*., 1998; Shinohara *et al*., 1992). Homologous recombination is the complete exchange of strands between two homologous DNA molecules. Recently, key steps in the mechanism of homologous DNA recombination and the functional details of the RecA in the catalysis were elucidated (Chen *et al*., 2008). Additionally, experiments revealing a relationship between the recombination proficiency and UV resistance of various mutants showed that there is a functional relationship between homologous recombination and DNA damage repair (Clark and Margulies, 1965; Clark and Volkert. 1978; Howard and Theriot, 1966; Wang and Smith, 1983). Further studies demonstrated that homologous recombination is crucially important and related to repairing double-stranded DNA breaks and restarting stalled replication forks (Cox, 1998; Cox *et al*., 2000; Courcelle *et al*., 2001b; Kowalczykowski, 2000; Lusetti and Cox, 2002). This is also related to SOS mutagenesis. The DNA damage repair mechanism is induced by the co-protease activity of RecA during the SOS response. A low fidelity DNA polymerase in bacteria, DNA polymerase V (Reuven *et al*., 1999; Tang *et al*., 1999), forms during the SOS

response to repair the double-stranded DNA break. This process, called translesion synthesis, is an error-prone DNA synthesis mechanism that produces random mutations (Kato and Shinoura, 1977; Steinborn, 1978). A very recent study revealed that RecA is a structural component of the active form of DNA polymerase V, which is a complex of UmuD′$_2$C-RecA-ATP (Jiang *et al*., 2009). Therefore, RecA has been found to have at least three confirmed functions: homologous recombination, co-protease activity and translesion synthesis.

2.7.1 Active and inactive RecA: two structurally different conformations

RecA is a ~350 residue bacterial protein. The *E. coli* RecA consists of a 30-residue-long amino-terminal α-β motif, an α/β ATPase core of 240 residues and a 64-residue-long globular carboxy-terminal-domain (CTD) (Figure 2.5) (Story *et al*., 1992). RecA binds to ssDNA in an ATP-dependent manner using the ATPase core and forms a helical nucleoprotein filament. The RecA filament exists in two different conformations (DiCapua *et al*., 1990; Morimatsu *et al*., 2002; Vanlook *et al*., 2003b). The inactive conformation forms the self-polymeric filament of RecA protomers in the absence of DNA molecules (Figure 2.5A). It is a wide and compressed helical filament with an average helical pitch of ~83 Å (DiCapua *et al*., 1990; Flory *et al*., 1984; Vanlook *et al*., 2003b; Yu *et al*., 2001). Another distinct conformation is the active RecA which forms an elongated filament in the presence of DNA and ATP (Figure 2.5B). The active RecA filament is a narrow and extended filament having a pitch value ranging from 91 to 97 Å (DiCapua *et al*., 1990; Flory *et al*., 1984; Morimatsu *et al*., 2000; VanLoock *et al*., 2003b; Yu *et al*., 2001). These two distinct conformations can also be found in archaeal RadA (Wu *et al*., 2004) and eukaryotic Rad51 proteins (Conway *et al*., 2004; Galkin *et al*., 2006; Hilario *et al*., 2009). The two different conformations of RecA are shown in the Figure 2.5. The inactive crystal structure shows that the regions that are involved in the DNA binding, called L1 and L2 loops, are disordered and could not be seen (yellow arrows, Figure 2.5A) (Story *et al*., 1992); whereas, the loop regions became ordered in the active crystal structure (yellow arrows, Figure 2.5B) (Chen *et al*., 2008).

Active filament formation is highly cooperative and its nucleation requires the assembly of five to six RecA protomers (Galleto *et al*., 2006; Joo *et al*., 2006; Kelley *et al*., 2001).

Structural details of the active RecA filament of *E. coli* have been reported (Figure 2.6) (Chen *et al*., 2008).

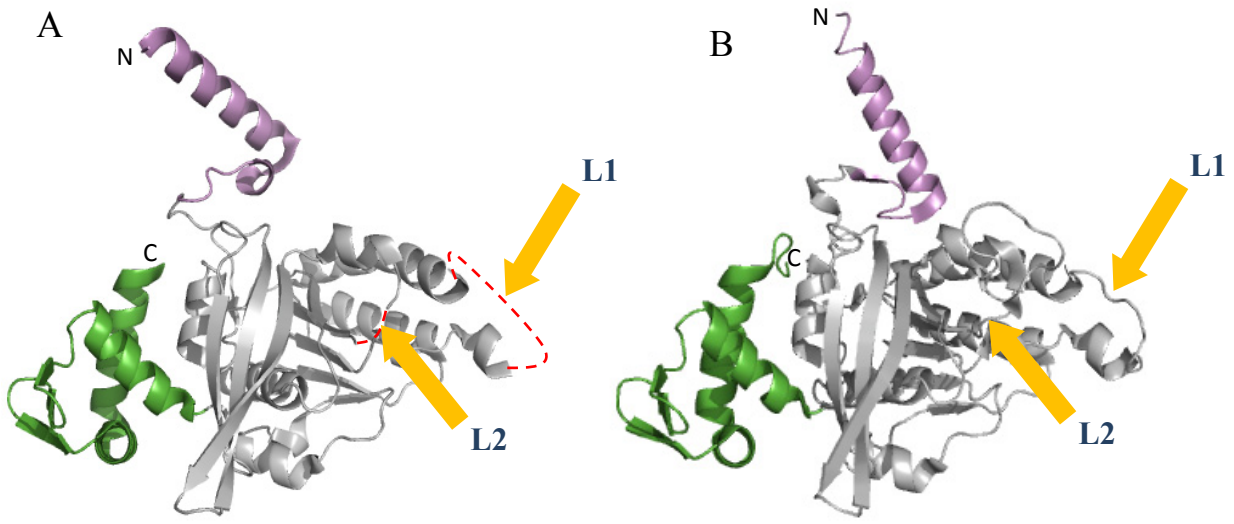


Figure 2.5 Crystal structure of inactive and active *E. coli* RecA monomer.

The inactive (A) and active (B) crystal structures of RecA monomer (PDB ID: 2reb, inactive monomer, Story *et al*., 1992 and PDB ID: 3cmu, active monomer, Chen *et al*., 2008). The structure of a RecA monomer has a large core domain (grey) and two smaller domains at the N- and C-termini (purple and green, respectively). The L1 and L2 loops are indicated with yellow arrows. These L1 and L2 loops were disordered (red dotted lines) in the inactive structure (A) and became highly ordered in the active structure (B). Figures were created with PyMOL.

According to the study by Chen *et al*. (2008), the nucleoprotein filament has a helical repeat average of 6.16 RecA per turn and a pitch of 93.96 Å. This description resembles the other predicted active filament structures (Bell, 2005; Bianco *et al*., 2005; Cox, 2007c; Flory *et al*., 1984; McGrew and Knight, 2003; Stasiak and DiCapua, 1982; Yu *et al*., 2001). The ssDNA-bound RecA filament is termed the presynaptic complex. The active RecA crystal structure (Chen *et al*., 2008) showed that ssDNA was located in close proximity and also helically wrapped around the axis of the nucleoprotein filament. It is in B-DNA-like conformation, but underwound with average helical parameters of 18.5 nucleotides per turn and 5.08 Å rise per base pair (bp). Exactly three nucleotides of the ssDNA are bound to a single RecA protomer. ssDNA made contact with the RecA protein through the L1 and L2 regions and

a portion of two α helices of the N-terminal (Chen *et al*., 2008). The L1 and L2 contact portions in the helix were followed by the N-terminal region whereas the L1 and L2 portions of RecA were found disordered in the inactive filament (Bell, 2005), whereas in the DNA-bound structure, they became highly ordered. The ATP binding pocket lies between the two ATPase cores of two RecA protomers in the presynaptic filament, which forms a completely buried environment.

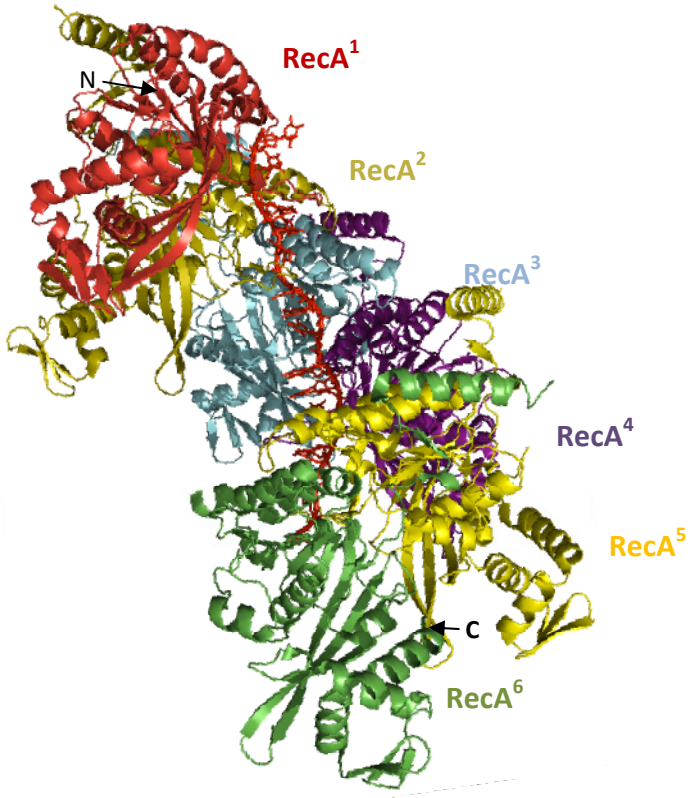


Figure 2.6 Crystal structure of *E. coli* RecA nucleoprotein filament (presynaptic complex).

Structure of the $RecA_6$-$(dT)_{18}$ complex (PDB ID: 3cmu, Chen *et al*., 2008). The six RecA protomers are numbered from the N-terminal RecA subunit to onwards. They ($RecA^1$-$RecA^6$) are colored dark red, golden, cyan, purple, yellow and green, respectively. The DNA backbone is marked by a red coil. Only 15 of the 18 nucleotides were found ordered (red) in the crystal strucuture of six protomers. Figure was created with PyMOL.

2.8 Mechanism of the RecA-mediated cleavage of LexA

LexA is resistant to intracellular degradation under physiological conditions (Neher *et al*., 2003), but cleavage is triggered by the interaction with activated RecA (Little, 1991). However, LexA slowly undergoes auto-cleavage in solution (Little, 1984). Self-cleavage can occur in a RecA-dependent manner *in vitro* at alkaline pH (Little, 1984). Active RecA stimulates LexA to auto-digest at a faster rate (Little, 1991). Two residues of the CTD of LexA, Ser119 and Lys156, act as catalytic residues during the cleavage (Ser/Lys dyad). This dyad cleaves the peptide bond between two other residues (Ala84 and Gly85) in the same domain. LexAs from different bacterial species and its homologs (UmuD and λ repressor, for example) consistently contain these conserved residues (Figure 2.4) and therefore, are thought to possess the same type of cleavage mechanism.

LexA remains predominantly in a non-cleavable conformation in the absence of active RecA, and the ε-amino group of Lys156 of LexA is favorably protonated and exposed to the aqueous environment. At this stage, Lys156 cannot act as a general base in activating the catalytic Ser119 residue. Thus, the self-cleavage of LexA is kept in check. RecA's co-protease activity facilitates the LexA self-cleavage by providing a hydrophobic pocket (Lin and Little, 1989), which offsets the energetic cost in the burial and neutralization of the ε-amino group of Lys156 in LexA (Figure 2.7) (Luo *et al*., 2001). The cleavable conformation of LexA, in which Lys156 is buried and deprotonated, increases its rate of self-cleavage by ~10^4-fold at neutral pH as explained by the conformation-equilibrium model (Roland *et al*., 1992). During auto-cleavage, Lys156 removes a proton from Ser119 and therefore makes it nucleophilic, which in turn allows it to attack the Ala84-Gly85 peptide bond (Figure 2.7) (Slilaty *et al*., 1992). The Ser/Lys dyad model is supported by mutational studies (Lin and Little, 1989; Lin and Little, 1988; Slilaty and Little, 1987). Replacing Ser119 or Lys156 with alanine (S119A or K156A mutations) produces non-cleavable LexA. It has been proposed that LexA can exist in two distinct conformations, cleavable and non-cleavable. In the non-cleavable form, the Ala84-Gly85 polypeptide chain stays ~20 Å away from the Ser/Lys dyad (Figure 2.3). However, it bends significantly in the cleavable conformation, presenting the cleavage site to the catalytic Ser119 and burying Lys156 (Luo *et al*., 2001). The buried Lys156 is exposed to an environment where a positive charge is not stable (Luo *et al*., 2001).

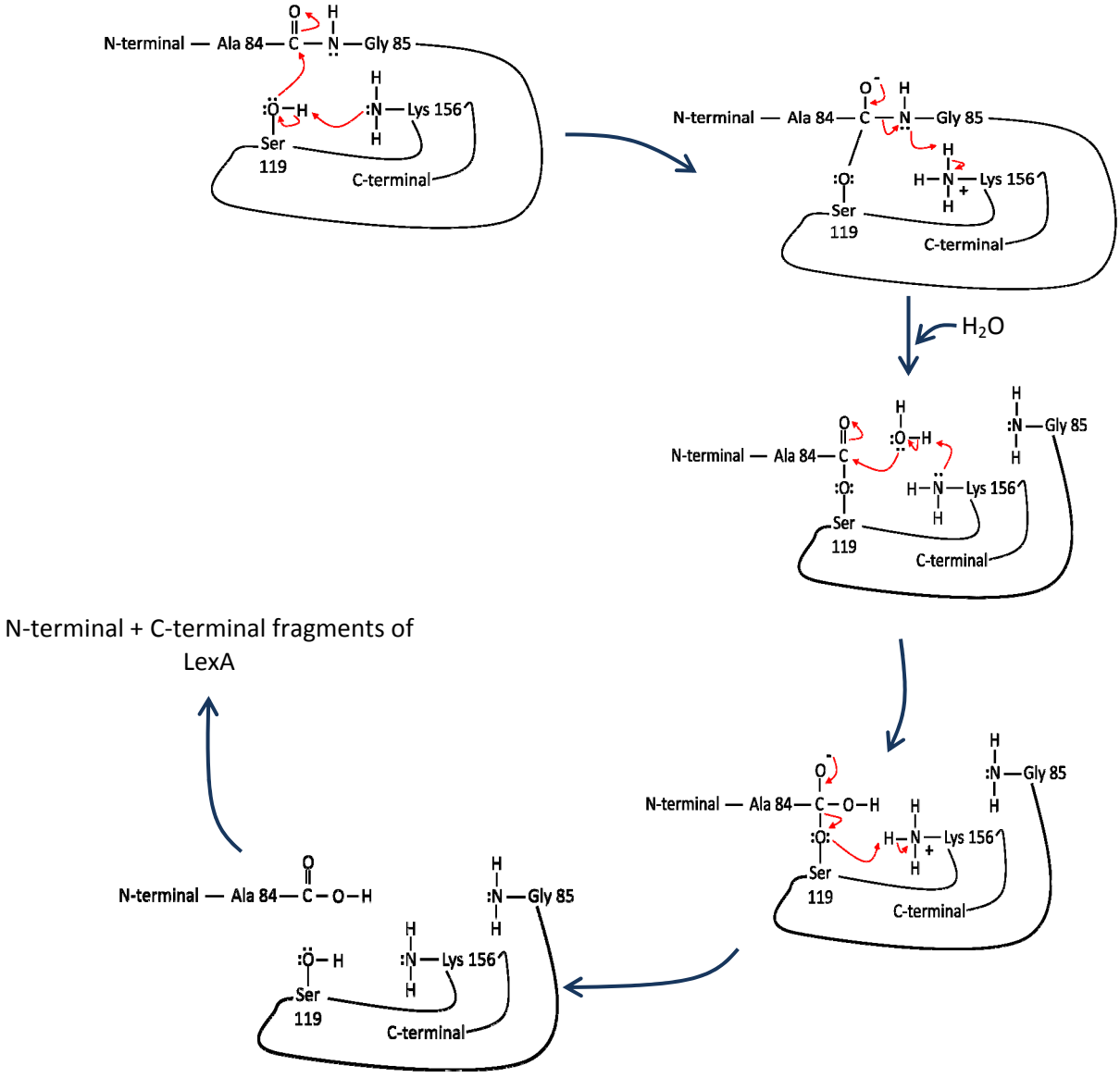


Figure 2.7 Cartoon drawing of the LexA self-cleavage catalysis mechanism.

Uncharged Lys156 pulls a proton from the hydroxyl group of Ser119 and enhance the nucleophilicity. The nucleophilic oxygen then attacks the carbonyl carbon of Ala84 and forms a transient bond between Ser119 and Ala84. In the next step, nitrogen from the α-amino group of Gly85 absorbs one proton from the ε-amino group of Lys156 and the peptide bond between Ala84-Gly85 is cleaved. The hydrolysis of the transient bond between Ala84 and Ser119 takes place in the next step by one incoming water molecule, and at the end of the reaction one N-terminal and one C-terminal fragments of LexA are produced. The red arrows show the direction of electron transfer. (This figure was modified from Slilaty and Little, 1987.)

A tetrahedral intermediate is formed during the catalysis process of classic Ser-His-Asp proteases like chymotrypsin. The intermediate is favourably stabilized by a structural feature known as the 'oxyanion hole'. Luo *et al*. (2001) showed that, the Ser-Lys proteases like LexA superfamily also possess the same mechanism. Figure 2.8 shows the catalytic site of the cleavable form of LexA. Ala84 O, lies within hydrogen-bond distance of two adjacent main chain amide nitrogens of Ser119 (2.8 Å) and Met118 (3.2 Å), and this location is the oxyanion hole of LexA. The conformation of oxyanion hole is stabilized by hydrogen bonding to the adjacent glycine main chain atoms (Gly117) to a solvent molecule and to a side chain of Asp127.

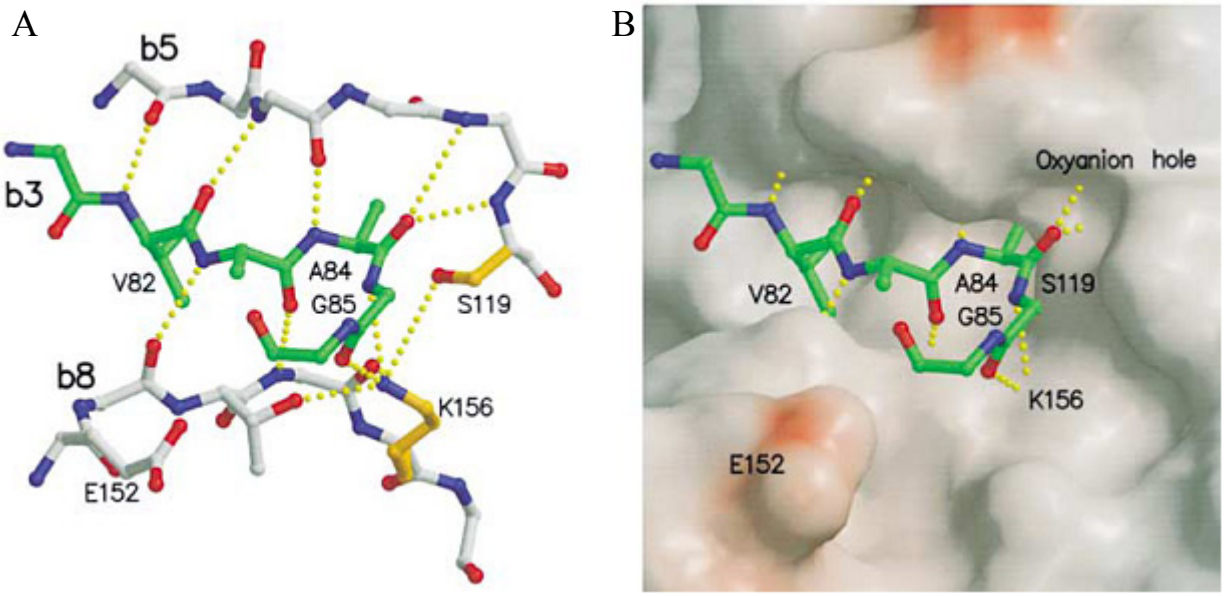


Figure 2.8 Stabilization of the catalytic site region peptide complex in the oxyanion hole of LexA.

A) A ball and stick model of the catalytic site of the cleavable form of the CTD of LexA. The carbon chain of the catalytic site region and the catalytic core are colored in green and grey, respectively. Oxygen and nitrogen are colored in red and blue regardless of their location. Hydrogen bonds are shown as yellow dashed lines. B) The catalytic site with electrostatic surface. The electropositive surface is colored in blue and electronegative surface is colored in red. The position of the oxyanion hole is pointed. Reprinted with the permission from Elsevier (Cell) (Luo *et al*., 2001).

Deprotonation of Lys156 is required for cleavage (Lin and Little, 1989) since auto-cleavage is efficient only at a high pH (Little, 1984). RecA does not participate directly in the

chemistry of cleavage reaction, and in particular, does not appear to affect the pK_a of Lys156 in the cleavable conformation. In fact, the energetic cost of burying the lysine terminal amino group may help to keep the auto-cleavage reaction in check. However, the crystal structures of the LexA C-terminal domain in the non-cleavable and cleavable forms are very similar, except for the movement of the cleavage site region (Figure 2.3). Luo *et al*. suggested that this argues against an allosteric effect by RecA (Luo *et al*., 2001). Instead, the authors postulated that RecA binds preferentially to the cleavable conformation of LexA, stabilizing this form and promoting cleavage (Luo *et al*., 2001; Ronald *et al*., 1992). However, Ronald *et al*. (1992) showed that less than 1 in 4000 LexA molecules are in the cleavable conformation used for RecA-dependent cleavage at pH 7.4 (Ronald *et al*., 1992). Therefore, it is a little impractical if RecA only binds with the cleavable conformation of LexA and thus the concentration of LexA drops tenfold in the cell. However, Kim *et al*. (2008) recently postulated that RecA can bind to the non-cleavable form of LexA as well and therefore acts, at least in part, through an allosteric mechanism during cleavage (Giese *et al*., 2008), i.e., converting LexA from the non-cleavable conformation to the cleavable conformation. Thus, the interaction of LexA with RecA-ssDNA shifts the equilibrium of the LexA concentration towards the cleavable conformation.

2.9 Overview of the structural interactions between LexA and RecA

The active RecA filament mediates self-cleavage of the LexA repressor. Though the role of RecA in LexA catalysis is indirect, LexA and RecA have to physically interact. The three dimensional structure of the LexA and RecA complex is not available to help understand precisely the interactions and the mechanism of LexA catalysis. However, several attempts had been undertaken to reveal the binding sites of LexA on the RecA protein. The apparent Km of LexA binding to RecA nucleoprotein filament has been estimated to be approximately 0.5 μM (Lin, 1988). However, a non-cleavable LexA mutant, such as K156A, was found to bind activated RecA more tightly than the wild-type (Slilaty and Little, 1987). This mutant has been used in several experiments to study the interaction between RecA and LexA.

Activated RecA forms an extended helical filament with ssDNA that has a higher helical pitch than the inactive filament. Interestingly, RecA ATPase activity can be activated by high salt in the absence of DNA and it also forms an extended helical filament (95 Å pitch)

(Pugh and Cox, 1988). This salt-activated RecA shows co-protease activity as well (Dicapua *et al*., 1990). Therefore, it is obvious that the extended helical filament of RecA is required for the cleavage of LexA. The simple explanation for this may be because LexA cannot fit into the compressed filament groove found in the inactive RecA filament. Attempts to fit the LexA difference density into inactive RecA filament reconstruction (Yu and Egelman, 1992) or into the inactive filament from the RecA crystal structure (Story *et al*., 1992) showed a huge overlapping density region (Yu and Egelman, 1993). Therefore, it is suggested that while RecA ATPase activity requires a highly specific conformation at the active site of catalysis, the catalysis of LexA cleavage and other repressors may be much less specific at the active site and depend upon a more general LexA orientation within the RecA groove or binding sites (Yu and Egelman, 1993).

Story *et al*. (1992) suggested that the binding sites of LexA and other repressors are in the "notch" between lobes of the adjacent RecA subunits in the filament. Residues 329 to 354 were not seen in the inactive crystal structure presumably due to a high degree of disorder (Story *et al*., 1992). These are the C-terminal residues of the *E. coli* RecA protein. Residues 229 and 243 in the 'notch' region of RecA have been proposed to be involved in LexA binding (Story *et al*., 1992). By performing mutational studies, the residues located at this site were found to differentially affect RecA-mediated cleavage of the LexA repressor or its homologs *in vivo*. For example, the R243L mutation prevents cleavage of the φ80 repressor and UmuD protein (Dutreix *et al*., 1989), and the G229S mutation prevents φ80 repressor cleavage (Ogawa and Ogawa, 1986). These residues (Gly229 and Arg243) are located at the inner surface of the pendulous lobe of the activated RecA and protrude into the deep helical groove. However, based on an electron microscopy (EM) study on the RecA/LexA complex, no density from LexA was found in the notch itself (Yu and Egelman, 1993).

It had been found that high concentration of ssDNA (Rehrauer *et al*., 1996) and dsDNA (Takahashi and Schnarr, 1989) can completely inhibit the RecA-mediated cleavage of LexA (DiCapua *et al*., 1992). Arg243 was reported to be at the site for secondary DNA binding to RecA (Mazin and Kowalczykowski, 1998). It has also been suggested that this site is an interaction site of dsDNA that helps releasing the complementary strands from the DNA-RecA filament complex during homologous recombination (Chen *et al*., 2008). A second LexA

binding site on RecA is located on a loop, which is called L1 (Story *et al*. 1992) and contains residues 156 and 165 (Yu and Egelman, 1993). This region was also disordered in the inactive crystal structure (Figure 2.5A) (Story *et al*., 1992), but became highly ordered in the active structure in the presence of DNA (Figure 2.5B) (Chen *et al*., 2008). In fact, the L1 loop and the C-terminal lobe (residues 268 to 330) have been implicated in dsDNA binding and are also in the binding vicinity of the LexA repressor. This observation is in agreement with the statement about the competitive nature of ssDNA/dsDNA and LexA for binding sites on the RecA filament. This can also explain why non-cleavable LexA (S119A mutant) acts as a competitive inhibitor of the DNA strand exchange activity of RecA (Harmon et al., 1996).

The iterative helical real space reconstruction (IHRSR) has been applied using electron microscopy (EM) (Egelman and Stasiak, 1993) and crystal images (Story *et al*., 1992) to compare the RecA-DNA-LexA complex and the RecA filaments (VanLoock *et al*., 2003b). It was known and mentioned earlier that the average helical pitch of an active RecA filament is ~94 Å (Chen *et al*., 2008; DiCapua *et al*., 1990; Flory *et al*., 1984; Morimatsu *et al*., 2000**;** VanLoock *et al*., 2003b; Yu *et al*., 2001), while it is ~ 82 Å in the inactive filament (DiCapua *et al*., 1990; Flory *et al*., 1984**;** VanLoock *et al*., 2003b; Yu *et al*., 2001). Though the main difference between the active and inactive RecA filament is the helical pitch, it was found from the filament reconstruction model that the pitch of the RecA-DNA-LexA complex was 84.7 Å, close to the 82.07 Å found in the inactive crystal structure.

However, it can be seen that though the helical pitch is similar, the two filaments are quite different at low resolution (Figure 2.9A-B) (VanLoock *et al*., 2003b). The modeled activated RecA filament model (VanLoock *et al*., 2003a) was docked onto the RecA-DNA-LexA complex; it was shown that subtracting the density of the RecA model from that of the actual RecA-LexA reconstruction reveals a large mass of density near the axis of the RecA filament (yellow mesh, Figure 2.9C-D). In addition to this, a small mass of additional density is found ~6 Å from residue 328, the last C-terminal residue of RecA resolved in the inactive crystal structure (Figure 2.10A). This density was not found in low resolution EM reconstructions (VanLoock *et al*., 2003b).

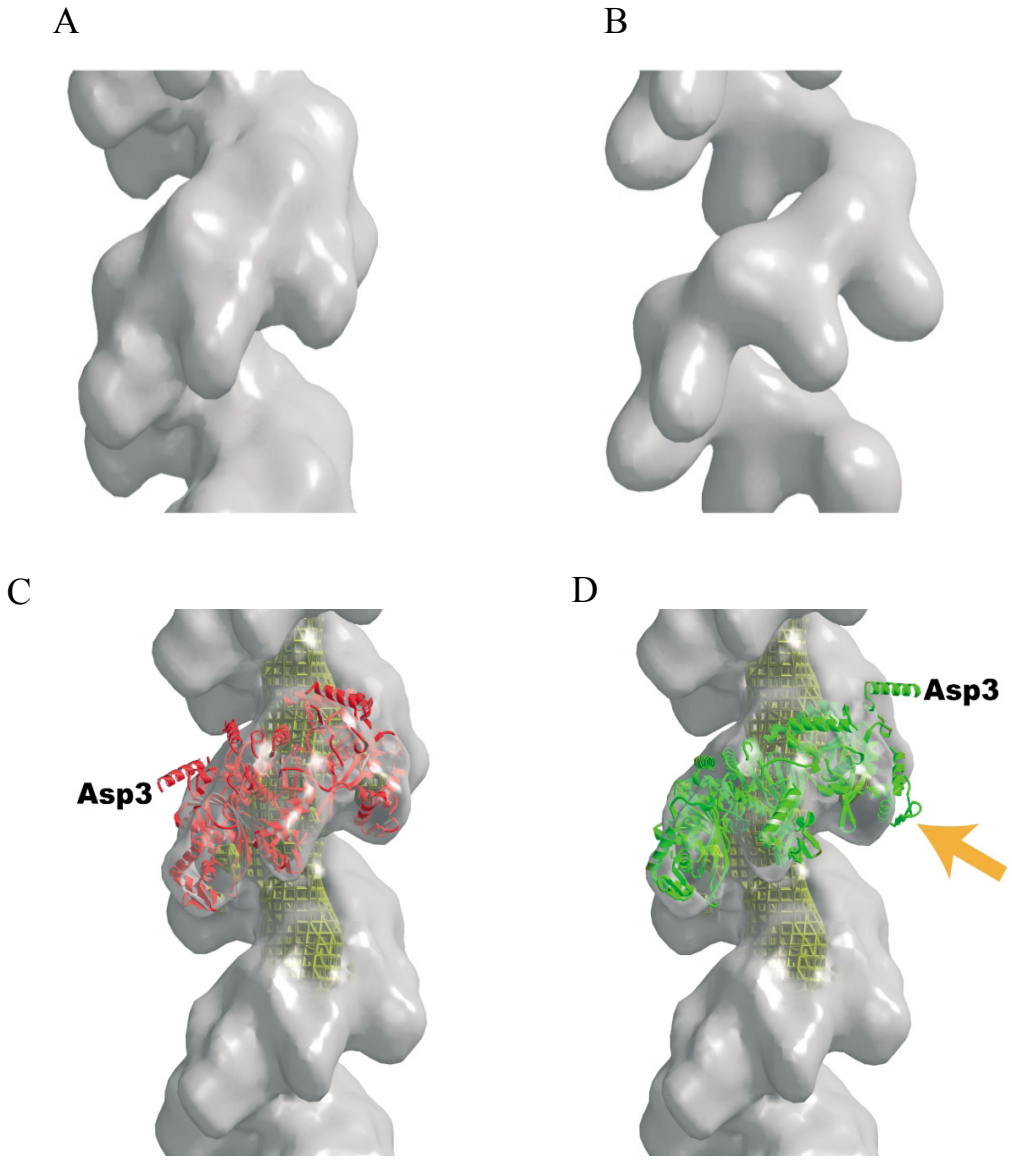


Figure 2.9 EM reconstruction of the RecA-ATP-DNA-LexA filament complex.

A is the low resolution surface model that was generated from the low resolution density of the inactive crystal structure of RecA with the difference density due to LexA, while B is the actual low resolution surface of the inactive crystal filament. C is a model for the LexA-bound RecA active filament (VanLoock *et al*, 2003a) and D is the LexA-bound RecA crystal structure of the inactive filament (Story *et al*., 1992). The orange arrow in D shows the protruded region of RecA from the density map, which indicates that the inactive crystal structure of RecA fits poorly with the reconstructed LexA-RecA filament. In both C and D, the difference density obtained by subtracting RecA from the LexA–RecA complex is shown with a yellow mesh. Reprinted with the permission from Elsevier (Journal of Molecular Biology) (VanLoock *et al*., 2003a).

The large density due to LexA was disconnected from the additional density found; therefore, it may not be due to LexA. A possible explanation for this additional density is that LexA binding near the filament axis may induce an allosteric stabilization of this region of RecA. Several other papers suggested that these C-terminal residues also act as an allosteric regulatory switch (Eggler *et al*., 2003; Lusetti *et al*., 2003a; Lusetti *et al*., 2003b). Consistent with these findings, it was found that, cleaving the residues from this region results in the induction of an allosteric conformational change involving the nucleotide-binding core (Yu and Egelman, 2001).

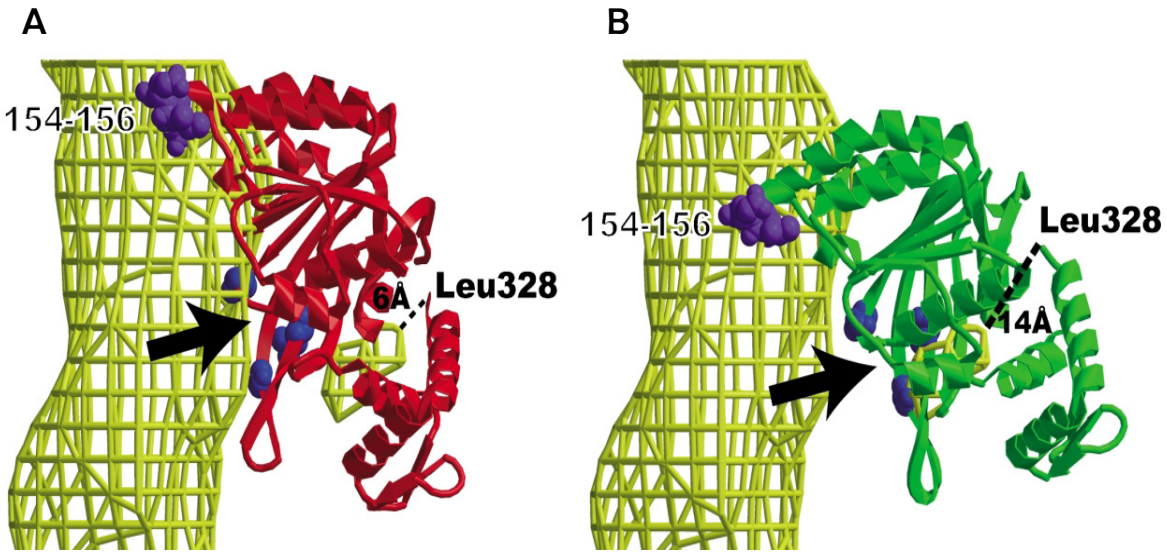


Figure 2.10 A single subunit from the fittings using both the RecA model (A, red) and the RecA crystal filament (B, green) along with the difference density (yellow).

RecA residues implicated in LexA binding are shown as spheres (154–156, purple; 67, 229 and 243, blue). Residues 67, 229 and 243 are immediately adjacent to the mass due to LexA in the model (black arrows in e and f), but are displaced from the LexA density when the inactive crystal filament was used. The distance between the most C-terminal RecA residue (Leu328) observed in the inactive crystal structure (Story *et al*., 1992) and a mass that is likely due to residues 329–354 (not seen in the inactive crystal structure) is indicated with a broken line. Reprinted with the permission from Elsevier (Journal of Molecular Biology) (VanLoock *et al*., 2003a).

Several residues in a RecA subunit were previously reported to make contact with LexA: 67 (Konola *et al*., 1998); 154-156 (Nastri *et al*., 1997); 229 (Ogawa and Ogawa, 1986);

and 243 (Dutreix *et al*., 1989). The larger difference density (yellow mesh, Figure 2.10C), presumably due to the presence of LexA close to the axis of the nucleofilament in the EM reconstruction model, can be compared to these contact regions (Figure 2.10). These residues in RecA are in close proximity to the LexA density (Figure 2.10A), whereas they are not in direct contact with the LexA mass in the inactive RecA crystal structure (Figure 2.10B).

Chapter Three: **Objectives**

The overall objective of this study was to produce a stable complex of two proteins from *Escherichia coli*, RecA and LexA, and to co-crystallize them. This goal can ultimately help solve the three-dimensional crystal structure of the RecA and LexA complex.

To achieve this goal, the following experiments were performed:

1. Producing non-cleavable LexA proteins with dimer interface mutants in order to purify a predominantly monomeric LexA in solution.
2. Screening out the predominantly monomeric LexA from the mutants produced. Two experiments were performed to accomplish this goal:
    a. Size exclusion chromatography and
    b. Dynamic light scattering (DLS).
3. Determining the binding strength of the LexA dimer interface mutants with RecA. For this, the co-protease inhibition assay was performed.
4. Attempting to co-crystallize the monomeric dimer-interface-mutants of LexA and RecA. Objective 4 was a part of the future studies. If objective 4 gave positive results, the current study would continue to research for PhD degree.

Chapter Four: **Materials and Methods**

4.1 Materials

Table 4.1 Materials and equipments used for the experiments.

Object	Provider
Cells	
Rosetta (DE3)	Novagene
XL1-Blue	Stratagene
DNA	
pET28a-c(+) vector	Novagene
ssDNA (dT45, dT22, dT20, dT18)	Integrated DNA Technology
Primers	Integrated DNA Technology
Media	
Agar	BD Biosciences
Bacto-Tryptone	BD Biosciences
LB Miller broth	BioShop, BD Biosciences
Peptone	BD Biosciences
Yeast Extract	BD Biosciences
Cloning enzymes and buffers	
10X DNA ligase buffer	MBI Fermentas
10X Thermopol reaction buffer	New England Bio Labs
NdeI	New England Bio Labs
Pfu DNA polymerase	MBI Fermentas
T4 DNA ligase	MBI Fermentas
Taq DNA polymerase	New England Bio Labs
Thrombin	Sigma
XhoI	MBI Fermentas
Other materials	
AMPPCP	Sigma
AMPPNP	Sigma
ATP	Sigma
Agarose	Invitrogen Life Technologies

Acetic acid (glacial)	EMD Chemicals Inc
Acrylamide (electrophoresis grade)	Sigma
Ammonium persulfate	EMD Chemicals Inc
Ammonium sulfate	EMD Chemicals Inc
Ampicillin (sodium salt)	BioShop
Boric acid	EM Science
Bradford reagent	Bio-Rad
Bromophenol blue	Alfa Aesar
Chloramphenicol	BioShop
Coomassie Brilliant Blue R-250	AnaSpec
dNTPs (dATP, dCTP, dTTP, dGTP)	Amersham Biosciences
Ethidium bromide	Sigma
Ethanol (95%)	Commercial Alcohols Inc
EDTA (ethylenediaminetetraacetic acid)	EMD Biosciences
Glycerol	EMD Biosciences
Hydrochloric acid	BDH Inc
HEPES (N-2-hydroxyenthylpeperazine-N'-2-ethane-sulfonic acid)	BioShop
Imidazole	Avocado Research Chemicals Ltd
IPTG (Isopropyl-thio-β-D-galactoside)	EMD Chemicals Inc
Kanamycin sulphate	BioShop
Lysozyme	Sigma
Magnesium acetate	BioShop
Magnesium chloride (hexahydrate)	EMD Chemicals Inc
Magnesium sulphate	EMD Chemicals Inc
MES [2-(N-morpholino)-ethane sulfonic acid monohydrate]	EMD Chemicals Inc
2-Mercaptoethanol	BioShop
Nickel sulfate (hexahydrate)	EMD Chemicals Inc
Potassium chloride	EMD Chemicals Inc
Sodium chloride	EMD Chemicals Inc
SDS (sodium dodecyl sulfate)	Calbiochem
Sucrose	EMD Chemicals Inc
Tricine	BioShop

Tris [Tris (hydroxymethyl) aminomethane]	EMD Chemicals Inc
Urea	EMD Chemicals Inc
Equipment and other	
375 Hotplate/Stirrer	VWR Scientific
ÄKTA Prime Protein Purification System	Amersham Biosciences
Allegra X-22R Centrifuge	Beckman Coulter
Avanti J-25 Centrifuge	Beckman Coulter
Centrifuge 5415D	Eppendorf
CP 124S (balance)	Sartorius
DynaPro MS800	Protein Solutions
Electroporator 2510	Eppendorf
HiPrep 16/60 Sephacryl S-100 High resolution	Amersham Pharmacia Biotech
HiPrep 16/60 Sephacryl S-300 High resolution	Amersham Pharmacia Biotech
Incubator	VWR Scientific
Innova 43 Incubator Shaker	Innova
Mastercycler Gradient	Eppendorf
Mini Vortexer	VWR Scientific
Orbit Environ Shaker	Lab-Line
QIAgen Miniprep Kit	QIAgen
QIAgen Gel Extraction Kit	QIAgen
Sonic dismembrator Model 500	Fisher Scientific
Symphony pH Meter	VWR Scientific
Ultrospec 2100 *pro*	Biochrom
UV illuminator	VWR Scientific
Resin	
DE52 anion exchanger (DEAE cellulose)	Whatman
His-Select Nickel Affinity Gel	Sigma

4.2 Common Procedures Used

4.2.1 Agarose gel electrophoresis

1-1.5% (w/v) agarose gels were prepared in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The solution was boiled until the agarose was completely dissolved in the buffer. Ethidium bromide (0.5 μg/mL) was added and mixed properly when the agarose solution cooled down. The cooled, liquified gels were then allowed to set in the gel cast by pouring and the cascade was properly sealed to prevent leaking. DNA samples were mixed with 1X loading buffer [0.04% (w/v) bromophenol blue, 5% (v/v) glycerol]. Different volumes of samples were loaded on the gels depending on the type of sample and purpose of the gel. DNA samples were resolved on the agarose gel at 120 V for 15-20 min in 1X TAE buffer. The gel-resolved samples were visualized and analyzed with a GelDoc UV illuminator. For the DNA gel extraction procedure, samples were viewed with a UV lamp at 365 nm and cut from the gel for purification.

4.2.2 Colony PCR (Polymerase Chain Reaction)

Colony PCR was performed with single colonies (colony forming units) of transformed XL1-Blue cells containing cloned plasmid to confirm that the correct gene was inserted into the vector. The transformed cells were grown on Luria-Bertani (LB) agar plates containing the appropriate antibiotics. Forward and reverse primers used for colony PCR had overlapping regions from the plasmid vector that would produce a PCR product containing the gene if it had been positively cloned. We used the pET28a-c(+) plasmid vector for all cloning purposes used with this technique. pET28a-c(+) has a T7 promoter site and a T7 terminator site (Figure 4.1B); we designed primers for these two sites of the vector. The primers are shown in the Table 4.2.

Table 4.2 Sequences of the primers used for colony PCR.

Construct	Sequence (5′-3′)
T7 Promoter	taa tac gac tca cta tag gg
T7 Terminator	gct agt tat tgc tca gcg g

Single colonies were picked from the LB plate and resuspended in 6 μL of double distilled water (ddH_2O). Four microliters of the resuspended colony were added to a PCR mixture with a final volume of 25 μL. The reaction volume consisted of 1 unit (U) of *Taq* DNA polymerase, 100 μM of each dNTP, 1X Thermo-Pol Reaction Buffer, 1 μM of each of the primers. The DNA containing the inserted gene was amplified by a PCR Thermocycler using the following cycles: 94°C for 2 min; 25 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 1 min; 72°C for 10 min. The PCR products were resolved with a 1% agarose gel and the DNA bands were viewed under UV light in the GelDoc.

4.2.3 Protein crystallization by vapour diffusion

Vapor diffusion is the most commonly used method to crystallize proteins. Water is allowed to evaporate from a protein solution to a larger reservoir solution; this makes the protein more concentrated in order to facilitate crystal formation. A drop (small reservoir) of protein solution is set on top of a larger reservoir, either hanging from a cover slide on top of the larger reservoir or sitting on another smaller well. The larger reservoir has a higher concentration of buffer and precipitant. This leads to the evaporation of water molecules from the protein drop to the well solution. Therefore, the concentration of protein in the drop increases and the protein tends to precipitate and form crystals (Rhodes, 1993; McRee, 1993). There are two ways to perform the vapor diffusion crystallization procedure, hanging drop method and sitting drop drop. They differ only in the way the drops are set. Notice that the crystallization environment is a closed system. The protein drops hanging from the cover slide on top of the well solution is the hanging drop method. On the other hand, a protein drop sitting on a smaller well is the sitting drop method. There are several commercial kits available to set up a crystal plate by the sitting drop method. Usually 96-well kits are used for the non-robotic sitting drop crystallization procedure and are what we used in our part of the co-crystallization trials.

4.2.4 LB Media Preparation

Luria-Bertani (LB) (Miller) broth was prepared in one of two ways. One way was to mix the ingredients [10 g Bacto-tryptone, 5 g yeast extract and 10 g NaCl] in a final volume of 1 liter of water. Another way was by dissolving commercially prepared LB Miller powder in water. The broth was autoclaved at 15 psi and 121°C for 30 minutes. Essential antibiotics were added to cooled autoclaved broth before culturing the cells. Agar medium was prepared by adding 15 g of Bacto-agar to 1 L of LB broth.

4.2.5 SDS-PAGE

Protein samples were prepared before running the protein gels. The proteins were mixed with 5X loading buffer [10% SDS (w/v), 10 mM β-Mercaptoethanol, 20% Glycerol (v/v), 0.2 M Tris-HCl, pH 6.8, 0.05% Bromophenol blue] to reach a final concentration of 1X. The protein samples were boiled for 5 minutes after adding the loading buffer and then loaded in the gels. Gels were first run at 30 V for 20 minutes and then at 120 V for 40 minutes. The gels were stained with staining buffer [0.25% (w/v) Coomassie Brilliant Blue R-250, 45% ethanol and 10% glacial acetic acid] for 2 hours and then destained with destaining buffer [40% ethanol and 10% glacial acetic acid] overnight.

Two types of protein gels were used in our study. We called the two types of gels Glycine-SDS-PAGE and Tricine-SDS-PAGE (Schägger, 2006). The principal difference between these two procedures was the use of Glycine or Tricine in the gel running buffer.

4.2.5.1 Glycine-SDS-PAGE

Glycine-SDS PAGE was performed by preparing the resolving gel, stacking gel and a running buffer. The ingredient compositions are listed in the Table 4.3.

Table 4.3 Ingredients and amounts for the components prepared for Glycine-SDS-PAGE.

12 % resolving gel	
H2O	10.2 mL
1.5 M Tris-HCl, pH 8.8	7.5 mL
20% (w/v) SDS	0.15 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	12.0 mL
10% (w/v) ammonium persulfate (APS)	0.15 mL
TEMED	0.02 mL
Stacking gel	
H2O	3.075 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL
20% (w/v) SDS	0.025 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 mL
10% (w/v) ammonium persulfate (APS)	0.025 mL
TEMED	0.005 mL
1X Running buffer	
Tris-Cl, pH 8.0	25 mM
Glycine	200 mM
SDS	0.1% (w/v)

4.2.5.2 Tricine-SDS-PAGE

Three types of buffers were prepared for this protocol. Two of them were running buffers (electrodes) and were called the ‘anode buffer’ and ‘cathode buffer’. Another one is the gel buffer, which is necessary for gel making. Anode buffer was poured outside the gel (the space in between the gels and the tank) and cathode buffer was poured in between the two gels. The buffer components are listed in the Table 4.4.

Table 4.4 Ingredients of the buffers used for Tricine-SDS-PAGE.

	Anode Buffer (10X)	Cathode Buffer (10X)	Gel Buffer (3X)
Tris (M)	1.0	1.0	3.0
Tricine (M)	----	1.0	----
HCl (M)	0.225	----	1.0
SDS (%)	----	1.0	0.3
pH	8.9	~8.25	8.45

The gels were prepared according to the following protocol.

Figure 4.5 Ingredients for Tricine-SDS-PAGE preparation.

		Stacking gel (4%)	10%	16%
AB-3:Acrylamide/Bis-acrylamide				
(48%/1.5% w/v)	(mL)	1	6	0
Gel buffer (3X)	(mL)	3	10	0
Glycerol	(g)	----	3	3
Add water to final volume	(mL)	12	30	0
10% APS	(µL)	90	150	0
TEMED	(µL)	9	15	10

4.3 Cloning

A truncated LexA, Δ_{68}LexAK156A, was previously introduced into the pET28a-c(+) vector (Novagen) in our laboratory by Gloria Qian. The first 68 amino acids were removed from LexA to truncate the DNA-binding domain and the K156A mutation was introduced to make the LexA mutants non-cleavable (termed as Δ_{68}LexAK156A). The map and the cloning region of the pET28a-c(+) vector is shown in Figure 4.1. The position of the LexA gene inserted is also marked in Figure 4.1A. The gene for kanamycine resistance helps the clone selection during cloning process. The cloning region shows the essential cutting sites, His-tag positions and the position of T7 promoter and T7 terminator (Figure 4.1B).

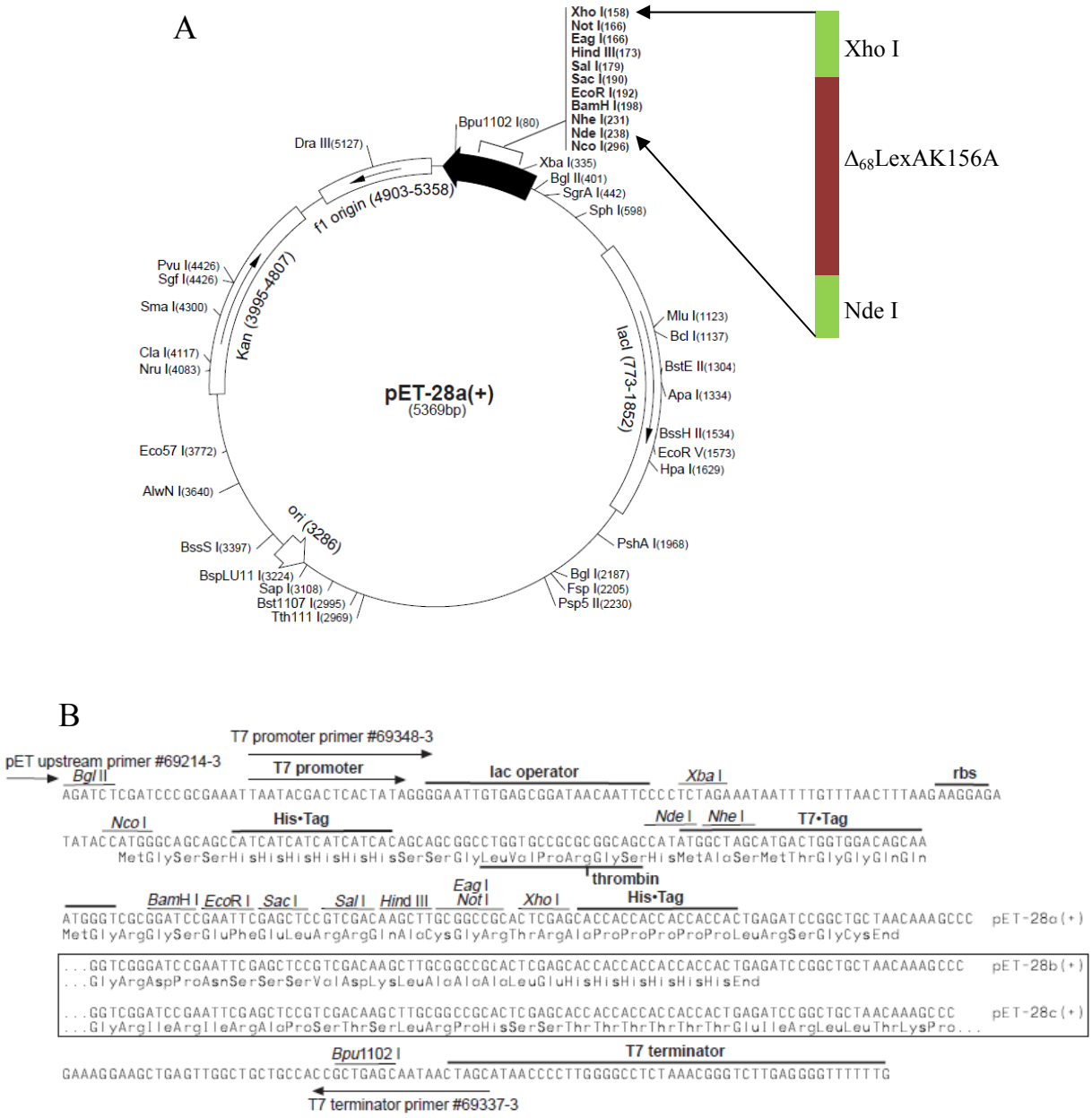


Figure 4.1 pET28a(+) plasmid vector map and cloning region in the vector.

A) The 5.4 kb pET28a(+) bacterial protein expression vector is shown here. It contains a short cloning/expression region (shown in Figure B). The vector encodes a Kanamycine resistence gene (KanR2). It has a short region containing several restriction cutting sites. The position of the Δ_{68}LexAK156A gene inserted in to the vector is shown here. Δ_{68}LexAK156A gene was inserted in between the *Xho I* and *Nde I* cutting sites (black arrows). B) The cloning/expression region of the pET28a-c(+) is shown. The region has a both N- and C- terminal His-Tag site. The cutting regions and T7 promoter and terminator regions are also given. Courtesy of Novagen (*www.emdchemicals.com/showBrochure?id=200905.345*).

Two β-11 strands of the CTDs of the LexA dimer interacts antiparallely in the dimer interface (Figure 4.2) (Luo *et al*., 2001). Three consecutive residues Val195, Ile196 and Arg197 are found in the C-terminal end positions of the β-11 stand which also takes part in the antiarallel β-11 strand interaction. Therefore, mutations were targeted for these three residues. The targeted mutations were Valine195 to Proline (V195P), Isoleucine196 to Tyrosine (I196Y) and Arginine197 to Proline and Glutamine (R197P and R197Q). V195P and R197P mutations were chosen to break down the hydrogen bonding between the two antiparallel β-11 strands and with other residues (Figure 4.2B-C). I196Y was chosen to produce steric hindrance in the dimer interface (Figure 4.2B) and R197Q was chosen to break down the hydrogen bonding with Gly124 (Figure 4.2C). The sequences of the forward and reverse primers for the mutations are listed in Table 4.6.

The forward primers had an *Nde I* restriction site and the reverse primers had an *Xho I* restriction site. Since the mutation sites were close to the end of the C-terminal end of LexA, mutations were introduced only in the reverse primers. The forward primer was designed by and obtained from Gloria Qian and was the same for all four mutations. No start codon was introduced in the forward primer because that would encode the hexa-histidine tag from the vector into the N-terminal site of the proteins. Table 4.6 contains the list of the primers used for cloning purpose.

Table 4.6 Sequences of the primers used for cloning of the LexA mutants. Restriction cutting sites and the coding sequences are shown in bold and italic letters, respectively. The mutation codons are underlined.

Name	Sequence (5′-3′)	Restriction sites
Δ_{68}LexA For	gga att c**ca /tat g***ca gga aga gga aga agg gtt*	*Nde I*
V195P Rev	ccg **c/tc gag** *tta cag cca gtc gcc gtt gcg aat <u>agg</u> ccc aac*	*Xho I*
I196Y Rev	ccg **c/tc gag** *tta cag cca gtc gcc gtt gcg <u>ata</u> aac ccc aac*	*Xho I*
R197P Rev	gtg **c/tc gag** *tta cag cca gtc gcc gtt <u>agg</u> aat aac ccc aac*	*Xho I*
R197Q Rev	gtg **c/tc gag** *tta cag cca gtc gcc gtt <u>ttg</u> aat aac ccc aac*	*Xho I*

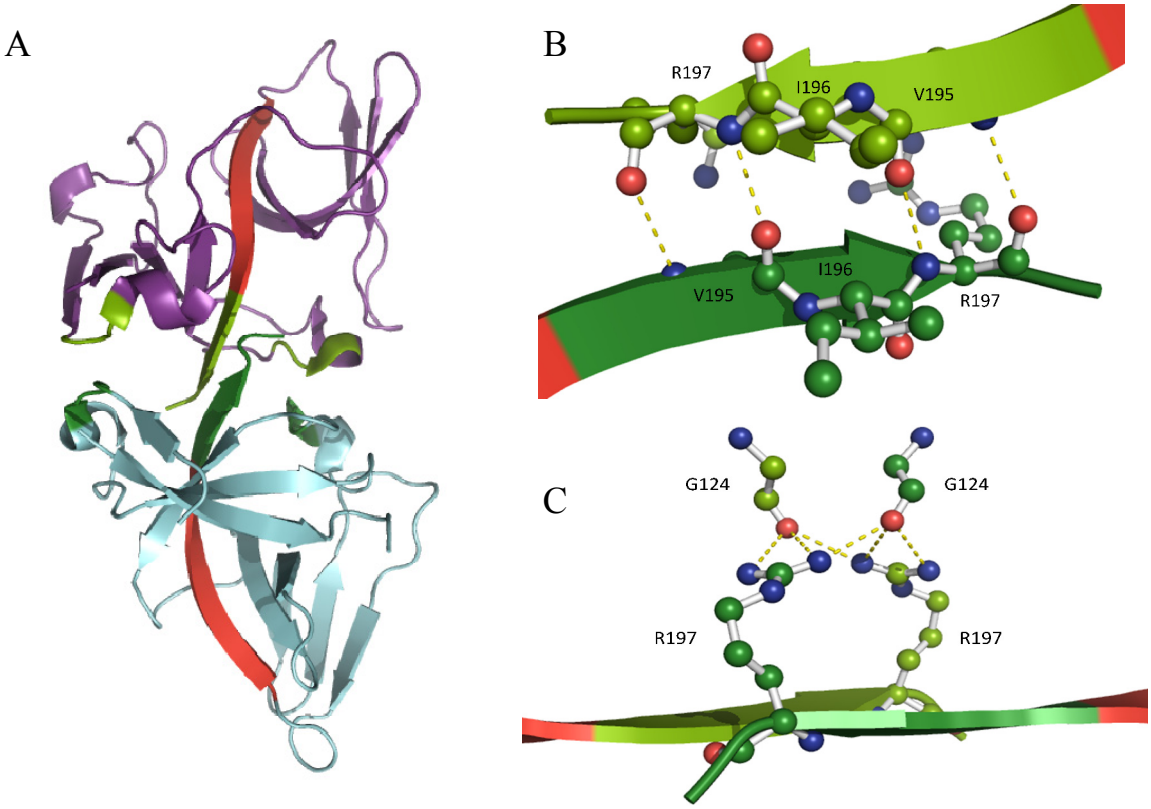


Figure 4.2 Dimer interface of *E. coli* LexA and the interactions involving antiparallel β-11 strands.

A) Two CTDs of LexA interacting in the dimer interface to form a dimer (PDB ID: 1jhh, Luo *et al*., 2001). Top and bottom monomers are shown in purple and cyan, respectively. ~1380 Å^2 area is buried in the dimer interface (Luo *et al*., 2001). The interface region (residues from two loops: Gln99 to Asp110 and Ser116 to Gly128, and part of the antiparallel β-11 strands) that are buried and interacting in the dimer interface are shown in green (light green in top monomer, dark green in bottom monomer). Two antiparallel β-11 strands are shown in red except for the part interacting in the dimer interface (green). Figure B and C are cartoon representation of the antiparallel β-11 strands showing the interactions. Residues Val195 to Arg197 are presented in ball and stick model in figure B. Polar interactions between these residues are shown in yellow dotted lines. Arg197 from one strand interact with Val195 of the other strand by two hydrogen bonds and *vice versa*. Figure C shows a different interaction between the Arg197 with the Gly124, donated from a loop (Ser116 to Gly128) in the dimer interface. Gly124 from one monomer forms two hydrogen bonds with the two side chain nitrogens of Arg197 from another monomer. One hydrogen bond was found between Gly124 and Arg197 from the same monomer. The residues that were targeted for mutations in the current experiment were Val195, Ile196 and Arg197 to interrupt the interactions in the dimer interface of two monomers of LexA. Figures were created by PyMOL.

The genes were amplified by the PCR technique as described by Sambrook and Russel (2001). *Pfu* DNA polymerase was used for the chain reaction. All the PCR products were extracted and purified from 1% agarose gels (w/v) with the Qiaquick gel extraction kit. Both the PCR products and the pET28a-c(+) vector were double-digested with *Nde I* and *Xho I* [2.5 µL 10X Buffer No. 4, 100X BSA, 1 µL *Nde I*, 1µL *Xho I* and 20 µL PCR products, incubated overnight at room temperature]. The cut vector was then ligated to the cut PCR products by T4 DNA ligase [1 µL Ligase buffer, 1 µL T4 Ligase, 6 µL digested fragments and 2 µL digested vector, incubated 5 hours at 37°C]. Ligated products were then transformed into XL1-Blue *E. coli* competent cells by electroporation with 1600 volts of electric impulse. After the electroporation, each batch of cells (50 µL) was first cultured in 400 µL of SOC culture medium [20 g Bacto-Tryptone, 5 g Yeast extract, 0.5 g NaCl, 2.5 mL of 1 M KCl, ddH_2O to 1 L]. After one hour of incubation at 37°C, the cultures were transferred to LB agar plates containing 35 µg/mL kanamycin (spread plate method) and grown overnight at 37°C. A few colonies were selected and marked on the plate after growth and picked to start colony PCR. This time, the T7 promoter and the T7 terminator (Table 4.2) primers were used and *Taq* DNA polymerase was used for the elongation process. The PCR products were resolved with 1% (w/v) agarose gels and the band sizes were compared with the ladder. The positive colonies were picked from the plates and grown in LB broth (with 35 µg/mL kanamycin) overnight at 37°C in the Orbit Environ Shaker at 150 rpm. Plasmids were purified from the grown XL1-Blue cells using the Qiagene plasmid extraction kit. Sequences were confirmed by sending the DNA samples to the DNA Laboratory of the Plant Biotechnology Institute (PBI), Saskatoon, Canada. Purified plasmids containing the desired genes were electroporated into Rosetta (DE3) cells as described above and grown in SOS medium for an hour at 37°C with occasional shaking. The culture was transferred to 10 mL of LB broth. After 1 hour of growth at 37°C with shaking, 35 µg/mL kanamycin and 30 µg/mL chloramphenicol were added to the medium and incubated at 37°C overnight at 150 rpm. 800 µL of grown culture were added to a small tube to create a stock and stored with 20% glycerol at -80°C.

The wild-type RecA and wild-type LexA genes were obtained from the lab and were previously cloned between the *Nde I* and *Xho I* restriction sites of the pET28a-c(+) vector. In contrast to the *lexA* genes, *recA* gene had C-terminal hexa-histidine tag to facilitate binding to a

Ni^{2+} charged affinity column. Two other LexA mutant genes, Δ_{68}LexAK156A and Δ_{68}LexAK156AI196K, were also obtained from the lab and purified same way as the other LexA mutants.

4.4 Expression of the proteins

With the sterilized pipette tip, a tiny amount of the frozen electroporated Rosetta (DE3) cells containing the LexA mutant genes was transferred from the stock to 50 mL of LB medium containing 35 μg/mL kanamycin and 30 μg/mL chloramphenicol and incubated at 37°C overnight in a shaking incubator. One liter of LB medium was inoculated with 25 mL of the overnight cultures. Throughout the culturing, 35 μg/mL kanamycin and 30 μg/mL chloramphenicol were always added to the growth. Inoculated cultures were grown at 37°C in the shaking incubator until the absorbance of the culture reached 0.5 at 590 nm wavelength. To induce protein expression, 0.25 mM IPTG was added to the cultures, which were incubated for an additional 3-4 hours under the same conditions. After induction, the cells were collected from the liquid culture by centrifugation at 4000 X g for 20 minutes at 4°C. The pellets were collected in a separate tube and stored at -20°C until protein purification. Wild-type RecA, wild-type LexA, Δ_{68}LexAK156A and Δ_{68}LexAK156AI196K proteins were also expressed by the same method.

4.5 Protein purification

The collected cell pellets were first taken out from the -20°C freezer and resuspended in binding buffer [0.5 M NaCl, 30 mM Tris-Cl, pH 7.8]. Lysozyme (10 mg/mL) was added and the cells were sonicated with a Sonic Dismembrator at 70% amplitude for a cycle of 3 minutes (pulse on) in a 3 second pulse and 6 second rest for 3-5 times until the cells were broken open. The sonicated products were centrifuged at 31000 X g for 20 minutes. Supernatants were collected and eluted from a DE52 anion exchange column to get rid of residual DNA. The anion exchange column was previously equilibrated with the binding buffer. The flowthrough from the anion exchange column was then passed through a nickel affinity column. The nickel affinity column was first charged by running binding buffer and then a 0.1 M $NiSO_4.6H_2O$

solution over the column. The unbound nickel was removed by running binding buffer over the column again. The column with the bound histidine-tagged protein was then washed with a sufficient volume (~200 mL) of washing buffer [10 mM Imidazole, 0.5 M NaCl, 30 mM Tris-Cl, pH 7.8] to elute all the unbound proteins and impurities. Bound proteins were then eluted from the column by washing with elution (stripping) buffer [0.5 M NaCl, 50 mM EDTA, pH 7.8]. The eluted proteins were incubated overnight with thrombin (1 U/mg) at room temperature to remove the histidine tag from the protein. The proteins were run on 13% protein gels (Glycine-SDS-PAGE) to confirm the cleavage. After cutting, the DE52 anion exchange column was run to get rid of the thrombin. The buffer salt gradient ranged from 0.05 M to 0.5 M NaCl. The protein fractions were collected and precipitated overnight with 35% (w/v) ammonium sulfate. The precipitates were collected by centrifugation at 31000 X g at 4°C. The precipitates were redissolved in elution buffer [0.1 M NaCl, 30 mM Tris-Cl, pH 7.8, 0.1% β-Mercaptoethanol] for gel filtration chromatography and eluted from the HiPrep Saphacryl S-100 column. The flow rate was set 1 mL/min. Protein fractions of the intended size were collected, while avoiding the void volume.

The histidine tag was not removed during the purification of wild-type RecA. The other procedures followed for RecA purification were the same as those for the LexA proteins, but RecA had to be eluted from a HiPrep Saphacryl S-300 column due to its larger size (37.8 kD) and its tendency to form a hexamer. The buffer used for this purpose was also different and contained 1 M Urea, 1 M NaCl, 30 mM Tris-Cl, pH 7.8 and 0.1% β-Mercaptoethanol. Protein fractions from the profile peak were collected.

All the proteins from the gel filtration elution were concentrated with Millipore centrifuge filter tubes (45 μm pore size) until the protein concentration reached 20 mg/mL. Finally, the purified proteins were stored at -80°C.

4.6 Size exclusion chromatography profile analysis and comparison

Gel filtration or size exclusion chromatography separates protein according to size; the larger proteins elute from of the column earlier. Therefore, multimers should be eluted before monomers. As a result, the volume of buffer needed to elute proteins should differ according to

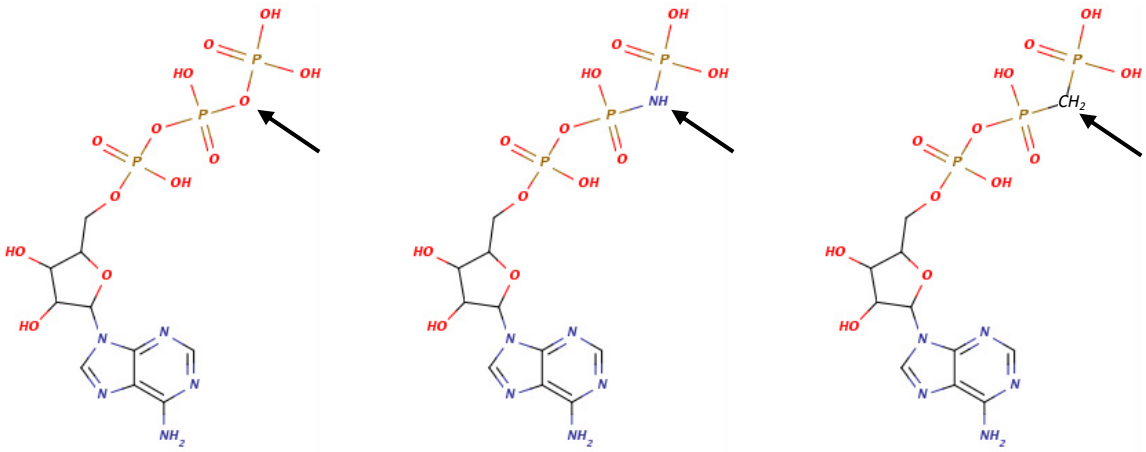
different forms of oligomerization. Therefore, the position of the peak of the protein in the elution profile should be different based on the monomeric, dimeric or multimeric forms. LexA mutants (Δ_{68}LexAK156A, Δ_{68}LexAK156AI196K, Δ_{68}LexAK156AV195P, Δ_{68}LexAK156AI196Y, Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AR197Q) were eluted through the HiPrep Saphacryl S-100 gel filtration column during purification. The elution profiles of the different mutants were saved and the volumes of buffer (mL) that were need for the elution of the LexA mutants were recorded. The volume profiles of the four LexA mutants (Δ_{68}LexAK156AV195P, Δ_{68}LexAK156AI196Y, Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AR197Q) were compared with the previously analyzed (in the current lab) known predominantly dimeric (Δ_{68}LexAK156A) and monomeric (Δ_{68}LexAK156AI196K) LexA mutants.

4.7 Dynamic Light Scattering (DLS) technique

Dynamic light scattering provides information about the size of any particle in solution by measuring the hydrodynamic radius. The percentage of the monomeric or oligomeric forms of the LexA mutants can therefore be measured in solution by using this method. Three different concentrations of each LexA mutants were analyzed: 200, 100 and 33.3 μM. The dilution buffer for the LexA mutants contained 50 mM HEPES-Tris, pH 7.8 and 0.1 M NaCl and was subsequently filtered with 0.45 μm pore size polyvinylidene difluoride filters (Millipore). The LexA mutants were diluted in the buffer, centrifuged for 4 minutes at 4000 X g and then filtered with a 0.02 μm Anodisk filter before starting the experiment. The DLS measurements were performed at 25°C with a DynaPro MS800 instrument. The instrument used an 824.8 nm laser diode, which was operated at a power of 55 mW and had a fixed scattering angle of 90 degrees. Scattered intensity was accumulated at intervals of 5 sec for a period of 6 minutes. The particle size distribution was analyzed with Dynamics V5.26.60, which was supplied by the manufacturer. The mass percentage of the different sized LexA mutants (monomeic, dimeric and tetrameric) in the solution was produce by the software at different concentrations and the dimer dissociation constant (K_d) was revealed from the concentration and mass percentage of the protein.

4.8 Co-protease assay of wild-type LexA

Wild-type RecA and LexA were allowed to interact in a reaction procedure called the co-protease assay. The prepared reaction solution consisted of 50 mM HEPES-Tris buffer, pH 7.4, 20 mM $MgAc_2$, 2 mM AMPPNP, 1 µM dT 45 (ssDNA), 10 µM wild-type RecA and 20 µM wild-type LexA. All the ingredients were first combined in a tube and mixed gently by pipetting. AMPPNP, an analog of ATP, was the last component added in order to start the reaction. The phosphodiester bond between the β- and γ -phosphate of ATP breaks down during the ATPase activity by proteins, such as RecA. AMPPNP, full name Adenylyl-imidodiphosphate, has a nitrogen (N) atom instead of oxygen (O) in between the β- and γ-phosphate group of ATP (Figure 4.3). The presence of the nitrogen atom makes the molecule resistant to phosphodiester bond breakdown and γ-phosphate cannot be released as a leaving group. Therefore, AMPPNP was used in the experiments stabilize the reaction rate. Replacing the same oxygen (O) atom with carbon (C) also makes the molecule hydrolysable by ATPase activity and the molecule is called AMPPCP. The structures of ATP, AMPPNP and AMPPCP are presented in Figure 4.3.



ATP AMPPNP AMPPCP

Figure 0.1 Structure of ATP and its analogs.

Chemical structures of the ATP, AMPPNP and AMPPCP are shown. Positions of oxygen (O), nitrogen (N) and carbon (C) atoms between the γ- and β-phosphate groups are indicated with the arrow in ATP, AMPPNP and AMPPCP, respectively. These figures were created with Online Chemical Editor (JChemPaint).

One negative control reaction solution was also prepared without any AMPPNP. The proteins were allowed to interact while the solution was kept at room temperature (~20°C) for 2 hrs. The reaction was stopped by adding 1X protein loading dye. Tricine-SDS-PAGE was performed to analyze the cleavage. 13% Tricine-SDS polyacrylamide gel was used and the gel was Coomassie stained. The gels were viewed and pictures were captured and analyzed by the Kodak GelDoc imaging system.

4.9 Time course co-protease assay

A time course co-protease assay was performed with the same reaction solutions and conditions as in the previous experiment. The reaction was also allowed to continue for 2 hours. However, samples were taken at 30 minutes intervals from 0 min to 120 minutes. Therefore, 5 total samples were collected from one reaction. The reaction was stopped at different intervals by adding 2X protein loading dye and placing the tube on ice until the protein gel was run. Tricine-SDS-PAGE was performed to monitor the cleavage of LexA with respect to time. Pictures were captured as described above and the band sizes were analyzed.

4.10 Co-protease inhibition assay

If the non-cleavable LexA mutants bind tightly to RecA, they will occupy the binding sites for wild type LexA. Therefore, RecA-mediated cleavage of wild type LexA will be inhibited, and the mutants will act as inhibitors of co-protease activity. The co-protease inhibition assay is the same as the co-protease assay except for the addition of inhibitors, i.e., the LexA mutants. For this purpose, 2 mM AMPPNP and 10 μM of inhibitors (LexA mutants) were added before addition of wild type LexA to the reaction solution. The mixture was incubated for 3 minutes at room temperature to allow the mutants to bind to RecA. Then, wild type LexA was added, mixed gently, and incubated for 2 hours at room temperature. Inhibitory capability was measured by running the samples on 13% Tricine-SDS acrylamide gels. A time course of inhibition was also performed by sampling the reactions in 30-minute intervals for a total time of 2 hours. All of the gel pictures were captured and the band sizes were analyzed.

4.11 Co-crystallization attempts of RecA and LexA

For the RecA and mutant LexA co-crystallization attempts, 24-well crystallization plates for the hanging drop method and 96-well plates for commercial crystallization kits for the sitting drop method were used. Protein solutions contained 10 μL of RecA (~20 mg/mL), 10 μL of LexA (~20 mg/mL), 1 mM ATP or analogues and 1 mM of ssDNA of different sizes. Well solutions contained different concentrations of buffers, precipitants, and salts. The conditions that were used for the hanging drop method are given in Table 4.7.

Two different commercial kits from Qiagene/Nextal were used, The Classics and The Pegs. The plates contained 96 wells for 96 different conditions for each kit. The compositions and ingredients of the kit solutions can be found on the Qiagen webpage (*http://www1.qiagen.com*).

Around 0.8 μL of protein solution and 0.8 μL of well solution were used to make one crystallization drop. The wells were sealed with grease-mounted cover slips for the hanging drop method and adhesion tape for the sitting drop method. The plates were incubated at room temperature and observed under a microscope at regular periods. The conditions and changes during the incubation period in each well were recorded. The conditions were repeated if crystals were found in any of the drops. We tried to purify the proteins in different buffers, such as buffers containing acetates instead of chlorides or potassium instead of sodium, to facilitate crystallization.

Table 4.7 Ingredients and conditions applied for the co-crystallization trials.

Crystallization Trials		
Protein Solution		
Components	**Varying components**	
LexA mutants	I196Y R197P	
ATP or analogues	ATP AMPPNP ADP ADP + AlF_4 AMPPCP	
ssDNA (dT## are nt numbers)	dT45 dT22 dT20 dT18	
Well Solution		
Components	**Varying components**	**Varying parameters**
Buffers	HEPES HEPES-Tris Tris MES	*pH* 6.0-8.5
Precipitants	PEG 550 PEG 1500 PEG 3350 K15 Ethylene Glycol	*Concentration* PEG and K15: 8-30% Ethylene Glycol: 20-60%
Salts	NaCl KCl NaAc KAc $MgCl_2$ $MgAc_2$	*Concentration* 0-0.2M

5.1 Mutagenesis, cloning, expression and purification of the RecA, wt LexA, and six LexA dimer interface mutants (Δ_{68}LexAK156A mutants)

RecA, wild type LexA and six of the LexA dimer interface mutants (Δ_{68}LexAK156A, Δ_{68}LexAK156AI196K, Δ_{68}LexAK156AV195P, Δ_{68}LexAK156AI196Y, Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AR197Q) were purified. Δ_{68}LexAK156AV195P, Δ_{68}LexAK156AI196Y, Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AR197Q mutants were freshly cloned and the other two mutants (Δ_{68}LexAK156A, Δ_{68}LexAK156AI196K) were collected as pre-cloned (Moya, 2006) in the lab. Expressions of all the proteins in Rosetta (DE3) cells were sufficient to purify substantial amount of proteins. All proteins were soluble in the buffer solution [0.5 M NaCl and 30 mM Tris (pH 7.8)] after cell disruption by sonication (Figure 5.1, lane 4). Supernatant from the cell lysate was applied to the anion exchange column DE52 to remove the negatively charged dissolved DNA fragments from the solution. Positively charged proteins flowed through the column (Figure 5.1, lane 5).

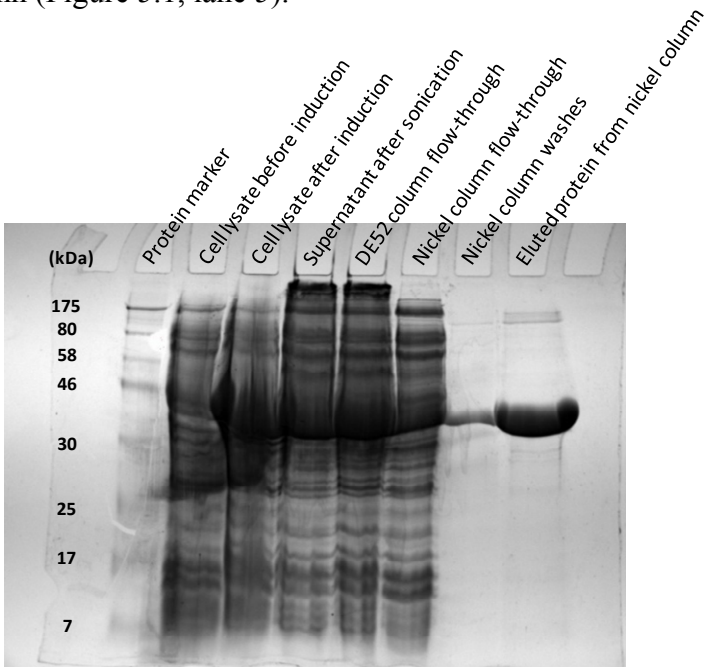


Figure 5.1 A typical SDS-PAGE gel of the primary purification steps of RecA.

This is a 12% Glycine-SDS polyacrylamide gel and the gel was stained with Coomassie Brilliant Blue R250. 20 µL of samples were loaded in each well.

Nickel chelating column chromatography produced reasonably pure protein, and size exclusion chromatography increased the purity to >90%. Samples from the different steps from cell disruption to nickel chelating column purification were collected and analyzed by Glycine-SDS-PAGE. Figure 5.1 shows different purification steps of wild type RecA. The purity gel shows the yield and purity of the proteins collected from nickel column flow through (Figure 5.2). 20 µL of protein samples were loaded in each well and the concentration of the proteins were not standardized. The yield and purity after this step differed from protein to protein.

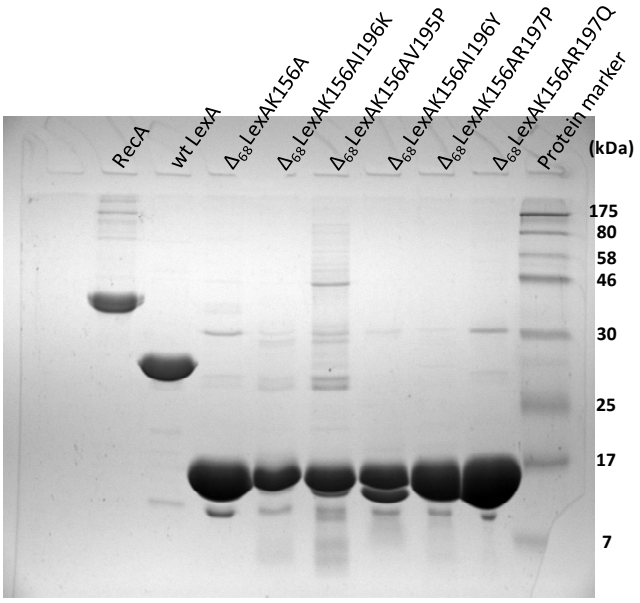


Figure 5.2 Purity of proteins after nickel chelating column chromatography.

The proteins were collected after the nickel chelating column run. 12% Glycine-SDS polyacrylamide gel was run and the gel was stained with Coomassie Brilliant Blue R250. 20 µL of proteins were loaded in each well. The concentrations were not standardized.

The final step of the purification before concentrating the protein was the gel filtration chromatography. The HiPrep Saphacryl S-100 column profile showed different elution volume for different mutants. Figure 5.3 shows the elution profile of Δ68LexAK156AV195P and two peaks can be seen during protein elution. The first early peak was the void volume due to the impurities of the proteins. It was eluted around 35 to 40 mL range usually. This peak was not

collected. The second peak was eluted at least after 50 mL of elution and was pooled for concentration.

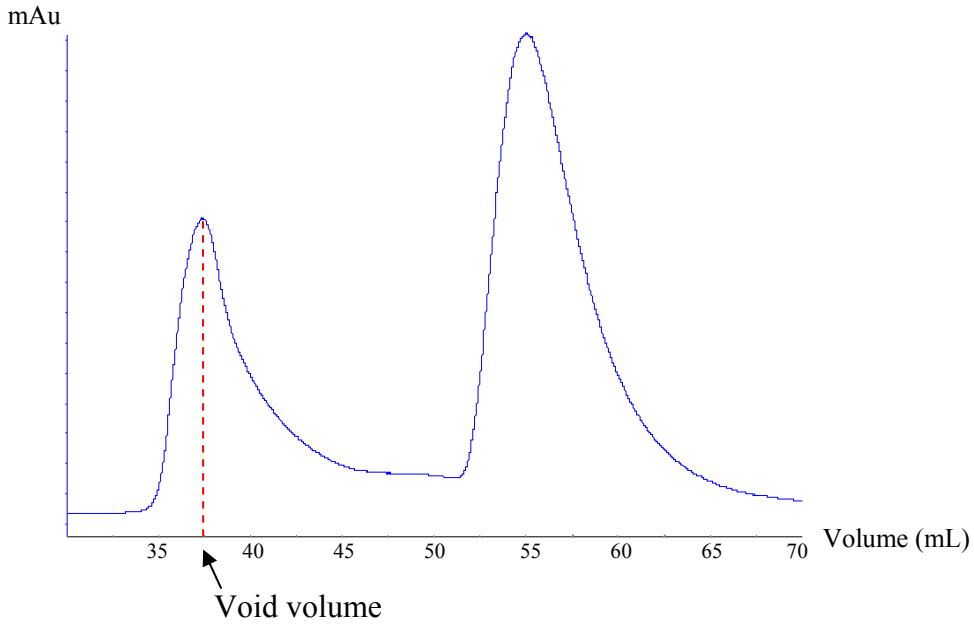


Figure 5.3 Gel filtration elution profile of Δ_{68}LexAK156AV195P.

The UV absorption unit (mAu) against buffer elution (mL) graph of the S-100 HiPrep Saphacryl column is shown. Two peaks represent the elution of proteins. The early peak was due to the impurities and the second peak represents the monomeric/dimeric form of the LexA mutants. The fractionation range (Mr) of the S-100 column for the globular protein is 1 X 10^3 – 1 X 10^5.

Purified LexA dimer interface mutants with N-terminal hexa-Histidine tags had a milky appearance in solution. When the His-tag was cut off from the mutants by thrombin treatment, the purified protein solutions became clear. This reveals that the aggregation of the LexA mutants was due to the N-terminal Histidine tag.

5.2 Gel filtration chromatography elution profile analysis

Size exclusion or gel filtration chromatography has become a remarkable tool for macromolecular purification since it was developed during the late 1950s (Hagel, 2001; Porath and Flodin, 1959). As the name indicates, size exclusion chromatography separates molecules according to size; therefore, this method can be used to measure the size of macromolecules.

This principle was used in our study to determine the sizes of the purified LexA mutants. Larger molecules, such as oligomers of LexA, elute earlier from the gel filtration column with a smaller solution volume than smaller molecules. As a result, polymers of a protein should elute from the column with a smaller solution volume than the monomers. This can indicate whether any protein is monomeric or polymeric.

In this experiment, the LexA mutants were applied to the gel filtration HiPrep Saphacryl S-100 column during purification, and the resulting gel filtration profiles were compared with the different LexA mutants. Two peaks appeared in most of the protein profile (Figure 5.4). The first peak appeared due to impurities of the protein. Proteins from the second peak were collected, which was assumed to give monomeric and/or dimeric proteins at the purification concentration. The profiles got from the gel filtration protein elution were used for the size determining experiment of the mutants.

The gel filtration graph profiles are shown in Figure 5.4. The Δ_{68}LexAK156A and Δ_{68}LexAK156AI196K mutants were analyzed previously in our lab (data not shown), and gel filtration profiles of dimeric and monomeric LexA species, respectively, were found. The mutant proteins produced in this study were compared with these two controls in the same buffer system. The V195P, I196Y and R197Q mutants had elution volumes that were similar to the dimeric control and are presumed to be polymers in solution. The elution profile of the R197P mutant was more similar to the previously characterized I196K monomer. Interestingly, both these mutants had a small bump/peak before the final peak at around 50 mL region. The I196K peak started earlier and showed an elongated buffer volume range which indicates that there may be more than one peak. R197P also showed a smalled peak before larger. This indicates that there may be more than one form of LexA present in these solutions. Note that the I196Y mutant was eluted with a little more solution than the dimeric control but less than the monomeric control (Figure 5.5).

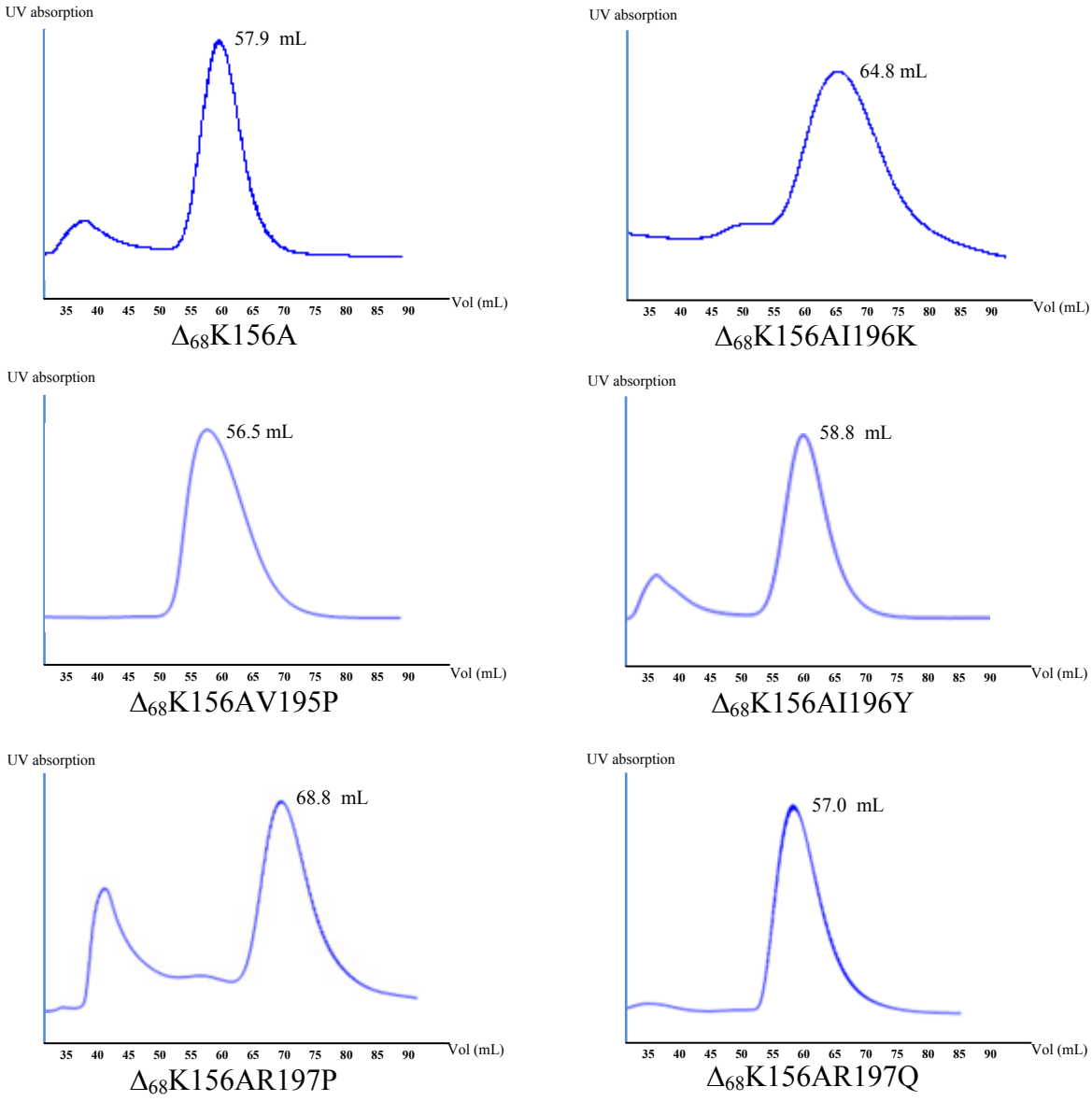


Figure 5.4 Gel filtration elution profiles of Δ_{68}LexA mutants.

The gel filtration elution profiles of six LexA mutants are presented. The volume (mL) of the buffer needed for the protein elution is shown on top of each peak. The elution volume (mL) was determined by pointing the volume needed for the appearance of the top of the peak. Some ghost peaks appeared due to the impurity or higher oligomerized state. HiPrep Sephacryl S-100 column was used for the protein elution. The fractionation range (Mr) of the S-100 column for the globular protein is 1 X 10^3 – 1 X 10^5.

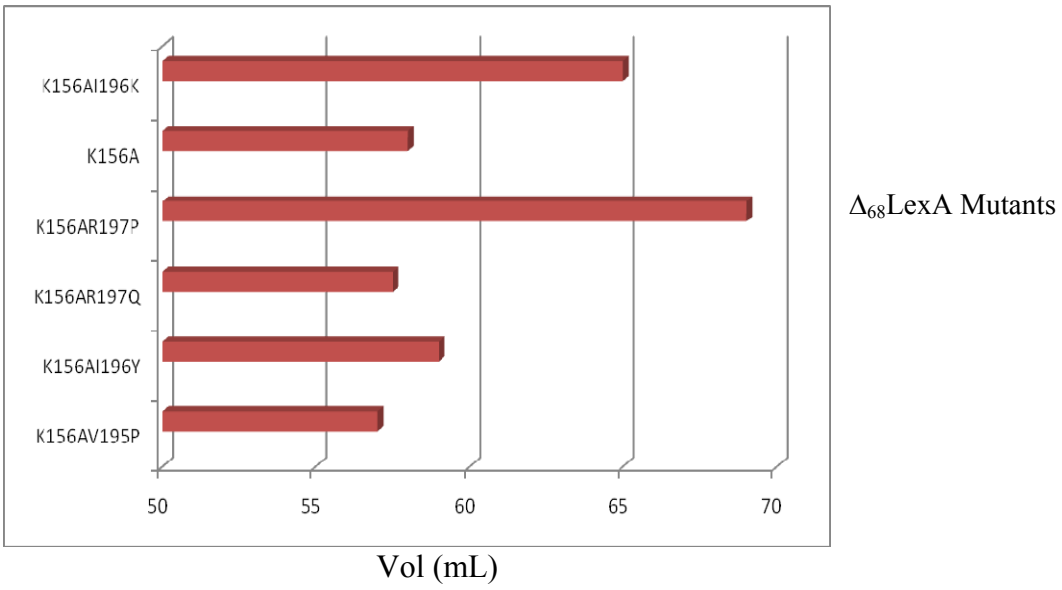


Figure 5.5 Graphical presentation of gel filtration chromatography sample elution volume profile.

The graph shows the comparative volumes (mL) of buffer that were required for the elution of different Δ_{68}LexAK156A mutants from the HiPrep Sephacryl S-100 column.

5.3 Dynamic Light Scattering (DLS) experiments

Dynamic light scattering is a more accurate technique to measure the size of particles or molecules in solution than gel filtration. Particles in solution undergo Brownian motion. If laser light is applied to a protein solution, the intensity of the light scattered by the particles fluctuates. The fluctuation of the light intensity depends on the size of the particles in the solution. Analysis of this fluctuation yields the velocity of the Brownian motion, and the size of the particles or molecules can be measured using the Stokes-Einstein equation. The size is determined from the hydrodynamic radius or diameter of the molecules in the solution, which refers how a particle diffuses in a fluid.

The particle size distribution analyzer provides the size of the protein molecules in molecular weight. Therefore, it is possible to determine the monomeric or polymeric form of

proteins from known molecular weights. Truncated mutant LexA monomers have a molecular weight of ~15 kDa.

The DLS data of the four Δ_{68}LexAK156A mutants at 33.3 µM concentration are presented in Figure 5.6 as pie charts. DLS data from all the mutants at different concentrations are tabulated in Table 5.1 and presented in the Figure 5.7. A previous study in our lab (data not shown) (Moya, 2006) and the current study showed that Δ_{68}LexAK156A is predominantly a dimer (~65% dimeric) at 33.3 µM. In the current study, Δ_{68}LexAK156AR197P was found as predominantly monomeric in solution at concentrations of both 33.3 µM and 100 µM (60% and 50% of the mass respectively) (Figure 5.6 and 5.7, Table 5.1). The Δ_{68}LexAK156AI196Y mutant was also 49% in the monomeric form at 33.3 µM. The other two mutants were predominantly dimeric or tetrameric at 33.3 µM and higher concentrations.

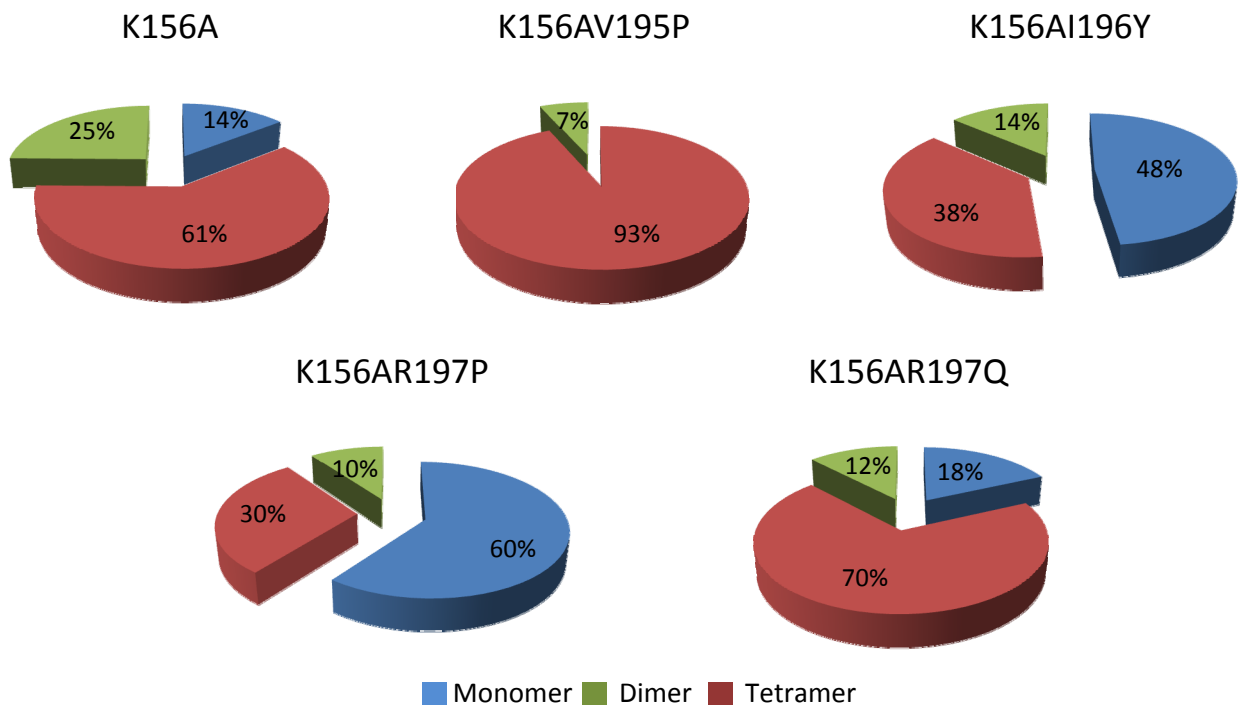


Figure 5.6 Percentages of the different forms of the Δ_{68}LexA mutants at 33.3 µM concentrations in solution.

The pie charts were generated with the data from the DLS profiles of the different LexA mutants. The R197P and I196Y mutants show high monomeric mass percentages in solution at 33.3 µM, whereas the other two mutants show higher oligomeric mass percentages.

Table 5.1 Dynamic light scattering data of five Δ_{68}LexA mutants.

Diffusion coefficient, hydrodynamic radius, molecular weigth, mass percentage and dimer dissociation constant (K_d) of different oligomeric forms of LexA mutants in three different concentrations are shown here. Dimer dissociation constant (K_d) values were estimated based on the mass distribution. K_d values at concentrations that showed absence or very low percentage of monomeric proteins were omitted.

Mutant LexA	Concentration (uM)	Diff (e-9cm^2/s)	Hydrodynamic radius (nm)	Molecular weight (kDa) *	% mass	K_d (µM)
K156A	33.3	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	13.96 59.26 24.08	2.18
	100	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	1.63 82.70 15.67	--
	200	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 80.66 19.34	--
K156AV195P	33.3	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 93.42 6.58	--
	100	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 87.75 12.25	--
	200	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 92.63 7.37	--
K156AI196Y	33.3	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	48.11 38.27 13.62	40.28
	100	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	19.84 63.36 16.80	12.42
	200	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	7.35 75.42 17.23	2.86
K156AR197P	33.3	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	60.40 29.60 10.00	82.04
	100	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	50.31 36.60 13.09	138.3
	200	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 66.07 33.93	--
K156AR197Q	33.3	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	18.23 69.57 12.20	3.2
	100	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	7.36 77.52 15.12	1.4
	200	1254 936.6 699.6	1.921 2.571 3.442	15.5 30.7 60.7	0.00 83.4 16.6	--

*15.5 kD = Monomer, 30.7 kD = Dimer, 60.7 kD = Tetramer.

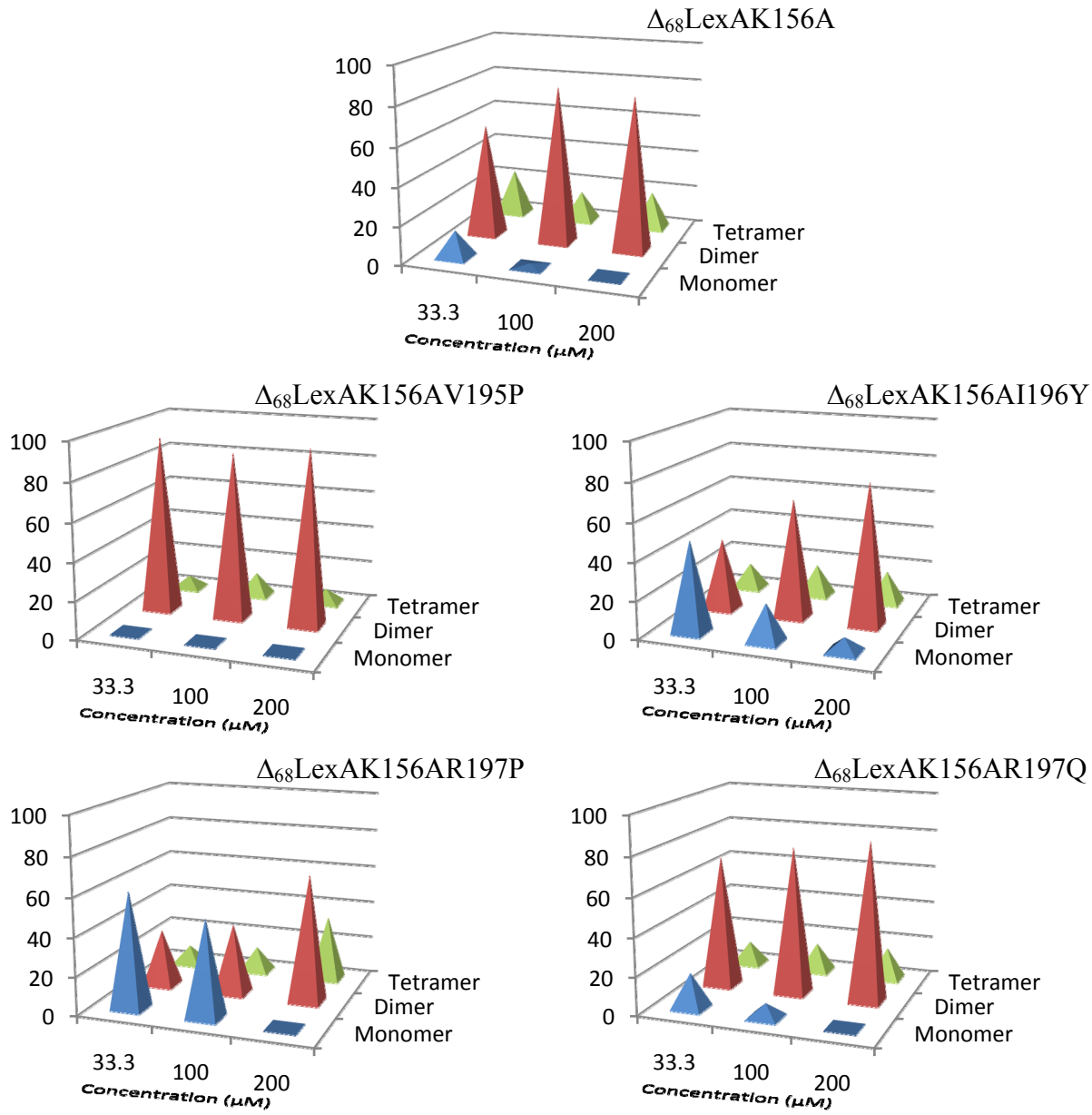


Figure 5.7 Oligomeric forms distribution of Δ_{68}LexA mutants at different concentrations by DLS.

The percentage of oligomeric states of the Δ_{68}LexA proteins are presented at different concentrations. The percentage, oligomeric forms and concentrations are labelled. Monomeric, dimeric and tetrameric forms are presented in blue, red and green, respectively. Three different concentrations of proteins were tried for this study: 33.3 μM, 100 μM and 200 μM. K156AI196Y and K156AR197P mutants showed that predominantly monomeric proteins were present at 33.3 μM concentration solution. K156AR197P shows more monomeric proteins even at 100 μM concentration. Mostly oligomeric forms were found in other mutants at different concentrations.

Dimer dissociation constant (K_d) of Δ_{68}LexAK156A for the dimeric form was estimated to be 2.18 μM (Table 5.1). This means Δ_{68}LexAK156A exists as a dimer at 33.3 μM concentration. This K_d value appeared to be close to the value reported by Kim and Little (1992), and the value reported for the C-terminal domain of the 434 phage repressor (Donner *et al*., 1998). This value is also close to another study performed previously in the current lab (~1 μM) (Moya, 2006, unpublished data). Δ_{68}LexAK156AR197Q mutant also showed K_d value <4 μM (3.2 μM) and therefore, assumed as predominantly dimeric. Additionally, Δ_{68}LexAK156AV195P would exist as a dimer even at lowest concentration tried (33.3 μM) since no monomeric protein was detected at that concentrations. On the other hand, Δ_{68}LexAK156AI196Y would exist as a monomer at 33.3 μM concentration, since the K_d for the dimer was ~40 μM. Whereas, Δ_{68}LexAK156AR197P showed a much higher K_d value of ~80 μM at 33.3 μM concentration and can be certainly predicted that it would exist as monomers at low concentrations. Interestingly, Δ_{68}LexAK156AR197P showed higher monomeric mass percentage than that of oligomeric even at 100 μM concentration.

Therefore, it was confirmed that Δ_{68}LexAK156AR197P was completely in the monomeric form, at least at concentrations as high as 100 μM. From the DLS experiment, we also found Δ_{68}LexAK156AI196Y as predominantly monomeric at 33.3 μM but not at higher concentrations.

5.4 Co-protease inhibition assay

RecA is termed a co-protease because it acts indirectly in catalysis of LexA cleavage (Little, 1991). Cleavage of wild type LexA occurs in between residues Ala84 and Gly85. As a result, two fragments produced after the digestion of LexA are residues 1 to 84 and residues 85 to 202. The two protein fragments can be detected on SDS protein gels. Therefore, the percentage of cleavage can be measured by analyzing band sizes and intensities of the intact and the cleaved products of wild type LexA from the SDS gel images.

Allowing the RecA protein to cleave wild type LexA in a reaction solution is called a co-protease assay. The co-protease inhibition assay is different in the sense that an inhibitor, i.e., a LexA mutant, is added prior to wild type LexA in the reaction solution. Three trials of the

co-protease inhibition assay were performed with a time course, and the results are shown in Figure 5.8. The band intensities and band areas of wild type LexA were analyzed with the Kodak GelLogic Image Analyzer. Figure 5.8 (A) shows the 2-hour time course of co-protease activity of LexA without any inhibitors, and samples from different times show that LexA was cleaved gradually with time.

The band area of intact LexA decreased with time as the product band areas increased (Figure 5.8A). ~74% estimated cleavage of wt LexA was observed after 120 minutes of the reaction without any inhibitors. On the other hand, cleavage ranged from 5-17% when incubated with the inhibitor mutants (Figure 5.8B-F). The wild type LexA was cleaved 5%, 7%, 13% and 17% in the presence of inhibitors Δ_{68}LexAK156AV195P, Δ_{68}LexAK156AR197Q, Δ_{68}LexAK156AI196Y and Δ_{68}LexAK156AR197P, respectively.

Notably, in the case of predominantly monomeric mutants (Δ_{68}LexAK156ARI196Y and Δ_{68}LexAK156AR197P), wild type LexA showed the most cleavage (15-17%) compared to the dimeric mutants. However, the percentage is still significantly smaller than in the absence of any inhibitors (~74%). Therefore, it can be concluded that Δ_{68}LexAK156AR197P inhibits RecA co-protease activity to some extend, which means it has some binding affinity to RecA as a monomer. Other mutants were better inhibitors than Δ_{68}LexAK156AR197P in terms of the percentage of LexA cleavage. The Δ_{68}LexAK156AI196Y mutant also showed good inhibitory affect, and this mutant was also found as predominantly monomeric at lower concentrations by DLS study. Actually visual observation of the band cleavages showed that Δ_{68}LexAK156AI196Y inhibited the LexA cleavage most though the band area measurement gave different results (13% wt LexA cleavage) (Figure 5.8D). This dispersion could be a result of errors during protein loading or gel staining variations. However, from the above results from monomeric protein determination and inhibition assay two mutants of LexA, Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AI196Y, were selected to perform co-crystallization trials.

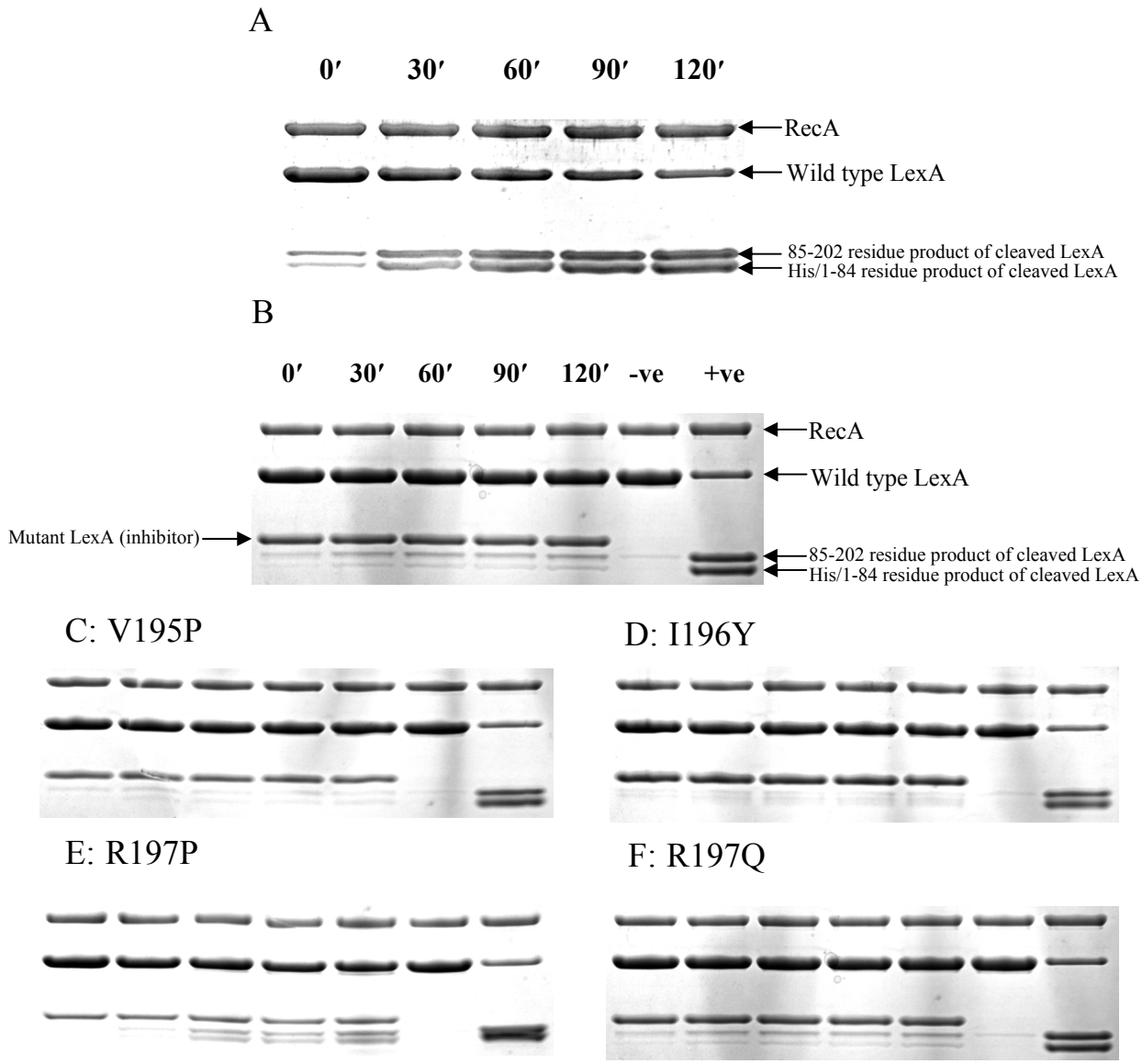


Figure 5.8 Co-protease assay of RecA and co-protease inhibition assays by different LexA mutants.

A) A typical co-protease timcourse assay of RecA. Cleavage of LexA is shown according to time (minutes). Wild type LexA cleaved gradually with time. The products were one His/1-84 residue peptide chain and one 85-202 residue peptide chain. The wild type LexA band narrowed gradually according to time, whereas the peptide product bands thickened. B) A typical co-protease inhibition time course assay. Inhibitors were the Δ_{68}LexAK156A mutants. RecA was incubated with the inhibitors prior to wild type LexA addition in the reaction mixture. Negative (-ve) control was performed without adding AMPPNP and positive (+ve) control was performed without adding inhibitors. C-F) Co-protease inhibition assay by the four Δ_{68}LexAK156A mutants. 13% Tricine-SDS polyacrylamide gels were run and the gels were stained with Coomassie Brilliant Blue R250. 8 μL of samples were loaded in each well.

5.5 Crystallization attempts

Two most common methods of protein crystallization, known as the hanging drop method and sitting drop method, are in the vapor diffusion category. Proteins undergo slow precipitation during the crystallization method. Vapor diffusion facilitates this procedure by allowing the solvent to evaporate from the protein solution and diffuse to another solution (well solution). RecA protein needs ss/ds DNA molecules and ATP or an analogue to become active. RecA only interacts with LexA in its activated conformation. Therefore, our co-crystallization trial of RecA and LexA contained different lengths of ssDNA and ATP or an analogue. RecA is an ATPase family protein and uses divalent cations (such as Mg^{2+}) during catalysis. Therefore, the crystallization solutions contained Mg^{2+} salts too.

The approach here with the hanging drop method was to start with a wide range of conditions containing different precipitants and salts at different concentrations. Unfortunately, no co-crystals were possible to produce. Some tiny crystals were found in some of the random conditions, but those crystals were not good enough for diffraction. It was presumed that the co-crystals should not resemble the size and shape of crystals of only the wild type RecA or mutant LexA alone. When repeated with the controls (LexA mutants and wild type RecA alone), the crystal sizes and shapes resembled the wild type crystals. In fact, no common trend in the crystallization conditions was identified. However, crystals were mostly found in solutions with 12-20% PEG 3350 or K15 and HEPES-KOH buffers with pH ranging from 7.0 to 8.0. KAc, KCl, NaCl, etc., and $MgCl_2$ or $MgAc_2$ salts at concentrations that ranged from 5 mM to 60 mM produced some but tiny crytals.

Crystallization kits with the sitting drop method were tried next to expand the range of conditions used. A total of 192 conditions were attempted with two different kits (The Pegs, The Classics, Nextal-Qiagen) each containing 96 kinds of solutions. The pattern of the results was similar to the hanging drop method. Some tiny crystals were obtained in some random conditions, but they were not reproducible and resembled the control crystals.

Both the RecA and the LexA mutants were purified with a different buffer system without any chloride salt, mostly using NaAc or KAc instead of NaCl or KCl. Crystallization trials with these proteins in conditions without any chloride also did not produce usable crystals.

Chapter Six: **Discussion**

6.1 Producing LexA dimer interface mutants

The number of LexA molecules in non-induced *E. coli* is approximately 1300, including 20% free and not bound to DNA (Sassanfar and Roberts, 1990). The C-terminal domains (CTD) of two LexA monomers interact with each other to form a dimer. DNA-bound LexA has an approximately 1000-fold higher dimerization stability compared to free LexA and the dimer dissociation constant (K_d) of LexA is in picomolar range (Mohana-Borges *et al*., 2000). Therefore, it is clear that LexA exists predominantly as a dimer under normal cellular conditions.

Interestingly, studies on the RecA-LexA interaction suggest that LexA monomers bind RecA tighter than the dimer (Schnarr *et al*., 1988). An attempt was previously made in our lab and tried to provide support for this phenomenon (Moya, 2006). A non-cleavable LexA mutant, Δ_{68}K156AI196K, was purified, and analyses showed that it was predominantly monomeric in solution and inhibited LexA self-cleavage to some extend but not better than the dimeric mutant Δ_{68}K156AV195D. However, co-crystallization attempts of RecA and the monomeric LexA mutant were unsuccessful in that case. Therefore, this project was started to identify more monomeric LexA mutants and to co-crystallize with activated RecA.

Crystal structure of LexA (Luo *et al*., 2001) revealed that residues from the anti-parallel β-11 strands and some other portions of the CTD make essential contacts that stabilize the dimeric form of LexA (Figure 4.2). A closer view of the anti-parallel β strands (Figure 4.2B-C) showed that residues from Val195 to Arg197 of β-11 participate the most in this interaction. Therefore, it was reasoned that, if mutations were made in this region, dimer formation would be possible to interrupt. For this purpose, four mutations were introduced in three consecutive residues (V195P, I196Y, R197P and R197Q). The mutated residues were selected because these might break down hydrogen bonds or produce steric hindrance between the two anti-parallel β-11 strands. A K156A-mutated and N-terminally truncated (68 residues) LexA gene (Δ_{68}LexAK156A) was used to start the experiment as a template to introduce the other single mutations. The Lys156 to alanine mutation makes the LexA non-cleavable, and the N-

terminally truncated version was used because this portion is not involved in any of the interactions or reactions in this study.

6.2 Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AI196Y are predominantly monomeric

Molecular weight of protein oligomers is the sum of the individual monomers that produce the oligomers. Monomeric or oligomeric form of the protein in a solution can be determined thus by analyzing the size of the protein in solution. Two experimental methods were used in this study to determine the size of the LexA mutants. Size exclusion chromatography is a method that separates molecules according to size. It is a very efficient and popular method of purifying macromolecules like proteins. The method was used here as a tool to measure the size of the LexA mutants produced, which would ultimately indicate the forms (monomeric or oligomeric) of the mutants. Another method used for this purpose is dynamic light scattering (DLS). DLS measures the hydrodynamic radius of a molecule in a solution, and size of the molecule (MW) can be determined from that. Protein molecules can be used as samples for DLS, and the size of the protein can be precisely calculated from the results.

Previously characterized LexA mutants, Δ_{68}LexAK156A and Δ_{68}LexAK156AI196K, were used as controls to compare the size exclusion chromatography elution profiles with the produced dimer interface mutants. Δ_{68}LexAK156A and Δ_{68}LexAK156AI196K were confirmed as predominantly dimeric and monomeric in solution, respectively (Moya, 2006). The results from the chromatography profiles showed that Δ_{68}LexAK156AR197P appeared lately and resembles the size of monomeric proteins (Figure 5.4, 5.5). Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AI196K showed similar kind of elution profile. Both of the proteins were eluted with a larger volume than the other proteins and interestingly, a smaller peak was found just before the larger peak (Figure 5.4). This indicates that there might be some proteins present in oligomeric forms in the protein solution. The Δ_{68}LexAK156AR197P mutant was also confirmed predominantly monomeric at both 33.3 μM and 100 μM from the DLS data (Figure 5.6, 5.7). The Δ_{68}LexAK156AI196Y mutant as well found as monomeric at 33.3 μM in the DLS data. However, it was close to the dimeric elution profile by size exclusion chromatography. Therefore, Δ_{68}LexAK156AR197P was confirmed to be predominantly monomeric and Δ_{68}LexAK156AI196Y was found monomeric at least at 33.3 μM. Interestingly,

the Δ_{68}LexAK156AV195P and Δ_{68}LexAK156AR197Q LexA mutants showed even higher oligomeric mass percentages in solution compared to Δ_{68}LexAK156A at 33.3 μM (Figure 5.6, 5.7), which may be because the dimer interface mutations unexpectedly made the individual LexA subunits interact better with each other.

Mutations V195P and R197Q reduced the K_d value (~2-4 μM) of the mutants and produced predominantly dimeric proteins in solution though those mutations were produced to break down the hydrogen bonds in the dimer interface. I197Y was assumed to produce steric hindrance between the strands in the interface and expectedly increase the K_d value to ~40 μM. Interestingly, R197P mutation increased the K_d value the most (~80 μM). Arg197 residue interacts not only with the Val195 of the opposite strand with two hydrogen bonds (4.2B) in the interface but it also interacts with Gly124 from the other monomer with two hydrogen bonds (Figure 4.2C). Actually this is the residue which shows most of the interactions (five hydrogen bonds, Figure 4.2B-C) in the dimer interface. It was possible that, replacing Arg195 with proline (P) broke down most of the interactions whereas glutamine (Q) could not. This might be the reason why the R197P mutation produced the most monomeric mutant.

6.3 Dimer interface mutants of LexA bind RecA tightly

Wild type LexA binds to the RecA nucleofilament at a Km of ~0.5 μM (Lin, 1988). However, non-cleavable LexA mutants, like K156A, interact with the active RecA filament tighter than the wild type (Slilaty and Little, 1987). Therefore, all of the mutants should bind to RecA tighter than wild type LexA. Affinities of the four LexA mutants for RecA was tried to determine.

An experiment called a co-protease inhibition assay was designed where the non-cleavable LexA dimer interface mutants were incubated with RecA in the reaction solution prior to addition of wild type LexA. LexA mutants would inhibit the co-protease activity if they bound to RecA tightly enough to block the binding site for LexA on the RecA nucleofilament. The results showed that both of the LexA mutants, Δ_{68}LexAK156AR197P (17% wild type LexA cleavage) and Δ_{68}LexAK156AI196Y (13% wt LexA cleavage), which were mostly monomeric in solution, inhibited the co-protease activity of RecA to a lesser extent than the dimers (5% and 7% of wt LexA cleavage by Δ_{68}LexAK156AV195P and

Δ_{68}LexAK156AR197Q, respectively). This result contradicts with the hypothesis: monomeric LexA binds to RecA tighter than the dimer (Schnarr *et al*., 1988). However, a previous study performed in our lab with two different dimer interface mutants (V195D and I196K) also showed that the monomers interact poorly with RecA during the inhibitory assay. Though range of the percentage of wild type LexA cleavage in the presence of different inhibitors did not vary a lot (only 5-17%), it was significantly higher than the percentage of LexA cleavage in the absence of any inhibitors (~74%). The reason why the monomeric LexA mutants did not inhibit the cleavage of wild type LexA more than the dimers is unclear. Portions of the dimer interface of LexA is a hydrophobic region and exposing the region by mutations could cause significant changes in the conformation at the dimer interface site and interrupts the RecA-LexA interaction. In spite of this, the previous studies by Schnarr *et al*. (1988) were believed to be consistent and this study continued with the next attempts to co-crystallize the monomeric Δ_{68}LexAK156AR197P and Δ_{68}LexAK156AI196Y proteins with RecA.

6.4 LexA monomers cannot stabilize the active RecA nucleoprotein filament during crystal packing

Protein crystallization is not always an easy task to perform. Sometimes it is not possible to crystallize even a single protein. Crystal structures of LexA (Luo *et al*., 2001) and inactive RecA (Story *et al*., 1991) were determined several years ago. The active structure of RecA was not solved in a conventional way because the active conformation is not stable during crystal packing. Instead, Chen *et al*. (2008) physically linked several RecA subunits (5 or 6) to produce a fusion protein to stabilize the active conformation for crystallization and structure determination. Therefore, it is reasonable that attempts to co-crystallize RecA and LexA would be even harder, since the active conformation of RecA is hard to stabilize. In addition, co-crystallization of multiple proteins is always a difficult task. In this case, it was thought that since RecA is a co-protease enzyme that physically interacts with LexA, LexA would have a high binding affinity for RecA and that mutations in LexA should make LexA and RecA bind even tighter. Therefore, if a LexA mutant binds tightly to the activated RecA conformation, it may stabilize the active form and make it possible to produce co-crystals.

The active structure of RecA showed that every three nucleotides of ssDNA bind to one subunit of the RecA filament (Chen *et al*., 2008). Therefore, the length of ssDNA should limit the size of the nucleoprotein filament. Because of this, different sized DNA molecules were chosen. There is another problem with RecA polymerization, since RecA polymerizes indefinitely if not blocked. Although it was thought that the DNA would limit the size of the nucleoprotein filament, it may not be sufficient. Therefore, DNA molecules were designed having a biotin group at the 5′ or 3′ end of the DNA. The biotin should block the self-polymerization of RecA. The concentrations of ATP or analogs (1 mM) and the ssDNA (1 mM) were fixed, and the types of ATP analogs and ssDNA were varied. Different types and concentrations of salts and precipitants were also tried. Unfortunately, no co-crystals of the activated RecA and LexA mutants were possible to obtain. As mentioned in the results section, some tiny crystals were found in random conditions without any identifiable trend. When compared with the LexA and RecA crystals alone, the shapes and sizes of the crystals were found similar. It was reasoned that if complex crystals were formed, they would have been completely different in size and shape from the crystals of LexA or RecA alone. Therefore, it can be concluded from this study that the developed monomeric LexA mutants do not have the ability to stabilize the active form of wt RecA and therefore cannot be co-crystallized.

6.5 Possible future works on LexA-RecA interactions

Two separate attempts in the current lab, including this work and by Moya (2006), on co-crystallization of RecA and LexA appeared unsuccessful. Therefore, this is the time to think about an alternative way to reveal the interactions between LexA and RecA and the details of the mechanisms of co-protease activity of RecA. It seems crystallization of RecA and LexA or its mutants together is too hard to accomplish. However, some of the options were not tried in these experiments what can be explored for the future studies on this topic. It was mentioned earlier that Chen *et al*. (2008) provided the structural details of the active RecA filament (Figure 2.6) and elucidated the mechanism of the homologous recombination. A quite interesting strategy was applied to obtain the RecA active filament and to limit the length of the filament. Multiple copies of the *E. coli recA* gene, corresponding to 1-335 residues, were fused in to a single reading frame with intervening 14-amino-acid linkers. To prevent the fusion

protein or concatemer from polymerizing, the N-terminal RecA had a deletion of 1-30 amino acids and the C-terminal RecA had Cys117Met, Ser118Val and Gln119Arg mutations. The fusion protein produced crystals with different length of ss/dsDNA, such as 15 nt long ($(dT)_{15}$) for 5 subunits RecA chain (termed $RecA_5$).

From the current study on the crystallization of LexA dimer interface mutants and RecA, it can be understood that these attempts are difficult to accomplish. On the other hand, there are reasons to believe that, the RecA fusion protein by Chen *et al*. (2008) can be applied to these attempts with the dimer interface monomeric mutants of LexA and even with the non-cleavable dimeric LexA as the fused RecA filament was proved to be crystallized in active form with different lengths of DNAs and with ATP or analogs. The fusion protein can be purified as described by Chen *et al*. (2008). The co-protease and co-protease inhibition assays with the RecA fusion protein can be performed. Any crystallization attempts of the LexA protein with the RecA fusion protein (from Chen *et al*., 2008) have not been reported yet. Therefore, there are opportunities to try the noncleavable and dimer interface mutants of LexA to co-crystallize with RecA fusion protein. Different length of the fused RecA chain can also be tried.

We mentioned earlier that VanLoock *et al*. (2003b) applied the iterative helical real space reconstruction (IHRSR) method using electron microscopy (EM) reconstruction of RecA-ATP-LexA-DNA complex (Egelman and Stasiak, 1993; VanLoock *et al*., 2003b) to compare the difference of fitting of inactive crystal structure of RecA (Story *et al*., 1992) and the model of RecA active filament (VanLoock *et al*., 2003a). Results showed that RecA active model fit better when the active (modelled) and inactive (crystal structure) RecA were docked on the RecA-ATP-LexA-DNA complex (Figure 2.9). VanLoock *et al*. had to use a model (VanLoock *et al*., 2003a) for the RecA active filament, as the crystal structure of active RecA was not available at that time. It would be interesting if the now-available active crystal structure of RecA was docked on the EM reconstruction complex to see how it fits. If the first attempt mentioned here is not possible to achieve, this study would be necessary to have some idea about the interactions of LexA and RecA.

Chapter Seven: **Reference List**

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