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THERMODYNAMICS OF DNA BINDING AND BREAK REPAIR BY THE POL I DNA POLYMERASES FROM ESCHERICHIA COLI AND THERMUS AQUATICUS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by
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ABSTRACT

Klenow and Klentaq are the “large fragments” of the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*. Examination of the DNA binding thermodynamics of both polymerases to replication versus repair substrates shows that Klenow binds primed-template DNA with up to 50X higher affinity than it binds to a nicked DNA, gapped DNAs, DNA with blunt-end or a 3’ overhang, while Klentaq binds all of these DNAs similarly. The presence of 5’ or 3’ phosphates has slightly different effects on DNA binding by both polymerases. In contrast, both polymerases bind mismatched DNA tighter than matched DNA, suggesting that they may share a similar mechanism to identify mismatched DNA, despite the lack of proofreading ability in Klentaq.

The effects of Klenow and Klentaq on ligation of DNA ligase were also studied. Both polymerases stimulate the intermolecular ligation activity of *E. coli* DNA ligase at concentrations sub-stoichiometric to the DNA concentration. This effect occurs with *E. coli* DNA ligase, but not for T4 and Taq ligases. Additionally, neither polymerase significantly enhances ligation of a substrate containing a single nick, suggesting that the polymerases bridge the two DNA ends during intermolecular ligation.

The nucleotide incorporation activities of both polymerases on substrates mimicking double-strand breaks (DSBs) were also examined. Both proteins are able to “repair” DSBs via alignment-based strand-displacement DNA synthesis. Moreover, their repair abilities have different dependences on 5’ phosphate and DNA ligase when DSBs contain non-cohesive ends. Additionally, both proteins mediated palindrome amplification alone when the short inverted repeats occur near DNA breaks, suggesting that short inverted repeats in prokaryotes may help in
DSB repair. 5’ phosphate at the matched break end is required for DSBs repair by both polymerases when one break end contains 3 consecutive mismatches.

Results of the electrophoretic mobility shift assay show that Klenow-DNA complexes are observed as slow or fast moving bands, or both while all Klentaq-DNA complexes are observed as slow moving bands. The protection of both ends of a DNA by Klenow from exonuclease digestion suggests that the slow moving bands may correspond to the 2:1 polymerase-DNA complex.
CHAPTER 1

GENERAL INTRODUCTION

DNA polymerases are enzymes that catalyze the incorporation of nucleotides into a DNA strand. They are best-known for their role in DNA replication, in which the polymerase synthesizes the new strand using an intact DNA strand as a template. Although there is a wide variation in the protein sequences, all DNA polymerases use an identical two-metal-ion catalyzing mechanism for their catalytic activity (1-2). All DNA polymerases can only add free nucleotides to a preexisting 3' OH of a primer, which is base paired with a template DNA containing the genetic information to be replicated. The polymerization reaction is catalyzed through the nucleophilic attack of the 3’ OH of the primer terminus on the α-phosphate of the incoming dNTP.

Based on sequence homology and structural similarities, DNA polymerases have been subdivided into seven families—A, B, C, D, X, Y and RT (1-7), each of them possesses different properties and plays various roles in DNA replication and/or DNA repair (8). According to their primary functions in vivo, some DNA polymerases are designated as replicative polymerases, whereas others are classified as repair polymerases. Thus, in addition to 5' → 3' polymerization activity, these polymerases may also possess 5' → 3' nuclease activity (family A), 3' → 5' exonuclease activity (families A, B, and D), lyase activity (X family), and RNaseH activity (RT family) (7). The A family polymerases possess a catalytic core, which performs highly accurate and processive DNA replication. On the basis of their primary role in DNA replication and maintenance during the regular cell cycle, the A family polymerases are divided into replicative and repair polymerases. T7 DNA polymerase and mitochondrial DNA polymerase γ are replicative members. Escherichia coli DNA Pol I, Thermus aquaticus Pol I, and Bacillus
*stearothermophilus* Pol I are usually classified as primarily repair polymerases. They are involved in filling in short gaps from excision repair of DNA damage, and in excising RNA primers while processing Okazaki fragments generated during lagging strand synthesis. The founder member of family A is DNA polymerase (Pol I) from *Escherichia coli* (9). Studies in this dissertation focus on the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus* (Taq polymerase). Therefore, in this review, I mainly concentrate on Pol I DNA polymerases.

1.1 DNA Polymerases I

The first isolated and characterized polymerase was DNA polymerase I from the mesophilic bacterium, *Escherichia coli* (10-12). *E. coli* Pol I is a single polypeptide with 928 amino acids and a 103 kDa molecular weight (13). It consists of three functional domains: the N-terminal 5' → 3' nuclease domain (residues 1 - 326), the 3' → 5' exonuclease (proofreading) domain (residues 326 - 519), and the C-terminal 5' → 3' polymerase domain (residues 520 - 928) (14-15). *E. coli* pol I is the best studied member of the family A polymerases. In addition to Pol I, four additional polymerases have subsequently been discovered in *E. coli* including polymerase II, III, IV, and V (16). DNA polymerase I and III were shown as the major replication enzymes. DNA polymerase III is a highly processive enzyme and plays an important role in DNA synthesis by extending the leading and lagging strands. DNA polymerase I is mainly involved in the lagging DNA strand synthesis, and is responsible for removal of ribonucleotides and subsequent replacement with deoxyribonucleotides during the processing of Okazaki fragments, and is involved in repair of damaged DNA. Although DNA polymerase I is not the primary replicative DNA enzyme in *E. Cali*, it is the most abundant DNA polymerase with about 400 molecules per cell, while there are only 10-40 molecules of polymerase III in the
Polymerase II, IV, and V are involved in repair of various types of DNA damage (18). The relative amount of different polymers in the cell can influence which polymers gains access to a replication fork or a damaged site (19).

*Thermus aquaticus* is a thermophilic bacterium, which was discovered and isolated by T. D. Brock and H. Freeze in 1969 from a hot spring in Yellowstone National Park (20). *Thermus aquaticus* is a bacterium, it can thrive or grow at temperatures around 75°C, while the optimal physiological growth temperature for *E. Cali* is 37°C (15). Chien, et al. first isolated Taq polymerase from *Thermus aquaticus* in 1976 (21). Subsequently, a high yield of Taq was obtained by cloning and over-expression of the Taq encoding gene in *Escherichia coli* (15, 22). Taq polymerase is also a single polypeptide, with 832 amino acids and a 94 kDa molecular weight. The overall sequence identity between Taq and *E. coli* Pol I is about 38% (23), while the sequence identity in the polymerase domain is 49% (3). Taq polymerase, like *E coli* Pol I, also is the most abundant DNA polymerase in *Thermus aquaticus* (17). Taq shows high similarity in structure and function to *E. coli* Pol I. It also consists of three different structural domains: the N-terminal 5' → 3' nuclease domain (residues 1 - 291), a nonfunctional proofreading domain (residues 292 - 423), and the C-terminal 5' → 3' polymerase domain (residues 424 - 832) (24).

The optimal temperature for Taq polymerase activity is 50-75°C, depending on reactive conditions used, and partial activity and structural stability are detected up to 100°C (15, 25). Due to the extreme thermostability of Taq and its ability to polymerize at elevated temperatures, it is widely used as a tool in the polymerase chain reaction (PCR) technique, for the *in vitro* amplification of DNA sequences.

Both *E. coli* Pol I and Taq polymerase are widely studied. Their characterizations are often extrapolated to all other family A DNA polymerases and sometimes other DNA...
polymerase families including the more complex eukaryotic DNA polymerases. Thus, they are used as model DNA polymerases and their studies are a basis for the understanding of other DNA polymerases. The 3’ exonuclease domain, responsible for correction of mis-incorporation, is inactive in Taq polymerase, while it is a main contributor to the high replication fidelity of *E. coli* Pol I (16). The 5’ nuclease domain is mainly responsible for the removal of RNA primers. It also involved in nucleotide excision repair (NER) by removing the damaged DNA nucleotides. *In vitro*, polymerase binds to nicked or gapped duplex DNA, and polymerase-catalyzed primer extension produces the substrate for 5’nuclease motif, resulting in nick translation. Thus, both polymerase and 5’ nuclease activities are essential for efficient nick translation (26). Some polymerases that lack the 5’-3’ exonuclease activity displace one strand of a duplex in order to synthesize a new strand. T7 DNA polymerase (27) and *Pseudomonas* LigD POL (28) are unable to catalyze strand displacement DNA synthesis by itself, while Klenow has a strong catalyzing ability for strand-displacement DNA synthesis (16, 29-30).

The studies of this dissertation primarily focus on the binding characteristics and functional repair properties of *E. coli* DNA polymerase I and Taq polymerase. Using biophysical and biochemical studies of both polymerases, we try to answer the following questions: 1) Do *E. coli* Pol I and Taq polymerases recognize replicative (primed-template DNA) and repair (e.g. gapped DNA) substrates differently? 2) How does gap-binding affinity of both polymerases correlate with their gap filling activity? 3) Do the two polymerases repair DNA double-strand breaks? If yes, what are the possible mechanisms of the repair? 5) How do the polymerases bind matched and mismatched DNA differently? And how does the affinity of the polymerases for mismatched DNA correlate to their repair capacities on DNA double-strand breaks? 6) How do the two polymerases selectively bind different DNA end structures differently? 7) How do the
polymerase and DNA ligase cooperate each other during repair process? And 8) what are the potential factors relevant to the formation of 1:1 or 2:1 polymerase-DNA complex?

1.1.1 Structures of Klenow and Klentaq Polymerases

Removal of the N-terminal 5’ nuclease domain from both *E. coli* Pol I and *Thermus aquaticus* Taq polymerase yield the Klenow (68 kDa) and Klentaq (62 kDa) “large fragments”, which retain full polymerization activity (31-32). Studies of Klenow and Klentaq demonstrate that they consist of two domains, the polymerase domain and the proofreading domain (31, 33). The proofreading domain is responsible for removal of mis-incorporated nucleotides in Klenow whereas it is inactive in Klentaq (34). Sequence alignment has shows 49 % sequence identity between two the polymerase domains of the two proteins (3).

Klenow denatures between 40-62°C depending on salt concentration and pH value while Klentaq is stable up to 100°C (25, 35). Previous thermodynamic studies of the unfolding of Klenow and Klentaq by chemical and thermal denaturation indicate that the stabilization free energy for Klentaq, 27 kcal/mol, is one of the largest among the monomeric proteins yet characterized, and that the difference in the stabilization free energy between Klentaq and Klenow is one of the largest ever determined for a homologous thermophilic-mesophilic protein pair (36).

Even though they have dramatic differences in thermostability, Klenow and Klentaq have highly similar crystal structures (Figure 1.1). The polymerase domain of both proteins resembles the morphology of a cupped half-open human right hand, which is a conserved architectural topology across all families of DNA polymerases (3, 6, 37-38). It consists of “fingers,” “thumb,” and “palm” subdomains, each of which plays an important role in the polymerization reaction. The fingers subdomain is involved in the binding of the incoming complementary dNTP and the
single strand portion of the template upstream of the primed-template junction, and delivering dNTP to the active site. The thumb subdomain directly interacts with the DNA duplex portion and is important in DNA binding and processivity (39). The palm subdomain contains the polymerization active site and orients the nascent primer strand for phosphodiester bond formation.

Figure 1.1: X-ray crystal structures of Klenow (1KFD (14)) and Klentaq (1KTQ (40)) DNA polymerases. Both polymerases have “half-open right hand” architectural topologies for their polymerase domains. The proofreading domain in Klentaq polymerase is inactive.

The proofreading domain of Klenow is located about 30 Å away from the polymerase active site, and can bind to the 3′-terminus of the primer (41). Klenow and Klentaq also share high similarity at the secondary structure level. The palm subdomain is comprised of an antiparallel β-sheet and its structure is relatively conserved within the DNA polymerase superfamily (2, 42). Unlike the palm, the other two subdomains, the thumb and fingers, are mostly α-helical, and their secondary structures are less homologous among the families, although they function similarly using analogous secondary structural elements (39, 42).
1.1.2 DNA Binding by Klenow and Klentaq

The catalytic mechanism for the addition of a nucleotide to the 3’-OH of the primer has been studied for Taq polymerase (43) and *E. coli* Pol I (44-46). Benkovic and his group have proposed a seven-step kinetic mechanism for the incorporation of nucleotides by Type I polymerases based on their extensive studies on the reaction catalyzed by Klenow (47). DNA substrate binding by the polymerase is the first step. It is followed by the binding of the incoming dNTP to form the “open” ternary complex. The third step is the conformational change of the ternary complex from “open” to “closed”. There are two potentially interesting conformational transitions identified during the switch from open to closed (46, 48). One is the significant movement of the finger subdomain due to the binding of the incoming dNTP. This movement does not take place normally with either mispaired dNTPs or ribonucleotides (rNTPs). This conformational change was revealed by studying the open and closed ternary crystal structures of Klentaq, and the conformational transition of Klenow-DNA complexes due to nucleotide addition using single-molecule FRET (smFRET) (49-50). The other major conformational step is a rearrangement of active site geometry. This step is observed to be slower than the fingers-closing movement and was blocked by mispaired nucleotides but not by complementary nucleotides regardless of whether they contain deoxyribo or ribo sugars. All of the above suggest that a DNA polymerase checks the complementarity of the incoming nucleotide to the template base first, and subsequently checks the correct sugar structure during incorporation (46-47). The fourth kinetic step is the chemical step where the nucleotide is added to the 3’-OH of the primer. Following the chemical step, a second conformational change occurs along with release of pyrophosphate. Following the release of pyrophosphate, the binary complex either dissociates or binds another nucleotide to start another incorporation round.
Several co-crystal structures of Klenow and Klentaq polymerases binding to DNA substrates have been determined (40, 42, 49, 51). There is an important difference in the way that Klenow and Klentaq interact with DNA in existing structures. Klentaq binds DNA in the polymerization mode with the 3’ primer terminus located at the active site of the polymerase domain, and has been trapped and observed in several of the kinetically intermediate steps. In contrast, co-crystal structures of Klenow have been observed only in the editing mode binding, where the 3’ single-stranded primer melted from the template interacts with the active site of the proofreading domain (Figure 1.2). However, biochemical studies show that polymerization mode binding by Klenow in solution is significantly populated (52-53). For both binding modes,

![Klenow and Klentaq structures](image)

**Figure 1.2:** X-ray crystal structures of Klenow (1KLN) and Klentaq (4KTQ) polymerases bound to DNA. Klenow polymerase is shown binding DNA in the editing mode (left figure) while Klentaq polymerase is shown binding DNA in polymerization mode (right figure) (49, 51). Klenow polymerase melts several base pairs at the primer-template DNA junction, and the single-stranded 3’ strand interacts with the proofreading domain. The template strand is shown in black in both structures. Primer is shown in blue (1KLN) and orange (4KTQ).
the duplex region of DNA binds between the “thumb” subdomain and the proofreading domain. A conserved region having two helices involved in binding of the duplex of DNA is found within the thumb subdomain (38). This region interacts with the phosphate backbone of the minor groove by rotating the thumb subdomain towards the palm subdomain during DNA binding. Deletion of the two helices causes a 4-fold decrease in processivity of Klenow (49, 54). The single-stranded template region binds in the “fingers” subdomain in the polymerization mode and at the RRRY motif in the editing mode (49, 55-57). The ternary complex crystal structure of Klentaq shows that Ser674 (equivalent to Ser769 of Klenow) and Arg746 (equivalent to Arg841 of Klenow) interact with the phosphate backbone of the single-stranded template overhang (49). Phe771 of Klenow binds the 2nd nucleotide of the template in the polymerization binding mode (57-59). The 3′-OH of the primer locates close to the catalytic residues of the polymerase active site in the polymerization binding mode. Most of the interactions between DNA and polymerase involve the phosphate backbone and the minor groove.

The 3′-OH of the primer shuttles to the exonuclease site by melting 3-4 bp DNA duplex at the primed-template junction in the editing binding mode (51). In editing binding mode, the single-stranded template is thought to be bound and stabilized by the RRRY motif (RRLY in Klenow), topologically located between the fingers and the 3′-5′ exonuclease domains, which is conserved across the polymerase family A, and is also the potential binding site for the ssDNA substrate (Figure 1.3) (56). On the contrary, the single-stranded template region of the primer template DNA should not interact with the RRRY motif in Klentaq since only the polymerization binding mode exists (Figure 1.4).

Structural studies have shown that the first four nucleotides of the 3′ primer terminus interact with the 3′ exonuclease site via hydrophobic interaction and hydrogen bonds. Leu 361,
Figure 1.3: Topological binding modes for Klenow with primed-template DNA and single-stranded DNA. This figure is adapted from Figure 8 from reference 56 (56). The binding of template (solid lines) and primer (broken lines) moieties of the primer template DNA is shown in A (editing mode) and B (polymerization mode). The RRRY motif (purple ellipse) has the sequence RRLY in Klenow. The proposed binding mode of ss-DNA is depicted in C.

Figure 1.4: Hypothesized binding modes for Klentaq with primed-template DNA and single-stranded DNA. Klentaq binds primer template DNA in polymerization mode (A). The proposed binding mode of ss-DNA is depicted in B, in which the 5’ end of ss-DNA interacts with RRRY motif in Klentaq and the exact location of the 3’ end in Klentaq/ss-DNA complex is not determined yet.
Phe 473 and His 660 of Klenow (not conserved in Klentaq) are involved in DNA binding at the 3’ exonuclease site (60). Biochemical studies suggested that the side chains of Arg 668 and Asn 845 favor interactions with correctly paired DNA terminus, while Asn 675, Arg 835, Arg 836, and Arg 841 contribute the negative energy to the both matched and mismatched DNA binding at the polymerase active site (61). The favorable binding energy contributed by Arg 668 may be due to the geometric specificity. Because the homologue of Arg 668 has a direct hydrogen bond with the minor groove edge of the primer terminal base in the co-crystal structures of Klentaq polymerase or T7 DNA polymerase (49, 55), analogous to Klenow, and hydrogen bond acceptors in the minor groove are similarly positioned in geometry in the four matched base pairs but are differently positioned in mismatched base pairs (62). On the contrary, Asn 845 forms a hydrogen bond with the sugar ring of the template nucleotide at this position (49, 55). The favorable energetic contribution of Asn 845 may be lost due to the non-proper geometry of the DNA when its terminus is mismatched. The group mutants comprising Asn 675 Ala, Arg 835 Ala, Arg 836 Ala, and Arg 841 Ala increase binding affinity of DNA at the polymerase site relative to the wild type. The improved polymerase site binding may be accounted for by the lesser distortion of the DNA substrate or of the protein itself upon the residue mutation (61), since co-crystal structures of homologous DNA polymerases bound to DNA substrates show that Asn 675 interact with the template strand at the position where the DNA switches from B-form to A-form geometry, and that the side chains of Arg 835, Arg 836, and Arg 841 interact with the bent single-stranded portion of the template (49, 55). The unfavorable binding energy contributed by these residues may optimize the chemistry of nucleotide incorporation and position the key regions of the DNA in proper juxtaposition relative to specificity–determining
side chains of the enzyme and accordingly allow the polymerase to discriminate matched from mismatched terminal base pairs (61).

Joyce has shown that shuttling of the 3’ primer terminus can occur via both intramolecular and intermolecular pathways (63). In the intramolecular pathway, DNA could shuttle between the two catalytic sites by sliding. While in the intermolecular pathway, DNA could move from one active site to the other via dissociation into free solution and re-association with the other active site. The use of the intermolecular or the intramolecular pathway depends on the competition between the polymerase or exonuclease reaction and DNA dissociation. Millar’s studies report that there is a binding equilibrium between both the pol and the exo site even for the completely matched DNA. Klenow and completely matched DNA binding is primarily in the polymerization mode (~86%) (64). Mismatches at the 3’ primer terminus favors the partitioning of the binding to the exo site, and a 4-base mismatch at the 3’ primer terminus leads to the binding of Klenow predominantly in the editing mode (64-65). In contrast, data from von Hippel and associates indicate that perfectly matched DNA binding by Klenow in the editing mode is populated (7-43%) and the proportion of participation of the primer end into the exo site is dependent on the thermodynamic stability of the base pairs at the P/T junction; 3 mismatches at the 3’ primer terminus cause Klenow to bind DNA completely in the editing mode; and 4 mismatches cause the DNA to not bind Klenow properly in the editing site (53).

Previous studies have suggested that single-stranded DNA (ss-DNA) is a substrate for editing mode binding by Klenow (41, 51, 60, 66-67). Recent studies by Modak et al. have suggested that ss-DNA binds both the RRRY motif and the exo site in Klenow (Figure 1.3, C) (56). Studies in our lab show that both Klenow and Klentaq can bind single-stranded DNA (68), even though the exo site in Klentaq is inactive (15). The results also show that Klenow binds ss-
DNA significantly tighter than Klentaq at the salt concentrations used and that more ions are released due to binding by Klenow relative to Klentaq (68). These findings suggest that the weak binding of ss-DNA by Klentaq may be due to its interaction only with the RRRY motif in Klentaq and either no interaction of the 3’ end of ss-DNA with Klentaq, or a different interaction than seen in Klenow.

1.1.3 Comparing the DNA Binding Thermodynamics of Klenow and Klentaq

Traditional techniques used to study the interaction of protein and DNA include fluorescence anisotropy, steady state fluorescence, the electrophoretic mobility shift assay, and filter binding. In our laboratory, we predominantly use the anisotropy technique to measure the DNA binding affinity of both Klenow and Klentaq (68-72).

Klenow and Klentaq polymerases have similar structures and functions. Thus, they are generally assumed to behave almost identically at the molecular level, such that characteristics of one protein from experimental or structural results are often extrapolated to the other. However, Klenow and Klentaq polymerases exhibit both similarities and differences in their DNA binding thermodynamics. According to studies from Joyce and associates, Klenow fragment contacts at least the first four unpaired template nucleotides when binding to primed-template DNA by the electrophoretic mobility shift assay (57). Crystal and co-crystal structures and solution studies have revealed that Klentaq binds DNA only in the polymerization binding mode, nine and seven nucleotides at the 5’ end of the template and the 3’ end of the primer of the duplex part of the primed-template DNA participate in contacts with the enzyme, and the single-stranded template contributes little to the binding affinity (40, 49, 52).

Klenow and Klentaq show different dependencies on temperature during DNA binding (70-71). DNA binding affinity by both proteins increases as the temperature increases up to
~40°C when binding to primed-template DNA. Above about 40°C, Klenow loses binding affinity due to denaturation and Klentaq binding affinity decreases. Furthermore, Klenow and Klentaq have different sensitivities to the salt concentration when binding to identical DNA (69). Klenow binds primed-template DNA 150-fold tighter than Klentaq at the same concentration of KCl. The two polymerases also show different binding preferences to different DNA structures (single-stranded DNA, primed-template DNA, and blunt-end double-stranded DNA) when examined at similar salt concentrations (68). Klenow binds primed-template DNA 8 times tighter than blunt-end double-stranded DNA whereas Klentaq binds them similarly at 75 mM KCl. These initial results suggested that Klenow and Klentaq may have different preferences when binding to repair versus replicative DNA substrates (and investigating this questions is the subject of Chapter 2 of this dissertation). Both proteins bind the blunt-end DNA, a substrate for the nonhomologous end joining repair pathway, indicating that they may be directly involved in repair of double-strand breaks (and this is the question investigated in Chapter 4).

1.1.4 Polymerization Activity

The chemical step of addition of nucleotides to the 3’-OH of the primer occurs through a two metal ion mechanism (39). Mutagenesis and biochemical studies have showed that residues Met 512, Arg 682, Asp 705, Lys 758, Tyr 766, Arg 841, His 881, and Asp 882 are critical for polymerase activity in E. coli Pol I (73-77). Comparison of the amino acid sequence of Taq to the sequence of E. coli Pol I show that only Met-512 is not conserved in the active site. However, Taq contains the functionally similar Leu residue at the analogous position, which can fulfill the role ascribed to E. coli Pol I Met-512 in primed-template binding, which stabilizes
Figure 1.5: The intermediate state of the two metal ion mechanism for polymerization catalysis. This figure is based on Figure 3 from reference 2 (2) and was created using the program ChemBioDraw. The two divalent metal ions (Me$^{2+}$) stabilize the pentavalent transition state. The two metal ions are in contact with the two conserved aspartate (Asp 705 and Asp 882) residues, the phosphates of the dNTP, the main chain oxygen (carbonyl), and two water molecules (black dots). Metal ion A induces the attack of the 3'-OH of the primer on the α-phosphate of the dNTP while metal ion B chelates the β- and γ-phosphates of the dNTP and stabilizes the negative charge of the oxygen.
DNA binding via mediating the conformational change (74). Structure and/or sequence alignment indicate that these residues are part of structural motifs that are conserved across the polymerase A family (5, 38). Crystallographic studies have shown that Asp 705, Asp 882 and Glu 883 in Klenow or Asp 610, Asp785 and Glu786 in Klentaq bind to Mg$^{2+}$ or Mn$^{2+}$ metal ions for catalysis of polymerization (5, 38, 51).

As the 3’-OH of the primer terminus nucleophilically attacks the α-phosphate of the incoming dNTP, one metal is responsible for the deprotonation of the 3’-OH of the primer, and the other facilitates stabilization of the pentacovalent transition state of the α-phosphate of the dNTP and the removal of pyrophosphate (1) (see Figure 1.5). Crystallographic studies have shown that either Mg$^{2+}$ or Mn$^{2+}$ in the metal ion binding sites of the polymerase domain activates polymerization activity (60), Mg$^{2+}$ probably is needed in PCR for the dNTPs.

### 1.1.5 Proofreading Activity

As discussed above, in Klenow, a mismatched nucleotide at the 3’-end of the primer can translocate from the polymerization domain to the proofreading domain without the dissociation of the DNA, where the mismatched nucleotide will be removed through hydrolysis of the phosphodiester bond (41-42, 51). This 3’ → 5’ exonuclease (proofreading) activity contributes to high replication fidelity (7, 78). One obviously different property between Klenow and Klentaq is that the proofreading domain of Klentaq is inactive. The faithful synthesis of DNA by *E. coli* Pol I is accomplished by the combination of incorporating correct nucleotides and removing wrongly incorporated nucleotides with the proofreading domain (16). Therefore, there is a significant difference in the fidelity of DNA replication between the two polymerases. The full-length *E. coli* Pol I synthesizes DNA with an error rate of $1.6 \times 10^{-7} – 1.5 \times 10^{-6}$ / bp (79). The average error rate for Klenow exo- is $2.5 \times 10^{-5} – 1 \times 10^{-4}$ / bp (80-81). Taq replicates DNA with
an error rate from $8.9 \times 10^{-5}$ to $1.1 \times 10^{-4}$ / bp (31, 82-86), while Klentaq polymerase has an error rate of $5.1 \times 10^{-5}$ / bp (31).

Figure 1.6: The proposed transition state of the two-metal ion mechanism for proofreading activity. This figure is based on Figure 1 from reference 87 (87) and was created using the program ChemBioDraw. It was adapted from the co-crystal structure of Klenow using the crystals of the Asp 424 Ala mutant of Klenow polymerase solved by Beese and Steitz (60). The attack of a hydroxide ion on the phosphorus is facilitated by the interactions with tyrosine (Tyr 497), glutamate (E357), and the metal ion A. Metal ion B stabilizes the O-P-O bond and facilitates the leaving of the 3'-hydroxyl group. Metal ions A and B (Me$^{2+}$) interact with the aspartate residues (Asp 355 and Asp 501). Metal ion B indirectly interacts with Asp 424 via water mediated hydrogen bond.

Biochemical and crystallographic studies have shown that residues Asp 355, Glu 357, Leu 361, Asp 424, Phe 473, and Asp 501 are essential for proofreading activity in Klenow (42, 88). The equivalent residues to Asp 355, Glu 357, and Asp 501 in Klenow are all missing in the
proofreading domain of Klentaq, and this accounts for the lack of editing activity (15). Klentaq contains the exact homolog of Asp 424 residue (15). Mutation of D424A in Klenow leads to a dramatic decrease in the 3′ → 5′ exonuclease activity of the polymerase but retention of the binding affinity of the exo site (88). Recent studies by Modak and associates indicate that Klentaq also contains an RRRY motif, which binds single-stranded DNA and/or the single-stranded template in editing mode binding by Klenow (56).

Like the reaction catalyzed by the polymerase domain, the reaction catalyzed by the proofreading activity also follows a two metal ion mechanism (60, 89) (see Figure 1.6). Two metal ions A and B are separated by 3.9 Å. Metal ion A interacts with Asp 355, Glu 357, Asp 501, and the 5′ phosphate of the dNMP, while metal ion B coordinates with Asp 355 and the 5′-phosphate of the dNMP (60). Asp424 also help stabilize metal ion B through two water-mediated H-bonds, and thereby mutagenesis or replacement of Asp424 with Ala results in the loss of metal ion B binding and the exonuclease activity, while Glu at this position retains considerable activity, presumably by interacting directly with metal ion B in a less optimal way. Although either Mg<sup>2+</sup>, Zn<sup>2+</sup> or Mn<sup>2+</sup> can fill the two metal ion binding sites, the exact metal ions used in vivo is not clear yet (60), but is generally thought to be Mg<sup>2+</sup>. According to the mechanism in Figure 1.6, metal ions play an important role for proofreading activity, and facilitate 1) formation of an attacking hydroxide ion, 2) the departure of the 3′-OH group, and 3) the stabilization of the transition state (41, 60, 89).

1.2 Gapped DNA Binding and Repair

Gapped DNAs are intermediates in excision repair, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and single-stranded break repair. Gaps can be filled by DNA polymerase I and sealed by ligase. The sizes of gap from different
pathways are dramatically different. NER is a major repair mechanism in both prokaryotes and eukaryotes (90). In the bacterium *Escherichia coli*, this process is initiated by nicking the DNA for a distance of 12 or 13 nucleotides on both sides of a lesion by the UvrABC repair nuclease, followed by removal of the damaged oligonucleotide by DNA helicase II to produce a gap (91-92). The main task of MMR is to remove base mismatches and small insertion/deletion loops (IDLs) introduced during replication. The single-stranded gaps from MMR patches are ~100 to >1000 nucleotides long (93-97). BER is the primary DNA repair pathway for correcting base damage caused by oxidation, alkylation, deamination, and depurinatiation/depyrimidination (98). There are two sub-pathways of BER: short-patch and long-patch removal which involve replacing 1 nucleotide (99-101) and 2–11 nucleotides (102-104), respectively. Most single-stranded breaks (SSBs) are accompanied by the loss of a single nucleotide at the site of the break and are thus actually single-nucleotide gaps (105).

1.2.1 Gapped DNA Binding by Different Polymerases

A critical event in gapped DNA repair by a DNA polymerase is the fact that the enzyme must recognize the damaged DNA containing different sizes of ssDNA gap, in the context of the large excess of the regular DNA. Thus characterization of the binding of the enzyme to DNA is of importance for understanding the recognition mechanism for gapped DNA.

The X family DNA polymerases, represented by DNA pol β, are involved in DNA gap repair synthesis. Pol β is a monomeric 39-kDa enzyme consisting of a C-terminal 31-kDa domain that includes the polymerase active site and an N-terminal 8-kDa domain that participates in DNA binding and harbors 5’-deoxyribose phosphodiesterase (lyase) activity (106-107). Like pol β, the full-length DNA polymerase λ (fPol λ), another member of the X-family DNA polymerases, also contains a 5’-deoxyribose phosphodiesterase (8 kDa) and a DNA
polymerase (31 kDa) domain (108-110). The affinity of pol β for gaps shows little or no dependence on the size of the ssDNA gap (111). Pol X from the African swine fever virus (ASFV) is another member of the pol X family (112-114). It shows a strong preference for ssDNA gaps having one and two nucleotides during gapped DNA binding in the presence of Mg$^{2+}$ (115).

1.2.2 Fill in of Different Sized ssDNA Gaps by Different Polymerases

Gap filling is also a critical step in gap repair. Most of DNA polymerases of family X have gap-filling activity. However, different polymerases may have different processivity or nucleotide incorporation efficiency. In Pols β (116), λ (117) and μ (118), there is a necessary spatial relationship between the polymerase domain binding at the primed-template junction and the 8-kDa domain binding to the 5′ end of a downstream DNA chain during repair synthesis to fill the gap. The simultaneous DNA binding by both the polymerase and the 8-kDa domain is crucial for processive gap filling synthesis. Consistent with this, both Pol β and Pol λ can only fill shorter gaps less than 5 nt or 6 nt in a processive manner with the requirement for 5′ phosphate at the 5′ end of the gap (109, 116-117, 119-121), whereas Pol α prefers long gaps of >30 nucleotides (122). The catalytic efficiency of pol β on 5′-phosphorylated 1-nucleotide gapped DNA is >500 times higher than on non-phosphorylated 1-nucleotide and 6-nucleotide (with or without 5′ phosphate) gapped DNAs, and 2,500 times higher than on primed-template with no gaps (123). Pol λ also shows higher incorporation efficiency on gapped DNA substrates than on primed-template DNA, and the 5′ phosphate of a downstream strand in the gap stimulates catalytic efficiency (124-125).
1.2.3 Gapped DNA Binding and Repair by Klenow and Klentaq

In Chapter 2 of this dissertation, I characterize the binding of Klenow and Klentaq to replicative and repair DNA substrates: primed-template DNA and gapped DNA with different sizes of ssDNA gap, to further understand the DNA substrate preference of these polymerases during DNA binding. The study shows that Klenow modestly prefers pt-DNA over gap binding while Klentaq binds to pt-DNA and gaps nearly identically. The affinity of Klenow for a gap is dependent on the size of the ssDNA gap. The 5’ phosphate has different effects on the binding of gapped DNAs to Klenow versus Klentaq, but does not affect gap fill-in or strand-displacement synthesis activities of either enzyme: 5’ phosphate tightens the binding of nicked DNA by Klenow but weakens the binding affinity for a long gap by Klentaq. Interestingly, the presence of a 3’ phosphate in the gap significantly weakens the binding to Klenow, but has very little effect on Klentaq binding. These results suggest that Klenow more significantly prefers replication over repair substrates and that Klentaq binds repair and replicative substrates nearly equivalently. Based on the published co-crystal structures of both polymerases and our data here, we further hypothesize that Klentaq can bind the gapped DNA at either side of the DNA gap, while Klenow binds preferentially in one orientation due to the 3’ end of the gap being pulled into the exo site.

1.3 Double-strand Breaks Repair

DNA double-strand breaks (DSBs) are particularly dangerous damage because failure to repair such damage will cause the loss of DNA integrity and/or death of the cells. Generally, DSBs are repaired by two fundamentally different processes, homologous recombination (HR) and non-homologous end-joining (NHEJ), which can be distinguished based on whether the DNA sequence is homologous at the break site (126-127). HR is the predominant DSB repair
pathway in dividing cells, where the sister chromatid is available to guide this type of repair. It relies on the pairing of one of the broken strands with a complementary region on the sister chromatid to repair DSBs and is restricted to late S/G2 phase of the cell cycle (126, 128). In contrast, NHEJ does not require homology and can rejoin broken DNA ends with little or no base pairing at the NHEJ junction and therefore can be either faithful, without loss of gene information, or unfaithful, with insertion or deletion of gene information. NHEJ can function throughout the cell cycle, but usually is dominant in G1/quiescent cells where a homologous DNA template is unavailable (127-130). The key protein factors in NHEJ, including the Ku70/Ku80 heterodimer, protein kinase DNA-PKcs, XRCC4 and DNA ligase IV, were all first identified in mammals. More recent studies have demonstrated the presence of functionally homologous factors in the lower eukaryote *Saccharomyces cerevisiae* (131), and more recently, in prokaryotes (132), indicating that the mechanism of NHEJ has been somewhat conserved throughout evolution.

However, NHEJ pathway does not exist in all prokaryotes, such as Ku and LigD gene are absent in *Escherichia coli* (133-134). The iterative PSI-BLAST database searches (135) were performed using the sequences of YkoV, MgKU70, Yku70p-Yku80p and LigD as queries respectively; the searches were run to convergence, with a profile inclusion threshold of expect (E) value of 0.001. No homology of Ku or LigD was detected in *Thermus thermophilus* HB27 and *Thermus thermophilus* HB8. The absence of Ku and LigD genes in *Thermus aquaticus* was assumed since both *Thermus thermophilus* HB27 and *Thermus thermophilus* HB8 with high homology to *Thermus aquaticus* missed Ku and LigD genes.
Figure 1.7: DSB repair by the NHEJ pathway in prokaryote. This figure is based on Figure 1 from reference 130 (130). A Ku homodimer binds to the ends of the DNA break and recruits LigD. The polymerase domain of LigD specially binds to a 5’-phosphate (P) and, together with Ku, promotes end-synapsis. The nuclease and polymerase activities of LigD, and possibly other factors, process the break termini, if required, to restore complementary ends. Finally, ligation of the nicks by LigD repairs the break.

Although the exact mechanism of NHEJ is remains unclear, three basic steps have been phosphorylation /desphosphorylation), and (iii) ligating the ligatable ends (130). Eukaryotic NHEJ is mediated by more than eight core factors in DSB repair process including the Ku70-Ku80 complex, DNA-PK, DNA ligase IV, XRCC4, Artemis and other factors (130). In prokaryotes, Ku and LigD are the critical agents of the NHEJ pathway, although it is possible
that other factors participate in this DSB repair pathway (Figure 1.7) (130, 136). The bacterial end-binding protein Ku entails (proximity) approximation of the broken DNA ends, followed by sealing of at least one of the broken strands by a specialized bacterial ATP-dependent DNA ligase, either LigD or LigC (136). Studies show that Mt (Mycobacterium tuberculosis) LigD contains end-processing, gap-filling and ligation activity required for the DSB repair (137-138).

Figure 1.8: End-processing steps in NHEJ of different DNA end structures produced by restriction enzymes. This figure is based on Figure 2 from reference 129 (129). A. Complementary or blunt ends can be directly ligated by DNA ligase. B. Blunt and 5' overhang ends need fill-in synthesis to create two blunt termini, which can be ligated. C. 5' overhang and 3' overhang ends are recruited by an alignment protein, which proximate termini to allow DNA synthesis by polymerase and, finally, ligation.
Studies have shown that the mechanisms of NHEJ are multi-fold and the outcomes depend on the initial structures of the DSBs and the available ensemble of end-processing and end-sealing components, which are not limited to Ku and LigD (Figure 1.8) (129, 139). For example, DSBs with complementary ends are directly joined by ligase. Blunt ends combined with 5’ protruding ends first must undergo fill-in synthesis, and then are ligated. For DSBs with separate 5’ and 3’ overhangs, the ends are aligned and gap filled, then sealed by ligase. It is widely believed that fully efficient NHEJ requires Ku in both eukaryotes and prokaryotes, where Ku binds DNA ends and subsequently stimulates both synapsis and ligation (140).

Studies have shown that most pol X family polymerases are involved in the eukaryotic NHEJ pathway (141-142). In mammalian cells, the POL X family consists of pol β, pol μ, pol λ, and TdT. All of these function in NHEJ except pol β. Both Klenow and Klentaq share many functional features with LigD and pol X family polymerases, especially polymerase μ. One notable property of both polymerases is that they have both template-dependent and template-independent polymerase activities (143-144). Both of them can add a single non-template nucleotide to the 3’ hydroxyl of blunt-ended duplex DNA substrate \textit{in vitro}. This function allows the formation of terminal microhomology sequences for annealing between the two DNA ends in instances in which no suitable microhomology exists. Since neither \textit{E. coli} nor \textit{T. aquaticus} have Ku or ligD homologies, we investigated the potential for Pol I type polymerases to serve this function. Potential involvement of Pol I polymerases in NHEJ was first postulated by King and associates basing on the ability of Klenow to use un-continued single-stranded DNA as a template for DNA synthesis (145-147). Repairs of DSBs with different end structures by Klenow and Klentaq polymerases are characterized in Chapter 4 of this dissertation. Both polymerases can repair DSBs via DNA fill-in and alignment-based strand-displacement synthesis using
3’melted single-stranded DNA from a second piece of DNA as a template, with different dependencies on DNA ligase. The structure of the broken ends determines the dependence of the DSB repair on DNA ligase. The potential effects of Klenow and Klentaq on ligation of a DNA ligase were also discussed in Chapter 3.

1.4 Effect of Mismatch on Double-strand Breaks Repair

Endogenous and exogenous DNA-damage agents are constant threats causing DNA damage in living organisms. Different types of DNA damage are believed to be repaired through lesion-specific repair pathways, such as DSB repair, NER, BER, or MMR (148). For each type of DNA damage, there are groups of proteins from a given pathway to recognize DNA damage sites, process them, and complete repair.

DSB is the most dangerous form that affects genome stability because it makes cells more prone to failure repair due to the little or no complementary pairs. DNA mismatch that occurs during DNA replication can be repaired by the proofreading activity of DNA polymerase or by the mismatch repair (MMR) machinery (149). Recent studies have demonstrated that DSB repair mechanisms and MMR mechanism are not completely separate and MMR machinery is highly involved in DSB repair. MSH2–MSH3 heterodimeric complex is involved in mismatch repair. It recognizes small loops of 1–13 nucleotides in length (150-151), and is also required during genetic recombination (152-153). Deficiency of MSH2 and MSH3 have been shown to impair the removal of non-homologous DNA ends, necessary for new DNA synthesis and ligation, in both gene conversion and single-strand annealing (SSA) (154), which mediates homologous recombination of DSBs (155-156). MSH2 and MLH1 may also influence the NHEJ pathway by inhibiting the annealing of DSB ends having mismatched termini (157-158). However, the effect of a nearby mismatch on repair of DSB has not been investigated previously.
Spontaneous mutations can occur since reactive oxygen species from endogenous oxidative metabolism and exogenous agents attack DNA in close area. Some mutations in close proximity to each other, such as base lesions and DNA single strand breaks (SSBs) may affect each other during repair (159). Some ‘complex’ lesions may delay repair and generate DNA double strand breaks and mutations (160-162). The effect of terminal mismatch on repair of DSB was discussed in Appendix 1. The results show that mismatch at one end of DSBs can have positive or negative effect on repair with dependence on the end structures. Mismatch lowers the repair efficiency of DSB having non-complementary 5’ overhang, while it promotes the repair of DSB having blunt end.

1.5 The Stoichiometry of the Polymerase-DNA Complexes

The studies of function and structure of DNA polymerase mainly focused on the fidelity of nucleotide (dNTP) incorporation into the 3’ terminus of primer. Relative less is known about DNA polymerase-DNA interactions and their corresponding functions. Considering that DNA polymerases have developed different patterns of substrate specificities by evolution (163), more information on the interactions of DNA polymerase and different types of DNA substrates are essential and helpful to understand the functions and mechanisms of DNA polymerases (164). Although most of the identified DNA polymerases are demonstrated as a monomeric form with or without interaction with DNA in crystallographic studies, the 2:1 polymerase-DNA complexes have been detected in solution by biophysical and biochemical methods (115, 165-166).

1.5.1 The Reported 2:1 Polymerase-DNA Complexes and Their Functional Relevance

The 2:1 Pol β-DNA complexes have been detected using various methods in different laboratories. Yang et al. have showed that Pol β formed a 2:1 complex with the biologically relevant gapped DNA and primed-template DNA, whereas it formed only 1:1 complex with
blunt-end double-stranded DNA and single-stranded DNA (non-natural DNA substrates of Pol β) by surface plasmon resonance measurements (167). By small-angle X-ray scattering (SAXS) and sedimentation velocity (SV), Tsai and coworkers reported that the 2:1 complex is the predominant form with excess amount of Pol β relative to gapped DNA, whereas the 1:1 complex is the major form with excess amount of gapped DNA (166). Their structural studies indicate that the two Pol β molecules bind to the same site of DNA and display different conformations in the 2:1 complex. Based on their structural model and the obtained optimal activity of polymerase when Pol β is in excess relative to DNA (168-170), they speculated that the two protein molecules in the 2:1 complex might function as 5’dPR lyase and the nucleotidyl transferase, respectively (166). The studies of interactions of ASFV pol X and gapped DNA showed that the protein initially used its total binding site (16±2 nucleotides) to form 1:1 complex and then only used its proper binding site (7±1 nucleotides) to 2:1 complex as the protein concentration increased (115). Two ASFV pol X molecules cooperatively binding to the ds-DNA can prevent dissociation from the DNA and thus allows the enzyme to examine the long patches of DNA before it encounters the damaged DNA and then change to the tight binding mode and fix the damaged DNA. The 2:1 interactions of replicative polymerases from the T4 bacteriophage (171), T7 bacteriophage (172-173), and E. coli (174-175) during DNA synthesis is due to dimerization. For these systems, protein dimerization at a replication fork provides a mechanism for coordinating leading and lagging strand synthesis during DNA replication (172-173, 175).

1.5.2 The Interactions of Klenow and Klentaq with DNA

Previous studies, albeit indirect, suggested there might be more than one Klenow molecule could bind to the same primed-template substrate (46, 176). The studies of interactions
of Klentaq and DNA by fluorescence anisotropy and isothermal titration calorimetry (ITC) have suggested that Klentaq formed 1:1 complex with DNA (68). Similar to Pol β and ASFV pol X, both 1:1 and 2:1 Klenow-DNA complexes in solution have been reported. And a 2:1 Klenow-DNA complex has been directly detected by fluorescence anisotropy titrations and analytical ultracentrifugation (165). The results from fluorescence anisotropy titration, DNA gel shift assays, and sedimentation equilibrium experiments suggest that the second Klenow molecule may bind to the upstream duplex part from the primed-template junction. Bailey et al hypothesized that the dimerization interface in the 2:1 complex is located in the exonuclease domain of Klenow based on that the 2:1 complex is the dominant form for the matched primed-template DNA (representing polymerization mode binding) and only the 1:1 complex is formed for the mismatched primed-template DNA (mainly representing editing mode binding) (165). Interactions of Klenow and Klentaq with DNA were further studied by gel shift titrations and protection assays of DNA from exonuclease by both proteins (Appendix 2). The results show that Klenow form 1:1 complex for replicative substrate (primed-template DNA) and 2:1 complex for repair substrate (with blunt end or 3’ overhang) and that Klentaq forms 2:1 complex for each kind of DNA substrates. The 2:1 complex is formed by two protein molecules binding at both ends of a linear DNA.

1.6 References


CHAPTER 2
INTERACTIONS OF REPLICATION VERSUS REPAIR DNA SUBSTRATES WITH THE POL I DNA POLYMERASES FROM E. COLEI AND T. AQUATICUS

2.1 Introduction

Different DNA polymerases partition differently between replication and repair pathways. In this study we examine if two Pol I family polymerases from evolutionarily distant organisms also differ in their preferences for replication versus repair substrates. The DNA binding preferences of Klenow and Klentaq DNA polymerases, from E. coli and T. aquaticus respectively, have been studied using a fluorescence competition binding assay. Klenow polymerase binds primed-template DNA (the replication substrate) with up to 50X higher affinity than it binds to nicked DNA, DNA with a 2 base single-stranded gap, blunt-ended DNA, or to a DNA end with a 3’ overhang. In contrast, Klentaq binds all of these DNAs almost identically, indicating that Klenow has a stronger ability to discriminate between replication and repair substrates than Klentaq. In contrast, both polymerases bind mismatched primed-template and mismatched blunt-ended DNA tighter than they bind the corresponding matched DNA, suggesting that these two proteins may share a similar mechanism to identify mismatched DNA, despite the fact that Klentaq has no proofreading ability. In addition, the presence or absence of 5' or 3' phosphates have slightly different effects on DNA binding by the two polymerases, but again reinforce Klenow's more effective substrate discrimination capability.

Pol I family DNA polymerases are involved in both DNA replication and repair activities within the prokaryotic cell. Pol I is an essential enzyme both in excision repair and in the

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processing of Okazaki fragments generated during lagging strand DNA synthesis (1). During DNA repair, Pol I plays an important role in the gap filling synthesis involved in nucleotide excision repair (NER) (2). Both Klenow and Klentaq completely fill ssDNA gaps of various sizes in duplex DNA (3-4). The thermodynamic analysis of Klenow and Klentaq binding to gapped DNA, however, has not been previously addressed. In this study we examine these two Pol I polymerases from evolutionarily distant organisms to ask if they differ in their preferences for interacting with replication versus repair substrates. The initial binding preferences of the polymerases for different types of "damaged DNAs" versus primed-template DNA (the reference replication substrate) should reflect the partitioning of the polymerases into repair versus replication pathways in vivo.

Klenow and Klentaq are the “large fragment domains” of the Pol I DNA polymerases from *E. coli* and *T. aquaticus*, respectively, and both are fully functional DNA polymerases on their own. Evolutionarily, *T. aquaticus* is more than a billion years older than *E. coli*, and this study asks if the DNA selectivity of the enzyme has changed over that evolutionary time. The most obvious difference between these two polymerases is the presence of 3’ exonuclease activity in Klenow and its absence in Klentaq, despite the fact that the two proteins retain high structural and sequence homology (5-10). Herein, we have characterized the interactions of Klenow and Klentaq with primed-template DNA versus different damaged DNA substrate analogs. Damaged DNA takes many forms. In this study we specifically examine gapped DNA, nicked DNA, and DNA with different DNA end structures that mimic potential DNA double-strand breaks. The role of the 5’ or 3’ phosphate in the binding of gapped DNA by both proteins was also examined.
It is found that Klenow polymerase has the ability to discriminate between primed-template DNA and different repair substrates during the initial DNA recognition step, while Klentaq barely distinguishes among the different potential substrates. Klenow has not evolved toward a strongly biased preference for one type of substrate over another, however, but has developed a $\leq 2.5$ kcal/mole discriminatory binding capability that Klentaq does not have.

Interestingly, for primed-template substrates, both polymerases bind mismatched DNA about 2-3X tighter than they bind matched DNA, suggesting that these two proteins may share a similar mechanism for identifying mismatched DNA, despite the fact that Klentaq has no proofreading ability. Such a capability may be related to a proposed role of DNA polymerases in protecting DNA ends after double-strand breaks (11-12).

2.2 Materials and Methods

2.2.1 Preparation of Oligonucleotides

Oligo-(deoxyribo)nucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and using extinction coefficients provided by manufacturer. Hairpin DNAs and gapped DNAs were prepared by self-annealing a single oligonucleotide by heating at 95°C for 5 minutes and then slowly cooling to room temperature. The DNA constructs used for experiments are shown in Table 2.1. 13/20 is a duplex DNA with 5’overhang and labeled with Rhodamine-X (ROXN) Ester at the 5’ end of the primer for fluorescence anisotropy (13-15). Hp39 is a hairpin DNA with a 5’ overhang, while hp57 is a hairpin DNA with a 3’ overhang and hp46 is a blunt-end hairpin DNA. Hp39m is the same size as hp39 but it contains 3 mismatches at the primed-template end, while hp46m is the same size as hp46 but contains 3 mismatches at the blunt end. Nick is a 52-mer double-hairpin oligonucleotide with one single-strand nick, gap2 is a 50-mer
double-hairpin oligonucleotide with a 2-base gap, and gap10 is a 54-mer double-hairpin oligonucleotide with a 10-base gap. Unlabeled DNA is used for competition assays. In some instances, the 5’ terminus of DNA was phosphorylated with unlabeled ATP experiments, as indicated. Gap 10 with a 3’ phosphate modification was purchased from Integrated DNA Technologies (Coralville, IA).

Table 2.1: Sequences of DNA constructs used in this study

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Sequences</th>
</tr>
</thead>
</table>
| 13/20      | 5’-TCGCAGCGGTCCA-3’
            | 3’-AGCGTCGGCAGTGATCCAAA-5’ |
| hp39       | AAGGCTACCTGCGATGA-3’
            | AGCCGATGCGTACTACCC-5’ |
| hp57       | AAGCAGCTGCTACCGCCACTAGCAAAAC-3’
            | GAGCGGATACGATCGGATGC-5’ |
| hp46       | AAGGCTACCTGATTAATG-3’
            | AGCGTGATGCGTACTATTAACC-5’ |
| hp39m      | AAGGCTACCTGCA CAG-3’
            | AGCGTGATGACGTACTACCC-5’ |
| hp46m      | AAGGCTACCTGATGAAATCAC-3’
            | AGCGTGATGACGTACTATTAACC-5’ |
| nick       | 5’-GCCTGTGAGGGATACCCACAGCCAGCCCTGAGGTACCTCAGACGCGTG-3’ |
| gap2       | 5’-GCCTGTGAGGGATACCCACAGCCAGCCCTGAGGTACCTCAGACGCGTG-3’ |
| gap10      | 5’-GCCAGTGTCAGTCACGTATATATATATATATATATATATATTCGGGAGACAGCGTGACGCTGC-3’ |

2.2.2 Preparation of Klenow and Klentaq Polymerases

Klenow Fragment (KLN) was purified as described previously (16-17). The Klenow clone used in this study contains the D424A mutation (Klenow exo-) and was provided by Catherine Joyce of Yale University. This mutant significantly decreases the 3’-5’ exonuclease
activity, but DNA binding to the proofreading site remains intact (18). The Klentaq clone was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Klentaq (KTQ) was purified as described previously (16). Protein concentrations were calculated by measuring absorption at 280 nm and using ε280 values of 5.88 x 10^4 M^-1 cm^-1 for Klenow and 7.04 x 10^4 M^-1 cm^-1 for Klentaq.

2.2.3 Fluorescence Anisotropy

The fluorescent DNA construct used for equilibrium DNA binding was the 13/20 primer template DNA shown in Table 2.1. 13/20 mer was fluorescently labeled and used at a concentration of 1 nM in the cuvette, [DNA] << K_d. The proteins are titrated into the DNA solution. The anisotropy increases as more protein-DNA complex is formed due to protein addition. The data are analyzed by fitting to equation 2.1 using the program Kaleidagraph to obtain the dissociation constant (K_d).

\[ \Delta A = \{\Delta A_T \cdot (E_T/K_d)/(1 + E_T/K_d)\} \]

where ΔA is the change in fluorescence anisotropy, ΔA_T is the total change in anisotropy, E_T is the total polymerase concentration at each titration point, and K_d is the dissociation constant for polymerase-DNA interaction.

2.2.4 Competition Experiments

Competition assays were used to study the binding affinity of Klenow and Klentaq to different DNA constructs, as described in detail previously (15, 19). Briefly, a competition assay is initiated by mixing 1 nM rhodamineX-labeled 13/20 mer DNA with polymerase at a concentration equal to or slightly higher than the Kd. 13/20 mer is labeled at the 5’ end of the primer (i.e. at the blunt end of the construct). As demonstrated previously, the fluorophore does not affect binding of the polymerase, and polymerase binding only alters anisotropy not steady-
state fluorescence (15-16, 19). Unlabeled competitor DNA is then titrated into the mixture, while maintaining the labeled DNA and the polymerase at constant concentrations. The anisotropy decreases as the unlabeled DNA competes with labeled DNA to bind the protein. See reference 15 for a detailed discussion of the competitive binding assay and requisite controls. In this study, competition assays were performed at 25°C with and without 5mM MgCl₂ in 10 mM Tris, pH 7.9, and in 50mM KCl for Klentaq and 150mM KCl for Klenow.

Competition curves are then fit to equation 2.2:

\[
\Delta A = \frac{\Delta A_T ([I]/K_I)/(1 + [I]/K_I + E_T/K_d)}{(1 + [I]/K_I + E_T/K_d)}
\]  
(Eq. 2.2)

where \(\Delta A\) is the change in fluorescence anisotropy, \(\Delta A_T\) is the total change in anisotropy, \([I]\) is the total competitor DNA concentration at each point during the titration, \(K_I\) is the inhibition constant for the competitive DNA binding, \(E_T\) is the total polymerase concentration, and \(K_d\) is the dissociation constant for polymerase DNA binding to the fluorescent 13/20mer DNA. \(E_T\) is kept constant along with the concentration of fluorescent 13/20mer DNA by including these components at constant concentration in the solution with the competitor DNA. All competition experiments were replicated at least three times. Nonlinear regression was performed using the program Kaleidagraph (Synergy Software).

The Gibbs free energy is calculated using equation 2.3,

\[
\Delta G = -RT \ln (1/K_d)
\]  
(Eq. 2.3)

where, \(\Delta G\) is the Gibbs free energy, \(R\) is the gas constant (1.987 cal K⁻¹ mol⁻¹), \(T\) is the temperature in Kelvin, and \(K_d\) is the dissociation constant for polymerase-DNA binding.

The relative binding affinity is calculated using equation 2.4,

\[
\text{Relative affinity} = \exp \left( (\Delta G_{hp39} - \Delta G_{gap})/RT \right)
\]  
(Eq. 2.4)
where, $\Delta G_{hp39}$ is the Gibbs free energy for the binding of the hairpin DNA with primed-template, and $\Delta G_{gap}$ is the Gibbs free energy for binding of various gapped DNAs. $R$ is the gas constant (1.987 cal K$^{-1}$ mol$^{-1}$), and $T$ is the temperature in Kelvin.

2.3 Results

2.3.1 Structures of the Oligonucleotides Used

Most of the DNA molecules used in this study to explore the binding preferences by Klenow and Klentaq have stable terminal hairpin structures flanking the duplex region to prevent blunt-end binding of the polymerase on one or both sides. Hp39 is a primed-template hairpin DNA, which is used throughout as the “reference DNA”, representing a “normal” replication-type substrate (20). The different DNA substrates that are compared to the binding of hp39 in this study include: 1) nicked DNA, 2) DNA with a 2-base single-stranded gap, 3) DNA with a 10-base gap, 4) blunt-end DNA, 5) blunt-end DNA with 3-mismatched bases at the end, 6) ptDNA with 3-mismatched bases at the primed-template junction, and 7) DNA with a 3' overhang instead of a 5' overhang. In addition to these comparisons, the effects of 5' or 3' phosphorylation within gapped and nicked DNA was also investigated.

2.3.2 Gap and Nick Binding by Klenow and Klentaq

Both Klenow and Klentaq are involved in DNA replication and gap filling synthesis (3, 21). One of the necessary steps in many DNA repair processes is the recognition of damaged DNA by a DNA repair polymerase (22-23). To examine DNA binding features of both polymerases in DNA repair and replication, we have used a fluorescence anisotropy competition assay to study the binding affinity of two polymerases to the gapped DNA. Because the two proteins have different salt dependences during DNA binding (16), the titrations of each protein were carried out at different salt concentrations to place them in similar binding affinity range.
Figure 2.1 shows representative competition curves for the binding of Klenow and Klentaq to different DNA substrates at 25°C. Curves are fit with Equation 2.2 from Materials and Methods to obtain $K_d$ values for each type of DNA construct. It is immediately obvious from Figure 2.1 that the affinities of Klenow for the different DNA constructs vary quite a bit, while the affinities of Klentaq for the different constructs are quite similar. Figure 2.2 B shows the free energies of binding of each of the constructs. In Figure 2.2 B, it can be seen that Hp39, the reference pt-DNA substrate, binds tightest to Klenow, and that the nicked DNA binds the weakest. Both gapped DNAs also bind more weakly to Klenow than does the pt-DNA, but as the gap length increases, the binding affinity to Klenow increases. In contrast, Figure 2.2 B shows that Klentaq polymerase binds pt-DNA, gapped-DNA and nicked-DNA all with similar binding affinities (see Table 2.2 for Gibbs free energies and $K_d$ values).

Figure 2.1: Equilibrium competition titrations for binding of Klenow (KLN,panel A) and Klentaq (KTQ,panel B) to different DNA structures. In both panels, primed-template DNA (Hp39) is shown with diamonds, gap10 DNA is shown with circles, and nicked DNA is shown with squares. Klenow titrations were performed at 25°C in 10 mM Tris, 5 mM MgCl2 and 150 mM KCl at pH 7.9. Klentaq titrations were performed at 25°C in 10 mM Tris, 5 mM MgCl2 and 50 mM KCl at pH 7.9. In order to plot each set of titrations on the same plot, only part of the collected data is shown for some of the constructs. Lines show the fit to Equation 1 as described in Materials and Methods.
Figure 2.2: Affinities of Klenow (KLN) and Klentaq (KTQ) for primed-templates, nicks, and gaps. Panel A shows schematics of the DNA constructs (DNA sequences are given in Table 2.1). Panel B shows the ∆G of binding for each construct. Error bars are the standard deviations on three titrations. Panel C shows the relative difference in binding free energy for each DNA construct, using the pt-DNA (Hp39) as a reference: so for example, ∆∆G_{nick} = ∆G_{nick} – ∆G_{ptDNA}. Positive ∆∆G values indicate that the compared construct binds more weakly than ptDNA.
Table 2.2: The binding constants (Kd) and free energies (ΔG) of binding of Klenow (KLN) and Klentaq (KTQ) polymerases to primed-template and gapped DNAs at 25°C in 10 mM Tris, 5 mM MgCl₂, and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9. Titrations are shown in Figure 2.1.

<table>
<thead>
<tr>
<th>DNA</th>
<th>KLN</th>
<th>KTQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd (nM)</td>
<td>ΔG (kcal/mol)</td>
</tr>
<tr>
<td>hp39</td>
<td>6.7 ± 0.5</td>
<td>-11.14 ± 0.04</td>
</tr>
<tr>
<td>nick</td>
<td>374.5 ± 89.8</td>
<td>-8.76 ± 0.13</td>
</tr>
<tr>
<td>gap2</td>
<td>163.5 ± 34.8</td>
<td>-9.25 ± 0.11</td>
</tr>
<tr>
<td>Gap10</td>
<td>27.3 ± 5.5</td>
<td>-10.31 ± 0.11</td>
</tr>
</tbody>
</table>

Figure 2.2 C and Table 2.3 illustrate the results as ΔΔG values, where each DNA construct is directly compared to the hp39 reference pt-DNA. All subsequent figures in this chapter will utilize this same ΔΔG format, always using hp39 pt-DNA as the reference against which all other DNA substrates are compared. Figure 2.2 thus shows that Klenow polymerase readily distinguishes these different DNA constructs, while Klentaq polymerase cannot really distinguish among them. If gapped DNA is representative of a repair-type substrate and pt-DNA represents a replication-type substrate, these data indicate that Klenow both distinguishes between replication and repair substrates, and prefers replication-type substrates by up to 2.4 kcal/mole.

Translating the ΔΔG values in Figure 2.2 C into relative affinity differences (e.g. $K_a^{ptDNA}/K_a^{nick}$) indicates that Klenow's affinity for pt-DNA is 50X greater than its affinity for nicked-DNA, 25X greater than its affinity for gap2-DNA, and 4X greater than its affinity for gap10-DNA, while the ΔΔG values for Klentaq binding show its negligible affinity difference among these different DNAs (see the relative affinity factor in table 2.3). Thus the affinity of
Table 2.3: The differences in free energies and the relative affinities between primed-template DNA and gapped/nicked DNA binding by Klenow and Klentaq at 25°C in 10 mM Tris, 5 mM MgCl₂, and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9.

<table>
<thead>
<tr>
<th>DNA</th>
<th>KLN</th>
<th>KTQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>hp39</td>
<td>ΔG (kcal/mol)</td>
<td>ΔG (kcal/mol)</td>
</tr>
<tr>
<td>nick</td>
<td>-11.14</td>
<td>0</td>
</tr>
<tr>
<td>gap2</td>
<td>-8.76</td>
<td>2.38</td>
</tr>
<tr>
<td>gap10</td>
<td>-10.31</td>
<td>0.83</td>
</tr>
</tbody>
</table>

ΔΔG = ΔG_{DNA} - ΔG_{pt} and so represents how much tighter the pt-DNA binds polymerase tighter than the gapped or nicked DNA does.

Relative affinity indicates the binding potential of polymerase to different DNAs when they exist as the same concentrations.

Klentaq for a gap is not especially dependent upon the size of the gap, similar to finding in the binding studies of gapped DNA by pol β (24). All relative binding relationships are the same in experiments done in the absence of Mg⁺² (data not shown). Klenow's increase in affinity as the gap length increases indicates that gaps slightly longer than 10 bases will likely appear equivalent to pt-DNA for Klenow.

Another aspect of Figures 2.1 and 2.2, which we have also noted in previous studies, is that they show how readily the fluorescence anisotropy assay detects even very small differences in binding free energies among different substrates (15, 19). For example, the widely spaced titration curves illustrated for Klenow binding to different DNA constructs in Figure 2.1A span a free energy range of 2.4 kcal/mole (as shown in Figure 2.2), while the still resolvable different titration curves for Klentaq binding to different DNAs in Figure 2.1B span a range of ≤ 0.15 kcal/mole.

2.3.3 Effect of 3’ Phosphorylation on Binding of Pol I Polymerases to a 10-base Gap

Depending on how a DNA gap or break is generated, the 3’ end of the DNA may or may
not be extendable by polymerase. To gain an understanding of the binding of gapped DNA with a nonextendable 3’ terminus, we determined the binding affinities of Klenow and Klentaq for gap10-DNA with a 3’ phosphate within the gap, in the presence and absence of magnesium (Figure 2.3). The 3’ phosphate decreases the binding affinity of gap10 by Klenow in the presence (ΔΔG of 0.9 kcal/mol) and absence of magnesium (ΔΔG of 1.5 kcal/mol). This makes reasonable physiological sense, since the polymerase cannot extend this DNA until the phosphate is removed. In contrast, however, addition of the 3’ phosphate only slightly decreases the binding affinity of gap10 by Klentaq (Figure 2.3), again suggesting that Klenow has an enhanced ability to discriminate among these different DNA structures relative to Klentaq.

It has previously been shown that removing the 3’ OH from the primer (replacing it with a 3’ H) did not affect the binding strength of Klenow to primed-template DNA (25). This result provided experimental evidence that the 3’ OH does not contribute to the initial binding free energy, while our results show that a 3’ phosphate decreases the binding affinity of Klenow for gap10. The inhibition by 3’ phosphate may be due to steric conflict within the active site. Previous studies showed that the three catalytically important carboxylates (Asp 882, Glu 883 and Asp 705 in Klenow; Asp 785, Glu 786 and Asp 610 in Klentaq) are 2-3 Å away from the 3’ terminus of the primer strand in the binary complex with DNA (7), but addition of phosphate group to the primer terminus will add about 4 Å.

The larger inhibitory effect of 3’ phosphate on binding of gap10 by Klenow relative to Klentaq (Figure 2.3 and 2.4) could be because there is an equilibrium between the polymerization mode binding and editing mode binding for Klenow (26-27). When the 3’ primer shifts to the exo site, the 3’ OH (hydroxyl group) is hydrogen-bonded to glutamic acid 357 (28). The replacement of 3’ hydroxyl with a phosphate group will preclude this interaction. In
contrast, Klentaq lacks 3’- 5’ exonuclease activity and binds DNA only in the polymerization binding mode (4, 29).

Figure 2.3: The effect of 3’ phosphorylation on the binding of a 10-base gap to Klenow (KLN) and Klentaq (KTQ) polymerases in the presence and absence of Mg$^{2+}$. Schematic representation of the substrates is depicted above panel A. Data are shown as ΔΔG values compared to the same DNA without a 3’-phosphate: ΔΔG = ΔG$_{\text{with-3’p}}$ - ΔG$_{\text{no-3’p}}$. Positive ΔΔG values indicate weaker binding of the phosphorylated gapped DNA.

Table 2.4: The binding constants (Kd) and free energies (ΔG) of binding of Klenow (KLN) and Klentaq (KTQ) polymerases to gap10 DNA in the absence and presence of 3’ terminal phosphate at 25°C in 10 mM Tris, 5 mM MgCl$_2$, and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9. ΔΔG=ΔG$_{3’p}$-ΔG$_{\text{no 3’p}}$ and a positive ΔΔG value indicates that how much the 3’ terminal phosphate weakens the binding of gap10 by both DNA polymerases.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>Mg</th>
<th>no 3’p</th>
<th>3’p</th>
<th>3’p-no 3’p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd (nM)</td>
<td>ΔG (kcal/mol)</td>
<td>Kd (nM)</td>
<td>ΔG (kcal/mol)</td>
</tr>
<tr>
<td>KLN Mg</td>
<td>27.3 ± 5.5</td>
<td>-10.31 ± 0.11</td>
<td>120.6 ± 13.1</td>
<td>-9.43 ± 0.06</td>
</tr>
<tr>
<td>KLN No Mg</td>
<td>22.4 ± 3.2</td>
<td>-10.43 ± 0.08</td>
<td>295.3 ± 46.3</td>
<td>-8.90 ± 0.09</td>
</tr>
<tr>
<td>KTQ Mg</td>
<td>38.5 ± 6.4</td>
<td>-10.10 ± 0.09</td>
<td>75.8 ± 5.6</td>
<td>-9.71 ± 0.04</td>
</tr>
<tr>
<td>KTQ No Mg</td>
<td>7.53 ± 0.4</td>
<td>-11.08 ± 0.03</td>
<td>10.83 ± 0.9</td>
<td>-10.86 ± 0.05</td>
</tr>
</tbody>
</table>
2.3.4 5’ Phosphate Effect on Binding of Gapped DNAs to Pol I DNA Polymerases

The 5’ end of DNA in vivo is frequently phosphorylated, hence we also evaluated how the presence or absence of 5’ phosphorylation within gapped and nicked DNA might alter binding affinity. For longer gaps, the 5’ side of the gap will be too far away to even interact with the polymerase, but for nicks and these shorter gaps, the 5’ side of the gap could easily be within interaction distance. Joyce and associates have shown that for pt-DNA constructs where the 5’ overhang is very short (1-2 bases), 5’ phosphorylation of the overhang can tighten binding affinity to Klenow by about 1 kcal/mole (30).

To evaluate the contribution of the 5’ phosphate of gapped DNA during the binding process, each oligonucleotide was phosphorylated with unlabeled ATP, generating pairs of DNA molecules that differ only with respect to the presence of a 5’ phosphate. Figure 2.4 shows small but differing 5’ phosphorylation effects on the binding of Klenow versus Klentaq in the presence of magnesium. The addition of a 5’ phosphate enhanced the binding of Klenow to the nicked DNA, but not to gapped DNA. In contrast, 5’ phosphorylation shows a small unfavorable effect on binding of gapped DNA by Klentaq (see Table 2.5 for Gibbs free energies and K_d values). Interestingly, the effects of 5’ phosphorylation on gapped DNA binding of both polymerases differs from its effect on the binding of eukaryotic Pol β polymerase, where 5’ phosphorylation moderately increases binding affinity for longer gaps, but not for shorter gaps (31-32).

The 5’ phosphate effects on the affinity of both polymerases for gapped DNA were also examined in the absence of magnesium (data not shown) and the results were nearly identical for Klentaq, and slightly dampened for Klenow (nicked DNA bound 0.35 kcal/mole tighter to Klenow in the absence of magnesium). This relative insensitivity of either polymerase to the
presence or absence of Mg$^{+2}$ again contrasts with Pol $\beta$, where Mg$^{+2}$ and 5' phosphorylation have antagonistic effects on binding affinity of that polymerase to gapped DNA (33).

Figure 2.4: The effect of 5' phosphorylation on the binding of nicks and gaps to Klenow (KLN) and Klentaq (KTQ) polymerases. Schematics of DNA constructs are shown in panel A. In Panel B, Data are shown as $\Delta\Delta G$ values compared to the same DNA without a 5' phosphate: $\Delta\Delta G = \Delta G_{\text{with-5'p}} - \Delta G_{\text{no-35p}}$. Negative values indicate stronger binding and positive $\Delta\Delta G$ values indicate weaker binding of the phosphorylated DNA relative to the unphosphorylated DNA.
Table 2.5: The binding constants \((K_d)\) and free energies \((\Delta G)\) of binding of Klenow (KLN) and Klentaq (KTQ) polymerases to gapped DNAs in the absence and presence of 5’ terminal phosphate at 25°C in 10 mM Tris, 5 mM MgCl\(_2\), and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9. \(\Delta \Delta G = \Delta G_{\text{with-5’p}} - \Delta G_{\text{no-5’p}}\) and so represents whether 5’ terminal phosphate either tightens (negative) or weakens (positive) the binding of DNA by Klenow and Klentaq.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>DNA</th>
<th>Kd (nM)</th>
<th>(\Delta G) (kcal/mol)</th>
<th>Kd (nM)</th>
<th>(\Delta G) (kcal/mol)</th>
<th>(\Delta \Delta G) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLN</td>
<td>nick</td>
<td>374.5 ± 89.8</td>
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<td>gap2</td>
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<td>191.6 ± 18.9</td>
<td>-9.16 ± 0.09</td>
<td>0.06 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>gap10</td>
<td>27.3 ± 5.5</td>
<td>-10.31 ± 0.11</td>
<td>26.4 ± 4.1</td>
<td>-10.33 ± 0.07</td>
<td>-0.02 ± 0.13</td>
</tr>
<tr>
<td>KTQ</td>
<td>nick</td>
<td>61.5 ± 6.1</td>
<td>-9.83 ± 0.06</td>
<td>62.5 ± 6.2</td>
<td>-9.82 ± 0.06</td>
<td>0.01 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>gap2</td>
<td>57.6 ± 6.0</td>
<td>-9.87 ± 0.06</td>
<td>69.4 ± 4.9</td>
<td>-9.75 ± 0.04</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>gap10</td>
<td>38.5 ± 6.4</td>
<td>-10.10 ± 0.09</td>
<td>64.1 ± 8.7</td>
<td>-9.80 ± 0.08</td>
<td>0.3 ± 0.12</td>
</tr>
</tbody>
</table>

### 2.3.5 Binding of Different DNA End-structures by Klenow and Klentaq

Figure 2.5 and Table 2.6 show the affinity differences between pt-DNA and the binding of two different unproductive DNA end-structures to Klenow and Klentaq. Pol I polymerases have been suggested to act in protecting DNA ends after double-strand breaks (11-12, 19). Figure 2.7 shows again that Klenow discriminates against the non-replicative structures more effectively than Klentaq does. In fact, if two-stranded constructs are used instead of hairpin constructs, the difference in affinity between pt-DNA binding and blunt-end binding is even smaller for Klentaq (≤ 0.3 kcal/mole), whereas it remains larger for Klenow (19). However, the differences in affinities between the replicative DNA structures and the blunt-ended or inverted-template-primer DNA structures are not large for either polymerase (≤ 1.1 kcal/mole for Klenow, ≤ 0.6 kcal/mole for Klentaq): a result that certainly supports a protective end-binding capability for both polymerases.
Figure 2.5: Binding of different DNA end-structures by Klenow and Klentaq polymerases. The top panel shows schematics of the different DNA structures. Data are shown as \( \Delta \Delta G \) values relative to binding of ptDNA (Hp39): \( \Delta \Delta G = \Delta G_{\text{structureA}} - \Delta G_{\text{ptDNA}} \). Positive \( \Delta \Delta G \) values indicate weaker binding of the compared DNA relative to ptDNA binding.

Table 2.6: The binding constants (Kd) and free energies (\( \Delta G \)) of binding of DNAs differing in end structures by Klenow and Klentaq polymerases at 25°C in 10 mM Tris, 5 mM MgCl\(_2\), and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9.

<table>
<thead>
<tr>
<th>DNA</th>
<th>KLN Kd (nM)</th>
<th>KLN ( \Delta G ) (kcal/mol)</th>
<th>KTQ Kd (nM)</th>
<th>KTQ ( \Delta G ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hp39</td>
<td>5.8 ± 0.3</td>
<td>-11.23 ± 0.03</td>
<td>31.5 ± 1.57</td>
<td>-10.23 ± 0.03</td>
</tr>
<tr>
<td>hp57</td>
<td>27.7 ± 1.32</td>
<td>-10.30 ± 0.03</td>
<td>47.21 ± 2.54</td>
<td>-9.98 ± 0.06</td>
</tr>
<tr>
<td>hp46</td>
<td>39.7 ± 2.06</td>
<td>-10.09 ± 0.03</td>
<td>91.1 ± 5.81</td>
<td>-9.60 ± 0.03</td>
</tr>
</tbody>
</table>
2.3.6 Binding of Mismatched DNA by Klenow and Klentaq

Figure 2.6 and Table 2.7 shows the binding of Klenow and Klentaq to DNA constructs with mismatched ends. Our laboratory and others have collected a large body of data on the binding of Klenow and Klentaq to different DNAs, but no laboratory has previously characterized a duplex DNA that binds with higher affinity than the "normal" primed-template substrate (although single-stranded DNA, under high salt concentrations, will bind more tightly than pt-DNA). In Klenow, mismatches at the 3’ primer terminus favors the partitioning of the binding to the exo site (34-35), and three consecutive mismatches at the 3’ primer terminus cause Klenow to bind almost entirely in the editing mode (34-35). Thus, when binding to Klenow, duplex DNAs containing three consecutive mismatches will bind with the duplex pulled open and the 3’ end of the primer pulled down into the exonuclease active site. The only existing protein-DNA co-crystal of Klenow polymerase shows the DNA binding in this "editing mode" orientation (6, 8).

Clearly, mismatched DNA binds to the proofreading site of Klenow more tightly than matched DNA binds to the polymerization site. But Figure 2.8 unexpectedly shows almost the same enhancement of affinity when mismatched DNA binds to Klentaq polymerase. In fact, this is the only non-replicative DNA in this study that Klentaq appears able to distinguish from pt-DNA. Klentaq, however, does not have proofreading ability, nor an exonuclease site, and has only ever been observed to bind DNA in the polymerization mode (10, 29). In fact, the key amino acids identified as being involved in Klenow's proofreading activity (36) are all missing in Klentaq. Thus, the origin for its tighter binding to the mismatched DNA relative to the matched DNA remains unknown. Again the free energy differences are rather small, both Klenow and Klentaq bind the mismatched DNA ≤ 0.7 kcal/mol (see Table 2.7) tighter than the matched
DNA, but this difference would predict that if these two proteins encountered equal concentrations of matched and mismatched DNA, they would both be 2-3 times more likely to bind to the mismatched DNA. Moreover, this small difference lends more support to the potential physiological role of Klenow and Klentaq polymerases can participate in the protection of the broken ends of damaged DNA (11-12).

Figure 2.6: Binding of mismatched DNA to Klenow and Klentaq polymerases. The top panel shows schematics of the different DNA structures. Data are shown as $\Delta \Delta G$ values relative to the same construct without mismatches: $\Delta \Delta G = \Delta G_{\text{mismatched}} - \Delta G_{\text{matched}}$. Negative values indicate tighter binding of the mismatched DNA constructs.
Table 2.7: The binding constants ($K_d$) and free energies ($\Delta G$) of binding of Klenow (KLN) and Klentaq (KTQ) polymerases to matched and mismatched DNAs at 25°C in 10 mM Tris, 5 mM MgCl$_2$, and 150 mM KCl (Klenow) or 50 mM KCl (Klentaq) at pH 7.9.

<table>
<thead>
<tr>
<th>DNA</th>
<th>KLN ($K_d$ (nM))</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>KTQ ($K_d$ (nM))</th>
<th>$\Delta G$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hp39</td>
<td>8.0 ± 0.1</td>
<td>-11.04 ± 0.01</td>
<td>32.9 ± 1.3</td>
<td>-10.18 ± 0.02</td>
</tr>
<tr>
<td>hp39m</td>
<td>2.7 ± 0.1</td>
<td>-11.67 ± 0.02</td>
<td>14.0 ± 0.9</td>
<td>-10.71 ± 0.04</td>
</tr>
<tr>
<td>hp46</td>
<td>30.2 ± 1.2</td>
<td>-10.25 ± 0.02</td>
<td>91.1 ± 5.81</td>
<td>-9.60 ± 0.03</td>
</tr>
<tr>
<td>hp46m</td>
<td>8.4 ± 0.6</td>
<td>-11.01 ± 0.04</td>
<td>30.8 ± 1.1</td>
<td>-10.24 ± 0.02</td>
</tr>
</tbody>
</table>

### 2.3.7 Fill-in and Strand-displacement Synthesis on Gapped DNAs

Primer extension and DNA strand-displacement synthesis activities of Klenow on nicked and gapped DNA substrates were tested in comparison with a standard primed-template substrate. Reactions were performed in the presence of 50 uM dNTPs with increasing amounts of input polymerase, with the DNA (250 nM) in excess over the enzyme. Figure 2.7 shows the fill-in and strand-displacement synthesis of Klenow on a nicked DNA and a 10-base gap. The 10-base gap (gap10) DNA was filled in at low levels of input Klenow to yield a 64-mer extension product (Figure 2.7 left panel, lanes 3 and 4), and the blocking strand was displaced at higher levels of input polymerase (≥5 nM) to synthesize a full length 88-mer product, consistent with previous studies (37). Klenow was also able to displace the blocking strand and synthesize a full length 78-mer product on the nicked substrate (Figure 2.7 right panel, lanes 5, 6, and 7) when the enzyme concentration was equal or higher than 5 nM. Figure 2.7 also shows that addition of 5’ phosphate to DNA did not show any effect on the primer extension and DNA strand-displacement synthesis activities, differing from the stimulation of 5’ phosphate on primer extension by PaePOL (38). These results show that the substrates used for binding studies in this chapter are enzymatically functional substrates for the polymerases. The results also suggest that
Pol I can mediate NER or SSB repair through gap filling and DNA strand-displacement synthesis without a requirement for ligase. Klentaq shows similar features for primer extension and DNA strand-displacement synthesis on the same DNA substrates (data not shown). The 5’ phosphate of gapped DNA did not influence the filling and DNA strand-displacement synthesis activities of Klentaq either.

Figure 2.7: The fill-in and DNA strand-displacement synthesis by Klenow. Reactions of 10 ul contained 150 nM of DNA (gap10 in left up panel, gap10 containing 5’-phosphate in left down panel, nick in right up panel, and nick containing 5’-phosphate in right down panel), and varying amounts of Klenow (KLN). Lanes 1-6 in left panels contained Klenow (0, 0.2, 0.5, 1, 5, and 30 nmol) and lanes 1-7 in right panels contained Klenow (0, 0.5, 1, 2, 5, 10, and 30 nmol). Reactions were incubated at 30 °C for 10min in the buffer containing 10 mM tris, 150 mM KCl, and 5mM MgCl2, pH 7.9. Substrate and fill-in or strand-displacement synthesis product sizes are as indicated.

2.4 Discussion

In this study, we have examined the thermodynamics of binding of two different Pol I DNA polymerases to different replicative or repair-type DNA substrates, and found that the evolutionarily older polymerase (Taq) does not really discriminate among the different potential DNA substrates, while the evolutionarily younger polymerase (E. coli) has the capability of distinguishing among these potential intracellular binding sites (Figure 2.8). The polymerase from E. coli generally binds preferentially to replicative-type substrates (pt-DNA > nicks, gaps,
or unusual end-structures), but the preferences are only on the order of up to 2.4 kcal/mole. This indicates that while the *E. coli* polymerase definitely prefers replicative (or long gap) substrates, it does not effectively (thermodynamically) exclude any of these substrates. The gradual decrease in the affinity of Klenow for shorter gaps leading to the nick also makes some sense from a physiological point of view, since after filling a gap Klenow is likely to dissociate from the nick and allow ligase to close the nick. Alternatively, Klenow also has the capability to continue forward via displacement synthesis at the nick.

Figure 2.8: Schematic binding preference of Klenow and Klentaq on different DNA. Klenow preferentially binds the replication-type substrates over the repair-type substrates (top panel). Klentaq binds all types of DNA substrate similarly.

Another conclusion from this data is that the single-stranded portion of the DNA is more important for Klenow binding than for Klentaq binding. This fact has been demonstrated in previous studies of primed-template DNA with different overhang lengths (19, 30). The data in this study extend these findings to include the single-stranded portion of the DNA within a gap, which also clearly contributes to the total binding free energy for Klenow, but not for Klentaq.
2.4.1 Model for the Primed-Template DNA Recognition by Klenow

On the basis of the results obtained in this work and the discussion in the previous section, we propose a possible mechanism of substrate discrimination by Klenow as depicted in Figure 2.9. If Klenow binds primed-template DNA in the polymerization mode, the total binding affinity is $E_t + E_{dsDNA}$, where $E_t$ is the affinity for the single-stranded template binding to the finger subdomain (27, 29) and $E_{dsDNA}$ is the affinity of duplex part binding to thumb subdomain (39). The interaction of the single-stranded template with the finger subdomain provides additional free energy of binding when the ss DNA extension of the primed-template DNA is long enough. As a result, the affinity of the enzyme for the primed-template DNA is amplified over the affinity for a double-strand blunt-end DNA. This mechanism for the primed-template DNA recognition was also supported by our lab’s previous studies (19). On the other hand, if there is a blocking strand complementary to the template strand, downstream of the 3’ primer terminus, the interaction of the single-strand template and polymerase ($E_{idsDNA}$) would be precluded, thus a DNA binds by Klenow weakly, such a nicked DNA.

Figure 2.9: A schematic model of the specific mechanism of primed-template DNA substrate recognition by *E. coli* Klenow polymerase.
2.4.2 Comparison of the 5’ and 3’ Phosphate on Binding of Gap10

Figure 2.10: Comparison of the effects of 5’ and 3’ phosphate on gap10 binding (replot the data for Figure 2.3 and 2.4). In Panel A, Data are shown as ΔΔG values compared to the same DNA without a DNA terminal phosphate: \( \Delta\Delta G = \Delta G_{\text{with-3’5’p}} - \Delta G_{\text{no-3,5’p}} \). Positive ΔΔG values indicate weaker binding of the phosphorylated DNA relative to the unphosphorylated DNA. Panel B shows potential schematic models for binding of gap10 by Klenow and Klentaq.

It is also notable that the effects of 5’ and 3’ phosphorylation of a 10 base gap on the binding of Klentaq are almost identical, in contrast to the somewhat different effects that phosphorylating opposite sides of the gap has on Klenow (Figure 2.10 A). This could indicate that Klentaq binds equivalently to either side of the gap (and thus is similarly inhibited by phosphorylating either side of the gap), while Klenow binds preferentially to the productive 3’-side of the gap (Figure 2.10 B), and thus is more inhibited by 3’ phosphate than by 5’ phosphate.
This possibility is also supported by the data on the binding of the two polymerases to DNA end-structures with a 5’ versus a 3’ overhang (Figure 2.5). Klentaq binds the DNA end structures with a 5’-overhang and 3’-overhang with nearly identical affinity, while Klenow binds them with significantly different affinities.

2.4.3 Potential Contribution of the Proofreading Site to Substrate Recognition

While the data clearly indicate that Klenow has a more sophisticated ability to discriminate among different DNAs than Klentaq does, a key question in the further interpretation of the data is: how much of this difference is due to Klenow's proofreading site? The Klenow exonuclease mutant used in these studies (and nearly all studies of Klenow in the past two decades) does not have exonuclease activity, but still binds DNA in the exonuclease site (18). The easiest/quickest answer to this question is the assumption that these differences must simply be due to the proofreading site, however, all but one set of DNA substrates are DNAs with fully matched duplex regions, where the partitioning into the proofreading site will be minimal (between 5 and 15%) ((27, 34, 40), Brown & LiCata, unpublished). In addition, the presence or absence of Mg\(^{2+}\), which is believed to alter the partitioning into the proofreading site (35), does not alter most of the quantitative results nor any of the qualitative results of this study (e.g. the data on 3’ phosphorylated gap binding in Figure 2.3 show the largest effect of Mg\(^{2+}\), but Klenow still shows significantly greater substrate discrimination than Klentaq in both the presence or absence of Mg\(^{2+}\). Despite these arguments, however, we cannot definitively rule out the potential participation of the proofreading site in Klenow's greater discrimination among these substrates.

Related to this discussion of the potential role of the proofreading site in Klenow, possibly the most unusual result in this study is the finding that both Klenow and Klentaq bind
mismatched DNA more tightly than matched DNA, and by about the same amount. Yet Klentaq
does not have a proofreading site, nor has it ever been observed to bind DNA outside the
polymerization site. This finding suggests that DNA end protection, such as after a double-
strand break, is an extremely important function for both polymerases. Protection of unmatched
end structures has been implicated in non-homologous end-joining (11-12). We chose to examine
DNA with a three-base mismatch because Klenow is known to bind such a DNA completely in
the editing mode, by pulling the primer strand into the proofreading site (5, 8, 27, 34, 40). The
topology of binding of mismatched DNA to Klentaq has not yet been addressed.

The importance of the end-binding/end-protection role of the polymerases is also
supported by the finding that both polymerases bind quite well to a DNA to an inverted end-
structure: an overhanging primer instead of an overhanging template. Many DNA binding
proteins are known to exhibit "non-specific" binding to DNA ends, but what is unusual in these
results is that the affinities of the polymerases for the different non-extendable ends are so close
to the affinities for the productive substrates: close enough that competition among all of these
different DNA "targets" for the 400 or so Pol I molecules within a prokaryotic cell would be very
effective.

2.4.4 Fill-in and Strand-displacement Synthesis by Klenow and Klentaq

The studies of gap filling and the influence of 5’ phosphate were started from Family X
polymerase such as DNA polymerase β (Pol β) (41), DNA polymerase λ (Pol λ) (42) to
mycobacteria LigD POL (38, 43) and Pol I DNA polymerase (3-4). Our result show that both
Klenow and Klentaq can completely fill in the gap with different sizes in ssDNA gap followed
by DNA strand-displacement synthesis without being influenced by the presence of 5’ phosphate
within the gap, even though 5’ phosphate shows effect on DNA binding by both proteins. Thus
the inhibitory effects of the phosphate on binding do not preclude functionality. Further studies on the kinetics of gap fill-in synthesis and strand-displacement synthesis could be needed, however, to determine if the binding differences translate into differences in enzymatic rates.

2.4.5 Comparisons with Other Polymerases

The characterization of eukaryotic polymerase Pol β carried out by Bujalowski and associates constitute the most extensive and detailed thermodynamic studies of polymerase binding to gapped-DNA carried out in any system to date (23-24, 33). Some of the differences between Pol I and Pol β gap binding were discussed above in Results. Bujalowski and associates have also studied gap binding by Pol X (44), and enzymatic studies of gap filling activity have been carried out for a number of DNA polymerases (38, 41-42, 45). Among all of these studies (present studies included), one thing is clear: there are no universal rules for polymerase gap binding. Pol X only functions on gaps where the single-stranded DNA portion is less than 4 nucleotides long (44). Both Pol β and Pol λ processively fill short gaps (less than 5 or 6 nucleotides) with a requirement for a 5’ phosphate at the 5’ end of the gap (41-42). Pol α prefers long gaps with >30 nucleotides of single-stranded DNA in the gap (41, 45). The presence of a 5’ phosphate stimulates the extension of the primer by PaePOL when the gap is more than 2 nucleotides long (38). For Pol β, the affinity of the polymerase is not especially dependent on the size of the gap, unless the gap is 5’ phosphorylated and then longer gap binding will be enhanced, and 5’ phosphorylation within the gap and the presence of Mg^{+2} are antagonistic for Pol β (33).

2.5 Concluding Summary

Our results here suggest that *E. coli* polymerase can discriminate between replication and repair substrates, but Taq polymerase does not. DNA phosphorylation within gapped-DNA has
different effects on the binding of *E. coli* versus *Taq* polymerases. Attempting to integrate the findings for these different polymerases quickly leads to the conclusion that characterizing gap binding behavior in one polymerase has virtually no predictive capability for any other polymerase. Gap binding characteristics clearly change among polymerases with different specializations and between prokaryotes and eukaryotes. In that sense the Klenow/Klentaq pair represents a unique comparison at the evolutionary onset of gap recognition: a comparison between two Pol I family polymerases where one has not yet developed the capability to distinguish gaps from other DNA structures, and one that is just beginning to make such distinctions.

### 2.6 References


CHAPTER 3

KLENOW AND KLENTAQ POLYMERASES STIMULATE DNA END JOINING BY E. COLI DNA LIGASE

3.1 Introduction

Klenow and Klentaq polymerases bind to different DNA ends and have been suggested to be potentially involved in DNA double-strand break repair. Herein, we show that both polymerases stimulate the intermolecular ligation activity of E.coli DNA ligase when present at concentrations lower than that of the DNA substrates. Concentration in excess of the substrates for either polymerase inhibits the ligation activity of DNA ligase. Moreover, longer incubation time improves the stimulation effect of both polymerases. Conversely, neither polymerase was able to stimulate the functionally homologous DNA ligases from bacteriophage T4 or Thermus aquaticus. Additionally, neither polymerase is able to significantly enhance ligation of a substrate containing a single nick, suggesting that the polymerases bridge the two DNA ends during intermolecular ligation.

DNA double-strand breaks (DSBs), resulting from a variety of endogenous cellular processes and exogenous DNA-damaging agents, are a lethal damage for cell or genomic stability in both prokaryotes and eukaryotes. DSBs are repaired by two major repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) (1-2). Recent work suggests that Ku and LigD are critical agents of the NHEJ pathway in prokaryotes, although it is possible that other factors participate in this DSB repair pathway (3-4) such as a DNA polymerase, which may be involved DNA end processing by fill-in synthesis or gap filling on pieces of bridged DNA, after which DNA ligase seals the nick to complete repair (4).

Not all prokaryotes, however, appear to possess an NHEJ pathway, as the Ku and LigD genes are absent from many bacterial species, including E. coli (5-6). For T. aquaticus, iterative
PSI-BLAST database searches (7) were performed using the sequences of YkoV, MgKU70, Yku70p-Yku80p and LigD as queries; The searches were run to convergence, with a profile inclusion threshold of expect (E) value of 0.001. No homology to Ku or LigD was detected in *Thermus thermophilus HB27* and *Thermus thermophilus HB8*, each having high similarity to *Thermus aquaticus*. Thus we concluded that there are likely no homology of Ku and LigD genes in *Thermus aquaticus* either.

Both Klenow and Klentaq show tight binding affinity to DNA with different end structures including 5’ or 3’ overhangs and blunt ends (8, Chapter 2 of this dissertation). Previous studies of potential NHEJ activity of Pol I showed that Klenow polymerase is able to use discontinuous templates. It can produce fill-in products between two DNA ends with microhomologies (9-11). Relatedly, work discussed in Chapter 4 of this dissertation demonstrate the ability of both Klenow and Klentaq to perform strand-displacement synthesis on two pieces of DNA with microhomologies. These activities have led to speculation on the role of Pol I DNA polymerase in DSB repair. Pol I DNA polymerase may play several direct roles, including protection of ends from degradation, bridging of DNA ends prior to joining, and recruitment of ligase to the junction site.

Pretreating of a linear DNA with blunt end and 3’-protruding single strand (PSS) end with Pol I polymerases could increase the transformation efficiency and the preservation of 3’-PSS when it was transformed to *E. coli* strain, MC1061, which has no known recombination or DNA repair defects (12). This increased junction formation at non-complementary ends suggests that Pol I DNA polymerase might act to facilitate ligation.

In this chapter, we tested the possible effect by adding either Klenow or Klentaq polymerase to DNA ligation assays. Different bacterial DNA ligases may function in specific
pathways or during different growing stages or may be functional under different growth conditions (13). *E. coli* DNA ligase consists of 671 amino acids and is a NAD\(^+\)-dependent ligase. It can close nicks and ligate two pieces of DNA with cohesive ends (14). Recently, the structure of *E. coli* DNA ligase complexed with nicked DNA has been solved (15). T4 DNA ligase, a 487 amino acid ATP-dependent ligase, is used routinely in molecular cloning for connection of both sticky and blunt ends. The structure of T4 DNA ligase is not solved yet. However, the structure of the bacteriophage T7 ATP-dependent ligase has been solved, which is assumed to have a similar fold to T4 ligase (16-18). Taq DNA Ligase is a 676 residue NAD\(^+\)-dependent ligase, which catalyzes the formation of a phosphodiester bond between juxtaposed 5´ phosphate and 3´ hydroxyl termini of two adjacent oligonucleotides, which are hybridized to a complementary target DNA. Taq DNA Ligase is active at elevated temperatures (45°C-65°C) (19-20).

Here we demonstrate the ability of Klenow and Klentaq to stimulate intermolecular DNA ligation. The effects of both polymerases on ligation are specific to *E. coli* DNA ligase with neither polymerase having significant effect on T4 or Taq ligase. This effect is possibly due to the ability of Pol I DNA polymerase to bridge DNA ends and/or make them easily accessible to DNA ligase.

### 3.2 Materials and Methods

#### 3.2.1 Materials

Oligonucleotides used in this study were obtained from Integrated DNA Technologies (Coralville, IA). The DNA substrate sequences are shown in Table 3.1. 5´ phosphorylated DNAs were purchased from Integrated DNA Technologies. Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and using extinction coefficients provided by the manufacturer. The DNA molecules constructed for this study have stable terminal hairpin
structures flanking the duplex region to circumvent the difficulty in analyzing the various products produced by multiple ligations. Hairpin structures are closed using a stable tetraloop (21). Hairpin DNAs were annealed from single-strand DNA by heating at 95°C for 5 minutes and slowly cooling down to ambient temperature.

Table 3.1: Sequences of DNAs Used in this Study.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hp27</td>
<td>AACGACGTCCGGGTTCGAGGCTGAGGGCC-3'</td>
</tr>
<tr>
<td>hp57</td>
<td>AACGGCTATGCTACCCGCTACGCAAACC-3'</td>
</tr>
<tr>
<td>nick</td>
<td>AACGGCTATGCTACCCGCTACGCAAACC-3'</td>
</tr>
</tbody>
</table>

Klenow (KLN) and Klentaq (KTQ) polymerases were purified in our laboratory (refer to Chapter 2). The Klenow clone used in this study contains the D424A mutation (Klenow exo-) and was provided by Catherine Joyce from Yale University. This mutant has only residual 3’-5’exonuclease activity, but retains DNA binding affinity for the proofreading site (22). Protein concentrations were measured at 280 nm and calculated by using ε280 values of 5.88 x 10⁴ M⁻¹ cm⁻¹ for Klenow and 7.04 x 10⁴ M⁻¹ cm⁻¹ for Klentaq. T4 DNA ligase, *E. coli* DNA Ligase, and Taq DNA ligase were purchased from New England Biolabs (Ipswich, MA).

3.2.2 Methods

Ligation reactions were performed in each ligase’s corresponding reaction buffer containing identical amounts of DNA substrates, a fixed amount of one of the ligases, and increasing amounts of polymerase or bovine serum albumin (BSA). DNA ligase, substrate, and reaction buffer were mixed, immediately followed by the addition of either polymerase or BSA. The reaction volume was 10 µl. Intermolecular ligations with two pieces of DNA having
complementary 5-base 3’ overhangs (hp27 and hp57) or intramolecular ligation with nicked DNA (nick) were performed at 25°C for *E. coli* and T4 ligases and 50 °C for Taq ligase for the indicated times. Ligations were stopped with 6 µl of a stop buffer containing 0.2% SDS, 10 mM EDTA, 90% formamide, and 0.1% bromphenol blue, and heating at 95 °C for 10 min, and then immediately put on ice for 5 min. A 4.5 µl of sample was loaded onto a 20% denaturing polyacrylamide gel containing 7.5 M urea and electrophoresed in TAE (80 mM Tris acetate, 2 mM EDTA, pH 8.0). Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a Bio-Rad gel imager.

The amount of ligated product formed was determined by quantitating the product on denaturing polyacrylamide gels using the program ImageQuant 5.1. Rectangles of the same size were drawn around all product bands, and equally sized rectangles were also drawn in the corresponding positions below each product band as background control. The difference in intensities between each product band and that of the corresponding background control yields the relative amount of ligation product formed. The standard deviation for each reading was reported by the program ImageQuant 5.1.

3.3 Results

3.3.1 Both Klenow and Klentaq Stimulate *E. coli* DNA Ligase Activity

We used hairpin DNA hp27 and hp57 containing complementary 5-base 3’ overhangs as the substrates to assess intermolecular end joining *in vitro*. Experiments were performed with highly purified Klenow/Klentaq and commercial *E. coli* DNA ligase. Figure 3.1 shows titrations of Klenow (left panel of Figure 3.1 A) and Klentaq (right panel of Figure 3.1 A) into reactions containing a constant level of *E. coli* DNA ligase. The reactions were performed in the presence of excess substrate so that the amount of product formation was below the ligation saturation
point. Upon addition of either Klenow or Klentaq to the ligation reactions, an increase in the amount of ligation product was observed (lanes 3-5 in left panel and lanes 2-5 in right panel of Figure 3.1 A).

Figure 3.1 B is the graphical analysis of the results from Figure 3.1 A. The graph depicts the about 150% enhancement of ligation product formation as a function of Klenow or Klentaq concentration for the tested ligase concentration. Stimulation increased with increasing concentrations of Klenow/Klentaq over the tested range. Furthermore, the stimulations of both proteins on ligation were similar within the tested range. Thus both polymerase-mediated stimulations of ligation rate were maximally 2.5-fold higher compared with reactions lacking polymerase.

3.3.2 BSA Does Not Enhance the Activity of E. coli DNA Ligase

To determine whether the stimulation of ligation is specifically contributed by the function of Pol I DNA polymerases, we titrated similar concentrations of bovine serum albumin (BSA) into reactions containing E.coli DNA ligase. The ligation product formation in the presence of increasing amounts of Klenow or BSA is shown (Figure 3.2 A). Increasing the concentration of BSA in the reaction had no significant stimulation upon ligation activity. Quantitation of these results shows that adding BSA actually inhibited the ligation by around 20% (Figure 3.2 B). Therefore, the stimulation of E. coli DNA ligase by either Klenow or Klentaq appears to be polymerase specific.

3.3.3 High Concentration of Klenow and Klentaq Inhibit Ligation of E. coli DNA Ligase

To further analyze the potential mechanism of stimulation, we also assessed the effects of higher than stoichiometric concentrations of Pol I DNA polymerase on ligation of E. coli DNA Ligase. The ligation product formation in the presence of increasing amount of either Klenow
Figure 3.1: Klenow and Klentaq stimulate *E. coli* DNA ligase activity. 10 µl reactions containing 250 nM of hp57, 500 nM of hp27, 0.3 units of DNA ligase (lanes 2-5 in the left panel of A and lanes 1-5 in the right panel of A) and varying amounts of protein (KLN=Klenow, KTQ=Klentaq) were performed. Lanes 2-5 in the left panel of A contained Klenow (0, 5, 15, and 50 nmol) and lanes 1-5 in the right panel of A contained Klentaq (0, 5, 10, 15, and 50 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for 6 min. Substrate and ligation product sizes are as indicated in nucleotides. Hp57 contains 5’ phosphate to allow ligation. Schematic representation of the substrates is depicted at the top of the figure. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement of ligation as a function of either Klenow or Klentaq.
Figure 3.2: Comparison of the effects of both polymerases and BSA on *E. coli* DNA ligase activity. Reactions of 10 ul contained 250 nM of hp57, 500 nM of hp27, 0.3 units of DNA ligase (lanes 2-5 in the left panel of A and lanes 2-6 in the right panel of A) and varying amounts of protein (KLN=Klenow, BSA=Bovine serum albumin). Lanes 2-5 in the left panel of A contained Klenow (0, 5, 15, and 50 nmol) and lanes 2-6 in the right panel of A contained BSA (0, 5, 10, 20, and 50 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for 6 min. Substrate and ligation product sizes are as indicated in nucleotides. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement/inhibition of ligation as a function of protein.
Figure 3.3: High concentrations of Klenow inhibit the activity of *E. coli* DNA ligase. Reactions of 10 ul contained 250 nM of hp57, 500 nM of hp27, 0.3 units of DNA ligase (lanes 2-8 of both panels in A) and varying amounts of protein (BSA=Bovine serum albumin, KLN=Klenow). Lanes 2-8 in both panels of A contained either Klenow or BSA (0, 5, 10, 15, 50, 300, and 500 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for 6 min. Substrate and ligation product sizes are as indicated. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement/inhibition of ligation as a function of either BSA or Klenow.

or BSA (from 5-500nM) is shown (Figure 3.3 A). As shown in Figure 3.1, stimulation increased with increasing amounts of Klenow when the number of Klenow molecules added is less than the number of DNA ends. But addition of concentrations of Klenow higher than the concentration of DNA ends inhibited ligation (compare lanes 5 and 8, Figure 3.3 A, right panel).
The similar effects of Klentaq for the similar concentrations are also shown (Figure 3.4). On the other hand, the same high concentrations of BSA had similar weak inhibition of ligation as low BSA concentrations (Figure 3.3 A, left panel).

Figure 3.4: High concentrations of Klentaq inhibit the activity of *E. coli* DNA ligase. Reactions of 10 ul contained 250 nM of hp57, 500 nM of hp27, 0.3 units of DNA ligase (lanes 2-7 A) and varying amounts of Klentaq. Lanes 2-7 of A contained Klentaq (0, 5, 20, 50, 300, and 500 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for 6 min. Substrate and ligation product sizes are as indicated. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement/inhibition of ligation as a function of either BSA or Klentaq.
We also tested the effect of high concentrations of both polymerases on nick sealing of a nicked DNA (Figure 3.5). Within similar concentration ranges, both Klenow and Klentaq do not significantly affect ligation as the concentration increases (Figure 3.5 A). Quantitation of these results shows that addition of either Klenow or Klentaq from 5 to 500 nM only changes the ligation efficiency by -20% to +30% compared to the ligation without polymerase addition (Figure 3.5 B).

Figure 3.5: Effect of high concentrations of Klenow and Klentaq on the activity of *E. coli* DNA ligase with a nicked DNA as a substrate. Reactions of 10 ul contained 150 nM of nicked DNA, 0.3 units of DNA ligase (lanes 2-8 in A) and varying amounts of either Klentaq or Klenow. Lanes 2-8 of top panel in A contained Klentaq (0, 5, 10, 20, 50, 300, and 500 nmol) and lanes 2-8 of top panel in A contained Klenow (0, 5, 15, 20, 50, 300, and 500 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for 6 min. Substrate and ligation products are as indicated. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement/inhibition of ligation as a function of either Klenow or Klentaq.
3.3.4 Klenow and Klentaq Do Not Stimulate T4 Ligase and TaqLigase

To examine the specificity of the functional interaction between Pol I DNA polymerase and *E. coli* DNA ligase, we determined whether Pol I could stimulate another NAD\(^+\)-dependent ligase, Taq DNA ligase, or an ATP-dependent ligase, T4 DNA ligase. Klenow (Figure 3.6 A) and Klentaq (Figure 3.7 A) were added to a reaction containing either the *Thermus aquaticus* DNA ligase or the bacteriophage T4 DNA ligase. Since the active temperature for Taq DNA ligase is 45-65°C, all Taq DNA ligase reactions were performed at 50°C. Considering the ligation efficiency of Taq ligase is lower than either *E. coli* or T4 ligase, high relative concentration of Taq ligase was used in all reactions.

Figure 3.6 A shows the titration of Klenow to T4 ligase and Taq ligase reactions. As increasing amounts of Klenow are added to the reaction, the changes in the ligation products formed by both ligases are small or negligible. Quantitation results show that Klenow enhances ligation of Taq ligase around 15-30% and does not affect ligation of T4 DNA ligase (Figure 3.6 B).

Figure 3.7 shows the effect of increasing amounts of Klentaq on ligation of either T4 ligase or Taq ligase. Similar effects to that of Klenow on ligation were observed. Klentaq does not significantly influence ligation by Taq ligase either, and slightly inhibits the function of T4 ligase. Even though, Klenow and Klentaq do not significantly affect the ligation of these two ligases, both polymerases display an interesting pattern where *E. coli* ligase is significantly enhanced, T4 ligase is inhibited or unaffected, and Taq ligase is in between T4 and *E. coli* ligases, but much more similar to T4 ligase. This suggests that there may be a very weak interaction between the polymerases and Taq ligase.
Figure 3.6: Klenow does not stimulate the ligation by T4 ligase or Taq DNA ligase. Reactions of 10 µl contained 250 nM of hp57, 500 nM of hp27, 0.3 units of T4 DNA ligase or 4 units of Taq DNA ligase, and varying amounts of Klenow. T4 ligase reactions were incubated at 25 °C for 6 min, Taq ligase reactions were incubated for 20 min at 50°C. Substrate and ligation product sizes are as indicated. The graphs represent the percent change of ligation as a function of Klenow. A, Reaction products resulting from either T4 ligase or Taq ligase are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement/inhibition of ligation as a function of Klenow.

As for *E. coli* ligase, the effects of high concentrations of Klenow and Klentaq on ligation of both T4 ligase and Taq ligase were also tested. Interestingly, both polymerases show inhibition effects on ligation of both DNA ligases when their concentrations are increased up to 300 or 500 nM (Figure 3.8, A, B, C, and D), even though they do not show significant effects on
ligation at lower concentration. These results suggest that Pol I DNA polymerase may inhibit the ligation of the three ligases through a similar mechanism, possibly just a simple situation of non-specific high protein concentration inhibition.

Figure 3.7: Klentaq does not stimulate the ligation by T4 ligase or Taq DNA ligase. Reactions of 10 µl contained 250 nM of hp57, 500 nM of hp27, 0.3 units of T4 DNA ligase or 4 units of Taq DNA ligase, and varying amounts of Klentaq. T4 ligase reactions were incubated at 25 °C for 6 min, Taq ligase reactions were incubated at 50°C for 20 min. Substrate and ligation product sizes are as indicated. The graphs represent the percent change of ligation as a function of Klentaq. A. Reaction products resulting from either T4 ligase or Taq ligase are shown on a 20% polyacrylamide gel containing 7.5 M urea. B. Percent enhancement/inhibition of ligation as a function of Klentaq.
Figure 3.8: High concentrations of Klenow and Klentaq inhibit the activity of Taq DNA ligase and T4 DNA ligase. Reactions of 10 µl contained 250 nM of hp57, 500 nM of hp27, 0.3 units of DNA ligase (Taq ligase in lanes 2-8 of A and B, and T4 ligase in lanes 2-8 of C and D) and varying amounts of protein (KLN=Klenow, KTQ=Klentaq). Lanes 2-8 in A, B, C, and D contained either Klenow or Klentaq (0, 5, 15, 50, 150, 300, 500 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for T4 DNA ligase or 50 °C for Taq DNA ligase for 6 min. Substrate and ligation product sizes are as indicated. A, Reaction products of Taq DNA ligase in the presence of Klentaq are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Reaction products of Taq DNA ligase in the presence of Klenow are shown on a 20% polyacrylamide gel containing 7.5 M urea. C, Reaction products of T4 DNA ligase in the presence of Klentaq are shown on a 20% polyacrylamide gel containing 7.5 M urea. D, Reaction products of T4 DNA ligase in the presence of Klenow are shown on a 20% polyacrylamide gel containing 7.5 M urea.
3.3.5 Neither Klenow nor Klentaq Significantly Enhance Ligation of a Nicked DNA

With two pieces of DNA as the substrate, Pol I DNA polymerase could recruit the two pieces of DNA together to form a temporary complex and accordingly give DNA ligase access to the ligation sites. Another possible explanation of Pol I-mediated stimulation, however, is that it helps *E. coli* DNA ligase access a nick more easily. Or both pathways could contribute to the stimulation effect of Pol I DNA polymerase on ligation by *E. coli* DNA ligase.

To examine the second possibility, we examined DNA with a single ligatable nick and double hairpin structures flanking the duplex region. If Pol I DNA polymerase stimulates ligation by helping ligase access the nick, we would expect to find that the increasing amounts of polymerase also would stimulate the ligation of the single nick by *E. coli* ligase. Figure 3.9 A shows a titration of Klenow into reactions containing T4 DNA ligase, *E. coli* DNA ligase, or Taq DNA ligase as indicated. As increasing amounts of Klenow (Figure 3.9 A, lanes 3-6) were added, the ligation efficiency of the three ligases was not significant changed. Figure 3.9 B shows a graph of the quantitation of the nick experiments overlapped with the double-oligo ligation data from Figure 3.1. The percent change in ligation due to increasing concentrations of Klenow lies in -20% to +20% range for the three ligases. In contrast, Klenow enhanced the ligation by *E. coli* DNA ligase of double pieces of DNA by 150% (diamond). Klentaq does not have an obvious effect on the ligation of a single nicked substrate either (Figure 3.10). The results are similar to the finding that Ku, one of the key NHEJ components, also does not enhance ligation at isolated single nicks (23). Combining these results with the stimulation of Pol I DNA polymerase on the connection of double pieces of DNA, it is likely that Pol I DNA polymerase stimulation involves recruitment of two pieces of DNA substrates.
Figure 3.9: Klenow does not significantly enhance ligation of substrate containing only a single nick. Reactions of 10 µl contained 150 nM of nicked DNA, 0.2 units of *E. coli* DNA ligase, or 0.1 units of T4 DNA ligase, or 0.8 units of Taq DNA ligase, and varying amounts of Klenow (KLN=Klenow). Lanes 2-6 in panel A contained Klenow (0, 5, 10, 15, 20, and 50 nmol) as indicated by the wedges. Either T4 ligase or *E. coli* DNA ligase reactions were incubated at 25 °C for 6 min, Taq ligase reactions were incubated at 50°C for 20min. Substrate and ligation products are designated as ‘nick’ and ‘closed’, respectively. Schematic representation of the substrates is depicted above the figure. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement of ligation by different ligases (*E. coli* DNA ligase = square, T4 DNA ligase = diamond, Taq DNA ligase = cross, and reverse triangle = *E. coli* DNA ligase on connection of double pieces of DNA), as a function of Klenow.
Figure 3.10: Klentaq does not significantly enhance ligation of substrate containing only a single nick. Reactions of 10 µl contained 150 nM of nicked DNA, 0.2 units of *E. coli* DNA ligase, or 0.1 units of T4 DNA ligase, or 0.8 units of Taq DNA ligase, and varying amounts of Klenow (KTQ=Klentaq). Lanes 2-6 in panel A contained Klentaq (0, 5, 10, 15, 20, and 50 nmol) as indicated by the wedges. Either T4 ligase or *E. coli* DNA ligase reactions were incubated at 25 °C for 6 min, Taq ligase reactions were incubated at 50°C for 20 min. Substrate and ligation products are designated as ‘nick’ and ‘closed’, respectively. Schematic representation of the substrates is depicted above the figure. A, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. B, Percent enhancement of ligation by different ligases (*E. coli* DNA ligase = square, T4 DNA ligase = circle, Taq DNA ligase = triangle, and reverse triangle = *E. coli* DNA ligase on connection of double pieces of DNA), as a function of Klentaq.
3.3.6 Klenow and Klentaq Enhance Ligation Rate over Time

To further analyze the stimulation effects of the polymerases, we determined the amount of ligation during repeated cycling of E. coli DNA ligase from substrate to substrate. To examine this, Klenow, E. coli DNA ligase, and two pieces of DNA were incubated at 25°C for 6 min, 10 min, and 20 min (Figure 3.11 A, B, and C). Continued enhancement of product formation is seen from increased incubation times. Figure 3.11 D shows a graph of the stimulation of Klenow at different reaction times. As the incubation time increases, the stimulation of Klenow on ligation of the double substrates increases, indicating that Klenow can repeatedly stimulate the ligation reaction. Figure 3.12 A and B show the ligation product formed by E. coli DNA ligase on double pieces of DNA in the presence of increasing amounts of Klentaq at different incubation times. Like Klenow, Klentaq shows stronger stimulation on ligation for longer time incubation (20 min) than that for shorter time incubation (10 min). The results here suggest that both polymerases could repeatedly align two pieces of DNA substrate for ligation by E. coli ligase. These results are consistent with the notion that stimulation is by improved bridging of the DNA substrates.

3.4 Discussion

Under appropriate conditions, both Klenow and Klentaq stimulate intermolecular DNA joining by E. coli DNA ligase. Previous work indicates that Pol I DNA polymerase can function as an alignment protein in vitro and perform continuous fill-in synthesis across two DNA oligonucleotides with microhomologies (9-10, 24). Prior studies also support a role of Pol I in the ligation step of DNA double-strand breaks (12). Exposure of the transforming linear DNA containing 3’ protuding single strands and blunt end to Taq or Klenow polymerase prior to transformation increased the transform efficiency into E. coli cells and reservation of 3’ PSS
Thus, in combination with these earlier studies, the Pol I polymerases are implicated as having significant roles in bacterial NHEJ and double-strand break repair via: 1) processing of DNA ends (25-26), 2) synthesis across and extension of aligned DNA substrates containing microhomologies (9-12, 22), DNA end protection (Chapter 2 and Yang & LiCata, 2011); and, 4) stimulation of DNA ligase activity (this Chapter).

Figure 3.11: Time of incubation effects on the Klenow mediated stimulation of *E. coli* DNA ligase. Reactions of 10 ul containing 250 nM of hp57, 500 nM of hp27, 0.3 unit of DNA ligase (lanes 2-5 in A and lanes 2-6 in B, and lanes 1-3 in C) and varying amounts of Klenow (KLN) were performed. Lanes 2-5 in A contained Klenow (0, 10, 15, and 50 nmol) and lanes 2-6 in B contained Klenow (0, 5, 10, 20, and 50 nmol), and lanes 1-3 in C contained Klenow (0, 20, and 50 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for different times. Substrate and ligation product sizes are as indicated. A, B, and C, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. D, Percent enhancement of ligation as a function of Klenow over time (6 min=circle, 10 min=square, and 20 min=triangle).
Figure 3.12: Time of incubation effects on the Klentaq mediated stimulation of *E. coli* DNA ligase. Reactions of 10 ul contained 250 nM of hp57, 500 nM of hp27, 0.3 unit of DNA ligase (lanes 1-5 in A and lanes 1-4 in B) and varying amounts of Klentaq (KTQ). Lanes 1-5 in A contained Klentaq (0, 5, 10, 15, and 50 nmol) and lanes 1-4 in B contained Klentaq (0, 10, 50, and 100 nmol) as indicated by the wedges. Reactions were incubated at 25 °C for different times. Substrate and ligation product sizes are as indicated. A and B, Reaction products are shown on a 20% polyacrylamide gel containing 7.5 M urea. C, Percent enhancement of ligation as a function of Klentaq over time (6 min=circle and 20 min=triangle).

Stimulation of ligation by Pol I can be attributed to the ability of Pol I to bridge two DNA molecules and stabilize an intermolecular association, because the effect of Pol I on ligation is only observed on the connection of double pieces of DNA and not on the nicked DNA substrate.
containing a single nick (Figure 3.1 and 3.9, 3.10). This stimulation is observed only with *E. coli* DNA ligase, not T4 DNA ligase or Taq DNA ligase (Figure 3.6 and 3.7), indicating that the ability to interact with the bridged intermediates is specific to *E. coli* DNA ligase among these three ligases. The structure of Pol I-bound DNA ends may be such that *E. coli* DNA ligase can fit well and perform ligation, but the other two ligases cannot.

*E. coli* DNA ligase consists of five domains; the N-terminal adenylation and OB fold domains, similar to T4 DNA ligase, including a Zn finger, HtH and BRCT domains (27). Sequence alignment results showed that *E. coli* DNA ligase and Taq DNA ligase are about 40% identical at the amino acid level, while there is only 2% identity between *E. coli* DNA ligase and T4 DNA ligase. Specificity for *E. coli* DNA ligase on double-oligo DNA connection suggests that the stimulation is due to specific interaction with Pol I, otherwise all three ligases should be similarly stimulated.

Additional, the smaller T4 DNA ligase containing two domains has lower affinity for DNA than the multidomain ligases (28). The weaker binding affinity of T4 ligase may also be one possible reason for that both Klenow and Klentaq do not stimulate its ligation under the conditions examined, and may also help explain why Taq DNA ligase shows weak stimulation effect on ligation compared to T4 DNA ligase.

The combination of the properties of Pol I described here and in previous research on DNA binding of Pol I suggest two potential pathways for Pol I to stimulate ligation (Figure 3.13). In the first potential pathway (Figure 3.13 A), at low concentration of protein, Pol I would align two pieces of DNA with microhomologies and stabilize the intermolecular interaction. Subsequently, a DNA ligase would come to the ligation site and Pol I could slide along DNA, allowing ligase access but continuing to stabilize the intermolecular association of the ends until
the nick is sealed. This is similar to the model for the stimulation of Ku on ligation of DNA ligase I (29), where Ku moves inward on DNA when DNA ligase I binds to a Ku-bound DNA, hence allowing ligation. Furthermore as Figure 3.3 and 3.4 showed, when the polymerase concentration is high compared to DNA substrate, nearly complete inhibition is achieved. This could be because Pol I binds almost all available DNA ends and prevents appropriate ligase access.

Figure 3.13 Hypothesized models for the role of Pol I in repair of DSB. A, the potential pathway for Pol I DNA polymerase to recruit two pieces of DNA together and provide the ligatable nick site for ligase. B, the potential pathway where Pol I DNA polymerase and DNA ligase bind the break DNA end separately and interact each other to improve ligation.

In the second potential pathway (Figure 3.13 B), at low concentration of Pol I, both Pol I and ligase have the opportunity to bind the DNA ends and then a potential interaction between Pol I and ligase could mediate the stimulation on ligation. Again, when the concentration of Pol I is high, the ligatable DNA ends are all bound by Pol I DNA polymerases and ligation is accordingly inhibited. The mechanisms for Pol I stimulation proposed here are different from the mechanism employed by PCNA, in which PCNA may stimulate ligation by improving binding
of DNA ligase I to the nicked site (30). However, our data indicate that both Klenow and Klentaq do not stimulate ligation on a nicked DNA substrate.

3.5 Concluding Summary

Stimulation of one protein by another is commonly observed in the reconstitution of DNA replication and repair in vitro. Our results suggest that Pol I, already known to participate in DNA replication and repair, also mediates biologically relevant stimulation of DNA ligase. Both Klenow and Klentaq stimulate the ligation of E. coli DNA ligase on double pieces of DNA, but not on a nicked DNA substrate. This effect does not occur with T4 DNA ligase or Taq DNA ligase. We suggest that the relative concentrations of reaction components when analyzed in vitro will influence the ability to stimulate. The component levels utilized in our reaction assays allowed us to detect the effects of Pol I. Our results suggest that Pol I could collaborate with DNA ligase during DNA repair in the cell.

3.6 References


CHAPTER 4

REPAIR OF DOUBLE-STRAND BREAKS BY POL I DNA POLYMERASE VIA ALIGNMENT-BASED STRAND-DISPLACEMENT DNA SYNTHESIS IN VITRO

4.1 Introduction

DNA double-strand breaks (DSBs) are one of the most dangerous forms of DNA damage and can cause genomic instability and cell death. In one typical pathway of DSB repair, the non-homologous end joining pathway (NHEJ), two pieces of DNA are recruited together by Ku analogues, followed by DNA end processing and nick sealing by a DNA ligase. However, the encoding genes of key components of the NHEJ pathway in prokaryotes, Ku and LigD, are absent from Escherichia coli and Thermus aquaticus. Thus, questions remain regarding whether there is another repair pathway besides homologous recombination (HR) involved in repair of DSB in such bacteria. By analyzing in parallel the repair of DSBs differing in end structures (blunt end, 5’ overhang, and 3’ overhang) by Klenow and Klentaq, with and without 5’ phosphate, in the absence and presence of DNA ligase, we demonstrate that both polymerases were able to do alignment-based strand-displacement DNA synthesis using 3’ melted single-stranded DNA from a second end as a template. Moreover, the activities of the two polymerases have different dependences on 5’ phosphate and DNA ligase. Both proteins are able to do alignment-based strand-displacement DNA synthesis on DSBs with complementary overhangs in both directions. In the absence of ligase, Klenow does not carry out DNA strand-displacement synthesis on DSBs with non-complementary 5’ overhangs or blunt ends. Addition of DNA ligase to the reaction mixtures restored DNA strand-displacement synthesis on DSBs. 5’ phosphate stimulates the repair of DSBs by Klenow. In contrast, repair activity of Klentaq on DSBs with non-cohesive ends depends on 5’ phosphatc, DNA ligase, and 3’ terminal nucleotide. Taken
together, our results indicate that pol I DNA polymerases may substitute for typical NHEJ components during DSB repair in *E. coli* and *T. aquaticus*.

DSBs (double-strand breaks) in DNA are a lethal type of damage for cell or genomic stability in both prokaryotes and eukaryotes, but evolution has developed several pathways to repair such deleterious lesions, thus maintaining the cell survival ability and genomic stability. DSBs are repaired by homologous recombination (HR) and non-homologous end-joining (NHEJ) (1-2). HR relies on the pairing of one of the broken strands with a complementary region with an intact DNA duplex to re-synthesize the broken ends. HR shows high fidelity during the DSB repair process (1). In the contrast, NHEJ does not require homology and can rejoin broken DNA ends with little or no base pairing at the junction of DSB and therefore is far more error-prone (1-2).

Approximately two decades ago, bacterial orthologues of Ku and LigD were suggested to cooperate during the repair process of DSBs (3-4). Experiments suggested that Ku and LigD are critical agents of the NHEJ pathway in prokaryotes, although it is possible that other factors participate in this DSB repair pathway (5-6). Generation and resolution of NHEJ intermediates frequently requires nucleolytic processing or DNA synthesis before the final ligation step. Multiple family X enzymes have been showed to participate in DSB repair, including yeast Pol IV (7), TdT (8) and Pol μ (9-10). Early studies suggested that the mechanisms of NHEJ are many, and that the outcomes depend on the initial structures of the DSBs and the available ensemble of end-processing and end-sealing components, which are not limited to Ku and LigD (11-12).

Both Ku and LigD gene homology are absent in *E. coli* (3, 13). Iterative PSI-BLAST database searches suggest that *Thermus aquaticus* also does not have homologies of Ku and
LigD genes (Chapter 3 in this dissertation). We want to ask if *E. coli* and *T. aquaticus* have other pathway to repair DSBs other than HR. We hypothesized a Pol I DNA polymerase could repair DSBs by aligning two ends of break together followed by strand-displacement DNA synthesis. The inspiration for this hypothesis came from the following sources. Earlier studies indicated that DNA polymerase might facilitate illegitimate recombination by stabilizing transient contacts by primer extension on discontinuous templates (14-16). Both Klenow and Klentaq have distinct double- and single-stranded DNA binding regions (17-19). There are important features that an alignment protein would likely have. The two proteins also show tight binding affinity to both primed-template DNA and different DNA end structures double-stranded blunt-end DNA (19). Klenow has a strong strand-displacement DNA synthesis activity (20).

To test the hypothesis, we examined alignment-based strand-displacement synthesis activity of Klenow and Klentaq on DSBs differing in end structures (complementary, non-complementary, blunt end) *in vitro*, and the effects of other factors, such as DNA ligase and 5’ phosphate, on the activity. We found that both Klenow and Klentaq could repair DSBs via alignment-based strand-displacement DNA synthesis and they had different dependence on DNA ligase and 5’ phosphate. Our results indicate Pol I DNA polymerase maybe a backup NHEJ pathway for DSBs to enhance the survival ability although there are no homology of the Ku and LigD genes in *E. coli* and *T. aquaticus*.

4.2 Material and Methods

4.2.1 Materials

All oligonucleotides used in this study were obtained from Integrated DNA Technologies (Coralville, IA). The DNA substrate sequences are shown in Table 4.1. Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and using extinction
coefficients provided by manufacturer. The DNA molecules constructed for this study have stable terminal hairpin structure flanking the duplex region to circumvent the difficulty in analyzing the various products produced by multiple end usages. Hairpin structures are closed using a stable tetraloop (21). Hairpin DNAs were annealed from single-strand DNA by heating at 95°C for 5 minutes and slowly cool down to ambient temperature. 5’ phosphate was added to

Table 4.1: Sequences of DNAs Used in this Study.

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<td>ss27un</td>
<td>5’-GAGAGGCTGGAATGACCGCTGGGCAAG-3’</td>
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substrates by using T4 polynucleotide kinase and unlabeled ATP, following the instructions provided by the company. Three different pairs of DSBs differing in end structures (complementary 3’ overhang, non-complementary 5’ overhang, and blunt end) were used to characterize the repair activity of both Klenow and Klentaq polymerases.

Klenow (KLN) and Klentaq (KTQ) polymerases were purified in our laboratory (refer to Chapter 2). The Klenow clone used in this study contains the D424A mutation (Klenow exo-)
and was provided by Catherine Joyce from Yale University. This mutant has only residual 3’-5’ exonuclease activity, but retains DNA binding affinity for the proofreading site (22). Protein concentrations were measured at 280 nm and calculated by using ε280 values of $5.88 \times 10^4$ M$^{-1}$ cm$^{-1}$ for Klenow and $7.04 \times 10^4$ M$^{-1}$ cm$^{-1}$ for Klentaq. T4 DNA ligase was purchased from the company Invitrogen.

4.2.2 Methods

Reactions were performed in 15 µl solutions with 300 nM of a short DNA, 150 nM of a long DNA, 0.5 µM of either Klenow or Klentaq polymerase, with or without the presence of T4 DNA ligase and 80 µM of dNTP. Reactions were carried out at ambient temperature (25 °C) for 20 min and were terminated by adding 5 µl of a stop buffer containing 0.2% SDS, 10 mM EDTA, 90% formamide, and 0.1% bromphenol blue, and heating at 95 °C for 20 min, and then immediately put on ice for 5 min. A 8 µl of sample was loaded onto a 20% denaturing polyacrylamide gel containing 7 M urea and electrophoresed in TAE (80 mM Tris acetate, 2 mM EDTA, pH 8.0). Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a Bio-Rad gel imager.

4.3 Results

4.3.1 Repair of DSBs with Complementary 3’ Overhang

To determine if both Klenow and Klentaq could align two complementary ends of a DSB and then do strand-displacement DNA synthesis, we set up reactions using two pieces of discontinuous DNAs (hp27 and hp57) with 5-base complementary 3’ overhangs and terminal hydroxyl groups as substrates as indicated in Figure 4.1 A. Below this are the possible products produced by polymerase on the DSB substrates. An 84-mer product is expected to form when direct ligation occurs (Figure 4.1 A, (I)). Two of 106- and 136-mer products are expected to form
when a polymerase aligns two broken ends together and catalyzes alignment-based strand-
displacement DNA synthesis in both directions with nicks sealed (79-mer products are expected
from only displacement synthesis) (Figure 4.1 A, (II) and (III)). The effect of 5’ phosphate on
DSB repair was also examined. The reactions consist of substrate DNA, either Klenow or
Klentaq, and dNTP. T4 DNA ligase was added to some cases. Reaction products were analyzed
on denaturing polyacrylamide gels.

Figure 4.1 B and C shows the repair activity of Klenow on DSB with 5’ hydroxyl and 5’
phosphate, respectively. We found that dNTP was necessary for the formation of a 79-mer
product (Figure 4.1 B, comparing lanes 3 and 5), indicating that the product was not from
ligation. The analysis of the product size revealed that the product came from alignment-based
strand-displacement DNA synthesis, where the two pieces of DNA substrates were recruited
together and Klenow did polymerization by unwinding the second piece of DNA as a template.
The addition of T4 DNA ligase resulted in extra products with larger sizes formed in low
intensity (Figure 4.1 B, comparing lanes 3 and 4), indicating that T4 DNA ligase might
contribute some to the repair reaction even though in isolation it could not join the two DNA
ends (Figure 4.1 B, lane 5). This was consistent with previous studies that T7 ligase was unable
to seal nicked DNA containing 5’ hydroxyl but was able to bind it (23).

To evaluate the contribution of the 5’ phosphate group, each DNA substrate was
phosphorylated with unlabeled ATP, we tested the repair activity of Klenow on the same DSBs
except with addition of 5’ phosphate in the absence and presence of T4 DNA ligase, respectively
(Figure 4.1 C). Three of 79-mer, 106-mer, and 136-mer products were obtained in the presence
and absence of T4 ligase. These products correspond to the predicted sizes formed by strand-
displacement DNA synthesis both with and without nick sealing. Due to the addition of 5’
Figure 4.1: Repair of DSBs with complementary 3'-overhangs by Klenow. A, Schematic representation of the DNA termini used in DSBs repair reactions and possible products. Substrate hp27 consists of 9 bp duplex part, a 4-nt hairpin and 5-nt 3'-overhang (top left) and substrate hp57 consists of 24 bp duplex part, a 4-nt hairpin and 5-nt 3'-overhang (top right). The products can come from direct joining of two pieces of DNA substrates (I) or alignment-based strand-displacement DNA synthesis (II and III) B, Repair of DSBs with 5'-OH. Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Both hp27 (300 nM) and hp57 (150 nM) were incubated with Klenow (0.5 µM). T4 DNA ligase and dNTP (80 µM) were added to some samples, as indicated (+). C, Repair of DSBs with 5'-phosphate. Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Both hp27 (300 nM) and hp57 (150 nM) were incubated with Klenow (1µM), dNTP (80 µM). T4 DNA ligase was added to one case, as indicated (+). All of reactions were incubated at 30 °C for 20 min.
phosphate to the substrate, a DNA-adenylate intermediate may be able to form, so that ligation product is also produced (Figure 4.1 C, lane 4, 80 nt/circle). 5’ phosphate and T4 DNA ligase enhance the repair activity of Klenow on this kind of DSB, but they are not required (compare lanes 3 of Figure 4.1 B and 4.1 C, lanes 4 of Figure 4.1 B and 4.1 C). The effect of 5’ phosphate on DSB repair is compatible to the requirement of polynucleotide kinase (PNK) for double-strand break repair (24). The formation of 106-mer and 136-mer products in the absence of T4 DNA ligase was surprising, because it has been proven that pol I DNA polymerase is unable to close the nick between the 3’ hydroxyl and 5’ phosphate group.

Figure 4.2: Repair of DSB with complementary 3’-overhangs by Klentaq. Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Both hp27 (300 nM) and hp57 (150 nM), with 5’ OH or 5’ phosphate as indicated, were incubated with Klentaq (0.5 µM). T4 DNA ligase and dNTP (80 µM) were added to some samples, as indicated (+). All of reactions were incubated at 30 °C for 20 min.
To examine how Klentaq repairs the same DSB with complementary 3’ overhangs, we did the same experiment by using Klentaq as an enzyme. Similar results were obtained (Figure 4.2). Klentaq repaired the DSB via strand-displacement DNA synthesis. Both 5’ phosphate and T4 DNA ligase were again shown to improve the DSB repair. The larger sized products resulting from simultaneous strand-displacement DNA synthesis and apparent nick sealing were also obtained in the absence of T4 DNA ligase when DSB contains 5’ phosphate.

4.3.2 Palindrome Amplification by Klenow and Klentaq

The formation of 106- and 136-mer products by both polymerases on the DSB with complementary 3’ overhang (hp27 and hp57) in the absence of T4 DNA ligase was unexpected, since Pol I DNA polymerase doesn’t have ligation function. A short inverted repeat, in proximity to a DSB, mediates the formation of a large DNA palindrome in mammalian cells (25). The products from strand-displacement DNA synthesis by Klenow and Klentaq on the DSB substrates we used contain short inverted repeat sequences at their ends, therefore palindrome amplification may occur by using those products as substrates. We chose the product 106/nick as a substrate to test whether both proteins could catalyze palindrome amplification. A nicked DNA substrate 106/nick with a single nick was prepared by annealing a 27-mer primer strand to the 3’ overhang of a 79-mer 3’ tailed hairpin duplex containing 5’ phosphate as depicted in Figure 4.3 A. Based on possible mechanisms for the formation of large DNA palindromes proposed by Steele, et al. (26), we suggest that there are two possible pathways for the formation of a 136-mer product by both proteins with the 106/nick substrate. One possible pathway is intramolecular recombination by which a double-hairpin DNA d-hp79 can form by folding back the 3’ overhang through base pairing after the ss-27 mer is removed (Figure 4.3 A). In the presence of dNTP, strand-displacement DNA synthesis by a polymerase on a substrate d-hp79 can form a 136-mer
Figure 4.3: Possible pathways of larger sized product formation by palindrome amplification. Polymerase aligns two pieces of DNAs, hp27 and hp57, and does strand-displacement DNA synthesis resulting in the production of 106/nick (nick DNA) formed. There is short reverted repeat sequence at the end of this nick DNA. This product is thus continuously used as a substrate for palindrome gene amplification. There are two possible pathways for palindrome gene amplification. A, intramolecular recombination pathway. The single strand 27-mer DNA is removed to yield the 3’ single-stranded overhang containing the short reverted repeat sequence (indicated as red color) (step 2). A hairpin is formed by intranstrand annealing within the short reverted repeat sequence (step 3). A new product is formed by fill-in and strand displacement DNA synthesis (step 4). B, Intermolecular recombination pathway. The single strand 27-mer DNA is removed to yield the 3’ single-stranded overhang containing the short reverted repeat sequence (step 2). Two molecules with long 3’ overhangs were aligned together by annealing of the single strands having short reverted repeat sequence (indicated as red color) (step 3). A new product is formed by strand fill-in and strand displacement DNA synthesis in both directions (step 4).
product. A second pathway is that the terminal short inverted repeat sequences can mediate a homologous recombination reaction that cause two copies of hp79 to anneal together, head to head, and following strand-displacement DNA synthesis form a 136 bp product (Figure 4.3 B).

To determine whether the short inverted repeat sequence at end of the DSB mediates palindrome amplification, we incubated Klenow with dNTP or dATP or neither. The reaction with 106/nick and T4 DNA ligase was used as a control. To confirm the palindrome amplification can be catalyzed by polymerase, a double hairpin DNA d-hp79 was also used as a control. The results from denaturing gel demonstrated that a 136-mer product was formed when either Klenow or Klentaq was incubated with 106/nick in the presence of dNTP (Figure 4.4 A lanes 3 and 6) compared to a 106-mer product formed due to ligation, whereas no products was formed when dATP substituted for dNTP or no dNTP was supplied (Figure 4.4 A lanes 2, 4, and 7), which is consistent with our model. In support of this point, the same size of product (136-mer) was formed when using d-hp79 substituting for 106/nick for substrate (Figure 4.4 A lanes 5 and 8).

As a control, no expected palindrome amplification product was formed when a ss27/79 without short inverted repeat was used as a substrate (Figure 4.5, lane 6).

Analysis of reaction products of Klenow on a native polyacrylamide gel (Figure 4.4 B), we only see about 70 bp products (Figure 4.4 B, lanes 3 and 5). This result is expected from formation of an intramolecular snap-back molecule followed by strand-displacement DNA synthesis, and is consistent with previous findings that formation of hairpin structures at inverted repeats is favored (27).
Figure 4.4: Molecular analysis of palindrome amplification mediated by Klenow and Klentaq. Reactions used 106/nick or d-hp79 (150 nM), Klenow (KLN) or Klentaq (KTQ) (0.5 μM), and dATP or dNTP (80 μM). The reaction with 106/nick and T4 DNA ligase was used as a control. All of reactions were incubated at 30 °C for 20 min. A, Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. B, Reaction products are shown on a 10% native polyacrylamide gel.
Figure 4.5: Negative control for palindrome amplification by Klenow. Reactions used 106/nick, ss27/79 or d-hp79 (150 nM), 0.5 µM of Klenow (KLN), and dNTP (80 µM). ss27/79 is an analogue of 106/nick, but does not contain short inverted repeats.

### 4.3.3 Repair of DSBs with Non-complementary 5’ Overhang

The experiments described above were carried out on DSB substrates with complementary 3’ overhangs. King, et al. and Clark, et al. demonstrated that a pol I DNA polymerase might function as the putative alignment protein for non-complementary end joining (14-15). Here we also determine if these two proteins could repair DSBs with non-complementary 5’overhangs via a similar pathway. The substrate structures are shown in Figure 4.6 A. Below the substrates are the possible products produced by polymerase on the DSB substrates. Figure 4.6 A, (I) shows the predicted product from direct ligation. Figure 4.6 A, (II) and (III) show the expected products formed via strand-displacement DNA synthesis only or
strand-displacement DNA synthesis by using 3’ end from either hp29-5 or hp39 as a primer plus nick ligation. We found that DSBs with non-complementary overhangs, in contrast to DSBs with complementary overhangs, were only repaired in the presence of T4 DNA ligase by Klenow (Figure 4.6 B, lanes 5, and 7). In addition, Klentaq can only repair the DSBs containing 5’ phosphate in the presence of DNA ligase (Figure 4.6 B, lane 9).

The repair activities of both proteins on this DSB were promoted by 5’-phosphate. Moreover, the fill-in synthesis products were always formed in the presence of dNTP (Figure 4.6 B, lanes 4-9), indicating that DSBs with non-complementary 5’ overhangs might require fill-in synthesis to make the blunt ends, which then are recruited together for further strand-displacement synthesis to complete repair. In the absence of DNA ligase, both proteins failed to repair the DSB having either a 5’ phosphate or hydroxyl group (Figure 4.6 B, lanes 4, 6, and 8). The requirement of DNA ligase for both proteins implies that DNA ligase play an important role during the bridge steps for this DSB prior to strand-displacement DNA synthesis.

4.3.4 Repair of DSBs with Blunt Ends

If the recessed 3’-OH of the DSBs had been extended to form blunt ends prior to alignment-based strand-displacement synthesis, then similar repair activities of both proteins on DSBs with blunt ends should be observed in the same conditions. Therefore, the repair of a DSB with blunt ends by Klenow or Klentaq was tested. The DNA constructs used and possible products were shown in Figure 4.7 A. The fact that Klentaq was unable to repair this DSB (data not shown) suggests that repair ability of this protein on a blunt-ended DSB is dependent on nucleotide at the 3’ terminal position of the DSB. Because the 3’ terminal bases of the blunt-ended DSB are G and C, respectively. In contrast, the 3’ terminal bases of hp29-5 and hp 39 after
Figure 4.6: Repair of DSBs with 7 nt non-complementary 5'-overhangs. A, Schematic representation of the DNA termini used in DSBs repair reactions and possible products. Substrate Hp29-5 consists of a 9 bp duplex part, a 4-nt hairpin and a 7-nt 5'-overhang (top left) and substrate Hp39 consists of a 14 bp duplex part, a 4-nt hairpin and a 7-nt 5'-overhang (top right). The products can come from direct joining of two pieces of DNA substrates (I) or alignment-based DNA Strand-displacement Synthesis (II and III) B, Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Both hp29-5 (300 nM) and hp39 (150 nM), with protruding 5’ OH or 5’ phosphate, were incubated with Klenow (KLN) or Klentaq (KTQ) (0.5 µM). For some samples, T4 DNA ligase and/or dNTP (80 µM) were added. All reactions were incubated at 30 °C for 20 min.
fill-in synthesis was T and G. The difference in ability of Klentaq to repair DSB with blunt end and 3’ recessed end could be attributed to its preference in bridging the gap with template 3’ terminal base T (15). On the other hand, Klenow can repair this DSB in the presence of DNA ligase (Figure 4.7 B). In contrast, it has been shown that at least two complementary bases are required by Klenow for elongating the 3’ terminus of double-stranded DNA using the bottom single-stranded oligonucleotide as a template (28). Our results, however, do not contradict these data, because experiments reported here were performed in the presence of DNA ligase, which will contribute much to the repair of DSB. 5’ phosphate improved the DSB repair by producing two additional larger sized products (Figure 4.7 B, lanes 2 and 4).

4.3.5 The Specific Pathway for Formation of Larger Repair Products with DSBs Containing Non-complementary 5’ Overhangs

Although the products from DSB repair reactions with hp29-5/hp39 substrates show expected size upon alignment-based strand-displacement DNA synthesis and nick ligation, the assay can not rule out the possibility that the products come from palindrome amplification by using the strand-displacement synthesized product as substrates. To determine whether the detected products resulted from strand-displacement DNA synthesis plus either ligation or palindrome amplification, experiments using DSB substrates with specific 5’ end modification by adding a phosphate group were performed. A selectively phosphorylated 5’ end of the substrates allowed us to predict the product formation pattern after DSB repair reaction (Figure 4.8 A). For the substrate hp29-5 containing 5’ phosphate, when alignment-based strand-displacement DNA synthesis occurred using the 3’ end of hp39 as a primer, a 82-mer product would form if only strand-displacement DNA synthesis occurs or a 128-mer product formed if both strand displacement DNA synthesis and palindrome amplification occur; and when
Figure 4.7: Repair of DSBs with blunt ends. A, Schematic representation of the DNA termini used in DSBs repair reactions and possible products. Substrate hp28 consists of a 12 bp duplex part and a 4-nt hairpin (left) and substrate hp46 consists of a 21 bp duplex part and a 4-nt hairpin (right). B, Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Both hp28 (300 nM) and hp46 (150 nM), with 5’ OH or 5’ phosphate as indicated, were incubated with Klenow (0.5 µM), dNTP (80 µM). For some cases, T4 DNA ligase was added to reaction. All reactions were incubated at 30 °C for 20 min.
alignment-based strand-displacement DNA synthesis occur using the 3’ end of hp29-5 as a primer, a 128-mer product will form if strand-displacement DNA synthesis plus ligation between 5’ phosphate of hp29-5 and the 3’ hydroxyl of hp39 after fill-in synthesis occurs or a 118-mer product will be formed if both strand-displacement DNA synthesis and palindrome amplification occur without ligation. For only the substrate hp39 containing 5’ phosphate, when alignment-based strand-displacement DNA synthesis occurred using the 3’ end of hp39 as a primer, a 118-mer product will form if strand-displacement DNA synthesis plus ligation occurs or a 128-mer product will form if both strand-displacement DNA synthesis and palindrome amplification occur without ligation; and when alignment-based strand-displacement DNA synthesis occurred using the 3’ end of hp29-5 as a primer, a 82-mer product will form if only strand-displacement DNA synthesis occurs or a 118-mer product will form if strand-displacement DNA synthesis plus palindrome amplification occurs.

As shown in Figure 4.8 B, a 118-mer product was formed by Klenow when only hp29-5 contained 5’ phosphate, and a 128-mer product was produced only when hp39 had 5’ phosphate. The sizes of these products are as predicted for alignment-based strand displacement DNA synthesis by using the 3’ end of the substrate having 5’ phosphate as a primer followed by palindrome amplification without nick ligation. Moreover, both 118-mer and 128-mer products were formed when both substrates contained 5’ phosphate. Similar experiments were done by using Klentaq substituting for Klenow, and analogous results were obtained. Together, the data shown in Figure 4.8 and 4.6 provide clear evidence that the large sized products came from palindrome amplification by using alignment-based strand-displacement DNA synthesized product as a substrate. 5’ phosphate may induce a polymerase to favor using the 3’ primer from a non-phosphorylated blunt end was a template for strand-displacement synthesis and a DNA
Figure 4.8: Substrate structures and resultant formed products with DNA substrates having different 5’ end configurations. A, The substrates with specific 5’ end configurations used in the experiments and expected products. B, Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Reaction mixtures containing polymerases (0.5 µM), both hp29-5 (250 nM) and hp39m (150 nM) with indicated configuration at the 5’ end, T4 DNA Ligase, and dNTP (80 µM) were incubated at 30 °C for 20 min.
ligase may help fold back the 3’ overhang containing short inverted repeat sequences to form a hairpin (29).

4.4 Discussion

DSBs induced by ionizing radiation usually do not have compatible ends that can be repaired by direct ligation, hence various end processing, such as exonuclease degradation, and/or fill-in DNA synthesis must be performed prior to joining ends (30). Clark, et al. demonstrated that a DNA polymerase could function as the putative alignment protein in non-complementary end joining (14). Recent studies further indicated that Klenow might mediate blunt end joining with a single-stranded DNA or a DNA end with a 3’ protruding single strand (15, 28, 31). The goal of this study was to further investigate how Klenow and Klentaq repair DSBs differing in their end structures and how other factors, such as a DNA ligase or 5’-phosphate, facilitate the repair event. The major findings reported here are that both proteins can repair DSBs via alignment-based strand-displacement DNA synthesis but have different dependence on DNA ligase and 5’ phosphate, and that short inverted repeats may help Pol I-mediated DSB repair.

4.4.1 Different Repair Ability of Klenow and Klentaq on DSBs with Different End Structures

Our data with the three pairs of DSBs hp27/hp57, hp29-5/hp39 and hp28/hp46, which differ in end structures, give rise to the following conclusions about DSB repair by Klenow and Klentaq: (i) both proteins are able to repair DSBs with complementary overhangs; (ii) the 3’ recessed terminus of a DSB is converted to a blunt end by fill-in DNA synthesis prior to repair; (iii) the repair ability of Klenow on a blunt-end DSB is less dependent on the end 3’ terminal base than that of Klentaq.
Our findings that both proteins repair compatible DNA ends with 3’ overhangs and 5’ terminal hydroxyl groups fit well with recent findings. First, ionizing radiation can produce 5’ hydroxyl termini when it breaks the DNA strand (32-33). Second, Klenow can bridge two piece DNAs with complementary overhangs together (28). Third, Klenow has a strong catalyzing ability for strand-displacement DNA synthesis (20, 34-35). All of these findings can be understood in light of the findings here that both Klenow and Klentaq polymerases can repair a DSB with complementary 3’ overhangs and 5’ hydroxyl via the alignment-based strand displacement DNA synthesis in the absence of other proteins.

Moreover, our results also show that both Klenow and Klentaq have reduced ability or inability to repair a DSB with non-cohesive hydroxyl-terminated ends. Since recessed 3’ hydroxyl ends will be converted to blunt ends by fill-in DNA synthesis prior to the alignment-based DSB repair, it was thought that the repair of this type of DSB should to same to the pair of hp28/hp46 with blunt ends. Interestingly, the two types of blunt DNA ends do not give identical repair results for these two proteins. Klenow can repair both types of DSB with 5’ hydroxyl in the presence of a DNA ligase whereas Klentaq can only repair the DSB of hp29-5/hp39 under the same condition (Figure 4.6 lane 5 and Figure 4.7 lane 2). We argue that the difference in repair ability of both proteins maybe due to their different preferences in template-independent nucleotide addition. It has been shown that Klentaq catalyzes template-independent nucleotide addition to the 3’-terminus of the blunt-end DNA with much stronger preference for incorporation of dA, while Klenow only shows moderate preference for incorporation of dA compared to other dNTP (15, 36). The pair hp29-5/hp39 contains 3’ terminal T and G, respectively, after being converted to blunt end, while the pair of hp28/hp46 contain 3’ terminal C and G, respectively. Therefore, if pol I DNA polymerases repair the blunt-end DSB via
strand-displacement DNA synthesis initiated by pairing the 3’ terminal nucleotide of one end to the template-independent extended nucleotide of the second end, Klenow’s relatively strong ability to effectively repair the blunt-end DSBs is reasonable. The ability of Klenow to repair the DSB with 3’ terminal C and G, differs from previous findings (15), but can be attributed to the presence of 5’-phosphate and T4 DNA ligase. The fact that Klenow and Klentaq have different activities on various DSBs is similar to the findings with family X polymerases, pol μ and pol λ, both of which are involved in DNA break-end joining reactions but they have different dependence on the sequence of the ends (37-38).

4.4.2 Short Inverted Repeat Sequences and DSB Repair

Somewhat unexpectedly, in several experiments where no DNA ligase is present, we recover the large products that seem to come from strand-displacement DNA synthesis combined with ligation. Experiments lacking 5’ phosphate or specially modified at one of the two ends by adding 5’ phosphate, demonstrated that those products were not from direct ligation and that they came from palindrome amplification using the alignment-based strand displacement DNA synthesis products as substrates (Figure 4.1 B, lane 4 and Figure 4.1 C lane 3; Figure 4.2, lanes 3 and 5; Figure 4.6 B, lane 5; and Figure 4.8). During palindrome amplification, the 3’ overhang of the molecule folds back and base pairs with a short inverted repeat, and the subsequent 3’ primed DNA synthesis leads to the formation of the large sized product. By our studies, we directly show that a short inverted repeat, next to a DSB, can mediate the formation of a large DNA palindrome, leading to genome rearrangement or DSB repair. We envision there is a mechanism in prokaryotes similar to that described in protists and mammalian cells: the repair of DSBs involving an intramolecular self-priming event at a short inverted repeat sequence near the DSB to generate a hairpin structure leading to a large palindrome formation (25, 39). One question
remains unaddressed in our model: what is a mechanism by which the single strand DNA is removed from a nicked DNA to generate the 3’ overhang with a short inverted repeat sequence? Our studies have shown that a pol I DNA polymerase alone mediated palindrome amplification, suggesting the polymerase also mediates this non-DNA synthesis-related strand-displacement. Large DNA palindromes have been observed in prokaryotes (40). It is conceivable that some short repeat facilitates DSB repair by pol I polymerase. Therefore, the existence of large amounts of short inverted repeat (IR) sequences in prokaryotes may have their origins in the evolution of an IR based DSB repair mechanism.

4.4.3 Effect of 5’ Phosphate on DSB Repair

The results of 5’ phosphate’s effect on the repair of DSBs with cohesive 3’ overhangs indicate that it can stimulate the intramolecular annealing within a short inverted repeat sequence, followed by strand-displacement DNA synthesis leading to the formation of 106- and 136-mer products in the absence of T4 DNA ligase (Figure 4.1 C, lane 3 and Figure 4.2 lane 5). However, the origin of 5’ phosphate’s contribution to intramolecular annealing remains to be resolved.

Klentaq was able to repair the DSB of hp29-5/hp39 with 5’ phosphate but not for the same DSB with 5’hydroxyl in the presence of T4 DNA ligase. This may be because 5’ phosphate of the blunt end contributes to its interaction with Klentaq or ligase. 5’ phosphate is required to form a DNA–adenylate intermediate, therefore is critical for the binding of viral ligases to nicked DNA (41). The ternary complex crystal structure of Klentaq shows that the 5’ phosphate group of the template nucleotide could pair with the incoming dNTP and interact with Ser674 (42).

Previous studies show that Klenow is able to synthesize DNA across a double strand breaks using a 3’ protruding single strand of a duplex DNA or a single strand DNA as a template.
due to the pairing of its terminal T with the non-template addition of dA to blunt end (15, 28). In our study, the pair of hp29-5/hp39 contains 3’ terminal T and G, respectively, after being converted to blunt end by fill-in DNA synthesis. The repair results show that both Klenow and Klentaq are able to synthesize DNA across a double strand break using either 3’ terminal T of the blunt end or 3’ template-independent extended A of the other blunt end as a template in the presence of 5’ phosphate and T4 DNA ligase. Further studies of the same DSB with a selectively phosphorylated 5’ end demonstrate that the addition of 5’ phosphate induces the enzymes to use the 3’ end of the DNA without 5’ phosphate as a template for DNA synthesis across the break (Figure 4.8). The addition of a 5’ phosphate contributed substantially to the binding affinity of DNA duplex with single-nucleotide and two-nucleotide template extensions by Klenow (43). Thus, with regard to whether the 3’ end with a non-template addition of a dA is used as a primer or a template for strand displacement DNA synthesis, we note these two mechanisms are not mutually exclusive, and both can make contributions to DSB repair. Moreover, 5’ phosphate may determine which 3’ end is to be used as a primer by changing relative binding affinity of the DNA substrates.

**4.4.4 Function of DNA Ligase during DSB Repair**

Our work establishes *in vitro* conditions that specially require pol I polymerase and DNA ligase to cooperate in repair of DSBs with non-cohesive ends (Figure 4.6 and 4.7). Binding affinities of different DNA to T4 DNA ligase are weak compared to Klenow or Klentaq, with estimated $K_d$ values in the micromolar range (29, 44). DNA ligase has a strong preference for binding to nicked substrate containing 5’-phosphate (23, 45). By combining our work and other findings, we raise the possibility of cooperation of these two different enzyme molecules, such as polymerase to hold the termini in juxtaposition, and ligase to stabilize the complex, which is
critical for the subsequent strand-displacement synthesis. This is consistent with previous studies that suggest that a DNA polymerase functions as the putative alignment protein during DSB repair (15, 28, 46).

Moreover, it is shown that the presence of T4 DNA ligase causes the formation of large sized products due to palindrome amplification followed the alignment-based strand-displacement DNA synthesis. A more likely explanation of the requirement of DNA ligase for large product formation is that DNA ligase might contribute to hairpin formation via folding back 3’ overhang upon base pairing within the short inverted repeats. This notion is supported by previous studies that showed that T4 DNA ligase at high concentration could link the unpaired 3’ hydroxyl group to the recessed 5’ phosphate group within a single molecule to form a stem-loop structure (29).

4.5 Concluding Summary

Double-strand breaks are severe DNA damage and can cause genome instability and cell death. In both eukaryotes and prokaryotes, DSB repair utilize mainly the HR and NHEJ repair machinery. Our results show that both Klenow and Klentaq polymerases can mediate DSB repair by a mechanism involving polymerase-dependent alignment of the broken end with fill-in and strand-displacement DNA synthesis and that the two polymerases have different dependence on DNA ligase and 5’ phosphate according to what bind of end structure is contained within the breaks. The ability of pol I DNA polymerase alone or cooperating with a DNA ligase to repair DSB in vitro reveals a potential alternative pathway for DSB repair in some prokaryotes which are missing the NHEJ key component genes: Ku and LigD. Palindrome amplification induced by the short inverted repeats at the DSB lead to the suggestion that the existence of large amounts short inverted repeat sequences in prokaryotes might provide a basis for DSB repair. These
findings provide potential additional explanation for the survival ability of *E. coli* and *T. aquaticus* cells after DSB damage.

### 4.6 References


APPENDIX 1

THE POTENTIAL INVOLVEMENT OF DNA MISMATCH IN DOUBLE-STRAND BREAK REPAIR BY THE POL I DNA POLYMERASES FROM E. COLI AND T. AQUATICUS

A1.1 Abstract

DNA is constantly being damaged by endogenous sources, such as reactive oxygen species, and exogenous agents, such as ionizing radiation. Different types of DNA damage are thought to be repaired through different repair pathways with distinct enzymatic machinery. However, recent studies have suggested that there is mechanistic overlap between mismatch repair and double-strand break (DSB) repair. In this study we examine the in vitro repair of DSBs with non-cohesive ends and a 3-base mismatch by Klenow and Klentaq polymerases. The presence of mismatch inhibits the repair of DSBs with non-complimentary 5’ overhangs or blunt ends by Klenow in the presence of T4 DNA ligase. In contrast, the repair capacity of Klentaq on the DSBs with blunt ends is improved by the presence of mismatch. 5’ phosphate is essential for the repair of the DSBs containing mismatches. The different effects of mismatch on the repair of DSBs by Klenow and Klentaq suggest that these polymerases may have different recognition mechanisms for DSB substrates. These data indicate that mismatch might enhance the repair of double-strand breaks under special conditions.

A1.2 Introduction

Ionizing radiation can generate different types of DNA damages including double-strand breaks, base damages, and mismatch which usually come from aberrant DNA replication (1). Different types of damage may be induced by different radiation energies and some damages induced can be clustered at the sites of energy disposition. Clustered damages will form when
multiple ionizations occur at the site of interaction with DNA (1-2). The combination of clustered damages is complex and can contain both DNA breaks and base damage (3).

DNA double-strand breaks (DSBs), such as those caused by ionizing radiation, are critical damages threatening the genomic stability (4). The work of Chapter 4 in this dissertation demonstrates that Klenow and Klentaq may repair DSBs via alignment-based strand-displacement synthesis. Mismatches are generated by wrong base incorporation or base insertion/deletion during DNA replication in organisms from bacteria to mammals. The DNA mismatch repair (MMR) pathway is responsible for correcting base substitution or small single-strand DNA loops due to base insertion/deletion by using special enzymes including MutS-/L-/H proteins in *Escherichia coli* (5-6). Much effort has been directed to understanding the repair of DSBs and MMR. Recently it was found that DSB repair pathways and MMR pathway are not separated, MMR interacting directly with DSB repair (7-9). The overlap of the DSB repair and MMR machinery is beneficial for cell surviving through heavy damages. However, it is poorly understood how a mismatch in proximity of a DNA break influences the repair of the DSB.

In this study, we show that mismatch at the junction of DSBs have different effects on the repair by Klenow and Klentaq. The repair of DSBs with non-cohesive ends by Klentaq was improved by having mismatch at one end of the break. In contrast, the repair of the same breaks by Klenow was not improved. Thus mismatch could potentially be involved in the sequential repair of adjacent damages consisting of breaks and mismatch in *Thermus aquaticus*.

**A1.3 Materials and Methods**

**A1.3.1 Materials**

All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and...
using extinction coefficients provided by the manufacturer. Hairpin DNAs were annealed from single-strand DNA by heating at 95°C for 5 minutes and slowly cool down to room temperature. 5’ phosphate was added to substrates by using T4 polynucleotide kinase and unlabeled ATP, following the instructions provided by the company. The DNA constructs used for experiments are shown in Table A1.1. Both hp29-5 and hp39 are hairpin-DNAs with 5’ overhangs, and hp39m3 is a hairpin DNA with a 3-base mismatch at the primer terminus. Both hp28 and hp46 are blunt-end hairpins, and hp46m3 contains a 3-base mismatch at 3’ primer terminus. Klenow Fragment (KF) and Klentaq (KTQ) polymerases were purified in our laboratory (refer to Chapter 2). T4 DNA ligase was purchased from New England Biolabs (Ipswich, MA).

Table A1.1: Sequences of DNAs Used in this Study.

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A1.3.2 Methods

A1.3.2.1 DNA Polymerase Extension Assays

Reaction mixtures (15 µl) contained 0.3 µM of DNA substrate, 0.5 µM Klenow or Klentaq and 50 µM dNTP in reaction buffer: 10 mM Tris-HCl, 150 mM KCl for Klenow or 50 mM KCl for Klentaq, and 5 mM MgCl2 (pH 7.9). The mixtures were incubated at 30°C for 20 min and quenched immediately with 5 µl of a stop buffer containing 0.2% SDS, 10 mM EDTA, 90% formamide, and 0.1% bromphenol blue, and heating at 95 °C for 6 min, and then immediately put on ice for 5 min before running a denaturing gel. The results were analyzed by electrophoresis through 20% polyacrylamide gel containing 7 M urea in TAE (80 mM Tris acetate, 2 mM EDTA, pH 8.0). Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a Bio-Rad gel imager.

A1.3.2.2 Reaction Conditions for DSBs Repair

A 15 µl reaction mixture consists of 250 nM of a short piece of DNA (blunt end or 3’ overhang or 5’ overhang), and 150 nM of a long piece of DNA (blunt end or 3’ overhang or 5’ overhang), 1 µM Klenow or Klentaq, and/or 80 µM deoxynucleotide triphosphates in a buffer containing 10 mM Tris-HCl (pH 7.9), and 150 mM KCl for Klenow or 50 mM KCl for Klentaq, and 5 mM MgCl2 in the presence or absence of T4 DNA ligase. All reactions were carried out at 30°C for 40 min and quenched immediately with 5 µl of a stop buffer containing 0.2% SDS, 10 mM EDTA, 90% formamide, and 0.1% bromphenol blue, and heating at 95 °C for 6 min, and then immediately put on ice for 5 min. The results were analyzed by electrophoresis through 20% polyacrylamide gel containing 7 M urea in TAE (80 mM Tris acetate, 2 mM EDTA, pH 8.0). Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a
Bio-Rad gel imager. In some instances, the 5’ terminus of DNA was phosphorylated with T4 polynucleotide kinase and unlabeled ATP.

**A1.4 Results**

### A1.4.1 Extension of Mismatch at 3’ Terminus

Mismatches produced from DNA replication by Pol I polymerase are supposed to be removed before new nucleotide incorporation and this event depends on the Pol I exonuclease domain. Klenow is able to discriminate paired and mispaired termini and extends mismatched termini to some extent (10). Klentaq was also found to have extension ability on primed-template with a single terminal mismatch (11). These results suggest that both polymerases may process the mismatched end of a primed-template DNA via mismatch extension prior to repair of DSBs containing terminal mismatches.

Three consecutive base mismatches were introduced at 3’ terminus of primer of hairpin DNA having a primed-template end or a blunt end. Extension from primer termini having a 3-base mismatch was tested (hp39m) (Figure A1.1 A). On a matched primed-template (Figure A1.1 B, lanes 2 and 8), both Klenow and Klentaq completely utilized the primer. On the primer terminus having 3 mismatches, both enzymes extended with relative efficiency, (Figure A1.1 B, lanes 3 and 9), with ratios of 0.34 and 0.14, respectively, compared to matched template (Figure A1.1 C). This is consistent with previous studies that have shown that both Klenow and Klentaq polymerases extend primed-template with the single terminal mismatch with low efficiency compared to matched primed-template (11-12).

Klenow has been previously shown to discriminate paired and mispaired termini and extend mismatches to some extent when the DNA substrate contains one mispair (10, 12-14). In the present experiments, Klenow extended the primer with 3 terminal mismatches much less
Figure A1.1: Discrimination against extension of mismatched termini by Klenow and Klentaq.
A, Substrates used for the assay. hp39 is a hairpin DNA with a 7-base 5’ overhang. Hp39m is analogous to hp39 but contains 3mismatched bases at the 3’ terminus of the primer. B, Extension from primers having zero or three mismatches. 0.5 uM Klenow (KLN) was incubated with matched (lane 2 for DNA with 5’ OH and lane 5 for DNA with 5’ PO4), or mismatched (lane 3 for DNA with 5’ OH and lane 6 for DNA with 5’ PO4) primer-termini. 0.5 uM Klentaq (KTQ) was incubated with the same substrates, matched (lane 8 for DNA with 5’ OH and lane 11 for DNA with 5’ PO4), or mismatched (lane 9 for DNA with 5’ OH and lane 12 for DNA with 5’ PO4) primed-termini. Control Experiments without enzymes are shown in lane 1, 4, 7, and 10. These gels were stained using SYBR Green. C, Quantitation of the extension reactions. Matched (matched primer-template end), Mis-m w/5’ OH (Mismatched primer-template end with 5’ terminal hydroxyl), Mis-m w/5’ PO4 (Mismatched primer-template end with 5’ terminal phosphate). Percents of primed-terminus utilization are calculated relative to the matched terminus.
efficiently than those with fully matched terminus and Klentaq had weak extension ability on the same mismatched DNA substrates. Klentaq discriminated better between paired and mispaired termini than did Klenow (Figure A1.1 C). Klenow utilized mismatched primed-template at a 3-4 fold lower relative ratio compared to a matched primed-template, while Klentaq showed a 7-8 fold lower relative ratio in extending mispaired terminus compared paired terminus. 5’ terminal phosphate did not affect the efficiency of mismatch extension by either polymerase. Combination of the relative tight binding affinity of mismatched DNA (Chapter 2) and weak extension activity of both proteins on a mismatched primed-template indicates that binding affinity is not mandatory for mismatch extension activity, consistent with previous findings (15). The possible mechanism for this may be similar to that of pol λ (16). In that mechanism, pol λ is unable to discriminate against the terminal mispair during DNA binding. However, the conformational change due to the incoming nucleotide binding is unlikely to occur, thus leading to the lower extending efficiency on mismatched substrates by pol λ.

A1.4.2 Effect of Mismatch on the Repair of DSBs with Non-complementary 5’ Overhang

The ability of Klenow to extend unpaired termini is worth considering relative to the repair of DSBs, since we showed that both Klenow and Klentaq repaired DSBs having 5’ non-complementary overhang via fill-in and DNA strand displacement DNA synthesis (Chapter 4). To understand if the activity of mismatch extension of both proteins contributes to the repair of DSB, we tested the repair of DSBs having noncomplementary 5’ overhangs and 3’ primer mismatches at one end by both proteins. The DNA substrates consist of the hairpin DNA hp29-5 containing a 7-nt 5’ overhang, and the hairpin DNA hp39m having three mismatches at 3’ primer terminus and a 7-nt 5’ overhang (Figure A1.2 A). Both substrates contained either 5’ phosphate or 5’ hydroxyl group. It is expected that 3’ mismatched terminus could be extended by
Figure A1.2 Reactant structures and resultant products formed with DNA substrates containing 5’ non-complementary overhangs and 3’ primer mismatches. A, The substrate structures and possible products due to mismatch extension and/or DNA strand-displacement synthesis. B, Reaction products were examined on a 20% polyacrylamide gel containing 7 M urea. Reaction mixtures containing polymerase (1uM), both hp29-5 (250 nM) and hp39m (150 nM) with indicated configuration at 3’ and 5’ ends and/or T4 DNA ligase, and/or dNTP (80 µM) were incubated at 30 °C for 40 min.
polymerase to produce a blunt end, followed by strand-displacement DNA synthesis to form a 82-mer or 118-mer product with the nick sealed by T4 DNA ligase (Figure A1.2 A, (I)), or a 82/128-mer product (Figure A1.2 A, (II)). Or mismatch makes melting DNA duplex easier, a 75-mer or 114-mer (nick sealed by ligase) product should be formed (Figure A1.2 A, (III)).

Reaction products were analyzed on denaturing gel. We found that DSBs with 5’ OH could not be repaired (Figure A1.2 B, left panel, lanes 5 & 6). Hp29-5 was converted to blunt end by fill-in synthesis (Figure A1.2 B, left panel, lanes 3, 5 & 6). Klenow extended the primer terminus having mismatch (Figure A1.2 B, left panel, lanes 4, 5, & 6). This is comparable with the extension of Klenow on substrate with one mismatch at 3’ terminus (12). But the extension capacity of Klenow did not mediate the repair of DSB in the presence of DNA ligase and dNTP (Figure A1.2 B, left panel, lane 6). In contrast, when the substrates with 5’ phosphate were used, Klenow was able to repair DSBs in the presence of DNA ligase by producing about 118 or 114-mer product (Figure A1.2 B, left panel, lane 8) compared to 118-mer and 128-mer products produced when the same DSB without mismatches at 3’ primer terminus was used as substrate (Figure A1.2 B, left panel, lane 11). The repair ability of Klentaq on the same DSB with mismatched ends was also tested. The similar results were obtained by forming 75-mer and about 118/114-mer products (Figure A1.2 B, right panel, lane 13). Taken together, the repair results of DSB having 5’ non-complementary overhang and 3’ primer mismatches suggest that 5’ phosphate and DNA ligase are essential for repair of this kind of DSB.

A1.4.3 The Specific Pathway for Repair of DSB with 3’ Primer Mismatches

The above results show that both polymerases may repair DSBs with non-complementary 5’ overhang through two different pathways (Figure A1.1 A, I & III), the specific repair pathway can be determined by using only one substrate containing 5’ phosphate (Figure A1.3). When only the substrate hp29-5 contains 5’ phosphate, a 82-mer product will be produced if
polymerase extends the 3’ mismatched terminus and followed by strand-displacement DNA synthesis (Figure A1.3 A, (I)) and a 128-mer product will be formed if subsequent palindrome amplification occurs via the short inverted repeats within the strand-displacement DNA synthesized product. A 114-mer DNA will be formed if polymerase elongates 3’ OH of hp29-5 by using the floating 3’ single-stranded primer of hp39m as a template in the presence of DNA ligase (Figure A1.3 A, (I)), with the possibility of subsequent palindrome amplification occurring after strand displacement synthesis is precluded because a nicked DNA with double hairpins and a 7-base 3’ flap is expected to form in this case. On the other hand, when only the substrate hp39m contains 5’ phosphate, a 118-mer product will be produced if polymerase extends the 3’ mismatched terminus followed by strand-displacement DNA synthesis in the presence of DNA ligase (Figure A1.3 A, (III)), otherwise a 75-mer DNA will be formed (Figure A1.3 A, (IV)).

The results show that Klenow and Klentaq have similar product formation patterns when we change the modification at 5’ terminus (Figure A1.3 B). A 114-mer product was produced when only hp29-5 contained 5’ phosphate, identical to the product when both hp29-5 and hp39m contained 5’ phosphate, suggesting that polymerase elongates the 3’ primer of hp29-5 to generate blunt end and then does strand-displacement DNA synthesis by unwinding hp39m (Figure A1.3 B, lanes 1, 3, 4, & 6). On the contrary, no product was formed when only hp39m contained 5’ phosphate (Figure A1.3 B, lanes 2 & 5), indicating that 5’ phosphate at the blunt end is essential for alignment-based strand displacement DNA synthesis by using the mismatched 3’ primer from the second end as a template. These results are also consistent with our and Joyce, et al.’s studies of 5’ phosphate effect on DNA binding affinity. 5’ phosphate increases the binding affinity of Klenow to DNA with short 5’ overhang (17), while it doesn’t show significant effect on binding of DSB with 7-base 5’ overhang by both Klenow and Klentaq (unpublished data).
A1.4.4 Repair of a DSB Having a Blunt End and 3’ Primer Mismatches

The repair of a DSB with a non-complementary 5’ overhang, selective 5’ phosphate modification and mismatch at one end by Klenow and Klentaq indicates that this kind of DSB is repaired by using the 3’ end of matched end as a primer and the floating 3’ mismatched end as a template to synthesize new DNA. To understand the mechanism better, we made a DSB with

![Diagram](image)

Figure A1.3 Substrates structures and resultant formed products with DNA substrates having different 5’ end configurations. (A). The substrate structures used in the experiments and expected products. (B). Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Reaction mixtures containing polymerase (1µM), both hp29-5 (250 nM) and hp39m (150 nM) with indicated configuration at 5’ end, T4 DNA Ligase, and dNTP (80 µM) were incubated at 30 °C for 40 min. Both Klenow and Klentaq repair DSBs with 3’ primer mismatches by using the floating primer of mismatched DNA substrate as the template for DNA strand-displacement synthesis.
a blunt end and 3’ primer mismatches at one end (Figure A1.4 A) and examined its repair by both proteins under similar conditions. We predicted that Klenow/Klentaq might repair this DSB by two possible pathways. First, polymerase repair DSB via strand-displacement synthesis by invading the floating 3’ primer from the mismatched end into the matched end. Second, polymerase could repair this DSB via strand-displacement synthesis by elongating the 3’ primer of the matched end using the floating 3’ terminus from the mismatched end as a template. Or polymerase could repair this DSB using the two pathways simultaneously. Both Klenow and Klentaq repair the DSB with 5’ phosphate in the presence of T4 DNA ligase by forming a 74 or 120-mer product, indicating that this DSB was repaired by polymerase via alignment-based DNA strand displacement synthesis using the mismatched 3’ primer as a template (Figure A1.4 B, lanes 5 & 10).

On the contrary, under the same conditions, Klenow repairs a 5’ phosphorylated DSB with a regular blunt end by forming 102-mer and 120-mer products via alignment-based strand displacement synthesis in both directions while Klentaq failed to repair the same DSB. These data showed that terminal mismatch impeded but did not prevent the repair of DSB by Klenow, whereas it promoted the repair of DSB by Klentaq. Mismatch effects on DSB repair by Klenow here is not compatible with suggestions by King, et al. (18), where it was thought that unpaired bases at the 5’ end of the DNA complementary to the primer would not prevent product formation. The repair of DSB through DNA synthesis using mismatched 3’ primer as a template suggests that mismatch may normally play a role in initiating strand separation for strand displacement DNA synthesis. Recently reported data support a fundamentally similar role for a 5’ flap of gapped DNA in the strand displacement DNA synthesis by Klenow (19).
A1.5 Discussion

Both Taq DNA polymerase and Klenow DNA polymerase can do fill-in DNA synthesis upon end joining of substrates with a blunt end and a 3’-protruding single strand (PSS) end in vitro (20). Both Klenow and Klentaq can extend a 3’-OH of primed-template with a single terminal mismatch (10-11). We have previously shown that both polymerases were able to repair DSBs via alignment-based strand-displacement DNA synthesis with different dependence.

Figure A1.4 Reactant structures and products formed with DNA substrates containing blunt ends and 3’ OH mismatches. (A) The substrate structures used in the experiments and possible products due to DNA strand-displacement synthesis. (B) Reaction products are shown on a 20% polyacrylamide gel containing 7 M urea. Reaction mixtures containing polymerase (1uM), both hp28 (250 nM) and hp46m/hp46 (150 nM) with 5’ PO4, T4 DNA Ligase, and dNTP (80 μM) were incubated at 30 °C for 40 min.
on 5’ terminal phosphate of substrate and T4 DNA ligase (Chapter 4), and the goal of the current study was to specifically address whether the terminal mismatch of DSBs affect their repair. The major findings reported here are that mismatch can affect DSB repair by supporting pre-made flapped substrate and the extent of influence is relevant to the strand-displacement activity of the polymerase.

A1.5.1 Mismatch Extension of a Primed-template DNA

Our results show that both Klenow and Klentaq have weak extension activities on the primed-template DNA (hp39m) with three consecutive mismatches at 3’ terminus. However, the potential mechanisms for the mismatch extension are not known yet. In our case, the unpaired base at the primer terminus was G, and the base on -1 position of the template was C. In this instance, primed-template realignment is a conceivable mechanism for extension of mismatched primer termini (Figure A1.5). Similar synthesis can occur with pol κ (21), with a one-nucleotide shorter product formed by looping out a template base. Since both Klenow and Klentaq can add bases to the 3’ hydroxyls of blunt-ended DNA duplexes in vitro (22), the mismatch extension product may come from direct bypass incorporation (Figure A1.5). More experiments are needed to determine whether mismatch extension is due to bypass incorporation or mediated by a slippage mechanism.

A1.5.2 Mismatch Effect on DSB Repair

Our work and that of others demonstrate that both polymerases can extend mismatched 3’primer. Both polymerases bind mismatched primed-template end tighter than matched DNA (Chapter 2 of this dissertation). King et al and Clark et al demonstrate that a DNA polymerase can function as a putative alignment protein for non-complementary end joining (23-24). The
presence of a 5’ flap on gapped DNA substrates clearly enhances the strand displacement DNA synthesis activity of Klenow (19). All of these observations imply that mismatch at one end of the DNA substrate can be tolerated, providing that the other end is complementary.

Figure A1.5 Two potential mechanisms of mismatch extension by Klenow and Klentaq. In direct mismatch extension (right), following a primer-terminal mispair, the incoming dTTP (indicated by red color) pairs with the correct templating base A. In extension by misalignment (left), the A-C mispair realigns so that the primer A becomes extrahelical and the primer-terminal G pairs with the next template base C. This is followed by the pairing of the incoming dATP (indicated by red color) with the subsequent complementary template base.

DSB should be relevant to its repair. Our data show that mismatch at one end of DSB did not disrupt the repair. Only a single product is formed during repair of both DSBs with mismatched and non-complementary 5’ overhangs by both proteins, compared with two products produced when the same DSB with a matched end is repaired. For Klentaq, but not Klenow, mismatch improves the repair capacity on the DSBs with blunt ends. Our data confirm a point mentioned by Ward that lesions may affect each other during repair (25). Our findings fit well with important recent in vivo findings. The number of microhomology-independent events was increased due to the deficiency of MMR protein Msh2 (26). It was suggested that that Mlh1 may function in NHEJ as part of a larger DNA repair complex by limiting the annealing of DNA ends containing non-complementary base pairs (27). These observations are compatible with the finding here that polymerase can repair DSB with terminal mismatches.

In contrast, it has been reported that mismatch might impair DSB repair (18). Our findings, however, do not contradict the data on which these previous statements were based,
because mismatches we report here lie at the 3’ primer terminus of a primed-templated duplex and mismatches reported previously exist between the 3’ protruding end of double-strand DNA and single-strand DNA. It has been shown that Klentaq catalyzed non-template nucleotide addition to the 3’ termini of blunt-ended DNA with much stronger preference for incorporation of dA, while Klenow only showed moderate preference for incorporation dA compared to other dNTP (24, 28). It was thought that Klentaq had a weaker strand displacement activity than Klenow, because in Klentaq the positions of Ser\textsuperscript{769}, Phe\textsuperscript{771}, and Arg\textsuperscript{841}, required for strand displacement DNA synthesis, are occupied by serine, histidine, and arginine, respectively(19, 29). Among those three residues, histidine does not interact with DNA substrate (29). The substrates hp29-5 and hp28 have 3’terminal dT and dC, respectively. Taken together, they can explain why terminal mismatch inhibits the repair of DSB by only allowing one direction of strand-displacement DNA synthesis while it promotes DSB repair with a 3’ terminal C at the blunt end by supporting a floating 3’ primer as a template for primed DNA synthesis. Because Klentaq can’t repair the blunt DSB with a 3’ terminal C via non-template added dA pairing with a 3’ terminal C while Klenow can.

Generally, there could be two possible pathways for repair of DSBs with mismatches. One is that a polymerase extends the recessed and mismatched 3’OH and then does alignment-based strand displacement DNA synthesis to complete repair. The other one is that a polymerase directly does alignment-based strand displacement synthesis using mismatched 3’ primer as a template. Size analysis of the products of our experiment demonstrate that both Klenow and Klentaq repair DSBs with mismatches through the second pathway. This finding provides support for the previous idea that some lesions promote the insertion of an incorrect nucleotide (30). We envision that the weak mismatch extension activity may account for why Klenow and
Klentaq polymerases don’t repair DSBs with mismatches via extending the mismatched terminus following strand-displacement DNA synthesis. Although the polymerases don’t repair the DSBs with mismatch at one end through mismatch extension \textit{in vitro}, the mismatch extension property of polymerase suggests a potential role as a ‘mismatch extender’ during non-homologous end joining (NHEJ), and possibly lesion bypass on its own or in cooperation with other DNA polymerases \textit{in vivo} (31). Further work will be required to determine if the mismatch extension ability of polymerase can facilitate NHEJ through end processing or lesion bypass.

The effect of mismatch on DSB repair may be due to two possibilities: Tight binding affinity of protein with mismatched DNA leading to an increased opportunity for mismatched primer being used as a template for strand-displacement DNA synthesis; Mismatch at the 3’ primer end of a DSB resembles the melting of the duplex portion and make it easy to start strand-displacement DNA synthesis. However, because the two possibilities produce identical products, we have not conclusively determined whether tight affinity or melted 3’ primer, or both contribute to the promotion of the mismatched DSB repair capacity of Klentaq.

\textbf{A1.5.3 Contribution of 5’ Phosphate and DNA Ligase on DSB Repair}

We find that both 5’ phosphate and DNA ligase are required for repair of DSBs with mismatches at one end. The observed crucial function might be due to the fact that 5’ phosphate increases the binding affinity of DNA by either polymerase or ligase. The increased binding affinity could lead to an increase in the stability of the transient complex of two-end bridging by specific proteins. Our experiments results show that the repair of DSBs with mismatch require the cooperation of polymerase and ligase. DNA ligase is critical for the joining of Okazaki fragments to complete DNA synthesis and is required to accomplish DNA repair pathways. We and others assumed that polymerases may bring two DNA end in a close proximity (18). Our study, combined with others imply that polymerase may play an important role in bridging two
DNA ends together and ligase may help stabilize the complex (Figure A1.6), although ligase and polymerase can cooperate in many ways by which two DNA ends can be aligned together, and thereby improve the efficiency of DSB repair. Both proteins bind matched and mismatched primed-template and blunt-end DNAs well (Chapter 2). The *Escherichia coli* DNA ligase binds nicked DNA tighter than the sealed duplex DNA or the single-stranded DNA (32). T4 DNA ligase mediates template-independent ligation (33).

![Figure A1.6 Functions of pol I DNA polymerase and DNA ligase in repair of DSB with terminal mismatches. Pol I DNA polymerase can bridge and bind both blunt and mismatched ends, but this complex is unstable prior to DNA ligase also binding. A DNA ligase may facilitate interaction by increasing the occupancy time of pol I polymerase at the break end and accordingly help the occurring of strand displacement DNA synthesis and nick ligation.](image)

5’ phosphate is required to form a DNA–adenylate intermediate, and therefore is critical for the binding of viral ligase to nicked DNA (34). The lack of a 5’ phosphate makes unstable binding of T7 ligase to nicked DNA (35). The presence of 5’ phosphate makes some contribution to DNA binding by Klenow and Klentaq. The binding affinity of Klenow to DNA with a short 5’
overhang (1 or 2 base overhang) was increased by 5’ phosphate (17). The ternary complex crystal structure of Klentaq showed that the 5’ phosphate group of the template nucleotide could pair with the incoming dNTP and interact with Ser674 (29). All these data indicate that 5’ phosphate contributes to the binding affinity of the DNA substrates used here by either polymerase or ligase, and thereby increases the staying time of polymerase and ligase at the break junction thus further promoting strand-displacement DNA synthesis.

A1.6 Concluding Summary

Mismatch is an inherent mutagenesis process in DNA replication, and double-strand breaks in DNA are one of most dangerous damages for genome stability. Our results show that terminal mismatch at one end doesn’t prevent the repair of DSB by Klenow and promotes the repair of the DSB with a blunt end by Klentaq. The involvement of mismatch in DSB repair process reveals a way that one type of DNA damage can help another DNA repair. These findings might reveal previously unrecognized role for mismatch damage, in that mismatch make some DSBs be more easily repaired by a special polymerase. Both polymerases have the capacity to synthesize DNA by using the floating 3’ overhang from a mismatched DSB end, leading to the suggestion that they might be the DSB repair enzyme of choice when mismatches and DSBs occur next to each other.

A1.7 References


APPENDIX 2

PRELIMINARY DATA ON THE INTERACTION STOICHIOMETRY OF POL I DNA POLYMERASES AND DNA

A2.1 Introduction

Upon associating with DNA, Pol I participates in either DNA replication or repair depending on the structure of the DNA substrate. In this investigation we use a combination of biochemical and biophysical techniques to probe the interaction stoichiometry of different polymerase-DNA complexes. Fluorescence anisotropy data show that Klenow binds DNA with different end structures differently while Klentaq binds them with nearly identical affinities. Electrophoretic mobility shift titrations carried out with various DNA substrates differing in end structures give rise to markedly different electrophoretic patterns. In the case of a linear DNA for which Klenow shows similar affinities at both ends, the Klenow-DNA complex is represented by a slow moving band when the protein to DNA ratio of the initial mixture is 4:1. However, for a DNA with which Klenow binds each end with a different affinity the complexes are represented by both fast and slow moving bands. In contrast, all Klentaq-DNA complexes are represented by a slow moving band at protein to DNA mixing ratios of 4:1 no matter what the DNA end structure. Protection assays of DNA from digestion by 5' and 3' exonucleases indicate that the fast and slow moving bands may correspond to 1:1 and 2:1 polymerase to DNA complexes, respectively. We hypothesized that the formations of the 1:1 complex and the 2:1 complex may be related to the affinities of protein to different DNA end structures. Similar binding affinities of protein at both ends make it preferentially form the 2:1 complex, while different binding affinities of protein to the two DNA ends make it prefer one end over the other and hence form a mix of 1:1 and 2:1 complexes.
A2.2 Materials and Methods

A2.2.1 Materials

Klenow Fragment (KF) and Klentaq (KTQ) polymerases were purified in our laboratory (refer to Chapter 2) DNA oligonucleotides were obtained from IDT (Coralville, IA, USA) (see Chapter 2 for DNA sample preparation). The DNA substrates used in this study are listed in Table A2.1. Exonuclease III was purchased from invitrogen (Carlsbad, CA) and T7 exonuclease was purchased from New England Biolabs (Ipswich, MA).

Table A2.1: Sequences of DNAs Used in This Study.

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A2.2.2 Methods

A2.2.2.1 Electrophoretic Mobility Shift Assay (EMSA)

All DNA constructs used in these experiments were unlabeled. Samples of 10 µL containing 0.3 uM DNA, were incubated for 25 min at 25 °C in the presence of 0-2.4 uM Klenow/ Klentaq. The control contains 0.3 µM DNA. The composition of the binding buffer is 10 mM Tris, 5 mM MgCl₂, 50 mM KCl for Klenow or 5mM KCl for Klentaq, at pH 7.9, 25°C. After incubation, the samples were then applied to 6% native polyacrylamide gel or 4-20% gradients gel (Bio-Rad) and electrophoresed in 1X TAE buffer (80 mM Tris acetate, 2 mM
EDTA, pH 8.0) for 50 min at a constant voltage of 100 volts at room temperature. Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a Bio-Rad gel imager.

**A2.2.2 Protection Assay of DNA from Exonuclease by Polymerases**

Reaction mixtures (13.5 uL) containing 66mM Tris-HCl (pH 8.0), 0.5 mM DTT, 25 mM KCl, 0.3 uM DNA and 0-4.8 uM Klenow were incubated at 25 °C for 20 min. Exonuclease III digestions were then performed by supplementing the DNA-containing mixtures (± Klenow) with 1.5 uL of a solution containing 6 mM MgCl₂ and 15 units of exonuclease III or 1.5 uL of a solution containing 50 mM MgCl₂ and 15 units of T7 exonuclease. The mixtures were then incubated at 25 °C for 5 min and quenched immediately with 0.2% SDS, 10 mM EDTA (heating at 95 °C for 6 min for running denaturing gel). The results were analyzed by electrophoresis through 10% native polyacrylamide gel or 20% polyacrylamide gel containing 7 M urea in TAE (80 mM Tris acetate, 2 mM EDTA, pH 8.0). Following electrophoresis, gels were stained with SYBR Green (Invitrogen) and imaged using a Bio-Rad gel imager.

**A2.3 Results**

**A2.3.1 Electrophoretic Mobility Shift Analysis of the Two Polymerases with DNA**

The interaction between each polymerase and the six DNA substrates was examined using an electrophoretic mobility shift titration. We incubated a constant concentration of pt-63/70 or ds-63 or pt-20/35 or gap10 or 17/27 or 17/27-4mm (0.3uM in each case) with increasing concentrations of Klenow (0-2.4 uM) and Klentaq (0-2.4 uM) and then analyzed the mixtures by native polyacrylamide gel electrophoresis, after which the gel was stained with SYBR green to visualize the DNA as shown Figure A2.1, Figure A2.2, and Figure A2.3, respectively. We have previously shown that at KCl concentrations higher than a specific concentration (>200 mM for
Klenow or >175 mM for Klentaq), the substrate preference for each of the polymerases is subject to change (1). For this reason all of the salt concentrations used in these experiments were between 5 and 50 mM KCl to ensure that the substrate binding hierarchy patterns remained consistent for both proteins.

Figure A2.1: Electrophoretic analysis of the interaction of Klenow/Klentaq with DNA. Samples were incubated for 25 min at 25 °C. A, Titration of 0.3 uM of the primer-template DNA pt-63/70 with Klenow (lanes 1-5) and Klentaq (lanes 6-10), respectively. B, Titration of 0.3 uM of the blunt-end double-strand DNA ds-63 with Klenow (lanes 1-5) and Klentaq (lanes 6-10), respectively. The protein:DNA ratio is indicated under each lane. The identities of the discrete bands are also indicated.

Klenow yielded one or two discretely shifted DNA complexes (designated complex A and complex B) throughout the course of the titration when binding to linear pt-63/70 containing the blunt end and the primed-template end, whereas Klentaq only yielded singly shifted DNA complex, this complex (designated complex B) migrates slower than complex A (Figure A2.1, A). Interestingly, the amount of a slower shifting bands corresponding to complex B is increased as the concentration of Klenow increases, suggesting that complex B may contain higher
stoichiometries of Klenow molecules bound to the DNA. The smearing between the complex A and complex B bands or ahead of the complex B band suggests that there is some degree of dissociating of protein from higher molecular weight to lower molecular weight over time. On the contrary, both Klenow and Klentaq yielded the single shift DNA complex (designated complex B) when binding to ds-63 only containing the blunt ends.

Klenow prefers the primed-template end of DNA more than the blunt end, and Klentaq does not show obvious preference to either end (Chapter 2 in this dissertation). Because Klenow displays similar binding affinity for both ends of the ds-63 DNA substrate, it may preferentially form a slower shifted complex at high concentrations, binding to both ends of the DNA. We further examined the binding of tp-20/35 containing the template-primer end (3’ overhang) and the blunt end; and gap10 containing the primed-template end and the template-primer end by both proteins (Figure A2.2). As expected, only singly shifted complex (designated complex B) is observed when the two proteins bind tp-20/35 since both proteins binds the template-primer end and the blunt end similarly. On the other hand, there are two discrete complexes formed at high concentration of Klenow with gap10, indicating that the difference in binding affinities between both ends of the DNA cause Klenow to preferentially form the faster shifted complex (Complex A) at a low protein to DNA ratio.

Our previous study of matched and mismatched DNA binding by Klenow shows that Klenow binds mismatched DNA tighter than matched DNA (by about 0.7 kcal/mol), while it binds the primed-template end of DNA tighter than the blunt end (by about 1.2 kcal/mole) (Chapter 2 in this dissertation). Therefore, Klenow prefers the mismatched primed-template end of DNA significantly over the blunt end. We predict that the faster shifting complex might be predominant at a high protein to DNA ratio when Klenow binds 17/27mer containing a primed-
template end with 4 mismatches. However, the slower shifting complex might be predominant when Klenow binds to normal 17/27mer. Figure A2.3 shows the comparison of the binding of the linear DNA 17/27 and 17/27-4mm by Klenow. At moderate concentration of Klenow, two shifting complexes (complex A and B) are formed for both DNA substrates (Figur A2.3, A).

However, at the highest concentration of Klenow, the slower shifting complex is predominant for the 17/27 substrate, while the faster shifting complex is predominant for the 17/27-4mm, confirming our previously expected hypothesis. The amounts of the slower and faster shifting complexes on the gel were quantified using the program Image Quant 5.1 and the ratio of the complex B to complex A was plotted as the function of the protein to DNA ratio (Figure A2.3, B). The ratio of complex B to complex A for the matched DNA is higher than that of the

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**Figure A2.2:** Electrophoretic analysis of the interaction of Klenow/Klentaq with DNA. Samples were incubated for 25 min at 25 °C. A, Titration of 0.3 uM of the template-primer DNA tp-20/35 with Klenow (lanes 1-4) and Klenataq (lanes 5-8), respectively. B, Titration of 0.3 uM of the gapped DNA gap10 Klenow (lanes 1-4) and Klentaq (lanes 6-9), respectively. The protein:DNA ratio is indicated under each lane. Lanes 5 and 10 contained 0.3 uM of gap10 without addition of any protein.
mismatched DNA, indicating that the big difference in binding affinity between the two ends of DNA might prevent the formation of the slower shifted complex (complex B).

Figure A2.3: Electrophoretic analysis of the interaction of Klenow/Klentaq with matched and mismatched DNA. Samples were incubated for 25 min at 25 °C. (A) Titration of 0.3 uM the matched DNA 17/27 (lanes 1-5) and the mismatched DNA 17/27-4mm (lanes 6-10) with Klenow in increasing concentrations, respectively. The protein:DNA ratio is indicated under each lane. The identities of the discrete bands are also indicated. The amounts of possible 2:1 and 1:1 polymerase-DNA complexes were quantified using the program Image Quant and were expressed as the ratio of 2:1 complex to 1:1 complex: these values are plotted as the function of the ratio of Klenow and DNA. The gel was obtained by Aqrty Bashyal.
A2.3.2 Correlation of the Exonuclease Activity of Klenow and the Klenow-DNA complex

Although the Klenow clone used in this study has a D424A mutation (Klenow exo provided by Catherine Joyce from Yale University), and the 3’-5’exonuclease activity is diminished (2), some residual 3’-5’ exonuclease activity has been seen by others (2-3) and our lab. Figure A2.4 B, left panel shows the exonuclease assay of Klenow on the blunt-end double-stranded DNA ds-20/20 over time. Some of the 20-mer strand of DNA was degraded to shorter DNA (designated digested DNA) when the sample had been incubated 1 hour. The 3’-5’ direction digestion changes one blunt end of the ds-20 to a primed-template end over time. Accordingly, the digestion at the 3’ terminus is expected to disrupt the binding pattern of Klenow on both ends of the DNA since Klenow prefers the primed-template end over the blunt end. As expected, long-term incubation of Klenow and ds-20 DNA caused complex B to be converted to complex A (Figure A2.4, B, lane 7). The converting of complex B to complex A, however, was prevented by addition of 2 mM EDTA (Figure A2.4, B, lane 8), which scavenges all traces of divalent metal ions necessary for the exo site activity of Klenow. These data provide additional support for a model in which Klenow prefers to form complex A when the binding affinities at both ends are significantly different.

A2.3.3 Klenow Protects DSB Ends from Exonucleases

The native gel shift reveals two discrete Klenow-DNA complexes on DNA substrates containing two ends with different binding affinities, indicating the potential 2:1 polymerase-DNA complex forms. Yet the gel shift does not exclude alternative scenarios, e.g. that Klenow homodimers bind sequentially to the same DNA end or access both DNA ends. To probe how the polymerase is deposited on a linear DNA, we incubated DNA with Klenow and then treated the polymerase-DNA complexes with E. coli exonuclease III (a 3’–5’ double strand DNA
Figure A2.4: The blunt-end double-stranded DNA ds-20/20 exonuclease digestion and binding by Klenow. (A) The DNA sequence used in these experiments. The exonuclease digestion of ds-20/20 by Klenow as the function of time (0, 1, 2, or 3 hours) is shown in (B, left panel) and the Klenow binding to ds-20/20 in Mg\(^{2+}\) and EDTA buffers after 10 minutes and 3 hours incubation time is shown in (B, right panel). The Klenow exonuclease digestion buffer contains 10 mM Tris, 5 mM MgCl\(_2\), and 50 mM KCl. Mg\(^{2+}\) buffer contains 10 mM Tris, 5 mM MgCl\(_2\), and 50 mM KCl while EDTA buffer is 10 mM Tris and 2 mM EDTA at 25°C, pH 7.9. These gels were stained using SYBR Green and were obtained by Hiromi Brown and Andy Wowor.

exonuclease) or T7 exonuclease (a 5’–3’ double strand DNA exonuclease). Whereas the free DNA was rapidly digested by exonuclease III (Figure A2.5, A, lane 2 or Figure A2.5, B, lane 2), the inclusion of Klenow afforded virtually complete protection from exonuclease (Figure A2.5, A, lane 8 or Figure A2.5, B, lane 5) relative to the control which only contains DNA (Figure A2.5, A, lane 1 or Figure A2.5, B, lane 1). Because protein binding at only one DNA end, or at
random internal sites, will not prevent end-resection by exonuclease III, we can confidently conclude that Klenow is bound stably at, or closely adjacent to, both DNA ends at high Klenow:DNA ratios.

Figure A2.5: Klenow protect ends. 0.3 uM DNA were incubated with different concentrations of Klenow for 20 min at 25 °C. Exonuclease digestions were then performed by adding 10 units of exonuclease to the mixture. The protection assay of pt-63/70 (A) or ds-63 (B) from exonuclease III (Promega) by Klenow was shown, and the protection assay of tp-20/35 from T7 exonulease (New England Biolab) by Klenow was shown in (C). C = control, which means only free DNA contained. The protein:DNA ratio is indicated under each lane. These gels were stained using SYBR Green.
Because Klenow preferentially binds the primed-template end, it is thus expected that Klenow might preferentially protect the primed-template end over the other end at a low protein concentration and protect both ends at a high protein concentration when the two ends of a DNA have different affinity, and protect both ends of a DNA equally at either low or high protein concentration when the two ends of a DNA have similar affinity. Figure A2.5, A shows the protections of the primed-template DNA pt-63/70 from exonuclease III by Klenow. At the low concentration of Klenow, both strands of the pt-63/70 were digested. Klenow preferentially protected the primed-template end and then both ends as the protein concentration increased by retaining the 70-mer strand first and then the 63-mer strand (Figure A2.5, A, lanes 7 and 8). In the contrast, Klenow did not show any significant preference in protecting the template-primer end and the blunt end of the tp-20/35 from the digestion of T7 exonuclease (Figure A2.5, C). This is not unexpected since Klenow binds the template-primer end and the blunt end similarly.

In summary, the protection results of DNA from exonuclease by Klenow suggest that the retarded bands designated complex A and complex B in the electrophoretic mobility shift assays may correspond to the 1:1 and 2:1 polymerase-DNA complexes, respectively.

A2.4 Discussion

A2.4.1 Advancement from previous reports of 2:1 Klenow-DNA complexes

Previous studies, albeit indirect, suggested there might be more than one Klenow molecule bound to the same primer template (4-5). In those studies, two discrete complexes, perhaps representing 1:1 or 2:1 polymerase-DNA complex, were observed by electrophoretic gel shift titrations, which were performed with Klenow and a primer-template DNA. Recently, Millar, et al. reported evidence from analytical ultracentrifugation experiments that Klenow mostly formed 1:1 complex with matched primed-template DNA at low protein concentration and predominantly formed 2:1 complex at high protein concentration(6). Millar et al. also
suggested that the second Klenow binding site may be located on the duplex part of DNA, upstream from the primed-template junction (6). This was because both the quantum yield of the fluorescein probe and the polarization anisotropy were enhanced due to the second Klenow molecule binding, and due to the fact that the short length of the single-stranded template region of DNA did not disrupt the 2:1 complex formation. The differences in our reported potential 2:1 complex studies are summarized as follows. First, the potential 2:1 complex in this study is formed by the two Klenow molecules binding at both ends of a linear DNA, at high protein concentration. Second, the observed stoichiometry of two enzyme molecules bound to the examined DNA may be related to the affinities of both DNA ends. This stoichiometry, suggested by gel shift studies and protection assay of DNA from exonuclease by protein, is induced at very high protein:DNA ratios, and the concentrations differ from the 1:1 stoichiometry found for protein complexes with analogous DNA substrates under equilibrium titration conditions (1).

**A2.4.2 Klenow Forms Two Different Complexes with DNA substrates Differing in Their End Structures**

In this report gel shift data suggest that Klenow forms a 1:1 complex with linear primed-template DNA at low protein concentration and a 2:1 complex at high protein concentration. This was further confirmed by the protection of DNA from exonuclease digestion by Klenow (Figure A2.5). Both ends of DNA are protected by Klenow at high protein concentration.

The binding studies of polymerase to different DNA end structures and gel electrophoretic titrations suggest that the formation of the 2:1 polymerase-DNA complex is related to the affinity of DNA end. Thus, when Klenow binds to a DNA with similar affinity at both ends, the formation of the 2:1 complex is favored. In contrast, when the first Klenow preferentially binds at the primed-template end of DNA, the second Klenow molecule has little or no apparent binding. Under these conditions, the 1:1 Klenow-DNA complex is favored. These
observations can be rationalized if the difference in binding affinity of both ends of a linear DNA plays an important role on the formation of the complexes. The similar affinity of both ends of DNA will cause Klenow to preferentially form the 2:1 complex. However, it remains difficult to identify the exact origins for the complexes of two protein molecules bound to one DNA molecule at a low protein to DNA ratio. For example, if we hypothesize that Klentaq, because it has similar binding affinity to different DNA ends, always binds both ends of DNA simultaneously, then by deduction the fast shifting complex (1:1 polymerase:DNA complex) should be dominant at a low protein to DNA ratio, whereas the slowly shifting complex (2:1 polymerase:DNA complex) may be predominant at a high protein to DNA ratio. Unfortunately, our results always show only slowly shifting complexes for Klentaq, inconsistent with the hypothesis. We can speculate that the preference for forming a 2:1 complex by Klenow/Klentaq with DNA containing the non-replicative ends may be due to the strong cooperativity between the two binding ends of a DNA with similar affinity. More experiments are needed to test this hypothesis. In addition, since Klenow’s residual 3’ exonuclease activity will convert a 63/63 mer DNA in to primed-template DNA, all of these results are also consistent with a model where the slow moving band (complex B) shifts to the fast band because the DNA is converted to a primed-template DNA rather than due to increased protein:DNA ratios in the reaction.

Our binding affinities studies (Chapter 2 in this dissertation) show that Klenow binds the mismatched primed-template end of DNA ~2.0 kcal/mol tighter than a blunt end. This difference predicts that Klenow will bind the primed-template end 41 times more likely if it encountered equal concentrations of the mismatched primed-template end and the blunt end. As the Klenow concentration increases, less of the 1:1 complex is converted to the 2:1 complex when Klenow binds the mismatched primed-template DNA compared to binding the matched
primed-template (Figure A2.3). In addition, the 3’-5’ digestion at one end of the blunt-end double-stranded DNA causes the 2:1 complex to be converted to the 1:1 complex (Figure A2.4). Taken together, these results are consistent with the idea proposed above that the difference in the affinity of both ends of a DNA causes Klenow to favor formation of the 1:1 complex.

**A2.4.3 Potential Models of the 2:1 Klenow-DNA Complex**

![Diagram showing potential models of the 2:1 Klenow-DNA complex.](image)

Figure A2.6: Model of Klenow binding to different DNAs as a function of the polymerase concentration, based on the results obtained in this work. Klenow can bind both ends of a DNA, where the two ends may have similar or different affinities to the protein. For a DNA containing the two ends with similar affinities to the protein, Klenow favors forming a 2:1 polymerase-DNA complex, while the 2:1 polymerase-DNA complex of a DNA containing two ends with significantly different affinities to the protein only dominate the distribution at high protein concentration.

A model for Klenow binding to DNA based on the results obtained in this work is depicted in Figure A2.6. Klenow binds the primed-template DNA end tighter than the blunt end and the template-primer end. For a linear primed-template DNA containing two ends differing in
binding affinity to protein, Klenow preferentially binds the preferred end at low protein concentration, leading to the mainly formation of the 1:1 complex (Figure A2.1, right panel). Because only the primed-template end is bound by Klenow, the primer is protected from the 3’-5’ digestion of exonuclease III (Figure A2.5, A, lane 7). As the protein concentration increases Klenow starts to bind the second end of the DNA. At a high Klenow concentration the complex with two Klenow molecules bound to a linear primed-template DNA dominates the distribution of the formed complexes. On the contrary, when Klenow binds DNA containing two ends with similar affinity Klenow favors forming the 2:1 polymerase- DNA complex (Figure A2.6, left panel). Again, this hypothetical model cannot currently account for the fact that Klentaq forms complex B even with hairpin DNAs that cannot form 2:1 complexes. Due to the above event this model must be considered speculative until further tests can be performed.

A2.4.4 Possible Functional Implications of Formation of the 2:1 Complex

What is the possible role of a 2:1 complex of Klenow or Klentaq polymerase with DNA containing two ends similar in binding affinity? Previously, a model for the role of cooperative binding to ds-DNA in the recognition of gapped DNA by ASFV polX was proposed (7). The model may be applicable to Klenow and Klentaq polymerases. If polymerase cooperatively binds to both ends of a non-replicative DNA (DNA with blunt end or 3’-overhang), it allows the enzyme to protect DNA for the next repair step. This is different from pol β, where the two protein molecules in the 2:1 complex are suggested to function as a 5’dPR lyase and a nucleotidyl transferase (8). It is also different from the dimerization of replicative polymerases from T4 bacteriophage (9), T7 bacteriophage (10-11), and E. coli (12-13) during DNA synthesis. For these polymerases protein dimerization at a replication fork provides a mechanism for coordinating leading and lagging strand synthesis during chromosome replication (10-11, 13).
A2.5 References


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