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Pol I DNA polymerases stimulate DNA end-joining by *Escherichia coli* DNA ligase

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**Abstract**

Klenow and Klentaq are the large fragment domains of the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*, respectively. Herein, we show that both polymerases can significantly stimulate complementary intermolecular end-joining ligations by *E. coli* DNA ligase when the polymerases are present at concentrations lower than that of the DNA substrates. In contrast, high polymerase concentrations relative to the DNA substrates inhibit the intermolecular ligation activity of DNA ligase. Neither polymerase was able to stimulate the DNA ligase from T4 bacteriophage. Additionally, nick-closure by *E. coli* DNA ligase (but not T4 ligase) is slightly stimulated by both polymerases, but only at about 10% of the magnitude seen for end-joining enhancement. The data represent one of the first observations of direct polymerase-ligase interactions in prokaryotes, and suggest that the polymerases stabilize the associated DNA ends during intermolecular ligation, and that such a complex can be taken advantage of by some, but not all, DNA ligases.

**1. Introduction**

Interactions between DNA polymerases and DNA ligases have been documented in a number of different eukaryotic systems, both in the context of base-excision repair and single-strand break repair [1–3]. In this study, we report a prokaryotic polymerase-ligase cooperation that functions in the context of DNA end-joining. On their own, DNA ligases can catalyze the end-joining of two DNA fragments with complementary ends and the repair of a single-stranded nick within double stranded DNA. In conjunction with different cooperating proteins, including ones that hold two DNA fragments together or that perform nuclease or polymerase dependent processing, DNA ligases can also end-join non-complementary DNA fragments in processes such as non-homologous end-joining (NHEJ) [4,5].

Both Klenow and Klentaq DNA polymerases (the large fragment domains of the Pol I polymerases from *E. coli* and *T. aquaticus*, respectively) show tight binding affinity to DNA fragments with different types of end-structures including 5’ overhangs, 3’ overhangs, and blunt ends [6]. Furthermore, previous studies of the end-joining abilities of Pol I DNA polymerases have shown that both *E. coli* and *T. aquaticus* DNA polymerases are able to use discontinuous templates to produce fill-in products between two DNA fragments [7–10]. This DNA synthesis-dependent end-joining activity has been demonstrated with fragments having fully complementary overlapping ends, with fragments having limited complementary regions that are interior to non-complementary end sequences, and with blunt ended fragments [7–10]. In vivo evidence for this synthesis-dependent end-joining activity in *E. coli* has also been described [10].

The present study demonstrates that *E. coli* and Taq Pol I DNA polymerases will also facilitate DNA end-joining activity in the absence of DNA synthesis by enhancing the complementary end-joining activity of *E. coli* DNA ligase when the polymerases are present at sub-stoichiometric concentrations relative to DNA fragments. The effects of both Pol I polymerases on ligation are specific to *E. coli* DNA ligase, with neither polymerase able to enhance T4 DNA ligase. We hypothesize that this effect is due to an ability of the Pol I DNA polymerases to stabilize association of the two DNA fragments combined with a productive interaction with *E. coli* DNA ligase.

**Abbreviations:** KLN, Klenow DNA polymerase; KTQ, Klentaq DNA polymerase; BSA, bovine serum albumin; NHEJ, non-homologous end-joining.

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2. Experimental procedures

2.1. Materials

Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The DNA substrate sequences are shown in Table 1. Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and using extinction coefficients provided by the manufacturer. All DNAs are 3’ phosphorylated. The DNA molecules used in 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 [11]. Hairpin DNAs were annealed from single-strand DNA by heating at 95 °C for 5 min and slowly cooling down to ambient temperature.

Klenow (KLN) and Klentaq (KTQ) polymerases were purified in our laboratory as previously described [12]. 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 [13]. Protein concentrations were measured at 280 nm and calculated by using ε280 values of 5.88 × 10⁴ M⁻¹ cm⁻¹ for Klenow and 7.04 × 10⁴ M⁻¹ cm⁻¹ for Klentaq. E. coli DNA ligase and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA).

2.2. Methods

Ligation reactions were performed in each DNA ligase’s corresponding commercially supplied reaction buffer containing identical amounts of DNA substrates, a fixed amount of one of the DNA ligases, and increasing amounts of one of the DNA polymerases or bovine serum albumin (BSA). The reaction volume was 10 µl. Intermolecular ligations with two pieces of DNA having complementary 5-base 3’ overhangs (hp27 and hp57 in Table 1) or intramolecular ligations with nicked DNA (Table 1) were performed at 25 °C for the times indicated in each of the figures. For end-joining reactions: 10 µl reactions contained 250 nM of hp57, 500 nM of hp27, 0.3 units of E. coli or T4 DNA ligase, and variable amounts of added polymerase or BSA as indicated in each figure. Higher concentration of the smaller hairpin were added simply to help with visualization on the gels. For nick closure reactions: 10 µl reactions contained 150 nM of nicked DNA, 0.2 units of E. coli DNA ligase, or 0.1 units of T4 DNA ligase, and increasing amounts of polymerase. Ligation reactions were stopped with 6 µl of a stop buffer containing 0.2% SDS, 10 mM EDTA, 90% formamide, and 0.1% bromophenol blue, by heating at 95 °C for 10 min, and then immediately putting on ice for 5 min. A 4.5 µl of sample of each stopped reaction 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. Each ligation reaction was performed 2–3 times.

The amount of ligated product on the denaturing polyacrylamide gels was quantitated using the program ImageQuant 5.1 (GE Healthcare). 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 controls. The difference in intensities between each product band and that of the corresponding background control yields the relative amount of ligation product formed, which is reported herein as an approximate enhancement of ligation in all figures. The variability in band quantification upon repeated trials was ±4–8%.

3. Results

3.1. Both Klenow and Klentaq polymerases stimulate E. coli DNA ligase activity at sub-stoichiometric concentrations

Hairpin DNA fragments (hp27 and hp57 in Table 1) containing complementary 5-base 3’ overhangs were used as the substrates to assess the DNA ligase catalyzed intermolecular end-joining to form a fully ligated 84 base construct in vitro. Fig. 1 shows the enhancing effects of increasing amounts of Klenow and Klentaq polymerases in reactions containing a constant amount of E. coli DNA ligase over differing reaction incubation times. Reactions were performed in the presence of excess starting DNA substrates relative to DNA ligase units (i.e. more DNA than the DNA ligase should react with in the time frame of the experiments). In addition, all polymerase concentrations used in Fig. 1 are well below stoichiometric concentrations relative to the DNA fragments (i.e. the starting concentrations of the DNA fragments are 250 and 500 nM while the highest polymerase concentration in these reactions is 50 nM). It can be seen that shorter incubation times (6 min) remain well below the point of substrate exhaustion, while the longer incubation times (20 min) are reaching substrate exhaustion and maximal product formation.

To rule out a generic protein effect or a molecular crowding effect, the ligation reactions were also characterized in the presence of increasing amounts of bovine serum albumin (BSA), which, as shown in Fig. 2, displays no enhancement of the DNA ligation activity, and in fact shows a slight (~20–25%) inhibition of ligation activity. To examine the specificity of the functional interaction between Pol I DNA polymerase and the NAD⁺-dependent E. coli DNA ligase, we examined whether the Pol I polymerases could simulate the ATP-dependent T4 DNA ligase. In contrast to their effects on E. coli DNA ligase, Fig. 2 shows that neither polymerase enhances

Table 1

| hp27 | AAGCCACGTCCCGGGTTTT-3’ |
| hp57 | AAGGGTCTGCTCAACCGCACTAGCAG3’ |
| nickedDNA | AAGCCACGTCCCGGGTTTT-3’ | 5’ |

*Regions of the sequences shown in bold are base-paired double-strands.
the activity of T4 DNA ligase, instead Klenow has little or no effect on T4 ligase while Klentaq inhibits T4 ligase by up to 40–50%.

3.2. High concentration of Klenow and Klentaq inhibit ligation of E. coli DNA ligase

To further explore the potential mechanism of stimulation, we examined the effects of significantly higher concentrations of polymerases – approaching stoichiometric values relative to the DNA fragments (in all end-joining experiments the DNA fragment concentrations are 250 nM for hp57 and 500 nM for hp27). Fig. 3 shows that in the presence of 500 nM of either polymerase, ligation of the two DNA fragments is inhibited, while the earlier demonstrated enhancement at lower concentrations is shown again in the 50 nM lanes. Similar inhibition was seen in the presence of 300 nM of either polymerase (data not shown). Fig. 3 also shows that high concentrations of BSA do not effect ligase activity—i.e. BSA neither enhances ligation at lower concentrations (Fig. 2) nor inhibits ligation at higher concentrations (Fig. 3).

Interestingly, Fig. 3 also shows that higher concentrations of Klenow and Klentaq polymerases also inhibited DNA end-joining by T4 DNA ligase, despite the absence of an enhancement effect on T4 ligase at lower polymerase concentrations. The 84bp ligated band is similar in intensity in the 0 and 50 nM [polymerase] lanes for T4 ligase in Fig. 3, while the 500 nM [polymerase] lanes for both polymerases show inhibition of T4 ligase. Similar inhibition of T4 ligase was also seen at 300 nM concentrations of both polymerases (data not shown). Quantitation of band intensities at the high polymerase concentrations in Fig. 3 indicated that the end-joining reactions are inhibited relative to the absence of polymerases by about 10–20% for both E. coli and T4 ligases (i.e. compare 0 polymerase lanes and 500 nM polymerase lanes).

3.3. Polymerases slightly enhance ligation of a nicked DNA by E. coli ligase

It is possible that Klenow and Klentaq polymerases could enhance the E. coli DNA ligase reaction via a direct polymerase-ligase interaction, or by forming a complex with both DNAs and stabilizing their non-covalent association, or both. The data suggest that both modes of action are involved, but that stabilization of the bi-molecular DNA association is a dominating effect. The fact that both polymerases stimulate E. coli DNA ligase but not T4 DNA ligase suggests a direct polymerase-protein interaction. To further examine these two possibilities, a double-hairpin DNA construct with a single interior nick was examined. In these reactions there is
no need for two DNAs to associate before the ligation reaction can occur. Fig. 4 shows that nick closure by *E. coli* DNA ligase is slightly enhanced at up to 20 nM concentrations of either polymerase, while T4 DNA ligase is slightly inhibited at all concentrations of either polymerase. Fig. 4 further supports the hypothesis of a direct favorable interaction between *E. coli* ligase and the polymerases, but both of these effects are within ±20–25% of the ligation activity in the absence of polymerases and are thus substantially more subtle than the 250–600% effects of the polymerases on end-joining activity by *E. coli* DNA ligase seen in Fig. 1. This >10 fold difference in the magnitude of the effects thus suggests that polymerase involvement in holding complementary fragments together in the reactions in Fig. 1 is a major part of the enhancement of DNA end-joining. Much higher polymerase concentrations (up to 500 nM of each polymerase) showed no further enhancement or inhibition of nick closure (data not shown).

4. Discussion

4.1. A prokaryotic version of a known eukaryotic protein-protein interaction

The data presented in this study demonstrate that both *E. coli* and *T. aquaticus* Pol I DNA polymerases can stimulate inter-
molecular DNA ligation by *E. coli* DNA ligase. Interactions between polymerases and DNA ligases have been directly documented previously in several eukaryotic systems within the context of different DNA repair processes, but not in prokaryotic systems. For example DNA polymerase I and DNA ligase I have long been shown to cooperate during base excision repair [1], where instead of mediating DNA end-joining, the DNA ligase catalyzes nick closure at completion of the repair process. A direct interaction between mitochondrial DNA polymerase γ and DNA ligase III has also been documented, again with mechanistic links to base excision repair [3]. Interaction between DNA ligase I/Iιx and poly (ADP-ribose) polymerase-1 (PARP-1) has been shown to be involved in single-strand break repair [2]. Interestingly, in prokaryotic systems, Ignatov and Kramarov have reported that adding ligase enhances the PCR amplification of long DNA sequences, and suggested that a polymerase-ligase interaction might be involved, but did not further characterize the hypothesized interaction [14].

Enhancement of *E. coli* DNA ligase by small molecules has been documented by a number of researchers. The original studies of *E. coli* DNA ligase by Lehman and associates demonstrated the enhancement of ligase activity by cations, especially ammonium ion [15]. Several different researchers have shown that molecular crowding agents and other small molecules enhance ligase activity [16–19]. The present report appears to be the first demonstration of a protein-based prokaryotic DNA ligase enhancement effect that is not crowding based. Beyond its potential fundamental roles in DNA replication and repair, enhancement of DNA ligation in general also has some biotechnological utility as evidenced by the patenting of small molecule enhancers of DNA ligation [18] and by the recent ligation efficiency enhancement studies of Suzuki et al. [20]. The approximately 2.5–6 fold (250–600%) enhancements of DNA ligase activity seen in the presence of Pol I polymerases in this study (Fig. 1) are similar to the range of cation enhancement effects originally described Modrich and Lehman [15] and similar to the range of enhancements described in the Kucera and Evans patent, which covers small molecules that enhance DNA ligation "by at least 25%" and up to about 9 fold [18].

4.2. Hypothetical model for Polymerase-DNA ligase cooperation

The data described herein suggest a simple model to explain the stimulation of ligation by the Pol I polymerases where at low concentrations of protein, one polymerase molecule could simultaneously bind both pieces of DNA, stabilizing the complementary association between the fragments. DNA ligase would then need to either interact directly with the polymerase to gain access to the ligation sites, or the ligation sites may be exposed in such a way in the putative polymerase-double DNA complex that *E. coli* DNA ligase can access them. At high polymerase concentrations, polymerases would be available to bind to individual DNA ends to form 1:1 (DNA-polymerase) complexes rather than 1:2 (DNA-polymerase-DNA) complexes. This would impede rather than enhance association of the DNA fragments, and result in the high concentration inhibition of ligation demonstrated in this study. The series of studies by Morgan and associates on DNA synthesis-dependent DNA end-joining by Pol I polymerases hypothesized a similar 1:2 DNA-polymerase-DNA complex to facilitate the end-joining results they observed [8,9]. Neither Morgan and associates, nor our laboratory, has (yet) been able to empirically demonstrate the existence of such a 1:2 complex, suggesting that, if it exists, it may be a transient or very low affinity complex.

4.3. Species specificity of the polymerase-DNA ligase interaction

The data in this study show that while both Pol I polymerases stimulate the activity of *E. coli* DNA ligase, neither stimulates the activity of T4 DNA ligase. This species specificity indicates that the mechanism of polymerase stimulation involves more than just holding the two DNA fragments together for longer than they might associate on their own — if this were the only mechanism for stimulation, both DNA ligases should be stimulated. This species specificity implies some sort of direct interaction and/or structurally specific characteristics of *E. coli* DNA ligase that allow it to cooperate/interact with the polymerases. It is notable that all of the documented eukaryotic polymerase-ligase interactions either demonstrate or postulate a direct protein-protein interaction between the two enzymes [1–3,7–9]. *E. coli* DNA ligase is a NAD+-dependent DNA ligase while T4 DNA ligase is an ATP dependent DNA ligase with only about 2% identity to *E. coli* DNA ligase [21,22]. Interestingly, the original cation enhancement effect seen for *E. coli* DNA ligase is also absent for T4 ligase [15]. The significant evolutionary/sequence distance between *E. coli* and T4 DNA ligases certainly make it plausible that the structure/function motif(s) on *E. coli* DNA ligase that allows it to cooperate/interact with the polymerases never evolved in T4 DNA ligase. Interestingly
for T4 ligase, in contrast to the absence of stimulation by polymerases at low stoichiometric ratios, higher concentrations of polymerases inhibited DNA end-joining by both *E. coli* and T4 DNA ligases (Fig. 3), further suggesting that when too many polymerase molecules are present and binding to individual DNA ends, any species of ligase may be occluded from accessing the DNA.

### 4.4. Possible relationship to non-homologous end-joining (NHEJ)?

Pursuant to DNA end-joining, DNA ligases are known to directly associate with several different types of partner proteins in different systems, a notable interaction being the Ku-LigD association involved in prokaryotic NHEJ, where Ku protein bridges the two DNA fragments and LigD (DNA ligase D) is a multifunctional enzyme having both polymerase and DNA ligase activities (as well as a 3’ phosphoesterase domain) (for reviews see [4,5]). In eukaryotic NHEJ, human Ku protein has also been shown to stimulate the end-joining activity of other human DNA ligases, and notably similar to the results shown herein for Klenow and Klentaq polymerases, Ku also does not significantly enhance ligation at isolated single nicks [23]. One of the hypothesized modes of action in the present study is also similar to the model for the stimulation of Ku on ligation of DNA ligase IV in mammalian cells, where Ku stabilizes the association of the two DNA fragments, then moves inward on DNA when DNA ligase IV binds to allow ligation [23]. Also similar in this mammalian Ku-Ligase IV interaction is the fact that higher (super-stoichiometric) concentrations of Ku inhibit Ligase IV activity [24].

It has been noted that *E. coli* does not contain a Ku homologue [25]. For *T. aquaticus*, we performed iterative PSI-BLAST database searches [26] using the sequences of Yk0v, MkgU70, Yku70p, Yku80p and LigD as queries, but no homologies to Ku or LigD were detected in *Thermus thermophilus* HB27 or *Thermus thermophilus* HBB, each having high similarity to *Thermus aquaticus*. Thus, it appears that there is not a homologous Ku-LigD or Ku-Ligase IV NHEJ system in *Thermus* bacteria either.

The data in the present study focus exclusively on DNA fragments with complementary ends, but such complementary double-strand breaks will also be repaired by the prokaryotic Ku-LigD or eukaryotic Ku-Ligase IV NHEJ systems. The data from this study combined with the previous studies by Morgan and associates on synthesis-dependent end-joining suggest that it may be fruitful to further examine whether in *E. coli* and *T. aquaticus*, the Pol I DNA polymerases may at least partially fulfill the NHEJ function that the Ku protein system performs in other systems, by facilitating DNA ligase end-joining repair of both homologous double-strand breaks (this study) and DNA-synthesis-dependent repair of non-homologous double-strand breaks [7–10].

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### Transparency document

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