Sealing of gaps in duplex DNA by T4 DNA ligase

Stefan V. Nilsson and Göran Magnusson

The Medical Nobel Institute, Department of Biochemistry, Karolinska Institute, S-104 01 Stockholm, Sweden

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ABSTRACT

Single-strand gaps in DNA molecules were found to be a substrate for T4 DNA ligase. Sealing of the gaps was optimal at the same conditions as ligation of blunt-ended DNA molecules. Spermidine at a concentration of 2 mM stimulated the ligation of gaps, as well as the joining of DNA molecules with cohesive and blunt ends. In addition, spermidine reduced the optimal ATP concentration. The ligation of single-strand gaps was a slow process, reaching a plateau after several hours at 25°C. Approximately 10% of circular duplex plasmid pBR322 DNA molecules with a gap of 1-5 nucleotides could be converted to a covalently closed form. When such molecules were used for transformation of E. coli cells deletion mutants were obtained at a high frequency. The size and position of the gaps and the deletions were equivalent, confirming that T4 DNA ligase was sealing the gaps.

INTRODUCTION

DNA ligases have been isolated from a variety of sources. The enzymes from E. coli and phage T4 which have been extensively studied (for a review see ref. 1), differ in their substrate specificity. The E. coli enzyme seals only abutted termini of base-paired DNA chains (2), whereas the T4 enzyme can catalyze some unconventional reactions like the joining of DNA and RNA chains (3), or fully base-paired double-stranded DNA molecules (4).

DNA ligases are indispensable tools in the construction of rearranged genomes. T4 DNA ligase is particularly useful in this work, since the enzyme catalyzes the joining of DNA molecules with both cohesive and blunt ends. In this paper we show that T4 DNA ligase can seal gaps in DNA molecules, and that the reaction, as well as the joining of DNA molecules, is stimulated by spermidine.
MATERIALS AND METHODS

Enzymes. DNA polymerase I (fraction 7) was purified as described by Jovin et al. (5). Exonuclease III was obtained as a by-product of the DNA polymerase I purification (5), and was further purified by DEAE-cellulose chromatography, to remove traces of endonuclease activity. T4 DNA ligase was purified from T4am122 infected E. coli BO cells according to Panet et al. (6). DNA ligase activity was assayed using DNA molecules with cohesive termini as a substrate. One unit of DNA ligase activity was defined according to New England Bio-Labs as the amount of enzyme joining 50% of BamHI gene-generated cohesive termini at a concentration of 0.12 μM during 30 min incubation at 16°C. Restriction endonucleases and nuclease S1 were obtained from New England Bio-Labs and Sigma Chemical Co., respectively.

DNA. Plasmid pBR322 DNA (7) was prepared from lysates of E. coli cultures (8) grown to stationary phase. Further purification was achieved by treatment with pancreatic RNAse (Sigma Chemical Co.) and proteinase K (E. Merck), extraction with phenol, ethanol precipitation and, finally, sedimentation in neutral sucrose gradients.

DNA with single-strand breaks was prepared by digestion with restriction endonucleases in the presence of ethidium bromide (9). Nicks were extended to gaps by treatment with DNA polymerase I (approximately 10 units per pmol of 5'-ends) in 70 mM Tris-HCl (pH 7.6), 70 mM MgCl₂, 10 mM β-mercaptoethanol for 30-120 min at 15°C (10). The enzyme was removed by extraction with phenol:chloroform (1:1). After ether extraction, DNA was used as a substrate for T4 DNA ligase, or in cases where gaps larger than 5 nucleotides were desired, further treated with exonuclease III. The digestion with exonuclease III was performed at 18°C in the medium described above, using approximately 15 units of enzyme per pmol of 5'-ends. The reaction was terminated by extraction with phenol:chloroform, followed by ether.

Incubations with T4 DNA ligase, carried out as detailed below, were terminated by heating at 65°C for 5 min. Before analysis by gel electrophoresis, sodium dodecyl sulfate and
EDTA were added to the samples at final concentrations of 0.1% and 10 mM, respectively.

Electrophoresis. Horizontal gels of 0.7% agarose (15x15x0.5 cm) were run in electrophoresis buffer (11) containing 0.5 μg per ml of ethidium bromide. DNA was visualized in U.V. light and quantitated by densitometer tracing of photographs. Vertical polyacrylamide 3-10% gradient gels (14x10x0.02 cm) were prepared in electrophoresis buffer without ethidium bromide.

RESULTS

Gapped duplex DNA used as a substrate for T4 DNA ligase. The results of preliminary experiments suggested that T4 DNA ligase can catalyze the sealing of single-strand breaks in duplex DNA where several nucleotides are missing. To study this reaction further a substrate was prepared consisting of double-stranded circular pBR322 DNA nicked by the restriction endonuclease Bam HI. The nicked DNA was exonuclease-treated to form gaps of 1-100 nucleotides, and then incubated with T4 DNA ligase under the conditions used for the joining of duplex DNA molecules with blunt ends. Fig. 1 shows the analysis by agarose gel electrophoresis of samples that were either run directly on the gel, or first digested with Bam HI. In gels containing ethidium bromide, closed circular DNA molecules (DNA I) migrate ahead of linear DNA (12). After treatment with Bam HI, approximately 5% of the gapped DNA molecules were converted to a form with the electrophoretic mobility of DNA I. The ligation product of DNA molecules with large gaps (Fig. 1 lanes 3,4) formed a double band, suggesting that gaps of certain sizes might be preferentially sealed. In a separate experiment the molecules with the mobility of DNA I were eluted from the gel, treated with phenol and still found to be resistant to Bam HI cleavage, but converted to linear DNA by digestion with another restriction endonuclease. The rate of ligation of nicks and gaps was widely different. With the amounts of enzyme used in these experiments nicks were ligated within seconds, whereas the sealing of gaps did not reach a plateau until after several hours.
Figure 1. Ligation of DNA molecules containing a single-strand gap. Plasmid pBR322 DNA containing a nick at the single BamHI site was treated with DNA polymerase I at 15°C for 45 min. Part of the DNA was further treated with exonuclease III for 10 and 20 sec, respectively, at 18°C. The gap-containing DNA was incubated with T4 DNA ligase (50 units/µg DNA) at 25°C for 12 hr in a medium consisting of 1 mM ATP, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.6), 10 mM β-mercaptoethanol. Before analysis by agarose gel electrophoresis, half of each DNA sample was digested with BamHI. The DNA substrates for the ligase reactions contained: a nick at the BamHI site (lane 1), a gap generated by DNA polymerase I alone (lane 2), or in combination with exonuclease III digestion for 10 sec (lane 3), or 20 sec (lane 4). Lane M shows the positions of native covalently closed circular (I) and relaxed circular (II) DNA. The position of linear DNA (III) is also indicated.

Effect of reaction conditions on the ligation of gaps. In initial attempts to find optimal conditions for the gap sealing activity of T4 DNA ligase the influence on the reaction rate of temperature, salt concentration and pH was investigated.

Temperatures from 5-37°C were tested and the ligase activity was found to be highest at 25-37°C, when measured as in the experiment of Fig. 1. In contrast, variation of the salt concentration from 10-150 mM NaCl, and of the pH from 6.8-8.0 had little influence on the reaction rate.

The effect of spermidine on the ligation of gaps was also investigated. A concentration of 1-2 mM spermidine increased the fraction of gapped DNA molecules that was converted into a covalently closed form approximately 2-fold. It appeared possible that in the presence of spermidine the optimal concentrations of ATP and MgCl₂ were altered. These parameters were therefore varied in the presence of 2 mM spermidine.
The result of the experiment presented in Fig. 2 shows that the highest rate of ligation was obtained with 0.1 mM ATP and 2-8 mM MgCl$_2$. These concentrations are lower than those used in the previous experiments.

Construction of deletion mutants by ligation of gaps in DNA. To prove that T4 DNA ligase catalyzes the sealing of gaps and to investigate the effect of the gap size on the ligation process, T4 DNA ligase treated DNA was used for transformation of E. coli cells. Clonally isolated preparations of DNA were then analyzed for the presence of deletions.

Plasmid pBR322 DNA was treated with Bam HI to introduce a nick at the recognition site of the enzyme. The nicked DNA was treated with DNA polymerase I in the absence of deoxy-nucleoside triphosphates to generate gaps with a size of a few nucleotides, and part of the material was further treated with exonuclease III for 1 or 2 min, to produce gaps of an estimated size of a few to several hundred nucleotides. The

![Figure 2. Influence of ATP and MgCl$_2$ concentrations on the ligation of gaps. Plasmid DNA containing a DNA polymerase I generated gap at the BamHI site was incubated with T4 DNA ligase (25 units/µg DNA) for 60 min at 25°C in 50 mM Tris-HCl (pH 7.6), 10 mM 8-mercaptoethanol and 2 mM spermidine. MgCl$_2$ was added to a final concentration of 1.6 (1), 8 (2) or 40 mM (3), in the presence of 0.1 mM (a) or 1.0 mM (b) ATP. Before agarose gel electrophoresis, the DNA samples were treated with BamHI. Lanes 4 and 5 show the ligation products of plasmid DNA containing a nick after and before BamHI cleavage, respectively.](image)
gap containing DNA was then incubated with T4 DNA ligase for 16 hr under optimal conditions. After ligation, remaining gaps were filled in by DNA polymerase I in the presence of deoxynucleoside triphosphates, and the DNA samples were digested with Bam HI to linearize molecules with an intact Bam HI site.

Following transformation of E. coli cells, plasmid containing bacteria were selected on ampicillin containing medium. Individual colonies were then tested for tetracyclin resistance, since deletions at the Bam HI site interrupt the tetracyclin resistance gene (13). All three DNA preparations yielded approximately 30% tetracyclin sensitive colonies, suggesting that gaps of various sizes could be sealed by ligation.

Plasmid DNA was prepared from individual clones and analyzed by restriction endonuclease digestion. Cleavage with Bam HI confirmed that most mutant genomes had lost the Bam HI site. Further analysis by HaeIII digestion showed (Fig. 3) that all mutants produced from gaps formed by DNA polymerase I alone had deletions that did not detectably alter the mobility of any DNA fragment on the gel (lane 1), whereas DNA molecules with larger gaps generated deletions of various sizes, up to approximately 100 base pairs. In Fig. 3 two mutants with deletions of approximately 5 (lane 2) and 30 base pairs (lane 3) are shown. Since no deletions were formed without ligase treatment of the gap containing DNA, we conclude that T4 DNA ligase is able to seal gaps with sizes from 1-2 to more than 100 base pairs with a similar efficiency.

**Effect of spermidine on the joining of DNA molecules by T4 DNA ligase.** The stimulatory effect of spermidine on the activity of T4 DNA ligase in closing gaps in DNA lead us to investigate the effect of the polyamine on other ligase catalyzed reactions.

DNA molecules with cohesive ends formed by cleavage with certain restriction endonucleases are efficiently joined by DNA ligases. To test the influence of spermidine on this reaction, pBR322 DNA cleaved with Bam HI at the single
Figure 3. Polyacrylamide gel electrophoresis of plasmid DNA digested with HaeIII. Plasmid DNA was prepared from clones of E. coli cells that had been transformed with DNA treated with T4 DNA ligase to seal gaps. The original gaps were generated by DNA polymerase I alone (lane 1) or in combination with exonuclease III digestion for 1 min (lanes 2 and 3). After digestion with HaeIII the DNA segments were separated by polyacrylamide gel electrophoresis. Lane M shows the fragments generated by cleavage of parental plasmid DNA. The size of the fragment containing the BamHI site (123 base pairs), as well as the size of the two neighbouring fragments is indicated.

recognition site was incubated with T4 DNA ligase in the presence of 2 mM spermidine. At the low DNA concentration used in the experiment circular monomers of DNA was the major reaction product. The result of the experiment shows (Fig. 4) that spermidine not only increased the rate of the reaction, but also the final amount of product formed. In a separate experiment (data not shown) the concentration of spermidine was varied. Again it was found that 1-2 mM spermidine was optimal in the stimulation of the ligase activity. At a concentration of 10 mM spermidine the formation of circular monomers was effectively inhibited. Instead, multimeric DNA molecules were formed. This result could be
Figure 4. Joining of DNA molecules with cohesive ends in the presence of spermidine. Plasmid DNA molecules linearized by BamHI digeston were incubated with T4 DNA ligase (100 units/μg DNA) at 15°C in a medium consisting of 0.1 mM ATP, 2 mM MgCl₂, 50 mM Tris-HCl (pH 7.6) and 10 mM β-mercaptoethanol with (○—○) or without (○—○) the addition of 2 mM spermidine. At the indicated time points samples were withdrawn and analyzed by agarose gel electrophoresis.

an effect of spermidine induced DNA condensation (14), which makes linear DNA molecules inflexible.

The effect of spermidine on the ligation of DNA molecules with flush ends was also tested. The substrate for this reaction was prepared by digestion of pBR322 DNA with the restriction endonuclease PvuII which leaves blunt ends. To avoid possible effects of specific DNA sequences at the ends of the molecules, the linear DNA molecules were treated with nuclease S1, and then incubated with DNA polymerase I in the presence of deoxynucleoside triphosphates to make the DNA molecules flush-ended again (15). Incubation with T4 DNA ligase and 1 mM ATP was done at a DNA concentration of 20 μg/ml, corresponding to a concentration of molecular ends of 15 nM, was performed in the presence or absence of 2 mM spermidine. The results are summarized in Fig. 5. It is clear that spermidine increased both the rate of blunt-end ligation and the amount of ligated DNA formed after 16 hr reaction. The dominating reaction product appeared to be a linear dimer, but significant amounts of circular monomers were also for-
Figure 5. Joining of blunt-ended DNA molecules in the presence of spermidine. Plasmid pBR322 DNA was linearized by digestion with PvuII and treated with nuclease S1 for 30 min at 25° C in the presence of 4.5 mM zinc acetate (25). Ragged ends were then repaired with DNA polymerase I. Incubation with T4 DNA ligase was done at 25° C as described in the legend to Fig. 4 with (o--o) or without (o---o) the addition of 2 mM spermidine. Samples were withdrawn at the indicated time points and analyzed by agarose gel electrophoresis.

med, particularly in the presence of spermidine.

DISCUSSION

The objective of this study was to develop a method for the introduction of small deletions in DNA. Such methods have been described (16,17,18) which all involve cleavage of DNA molecules in vitro and subsequent joining of the ends, either in vitro or in vivo. Using these methods the ligation of blunt DNA termini is required in many cases. Although this reaction is catalyzed by T4 DNA ligase, an apparent $K_m$ of 50 μM for 5'-termini (19) makes the process very inefficient at the low DNA concentrations used to avoid the formation of multimers.

Heteroduplexes of DNA with a deletion in one of the strands generate both wild type and mutant progeny when introduced to cells (20). When such heteroduplexes of plasmid pBR322 DNA were used for transformation of E. coli cells, approximately half of the recipient cells were found to carry progeny plas-
mid with the deletion, whereas very few cells carried both wild type and mutant plasmid (unpublished experiments). It was therefore obvious that the sealing of gaps in circular pBR322 DNA, leading to the formation of a heteroduplex equivalent, would result in the generation of deletion mutants after transformation of E. coli cells.

Treatment of circular plasmid DNA containing a gap in one of the strands with T4 DNA ligase led to the formation of DNA molecules, with the electrophoretic mobility of covalently closed circular DNA, which were resistant to Bam HI digestion (Fig. 1). The optimal temperature of this reaction was 25-37°C. Even at those temperatures, however, the reaction was slow and did not reach a plateau until after several hours. The relatively low yield of product made a quantitative assay of this ligase activity difficult. Moreover, we did not have a homogeneous substrate, since the methods used in this study did not allow the preparation of DNA molecules with gaps of defined size. This problem is illustrated by the experiment shown in Fig. 2, where much of the linear DNA resulted from Bam HI cleavage of DNA that had an intact cleavage site. Because of these difficulties, the fraction of ligated gaps in different experiments cannot be compared directly.

The optimal conditions for the ligation of gaps and joining of blunt-ended DNA molecules were similar. Spermidine stimulated both processes (Figs. 2 and 5), as well as the circularization of DNA molecules with cohesive ends (Fig. 4). In the presence of the polyamine ligation of blunt-ended DNA proceeded at similar rates using 0.1 or 1.0 mM ATP, whereas the sealing of gaps was optimal at the lower ATP concentration (Fig. 2).

Spermidine at a concentration of 1 mM was previously shown to stimulate T4 DNA ligase in the joining of synthetic oligonucleotides (21,22). In contrast, spermidine at a 10-fold higher concentration inhibited these reactions, as well as the T4 DNA ligase mediated joining of DNA molecules with blunt, but not with cohesive, ends (23). In addition to DNA ligase spermidine has been shown to stimulate various enzymes which use DNA as a substrate. These effects
probably result from binding of spermidine to DNA which leads to charge-neutralization and condensation of double-stranded molecules (14), and to the aggregation of DNA at spermidine concentrations above 2 mM (24).

The ability of T4 DNA ligase to seal single-strand gaps in DNA was proved by the generation of deletion mutants of plasmid pBR322. From these experiments (Fig. 3) it appeared that T4 DNA ligase can seal gaps varying in size from 1-100 nucleotides. After transformation of E. coli cells with circular DNA molecules containing the equivalent of a heteroduplex loop, deletion mutants of the plasmid were isolated. To obtain a high frequency of mutants, the removal of unligated circular DNA is essential, since these molecules are repaired to wild type plasmid in transformed cells. In the experiment aiming at the removal of the single Bam HI site of pBR322 DNA this was done by Bam HI digestion after filling-in of unsealed single-strand gaps with DNA polymerase. Following this protocol the mutation frequency was 30-40%.

When selection of mutants by restriction enzyme digestion was impossible the unligated DNA molecules were instead made single-stranded by exonuclease III treatment, and then fragmented by a brief nuclease S1 digestion. Using this procedure deletion mutants were isolated at a frequency of 5-10%.

The deletions had the same size range as the gaps, suggesting that the DNA strand shortened by ligation was used directly as a template for DNA synthesis during repair of the heteroduplexes. The size of the gaps formed by DNA polymerase I in the absence of deoxynucleoside triphosphates have been estimated to be less than 5 nucleotides. This is consistent with the size of the deletions which was smaller than 6 base pairs. In contrast, gaps formed by exonuclease III digestion had a heterogeneous size which, however, was proportional to the digestion time. Gaps formed by incubation with exonuclease III for 2 min, did not produce mutants with deletions larger than 100 base pairs (Fig. 3), although some of the molecules had considerably larger gaps, as judged by a separate analysis. It is conceivable that large gaps are sealed only if the
single-stranded region of the uninterrupted DNA chain forms base-paired loops which juxtapose the free ends of the other chain. The electrophoretic mobility of the ligation product shown in Fig. 1 might result from such an effect. However, the sealing of gaps does not require the presence of specific DNA sequences, since we have generated deletions at many sites. Furthermore, the free ends of the DNA chain do not have to be abutted, since gaps of two nucleotides could be sealed. An alternative explanation for the absence of plasmids with large deletions is that the bacteria do not take up or degrade molecules with large loops.

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REFERENCES