Modification of *Escherichia coli* DNA Ligase by Cleavage with Trypsin*

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Limited treatment of *Escherichia coli* DNA ligase with trypsin results in rapid loss of DNA joining activity. However, the ability to react with DPN to form the covalent enzyme-AMP intermediate is unaffected. The cleaved enzyme is also unable to catalyze the formation of DNA-adenylate, the second covalent intermediate in the ligase-catalyzed reaction. These findings demonstrate that portions of the DNA ligase molecule that are required for phosphodiester bond formation are not required for at least one of the partial reactions catalyzed by this enzyme.

*Escherichia coli* DNA ligase catalyzes the synthesis of phosphodiester bonds between adjacent 3'-hydroxyl and 5'-phosphoryl termini in duplex DNA. The reaction proceeds in a sequence of three steps involving two covalent intermediates, enzyme-AMP and DNA-adenylate (1).

After extensive dialysis of a partially purified preparation of *E. coli* DNA ligase, Zimmerman and Oshinsky (2) observed two altered forms of the enzyme, one with a significantly reduced molecular weight. Both forms were able to react with DPN to yield enzyme-AMP, the first of the two intermediates. The enzyme-AMP could then react with NMN to regenerate DPN to yield enzyme-AMP, the first of the two intermediates. The subsequent demonstration that the enzyme consists of a single polypeptide of molecular weight 75,000 (3) ruled out the possibility that these altered forms might represent subunits that dissociated during dialysis. Another and perhaps more likely explanation is that the altered forms were products of proteolysis. We have found, in fact, that the DNA joining activity of *E. coli* DNA ligase is destroyed much more rapidly by proteolytic digestion than is the ability to form enzyme-AMP (4). In an effort to understand the nature of the modification caused by such treatments, we have undertaken the isolation and partial characterization of the products of limited proteolysis of *E. coli* DNA ligase.

**Experimental Procedure**

*Materials*—The *Escherichia coli* DNA ligase preparations used were Fraction VII and Fraction VII rechromatographed on DEAE-Sephadex; specific activity, 10,000 units/mg. Both were homogeneous as judged by polyacrylamide gel electrophoresis and by analytical sedimentation (3). &-1-Tosylamido-2-phenylethyl chloromethyl ketone trypsin obtained from Worthington (219 units/mg) was dissolved in 0.001 N HCl at a concentration of 80 μg/ml and frozen in small aliquots which were thawed as needed. Exogenous trypsin inhibitor (Worthington code S.I.) was dissolved in distilled water to 1 mg/ml and stored frozen. DEAE-Sephadex A-50 and Sephadex G-50 (Pharmacia) were equilibrated according to the recommendations of the manufacturer. The synthesis of [3P]DPN has been described previously (3), as has the preparation of [*H*]-labeled d(A-T) 1000 copolymer (5) and d(A) 1000 (6). [2-14C]Nicotinamide-DPN (50 mCi/mmol) was purchased from Amersham/Searle. d(pT) 1000 and d(pT) 100, with a dideoxythymidylate residue at the 3' terminus; d(A) 1000 and d(A) 100, the alternating copolymer of deoxyadenylate and deoxythymidylate, with a number average chain length of 1000 nucleotides.

Limited treatment of *Escherichia coli* DNA ligase by trypsin results in rapid loss of DNA joining activity. However, the ability to react with DPN to form the covalent enzyme-AMP intermediate is unaffected. The cleaved enzyme is also unable to catalyze the formation of DNA-adenylate, the second covalent intermediate in the ligase-catalyzed reaction. These findings demonstrate that portions of the DNA ligase molecule that are required for phosphodiester bond formation are not required for at least one of the partial reactions catalyzed by this enzyme.
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Procedures for the measurement of the rate of exchange between DPN and NMN, and the formation of DNA-adenylate have been described (1).

Release of \(^{32}\text{P}\)AMP from enzyme-AMP was measured in a reaction mixture (0.1 ml) that contained 10 mM Tris-HCl (pH 8.0), 4 mM MgCl\(_2\), 1 mM EDTA, 0.05 mg/ml of bovine serum albumin, 12 mM (NH\(_4\))\(_2\)SO\(_4\), 0.051 mM d\((\text{PA})_{500}\), 0.031 mM d\((\text{dT})_{500}\) (concentration of nicks of 0.12 \(\mu\)m), and enzyme-\(^{32}\text{P}\)AMP. The reaction mixture was incubated for 30 min at 20\(^\circ\), after which it was chilled and the DNA precipitated by the addition of 5 \(\mu\)l of 10 mg/ml of bovine serum albumin and 0.4 ml of 10% trichloroacetic acid. After 2 min at 0\(^\circ\), the precipitate was collected by centrifugation, and the radioactivity of 0.3 ml of the supernatant fluid was determined using a Triton-toluene scintillation fluid.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to a modification of the procedure of Laemmli (7) using a slab gel apparatus based on the design of Reid and Bieleski (8). The gel contained 0.35 M Tris-HCl (pH 8.8), 15% acrylamide, 0.125% bisacrylamide, 0.1% sodium dodecyl sulfate, and 0.05% ammonium persulfate. The lower tray buffer contained 0.1 M Tris-HCl (pH 8), the upper tray buffer was 0.053 M Tris-glycine (pH 9.5) and 0.1% sodium dodecyl sulfate. Samples were denatured in 1% sodium dodecyl sulfate containing 1% 2-mercaptoethanol and loaded onto gels in 20% glycerol. Gels were dried by the method of Fairbanks et al. (9) and autoradiographed in contact with Kodak x-ray film.

RESULTS

Enzyme-AMP Formation by Tryptic Digest of DNA Ligase

Under conditions of limited proteolysis (enzyme to trypsin ratio, 50:1 by weight), phosphodiester bond synthesis catalyzed by DNA ligase was abolished rapidly. In contrast, enzyme-AMP formation was only slightly affected (Fig. 1). However, after prolonged incubation with trypsin (>20 min), both joining activity and the capacity to form enzyme-AMP were lost (data not shown). DNA ligase incubated without trypsin retained approximately 70% of its joining activity throughout the course of the reaction.

Incubation of the digest with \(^{32}\text{P}\)AMP-labeled DPN sulfate was carried out according to a modification of the procedure of Laemmli (7) using a slab gel apparatus based on the design of Reid and Bieleski (8). The gel contained 0.35 M Tris-HCl (pH 8.8), 10% acrylamide, 0.12% bisacrylamide, 0.1% sodium dodecyl sulfate, and 0.05% ammonium persulfate. The lower tray buffer contained 0.1 M Tris-HCl (pH 8), the upper tray buffer was 0.053 M Tris-glycine (pH 9.5) and 0.1% sodium dodecyl sulfate. Samples were denatured in 1% sodium dodecyl sulfate containing 1% 2-mercaptoethanol and loaded onto gels in 20% glycerol. Gels were dried by the method of Fairbanks et al. (9) and autoradiographed in contact with Kodak x-ray film.

\(^{32}\text{P}\)AMP-labeled peptides were formed which migrated more rapidly than native enzyme-AMP (molecular weight, 75,000). Occasionally, preparations of untreated enzyme showed a second band of \(^{32}\text{P}\) (Fig. 2); the amount of this material was variable and could represent slight degradation of the native enzyme during storage. Based on \(^{35}\text{S}\)-labeled phage \(\lambda\) proteins as standards, the two major digestion products had molecular weights of 50,000 and 64,000, respectively.

The \(^{32}\text{P}\)AMP-labeled peptides could also be separated from each other using DEAE-Sephadex chromatography (see Fig. 3).
DNA. Intact ligase or its cleavage products were incubated with and from intact DNA ligase by chromatography on DEAE-Sephadex (Fig. 3). Analysis of the two 32P-labeled products, I and II, were identical with that found with the native enzyme. The progress of the reaction can be measured by monitoring the increasing specific radioactivity of the NMN and decreasing specific radioactivity of the DPN. As shown in Table I, the exchange rates observed with both cleavage products, I and II, were identical with that found with the native enzyme.

**Inability of Tryptic Digest of DNA Ligase to Form DNA-Adenylate**—The second step in the overall joining reaction catalyzed by DNA ligase consists of the covalent DNA-adenylate intermediate (10). DNA-adenylate can be made to accumulate under special conditions (1, 10, 11) for example, by using 3'-dideoxy-terminated d(pT)160-dd(pT)1, annealed to poly[d(pA)3000], as substrate, to prevent subsequent phosphodiester bond synthesis. However, the rate of DNA-adenylate formation under these conditions is very much lower than the rate of joining observed with d(pT)160 lacking the 3'-dideoxy groups (1). Neither I nor II was able to catalyze DNA-adenylate formation even at the low rate characteristic of the untreated enzyme (Table II). When 3'-hydroxyl (dT)160 was used under identical conditions, no DNA adenylate was formed with either intact or digested enzyme (data not shown).

Our failure to detect joining or DNA-adenylate formation, under conditions where the generation of enzyme-AMP remained unimpaired, suggested that proteolytic cleavage of DNA ligase resulted in a decreased capacity to interact with DNA. To investigate this possibility, the DNA-dependent release of [32P]AMP from enzyme-AMP was examined. Inasmuch as the release of AMP from enzyme-AMP in the presence of DNA reflects a single catalytic event, the use of [32P]AMP of very high specific radioactivity provides a more sensitive test of the interaction of ligase and its digestion products with DNA than the joining reaction. The results shown in Table III indicate that no detectable AMP was released from the adenylated tryptic digestion products upon the addition of DNA, suggesting that the fragments are indeed unable to interact with DNA.

**DISCUSSION**

It is clear that portions of the DNA ligase molecule that are required for the overall reaction are not required for at least one of the partial reactions that it catalyzes. A similar situation has been observed for DNA polymerase I in which the polymerase and 3'→5'-exonuclease can be physically separated from the 3'→5'-exonuclease by subtilisin cleavage (12, 13).

In the case of *E. coli* DNA ligase, the cleavage products are fully capable of reacting with DPN to form enzyme-AMP. Furthermore, the rate of DPN-NMN exchange is the same as that for the intact enzyme. Since the rate of exchange depends on the slowest step in the exchange reaction, and that rate is the same for both native and modified enzyme, the defect in joining cannot be due to a reduced rate of enzyme-AMP formation. We therefore conclude that the critical defect, and that which results in the elimination of joining activity, exerts

**Table I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration</th>
<th>Percent 32P in DNA-NMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.54 pmol</td>
<td>79</td>
</tr>
<tr>
<td>II</td>
<td>0.54 pmol</td>
<td>78</td>
</tr>
<tr>
<td>Intact enzyme</td>
<td>0.54 pmol</td>
<td>94</td>
</tr>
<tr>
<td>No enzyme</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>Intact enzyme</td>
<td>5.4 pmol</td>
<td>49</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration</th>
<th>DNA-adenylate formed pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.2 pmol</td>
<td>0.07</td>
</tr>
<tr>
<td>I</td>
<td>0.6 pmol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>II</td>
<td>0.6 pmol</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration</th>
<th>AMP released pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.78 pmol</td>
<td>0.42 pmol</td>
</tr>
<tr>
<td>I</td>
<td>0.68 pmol</td>
<td>0.01 pmol</td>
</tr>
<tr>
<td>II</td>
<td>0.48 pmol</td>
<td>0.01 pmol</td>
</tr>
</tbody>
</table>

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References (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13)
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its effect at some step subsequent to the formation of enzyme-AMP.

The second step in the ligase reaction consists of the transfer of AMP from enzyme-AMP to the 5'-phosphoryl terminus at a nick in duplex DNA to form DNA-adenylate. The proteolytic digestion products, in contrast to the native enzyme, did not catalyze the formation of DNA-adenylate at a measurable rate. This failure could be due to the inability of the modified enzyme to carry out some catalytic event essential to DNA-adenylate formation, or it might be at the level of the interaction of the adenylylated peptides with DNA. The results of a steady state kinetic analysis of the overall joining reaction are consistent with a ping-pong mechanism (1). Therefore, it is not unreasonable to expect that an enzyme molecule that has lost the ability to interact with DNA might retain the ability to react normally with DPN. Measurements of the release of AMP from enzyme-AMP in the presence of DNA were carried out at concentrations of nicks in excess of the $K_m$ observed for the intact enzyme. Thus, if the defect in joining were due to a decreased affinity of the modified enzyme for nicks, this more sensitive assay should permit detection of some reduced level of joining. Although the results of the measurements were not conclusive (the inability to release AMP might still be due to some catalytic event that is defective), they did tend to suggest a defect in binding of the tryptic cleavage products to DNA. Attempts to demonstrate directly binding of uncleaved enzyme-AMP to nicked DNA were unsuccessful since we were unable to define conditions under which binding would occur without joining and release of the enzyme from DNA.

Zimmerman and Oshinsky (2) reported that one of the two modified forms of ligase they had observed was able to release AMP from enzyme-AMP upon addition of very high concentrations of DNA. Our results may not necessarily be in contradiction with their findings, as tryptic hydrolysis may result in a product which is different from the modified forms generated under their conditions.

Measurement of the formation of enzyme-AMP is a convenient way to assay for DNA ligase (2, 14). Since significant enzyme-AMP formation may occur under conditions in which joining activity is abolished, it is obvious that some caution should be exercised in the use of this assay in enzyme purification, for example.

REFERENCES
Modification of Escherichia coli DNA ligase by cleavage with trypsin.
S M Panasenko, P Modrich and I R Lehman


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