Structure of the DNA Ligase-Adenylate Intermediate: Lysine (ε-amino)-Linked Adenosine Monophosphoramidate*

(nucleotidyl group transfer/covalent catalysis/phosphoamides/T4 phage/E. coli)

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ABSTRACT Proteolytic degradation of the Escherichia coli DNA ligase-adenylate intermediate releases adenosine 5′-monophosphate linked to the ε-amino group of lysine by a phosphoamide bond. Measurements of the rate of hydroxylaminolysis of the ligase-adenylate provide further support for a phosphoamide linkage in the native enzyme. Lysine (ε-amino)-linked adenosine monophosphoramidate has also been isolated from the T4 phage-induced ligase-adenylate intermediate. These results indicate that an initial step of the DNA ligase reaction consists of the nucleophilic attack of the ε-amino group of a lysine residue of the enzyme on the adenyl phosphorus of DPN or ATP that leads to the formation of enzyme-bound lysine (ε-amino)-linked adenosine monophosphoramidate.

The synthesis of phosphodiester bonds in nicked bihelical DNA by the DNA joining enzyme (ligase) of Escherichia coli is believed to occur in a sequence of at least three partial reactions (1–3). The first of these consists of the adenyllylation of the enzyme by DPN, with the concomitant release of NMN. The adenyl group is then transferred from the enzyme to the 5′-phosphoryl terminus at the nick to generate a new pyrophosphate bond. In the final step of the sequence, the 5′-phosphate of DNA is attacked by the apposed 3′-hydroxylate group to form a phosphodiester bond, and AMP is eliminated.

The adenylated enzyme will support the formation of a phosphodiester bond in the absence of DPN and can be de-adenylated by NMN with the formation of DPN. Earlier studies have indicated that the adenyl group is covalently bonded to the enzyme, since the complex can be isolated by acid precipitation or by zone centrifugation in sucrose gradients of high ionic strength. Furthermore, this bond will withstand boiling at neutral or alkaline pH and treatment with acid at 0°C; however, it is cleaved by exposure to dilute acid at room temperature or above (2, 3). The acid lability and alkaline stability of the complex suggest that the nucleotide is bound to the enzyme by a phosphoamide bond (4).

Abbreviations and Nomenclature: IMP, inosine 5′-monophosphate; NMN, nicotinamide mononucleotide. The nomenclature of the nucleotide–adenosine acids and of the bond types involved in their linkage is that of Shabarova (4). AMP (5′–N′) lysine describes adenosine 5′-monophosphate linked by a phosphoamide bond to the ε-amino group of lysine. AMP (5′–N′) histidine refers to adenosine 5′-monophosphate linked to an imidazole nitrogen of histidine by a phosphoamide bond.

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Here we present evidence that the adenyllyl group is in fact attached to the ε-amino group of a lysine residue in the enzyme by a direct P to N bond. This finding indicates that an initial step of the DNA ligase reaction consists of the nucleophilic attack of the ε-amino group of a lysine residue on the adenyllyl phosphorus of DPN, leading to the formation of enzyme-bound lysine (ε-amino)-linked adenosine monophosphoramidate.

MATERIALS AND METHODS

Enzymes. E. coli polynucleotide joining enzyme (Fraction V) was isolated from E. coli 1100 according to the method of Oliveira and Lehman (5). T4 phage-induced polynucleotide ligase (6) was the gift of Dr. C. C. Richardson. Lyophilized trypsin, pronase (grade B), and aminopeptidase M were obtained from Worthington Biochemical Corp. Calbiochem, and Rohm and Hass, respectively. Adenosine deaminase, purified by the method of Wolffend et al. (7), was the gift of Mr. Peter Lobban.

Nucleotides and Phosphoramide. [32P]AMP was synthesized by the method of Symons (8) and [32P]DPN was synthesized from the labeled AMP by the method of Shuster et al. (9). [32P]AMP was converted to [α-32P]ATP by the method of Setlow and Kornberg (10). IMP (5′ → N′) histidine was synthesized by the reaction of IMP-morpholidate (Calbiochem) and L-histidine at pH 5.5. The compound, which was purified by paper chromatography and electrophoresis, migrated as a monooanion at neutral pH during paper electrophoresis and had an ultraviolet spectrum identical to that of IMP. It had a hypoxanthine:phosphate:histidine ratio of 1.02: 1.00: 1.07 and gave a negative Pauly (diazosulfanilic acid) test for free imidazole (11) until acid hydrolyzed. The compound gave a positive ninhydrin reaction and was insensitive to alkaline phosphatase before acid hydrolysis. Free IMP and histidine were released when it was incubated with 0.1 N HCl for 1 hr at 37°C. The NMR spectrum of the compound indicated that the nucleotide phosphorus was attached to an imidazole nitrogen of the histidine by a P-N bond (12). IMP (5′→N′) lysine was synthesized by a modification of the method of Moffat and Khorana for the synthesis of nucleotide morpholidates (13); N-α-carbobenzyloxy-L-lysine benzyx ester (Cyclo Chemical Co.) was used in place of morpholine. The carboxylated compound was hydrogenated in 8% triethyamine in methanol with palladium on charcoal at atmospheric pressure for 8 hr at 0°-4°C (14) and was purified by paper electrophoresis. The ultraviolet spectrum of the
product was that of IMP, and it behaved as a monoanion during electrophoresis at neutral pH. It showed a positive ninhydrin reaction, was insensitive to alkaline phosphatase before acid hydrolysis, and released IMP and lysine when treated with 0.1 N HCl for 1 hr at 37°C. The ratio of hypoxanthine: phosphate: lysine was 0.95: 1.00: 0.92. The NMR spectrum indicated a direct P-N bond on the e-amino group of lysine.

**Ligase-Adenylyl.** *E. coli* ligase–[^32P]AMP was prepared by incubating 140 units (5) of enzyme in a reaction mixture of 1 ml that contained 10 mM Tris-HCl (pH 7.9), 1 mM ammonium sulfate, 1 mM EDTA, 5 mM MgCl₂, 1.2–14 μM[^32P]^AMP–DPN (3–30 × 10⁴ cpm/pmol) and 5% glyceral for 10 min at room temperature. The reaction was terminated by the addition of 25 μmol of EDTA and the enzyme–[^32P]AMP was isolated by gel filtration (3) in 50 mM Tris-HCl (pH 9.0) on a 0.8 × 29 cm column of Sephadex G-50. Alternatively, the reaction mixture was precipitated with 5% trichloracetic acid, centrifuged at 5000 × g for 5 min, dissolved in 1 ml of 20 mM NaOH–20 mM sodium pyrophosphate, and reprecipitated with 5% trichloracetic acid. We are uncertain as to the number of adenyllyl groups bound per molecule of ligase because of the impurity of the enzyme preparations used. Alkaline hydrolysis of *E. coli* ligase–[^32P]AMP was in a sealed tube containing the trichloracetic acid-precipitated material, which had been dissolved in 300 μl of 4.6 M NaOH. Hydrolysis was for 2–6 hr at 100 or 115°C. The sample was cooled, neutralized with glacial acetic acid, and adjusted to 0.1 M sodium phosphate (pH 8.0). The precipitate that formed was removed by centrifugation and the supernatant was treated with charcoal. The charcoal was washed twice with water and the nucleotides were eluted with 50% ethanol adjusted to pH 11.5 with triethylamine.

**RESULTS**

**Cleavage of *E. coli* ligase–[^32P]AMP by hydroxylamine**

*E. coli* ligase–[^32P]AMP, which had been purified by gel filtration, was incubated with 3.86 M hydroxylamine at pH 4.75, with 4 M sodium acetate at pH 4.75, or with 0.2 M hydroxylamine at pH 7.5 (Fig. 1). The enzyme-AMP was more than 90% cleaved by the 3.86 M hydroxylamine in 25 min at 37°C (A). In contrast, less than 10% of the AMP was released in the control incubated at the same temperature, pH, and ionic strength in the absence of hydroxylamine (B). Neutral hydroxylamine split only 23% of the complex in 25 min (C), under conditions that are known to cleave quantitatively the acetyl phosphate anhydride bond of acetyl-AMP in 5 min (17).

Cleavage by acidic hydroxylamine suggests that a phosphoamide, rather than a phosphoester, bond links the nucleotide to the enzyme (4). The limited extent of cleavage by neutral hydroxylamine suggests that a mixed anhydride is probably not involved in the linkage (17). These results do not eliminate the involvement of other bond types in prior or subsequent steps of the joining reaction.

**Isolation of IMP (5→N) lysine from *E. coli* ligase-AMP by proteolytic digestion**

Sequential digestion of the heat-denatured enzyme–[^32P]AMP with several proteolytic enzymes led to the isolation of a lysine residue bearing the labeled nucleotide on its ε-amino group. Incubation of the enzyme–[^32P]AMP with trypsin released little[^32P]P from the polypeptide, which remained at the origin during electrophoresis (not shown). Pronase treatment of the trypsic digest converted the polypeptide material to products that migrated at neutral pH as dianions, as well as to some Pi (Fig. 2,A). When the mixture was hydrolyzed with aminopeptidase M, a labeled monoanionic product was produced, together with AMP and additional Pi (Fig. 2,B). The proteolytic conversion of a dianionic species to a monoanionic suggests that some of the material in the pronase digest de...
rives extra negative charge from an acidic amino-acid residue in the peptide attached to the nucleotide. Since the compound migrates almost as rapidly as AMP, either very few amino-acid residues are linked to the residue bearing the AMP or more than one acidic group is present. Adenosine deaminase quantitatively converted the monoanionic product of the aminopeptidase M digestion to a compound that migrated with IMP \( (5'\rightarrow N^2) \) lysine (Fig. 2, C), a result that suggested that it was AMP \( (5'\rightarrow N^2) \) lysine. In two experiments, the yield of IMP \( (5'\rightarrow N^2) \) lysine was 31 and 33% of the acid-precipitable \(^{32}P\) originally present.

The \(^{32}P\)IMP derivative was isolated from a proteolytic digest by charcoal adsorption, desorption, and preparative electrophoresis. It comigrated with authentic IMP \( (5'\rightarrow N^2) \) lysine at several pH values during electrophoresis, and in a number of two-dimensional thin-layer systems that utilize ion-exchange and partition chromatography for separations (Table 1). Examination of radioautographs of the thin-layer chromatographic plates showed that the shapes of the darkened film spots exactly matched those due to the reference IMP \( (5'\rightarrow N^2) \) lysine. Several of the systems used clearly separated the labeled material from IMP \( (5'\rightarrow N^{32}P) \) histidine.

**Table 1.** Electrophoretic and chromatographic properties of the isolated \(^{32}P\)-labeled nucleotide–amino acid and of reference compounds

<table>
<thead>
<tr>
<th>Separation system</th>
<th>Migration of (^{32}P)-labeled derivative relative to reference compounds</th>
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<tr>
<td>Electrophoresis</td>
<td>pH</td>
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<tr>
<td>Acetic acid</td>
<td>2.5</td>
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<tr>
<td>Pyridine acetate</td>
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<tr>
<td>Triethylammonium bicarbonate</td>
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<td>Sodium borate</td>
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<td>Two-dimensional thin-layer chromatography</td>
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<td>PEI-cellulose - 1</td>
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<td>PEI-cellulose - 2</td>
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<td>DEAE-cellulose</td>
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Electrophoresis was with a Savant high-voltage flat-bed apparatus, cooled with running tap water, at 3.5–4.5 kV. Picric acid, a visible marker, was run 16–20 cm from the origin towards the anode. The following buffers were used: 6.67% acetic acid (pH 2.5), 2.2% acetic acid–0.22% pyridine (pH 3.5), 0.67% pyridine–0.27% acetic acid (pH 5.3), 50 mM triethylammonium bicarbonate adjusted to the desired pH by the addition of \( \mathrm{CO}_2 \) (pH 7.8 and 8.2), and 1.27% sodium borate decahydrate (pH 9.2). The electropherograms were dried, cut out into 1-cm strips, and counted in a gas-flow counter. Polyethyleneimine (PEI)–cellulose TLC: we used the solvent systems and procedures described by Randerath and Randerath (31) for the separation of complex nucleotide mixtures (procedure 1) and for the separation of nucleoside monophosphates and nucleotide sugars (procedure 2). The solvent systems of Furlong (32) were used for diethylaminoethyl (DEAE)–cellulose TLC, except that 0.2 M rather than 0.38 M ammonium formate (pH 6.0) was used. The plates were washed for 10 min in anhydrous methanol before the second dimension was developed, and 0.5 M sodium formate buffer (pH 3.0) replaced 0.5 M ammonium formate. For chromatography on cellulose, propanol–water-ammonia (33) was used for the first dimensions, and isobutyric acid–ammonia–EDTA (34) was used for the second. Compounds were located and identified as described in Methods. A + indicates comigration of \(^{32}P\) and marker compounds.

![Fig. 2. Electrophoretic analysis of the proteolytic products of E. coli ligase–[\(^{32}P\)]AMP.](image-url) Electrophoresis was performed as described in Table 1 in 50 mM triethylammonium bicarbonate (pH 7.9). Ligase–[\(^{32}P\)]AMP (0.9 ml, 1.79 \times 10^6 cpm), which had been isolated by gel filtration in 50 mM Tris-HCl (pH 9.0), was made 10 mM in 2-mercaptoethanol and heated at 100°C for 3 min. To the cooled solution, 0.9 mg of trypsin in 30 \( \mu \)l of 50 mM Tris-HCl (pH 9.0) was added, and the mixture was incubated at 37°C for 11 hr. It was then heated at 100°C for 3 min and cooled. Pronase (10 \( \mu \)l of a 23.8 mg/ml solution), which had been treated to destroy nuclease (36), was added to the trypptic digest and the mixture was incubated at 37°C for 1.5 hr. The digest was heat inactivated as before, and after the addition of 10 \( \mu \)l of a 4.0 mg/ml solution of aminopeptidase M, was incubated for an additional 4 hr at 37°C. The digest was again heat inactivated. It was adjusted to pH 7.6–7.8 with 1 N HCl, and, after the addition of 20 \( \mu \)l of a 1 mg/ml solution of adenosine deaminase (1000 U/ml), was incubated at 37°C for 1 hr. Samples (20 \( \mu \)l) were withdrawn and spotted on paper for electrophoresis after Pronase (A), aminopeptidase M (B), and adenosine deaminase (C) treatment. Adenosine 3'5'-cyclic monophosphate (cAMP) was used as a reference compound to indicate the rate of migration of a monoanionic nucleotide at pH 7.9.
Isolation of IMP (5'→N) lysine from T4 polynucleotide ligase-[32P]AMP

Phage T4 induces the synthesis of a ligase that differs from the E. coli enzyme, in that it utilizes ATP instead of DPN as a cofactor (22). An adenylylated enzyme intermediate is also formed by the T4 ligase with the adenylyl residue derived from ATP (15). When heat-denatured T4 ligase-[32P]AMP was subjected to the same proteolytic degradation procedure used for the E. coli enzyme, IMP (5'→N') lysine was isolated. Trypsin released anionic material from the labeled polypeptide (not shown). Pronase converted the tryptic peptides to two main components, one of which migrated near AMP and the other in the region of a monoanionic nucleotide (Fig. 3, A). Finally, aminopeptidase M converted nearly all of these components to a monoanionic material (Fig. 3, B), presumably AMP (5'→N') lysine, since adenosine deaminase converted it to the corresponding IMP derivative (Fig. 3, C). The isolated IMP derivative comigrated during electrophoresis with authentic IMP (5'→N') lysine at pH 3.4, 7.5, and 9.2, and in the two-dimensional ion-exchange chromatographic systems described in Table 1. Pronase released polypeptides from the tryptic digest that were more negatively charged at neutral pH than would be expected from the addition of neutral amino acids to the adenylylated lysine. This suggests that both the T4 and the E. coli DNA ligases have acidic amino acids near the adenylylated lysine residue.

DISCUSSION

The identification of lysine (ε-amino)-linked adenosine monophosphoramidate in digests of the E. coli ligase-AMP intermediate suggests that an initial step of the reaction catalyzed by this enzyme occurs by a nucleophilic attack of the ε-amino group of a lysine residue on the adenylyl phosphorus of DPN. The E. coli DNA ligase is, to our knowledge, the first instance in which the ε-amino group of a lysine residue has been implicated as a nucleophilic catalyst in an enzyme-catalyzed nucleotidy1 group transfer. The ε-amino group of lysine is probably cationic at physiological pH values, so that the conversion of the primary ammonium group to a phosphoamidate bond would require the removal of a proton from the nitrogen. Anionic amino acid(s) that may exist in proximity to the lysine could serve in this capacity. The attack may also be facilitated by the chelation of magnesium ion by the pyrophosphoryl group of DPN, thereby rendering the phosphorus atom more susceptible to nucleophilic attack (23). Since the phosphoamidate nitrogen may be protonated to some extent at neutrality (24), nucleophilic attack on the adenylyl phosphorus by the anionic oxygen of the DNA 5'-phosphate to give the DNA-adenylate intermediate (2) might be expected to proceed readily. The final step in the reaction is presumed to occur by a nucleophilic attack on the activated DNA 5'-phosphoryl group by a 3'-hydroxy group to form a phosphodiester bond. It would therefore appear that the sequence of bonds broken and formed in the ligase reaction is: pyrophosphate → phosphoamidate → pyrophosphate → phosphodiester.

Both Baddiley et al. (25) and Shabarova (4) have proposed enzymatic models for nucleotidy1 group transfer that involve a phosphoamidate bond between the nucleotide and the imidazole nitrogen of a histidine residue in the protein. Our work substantiates their suggestion of an enzyme-nucleoside phosphoramidate. However, what is found with the E. coli and T4 DNA ligases is that the ε-amino group of lysine, rather
than the imidazole nitrogen of histidine, is the site of attachment of the isolated intermediate. Although the recovery of lysine (ε-amino)-linked isoinine monophosphoramidate after both alkaline and proteolytic degradation of ligase–AMP strongly suggests that a lysine residue is the primary site of attachment of AMP to the enzyme, it is important to point out that the possibility of migration of the adenyl group from a histidine residue during the course of the isolation cannot yet be eliminated.

The T4-induced polynucleotide ligase also forms an ε-amino lysine-linked phosphoramidate, but the adenylated group is derived from ATP rather than DPN. Presumably the reaction proceeds from the enzyme–AMP intermediate via a pathway similar to that used by the E. coli ligase, since synthetic polynucleotide–adenylate can be used by the T4 enzyme to synthesize a phosphodiester bond in the absence of ATP (26, 27).

Although the E. coli and T4 DNA ligases represent the first instances of the involvement of enzyme-bound adenylates in a covalent enzymatic catalysis, there are several cases in which adenylation of an enzyme produces a striking change in its intrinsic activity, for example, the adenyllylation of specific tyrosine residues of E. coli glutamine synthetase (28) and of unknown sites of E. coli RNA polymerase (29). It should be noted that nucleoside phosphoramidates have been isolated from ribosomal RNA (30), but that the significance of these compounds is unknown.

We acknowledge the excellent assistance of Mrs. Janice R. Chien. We thank Mr. Peter E. Lobban, Drs. A. L. Nussbaum and C. C. Richardson for generous gifts of materials, Dr. M. R. Bramwell for determining NMR spectra, and Dr. R. V. Gemmer for performing the catalytic hydrogenations. This work was supported by a grant from the National Institutes of Health (GM-06196). R. I. G. was supported, in part, by a USPHS post-doctoral fellowship.

Correction. In the article “Nucleic Acid Polymerases of the Developing Chicken Embryo: A DNA Polymerase Preferring a Hybrid Template”, by Stavrianopoulos, Jannis, G., Karkas, John D. & Chargaff, Erwin, which appeared in the September 1971 issue of Proc. Nat. Acad. Sci. USA 68, 2207–2211, the following changes should be made. On p. 2209, Table 4, third line of legend, “2.4 pg of DNA polymerase” should read “0.024 μg of DNA polymerase.” On p. 2210, line 3 from bottom of the legend to Fig. 3, “pmol/mg of protein” should read “nmol/mg of protein.”

Correction. In the article “Cell Cycle-Dependent Immune Lysis of Moloney Virus-Transformed Lymphocytes: Presence of Viral Antigen, Accessibility to Antibody, and Complement Activation”, by Lerner, Richard A., Oldstone, Michael B. A. & Cooper, Neil R., which appeared in the October 1971 issue of Proc. Nat. Acad. Sci. USA 68, 2584–2588, the following changes should be made. On p. 2584, right-hand column, third line from bottom, “5 X 10^1 cells” should read “5 X 10^3 cells.” On p. 2587, legend to Fig. 3, the labels “(upper panel)” and “(lower panel)” should be reversed. Also, it should be noted that the anode is to the left.

Correction. In the article entitled “Structure of the DNA Ligase–Adenylate Intermediate: Lysine (ε-amino)-Linked Adenosine Monophosphoramidate,” by Gumport, Richard I. & Lehman, I. R., which appeared in the October 1971 issue of Proc. Nat. Acad. Sci. USA 68, 2559–2563, the following change should be made. On page 2559, line 9 of the first paragraph should read: “phosphate of DNA is attacked by the apposed 3’-hydroxyl,” not “3’-hydroxylate.”