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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 adenylyl 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 adenylylation of the enzyme by DPN, with the concomitant release of NMN. The adenylyl 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 adenylylated enzyme will support the formation of a phosphodiester bond in the absence of DPN and can be deadenylylated by NMN with the formation of DPN. Earlier studies have indicated that the adenylyl 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).

Here we present evidence that the adenylyl 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 adenylyl 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 Olivera 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 Wolfenden *et al.* (7), was the gift of Mr. Peter Lobban.

Nucleotides and Phosphoramidates. [^{32}P]AMP was synthesized by the method of Symons (8) and [^{32}P]DPN was synthesized from the labeled AMP by the method of Shuster *et al.* (9). [^{32}P]AMP was converted to [α-^{32}P]ATP by the method of Setlow and Kornberg (10). IMP (5′ → N^{imid}) 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 monoanion 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^{ϵ}) lysine was synthesized by a modification of the method of Moffat and Khorana for the synthesis of nucleotide morpholidates (13); *N*-α-carbobenzoxy-L-lysine benzyl ester (Cyclo Chemical Co.) was used in place of morpholine. The carbobenzoxylated compound was hydrogenated in 8% triethylamine 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

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

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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 ϵ-amino group of lysine.

Ligase–Adenylate. E. coli ligase–[^{32}P]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 $MgCl_2$, 1.2–14 μM [^{32}P]-DPN (3–30 $\times$ 10^3 cpm/pmol) and 5% glycerol for 10 min at room temperature. The reaction was terminated by the addition of 25 μmol of EDTA and the enzyme–[^{32}P]AMP was isolated by gel filtration (3) in 50 mM Tris·HCl (pH 9.0) on a 0.8 $\times$ 29 cm column of Sephadex G-50. Alternatively, the reaction mixture was precipitated with 5% trichloracetic acid, centrifuged at 5000 $\times$ *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 adenylyl groups bound per molecule of ligase because of the impurity of the enzyme preparations used. Alkaline hydrolysis of *E. coli* ligase–[^{32}P]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.

T4 ligase–[^{32}P]AMP (9.4 $\times$ 10^5 cpm in 2.1 ml after isolation by gel filtration) was prepared from 10 units of T4 polynucleotide ligase according to the procedure of Weiss and Richardson (15) in 0.5 ml, with [α-^{32}P]ATP (14.3 $\times$ 10^3 cpm/pmol) present at a final concentration of 5.4 μM.

Chromatography. Two-dimensional thin-layer chromatography (TLC) was run on plastic sheets coated with polyethyleneimine-, diethylaminoethyl-, and unsubstituted-cellulose (Brinkmann Instruments). Reference compounds were identified by their quenching of ultraviolet light and labeled compounds by radioautography. Phosphate compounds were visualized by the method of Bandurski and Axelrod (16). ^{32}P was determined in a Nuclear-Chicago gas-flow or scintillation counter.

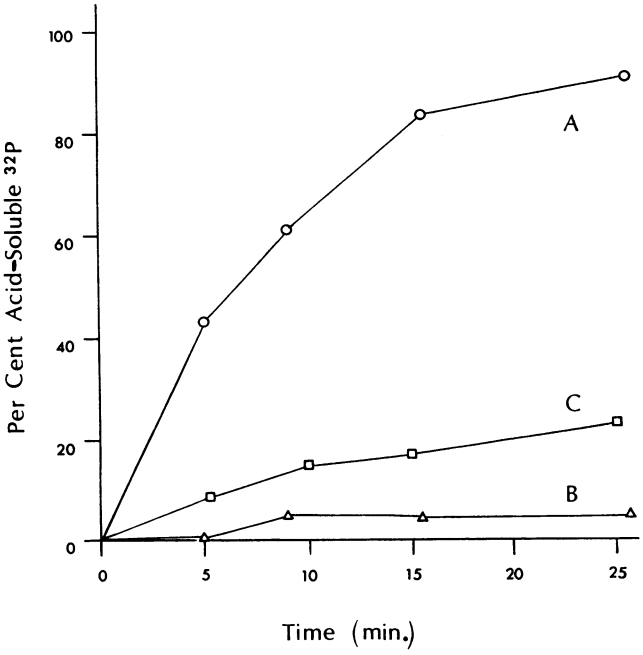

FIG. 1. Hydroxylaminolysis of *E. coli* ligase–[^{32}P]AMP. Solutions (100 μl) containing 3.86 M hydroxylamine hydrochloride (pH 4.75) (*A*), 4.0 M sodium acetate (pH 4.75) (*B*), or 0.2 M hydroxylamine hydrochloride (pH 7.5) (*C*) were added to tubes containing 10 μl of ligase–[^{32}P]AMP (14,950 cpm total) in 0.05 M Tris·HCl (pH 9.0). The reactions were run for the indicated times at 37°C, terminated by adding 0.5 ml of cold 0.1% bovine plasma albumin that contained 2 mM EDTA, and precipitated with 0.1 ml of cold 50% trichloracetic acid. They were centrifuged for 5 min at 20,000 $\times$ *g* in the cold after standing 5 min on ice. A 400-μl aliquot of the supernatant fluid was counted in a toulene-based scintillation mixture containing Triton X 100 (35) to determine acid-soluble ^{32}P. NH_2OH (3.86 M) was prepared by dissolving 27.8 g of NH_2OH·HCl in 100 ml of cold water and adding 3.8 ml of 45% KOH, to bring the pH to 4.75. NH_2OH (0.2 M) was prepared by mixing equal volumes of cold 4 M NH_2OH·HCl and 4M KOH, and diluting 10-fold with 0.1 M potassium phosphate (pH 7.5).

RESULTS

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

E. coli ligase–[^{32}P]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$^\epsilon$) lysine from *E. coli* ligase–AMP by proteolytic digestion

Sequential digestion of the heat-denatured enzyme–[^{32}P]-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–[^{32}P]AMP with trypsin released little ^{32}P from the polypeptide, which remained at the origin during electrophoresis (not shown). Pronase treatment of the tryptic 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 monoanion 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′ → N$^{\epsilon}$) lysine (Fig. 2, *C*), a result that suggested that it was AMP (5′→N$^{\epsilon}$) lysine. In two experiments, the yield of IMP (5′→N$^{\epsilon}$) 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′→N$^{\epsilon}$) 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′→N$^{\epsilon}$) lysine. Several of the systems used clearly separated the labeled material from IMP (5′→N^{imid}) histi-

TABLE 1. *Electrophoretic and chromatographic properties of the isolated ^{32}P-labeled nucleotide-amino acid and of reference compounds*

Separation system		Migration of ^{32}P-labeled derivative relative to reference compounds: IMP (5′→N$^{\epsilon}$) lysine	IMP (5′→N^{imid}) histidine
Electrophoresis	*pH*		
Acetic acid	2.5	+	−
Pyridine acetate	3.5	+	−
Pyridine acetate	5.1	+	−
Triethylammonium bicarbonate	7.5	+	+
Triethylammonium bicarbonate	8.2	+	+
Sodium borate	9.2	+	
Two-dimensional thin-layer chromatography			
PEI-cellulose - 1		+	−
PEI-cellulose - 2		+	−
DEAE-cellulose		+	−
Cellulose		+	

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 CO_2 (pH 7.8 and 8.2), and 1.27% sodium borate decahydrate (pH 9.2). The electropherograms were dried, cut 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.

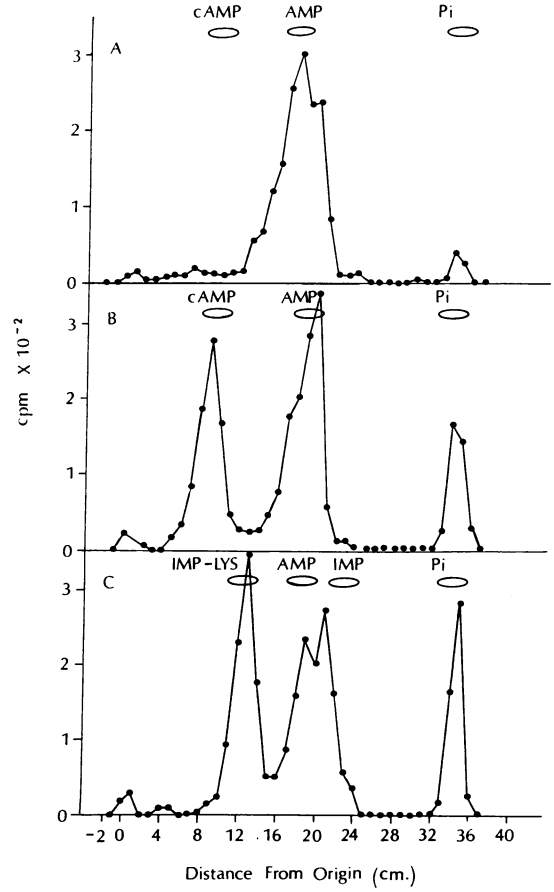

FIG. 2. Electrophoretic analysis of the proteolytic products of *E. coli* ligase–[^{32}P]AMP. Electrophoresis was performed as described in Table 1 in 50 mM triethylammonium bicarbonate (pH 7.9). Ligase–[^{32}P]AMP (0.9 ml, 1.79 × 10^{6} cpm), which had been isolated by gel filtration in 50 mM Tris·HCl (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 μl 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 μl of a 23.8 mg/ml solution), which had been treated to destroy nucleases (36), was added to the tryptic 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 μl 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 μl of a 1 mg/ml solution of adenosine deaminase (1000 U/mg), was incubated at 37°C for 1 hr. Samples (20 μl) 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.

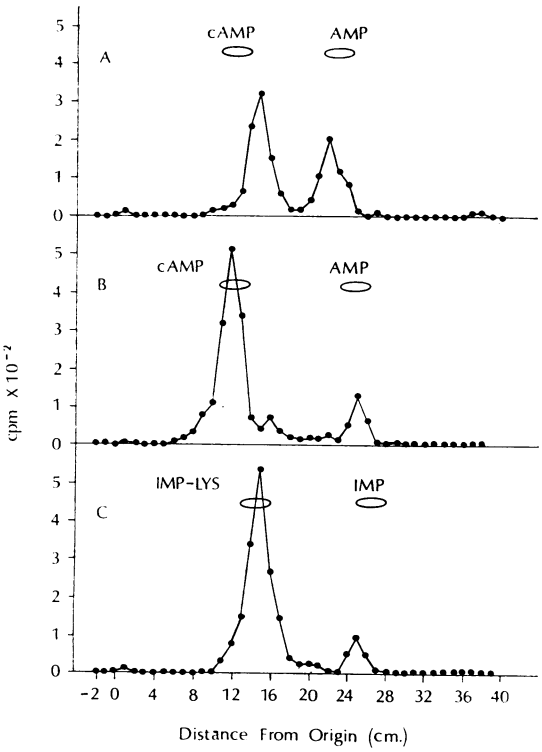

FIG. 3. Electrophoretic analysis of the proteolytic products of T4 ligase–[^{32}P]AMP. The labeled ligase–[^{32}P]AMP intermediate, formed as described in *Methods*, was isolated by gel filtration in 50 mM Tris·HCl (pH 9.0) and subjected to proteolysis as described in the legend to Fig. 2. Samples (40 μl) were withdrawn for electrophoresis in 50 mM triethylammonium bicarbonate (pH 7.9) after Pronase (*A*), aminopeptidase M (*B*), and adenosine deaminase (*C*) treatment.

dine. In addition, kinetic studies of the acid hydrolysis of the [^{32}P]IMP derivative and the known IMP-amino acids in 0.3 N HCl at 37°C showed that the labeled material was hydrolyzed at the same rate as the lysine derivative ($t_{1/2}$ = 15 min), and more slowly than histidine–IMP ($t_{1/2}$ = 8 min).

Treatment of the labeled compound with 0.1 N HCl for 1 hr at 37°C converted all of the label to free [^{32}P]IMP.

Isolation of IMP (5′→N$^{\epsilon}$) lysine from *E. coli* ligase–AMP by alkaline hydrolysis

The alkali stability of P-N bonds has permitted the isolation, in good yield, of *N*-phosphoaminoacids from several phosphorylated enzymes by vigorous alkaline digestion (18–21). Alkaline hydrolysis of *E. coli* ligase–[^{32}P]AMP for 5–6 hr at 115°C in 4.6 M NaOH yielded very small amounts (1–3%) of material which comigrated with IMP (5′→N$^{\epsilon}$) lysine in several of the systems used in Table 1. The alkaline treatment converted all adenosine compounds to inosine derivatives by deamination. Nucleotide–peptide fragments were released by hydrolysis at lower temperatures or for shorter times. Although the P-N bond of 3-phosphohistidine is very stable to alkaline hydrolysis (18), the stability of nucleoside phosphoramidates depends upon the ability of functional groups on the amino acid or peptide to protonate the nitrogen of the P-N bond (4). Our results indicate that the bond linking AMP to lysine is relatively labile to vigorous alkaline hydrolysis, but that a small amount of the IMP derivative can be recovered.

Isolation of IMP (5′→N$^{\epsilon}$) lysine from T4 polynucleotide ligase–[^{32}P] 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–[^{32}P]AMP was subjected to the same proteolytic degradation procedure used for the *E. coli* enzyme, IMP (5′→N$^{\epsilon}$) 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$^{\epsilon}$) 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$^{\epsilon}$) 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 nucleotidyl 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 phosphoamide 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 phosphoamide 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′-hydroxyl group to form a phosphodiester bond. It would therefore appear that the sequence of bonds broken and formed in the ligase reaction is: pyrophosphate → phosphoamide → pyrophosphate → phosphodiester.

Both Baddiley *et al.* (25) and Shabarova (4) have proposed enzymatic models for nucleotidyl group transfer that invoke a phosphoamide 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 inosine 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 adenylyl 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 adenylyl 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 polydeoxynucleotide–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 covalent enzymatic catalysis, there are several cases in which adenylylation of an enzyme produces a striking change in its intrinsic activity, for example, the adenylylation 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.

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