Deoxyribonucleic Acid Ligase

A STEADY STATE KINETIC ANALYSIS OF THE REACTION CATALYZED BY THE ENZYME FROM ESCHERICHIA COLI*

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SUMMARY

The DNA-joining reaction catalyzed by the Escherichia coli DNA ligase is markedly enhanced by low concentrations of monovalent cations, NH₄⁺ being most effective. The Kₘ for NH₄⁺ is about 1 mM, and at saturating concentrations it increases the true vₘₐₓ by 20-fold. Under these conditions the Kₘ for diphosphopyridine nucleotide is 7 μM, the Kₘ for single strand breaks is 0.03 to 0.06 μM, and the turnover number is 25 sealing events per min.

The DNA-joining reaction obeys ping-pong kinetics, thus providing kinetic evidence for the participation of a covalent intermediate, presumably ligase-adenylate. The rate of the ligase-catalyzed diphosphopyridine nucleotide-nicotinamide mononucleotide exchange reaction is unaffected by NH₄⁺, indicating that the activation occurs at a step subsequent to the formation of ligase-adenylate; furthermore, the exchange reaction is faster than the rate of DNA-joining, demonstrating that ligase-adenylate can be formed at a rate sufficient to be an intermediate in the over-all reaction. The rate of release of adenosine 5'-monophosphate from synthetic DNA-adenylate in the absence of NH₄⁺ is greater than the rate of DNA-joining, as expected for a kinetically significant intermediate. However, in the presence of NH₄⁺, the rate of adenosine 5'-monophosphate release is less than that of the over-all reaction. Since NH₄⁺ markedly increases the apparent rate of dissociation of ligase-adenylate from DNA, the result may reflect reversal of the reaction to form ligase-adenylate from ligase and DNA adenylate and dissociation of this form of the enzyme from the DNA.

Synthesis of phosphodiester bonds in duplex DNA by the DNA ligase of Escherichia coli appears to proceed in a sequence of three steps involving two covalent intermediates (Fig. 1). Ligase-adenylate, in which AMP is linked to the ε-amino group of a lysine residue of the enzyme through a phosphoamidate bond (1), is readily generated upon incubation of the enzyme with DPN in the absence of DNA (2, 3). Once formed, the adenylated enzyme can synthesize phosphodiester bonds in DNA without added DPN (2, 3). The second covalent intermediate is DNA-adenylate, in which AMP is bound in a pyrophosphate linkage to the 5'-phosphoryl terminus of the DNA to be joined. This activated DNA is more difficult to detect since it does not accumulate under steady state conditions; however, it has been observed after short incubations at low temperature in the presence of large amounts of enzyme (4). The DNA-adenylate isolated from such reaction mixtures is a substrate for the unadenylated form of the ligase and leads to the formation of a phosphodiester bond, again in the absence of DPN. The demonstration that a synthetic poly(d1)-adenylate is a substrate for the enzyme (5) provides additional support for the structure of DNA-adenylate and for its involvement in the ligase reaction.

These studies have provided good evidence for the minimal mechanism shown in Fig. 1. However, they have not rigorously established that ligase-adenylate and DNA-adenylate are kinetically significant intermediates on the main reaction path of the DNA-joining reaction. We have therefore undertaken a detailed kinetic analysis of the over-all joining reaction, and several of the partial reactions catalyzed by the E. coli DNA ligase.

EXPERIMENTAL PROCEDURE

Materials

Enzymes—The E. coli DNA ligase used in these experiments was the homogeneous preparation (Fraction VII) described in the previous paper (6). The ligase was diluted as described previously (6), except that (NH₄)₂SO₄ was omitted from the diluent in those experiments in which the effects of monovalent cations were investigated. T4 polynucleotide ligase, Fraction VII (7), was a gift from Dr. Vittorio Sgaramella. Polynucleotide kinase was purified as described by Richardson (8). E. coli alkaline phosphatase (Warthington BAPC) purified to remove endonuclease (9) was a gift from Dr. Wai Mun Huang, Stanford University. E. coli DNA polymerase I (10) and the “large” proteolytic fragment of this enzyme (11) were provided by Drs. A. Koruberg and D. Brutlag, Stanford University. Calf thymus terminal deoxynucleotidyl transferase (12) was a gift from Dr. Robert Ratliff (Los Alamos Scientific Laboratory, Los...
FIG. 1. Mechanism of the reaction catalyzed by the E. coli DNA ligase.

Almos, New Mexico). BSA† (A grade), was purchased from Calbiochem.

Nucleotides and Substrates—Unlabeled deoxyribonucleoside triphosphates, NMN, and DPN (chromatopure grade) were purchased from P-L Biochemicals. Dideoxythymidine was a gift from Dr. J. Moffat, Syntex Research. ddTMP was prepared from the nucleoside by the method of Russell and Moffat (13) and converted to the triphosphate (ddTTP) as described by Hoard and Ott (14).

[4-3H]Nicotinamide-DPN (50 mCi per mmole) was purchased from Amersham-Searle. [γ-32P]ATP (6 to 14 Ci per mmole) was prepared by the method of Glynn and Chappell (15). [α-32P]ddTTP was synthesized as described by Atkinson et al. (16). [32P]AMP and [32P]DPN (labeled in the adenyllyl moiety) were prepared as described previously (6).

d(pA)5100 and d(pA)6900 were prepared according to Riley et al. (17). [32P]d(pA-pT)₁₄₀ was synthesized as described previously (18).

A[p³²P]-p(dT)₇₋₄₄ was prepared as a modification of the method of Hall and Lehman (5). The triethylammonium salt of [³²P]AMP-morpholidate (0.39 μmole, 4 Ci per mmole), prepared by the method of Moffat and Khorana (19), was condensed with the tributylammonium salt of d(pT)₄ (0.16 μmole of oligomer) in 0.3 ml of anhydrous pyridine for 84 hours at 40°. The reaction mixture was taken to dryness, dissolved in 1.5 ml of 0.05 M Tris-HCl (pH 8.0), and treated with 90 μg of E. coli alkaline phosphatase for 2 hours at 37°. The A[p³²P]-p(dT)₁₋₄₄ was then isolated by chromatography on a DEAE-Sephadex A-25 column (12 cm × 0.5 cm², bicarbonate cycle equilibrated with H₂O), elution was with a 100-ml linear gradient of triethylammonium bicarbonate (pH 7.8, 0 to 1.2 M) at a flow rate of 7 ml per hour. The molar extinction coefficient of the adenylylated oligomer at 260 nm was 56,000 and the overall yield relative to the starting d(pT)₄ was 40%. The A[p³²P]-p(dT)₁₋₄₄ was extended to a number average chain length of 240 thymidylate residues using calf thymus terminal deoxynucleotidyltransferase as described by Kelly et al. (20), except that the concentration of dTTP was 3.1 mM, the enzyme concentration was 180 μg per ml, and incubation was at 35° for 70 min. The reaction was terminated by adding EDTA to 10 mM and heating to 70° for 10 min. After removal of the denatured protein by centrifugation, the adenylylated polymer was purified by filtration through a Sephadex G-50 column (41 cm × 0.74 cm), less than 1% of the ³²P in the isolated polymer was sensitive to alkaline phosphatase. For purposes of comparison with the A[p³²P]-p(dT)₁₋₄₄ (see below) [5'-³²P]d(pT)₁₄₀ was also prepared. (dT)₁₋₄₄ was extended to a number average chain length of 240 thymidylate residues under exactly the same conditions as described above for synthesis of the adenylylated d(pT)₁₄₀. The isolated...
polymers were then labeled with \( ^{32}P \) at the 5'-terminus using \([\gamma -^{32}P]ATP \) and polynucleotide kinase (9) without prior phosphorylation.

\([\gamma -^{32}P]d(pT)_{160} \) and \((dT)_1-d(pT)_{160} \) were prepared by methods very similar to those used for the synthesis of the \( d(pT)_{160} \) oligomers. \((dT)_1-d(pT)_{160} \) was terminated with a ddTMP residue by incubation with ddTTP and calf thymus terminal deoxyribonucleotidyltransferase (16). The essentially complete addition of deoxythymidylate to the termini was demonstrated in two ways. First, parallel reactions using \( \alpha -^{32}P \) ddTTP showed that 0.94 mole of ddTMP had been added per mole of the \((dT)_1-d(pT)_{160} \) oligomer. Secondly, when the terminally oligomer was annealed to a 10-fold excess of \( d(pA)_{1200} \), the initial rate of polymerization of ddTTP with the large fragment of \( E. \ coli DNA \) polymerase I (11) (in the presence of an excess of enzyme over 3' termini) was only 3% of that observed with the oligomer lacking a deoxythymidine terminus, thus indicating that at least 97% of the 3' termini had received a ddTMP residue. \((dT)_{160}-dd(pT)_{1} \) was prepared from \((dT)_1-d(pT)_{160}-dd(pT)_{1} \) using polynucleotide kinase (9) and unlabeled ATP. To verify that the 5' termini had been phosphorylated, it was shown that they could not be further phosphorylated by \( [\gamma -^{32}P]ATP \) in the presence of polynucleotide kinase unless they were pretreated with alkaline phosphatase.

**Methods**

**Enzyme Reactions**—All reactions involving the \( E. \ coli DNA \) ligase were conducted at 30° in a buffer composed of 0.015 M Tris-HCl (pH 8.0), 4 mM MgCl2, 1 mM EDTA, 100 \( \mu \)g per ml of BSA (this composition takes into account the contribution by the enzyme diluent). Substrates and monovalent cations were present at the indicated concentrations. Ammonium salts were diluted from stock solutions neutralized to pH 7.5 with \( NH_4OH \). \( CH_3NH_2Cl \) and \( CH_2CH_2NH_2Cl \) were prepared by neutralization of methylamine and ethylamine (Eastman) with HCl. KCl, NaCl, and \( NH_4Cl \) were Baker Reagent grade. RubCl (kindly provided by Dr. David Hogness, Stanford University) and CsCl (Harshaw) were optical grade.

DNA-joining reactions using \( [^{3}H]d(pA-pT)_n \) or \( d(pA)_n-d(pT)_{160} \) were carried out as described previously (18, 21) except that the buffer composition was slightly changed as indicated above. In reactions employing \( d(pA)_n-d(pT)_{160} \), the \( d(pA)_n \) and \( d(pT)_{160} \) were present at equimolar nucleotide concentrations. Formation of product was monitored by adsorption of a portion of the reaction mixture to 1.5 cm squares of DEAE-paper (Whatman). The chro- 

**Results**

The results of a steady state kinetic analysis of the over-all reaction are presented in Fig. 2 and Table 1. The DNA substrate used, \( [^{3}H]d(pA-pT)_{160} \), assumes a double helical conformation because of its capacity to form intramolecular hydrogen bonds and is converted to a circular form by the ligase (27). Because each \( d(pA-pT)_{160} \) molecule contains the equivalent of one single strand break, the use of this copolymer avoids complications that might arise from a DNA substrate with several breaks per molecule that is able to react several
FIG. 2. Bisubstrate kinetics of E. coli DNA ligase. Assay mixtures (0.36 ml) containing the standard components ("Experimental Procedure"), 0.01 M (NH₄)SO₄, and the indicated concentration of substrates were prewarmed to 30°. The reactions were initiated by adding 0.04 ml of enzyme (0.64 ng), and 0.1-ml samples were removed at 2.5- to 5-min intervals and heated to 100° for 2 min to terminate the reaction. The exonuclease III-resistant product was determined as described under "Experimental Procedure." Rates were calculated from the linear portion of the time course. The lines shown were determined by weighted regression analysis (26).

TABLE I
Weighted regression analysis of bisubstrate kinetic analysis of DNA-joining reaction

The kinetic data presented in Fig. 1 was subjected to a weighted least squares fit according to Wilkinson (26). Weighting factors of v^4 were used.

<table>
<thead>
<tr>
<th>Concentration of fixed substrate</th>
<th>Variable substrate</th>
<th>Slope ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPN</td>
<td>0.36 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>d(pA-pT)ₙ</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>d(pA-pT)ₙ</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>d(pA-pT)ₙ</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.36 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Secondary plots (not shown) of the intercept values of the two graphs of Fig. 2 against the inverse of the fixed substrate concentrations yielded straight lines that intersected the ordinate at very similar points, corresponding to the inverse of the true V_max (28). The turnover number obtained from the experiment in which d(pA-pT)ₙ was the fixed substrate was 25 sealing events per min per ligase molecule, while that obtained from the data in which DPN was the fixed substrate is 20 min⁻¹.

Secondary plots also permitted determination of K_m values of 7 µM for DPN and 0.025 µM for 5'-phosphoryl termini in d(pA-pT)ₙ.

to 100° for 2 min to terminate the reaction. The exonuclease III-resistant product was determined as described under "Experimental Procedure." Rates were calculated from the linear portion of the time course. The lines shown were determined by weighted regression analysis (26).

TABLE II
Effect of monovalent cations on DNA-joining reaction

Standard reaction mixtures ("Experimental Procedure") contained in 0.1 ml, d(pA)₉₀₀ 5'-32P[d(pT)₂₄₀ (0.05 µM in 5'-32P), 310 µM DPN, 0.60 ng of E. coli ligase and the indicated salts in a concentration range of 0 to 10 mM. After 15 min at 30°, the reactions were terminated by heating to 100° for 2 min, then 0.05 ml of Tris-HCl (pH 9), 4 mM ZnCl₂, was added followed by 50 µg of E. coli alkaline phosphatase. After 20 min at 80°, the phosphatase-resistant 32P was determined by spotting 100 µl samples on DEAE-paper as described under "Experimental Procedure." Blank values (no ligase) were determined in the presence of the different concentrations of the various salts and the data were corrected for these blanks which were <10% of the test values.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Relative activity at 1.5 mM salt</th>
<th>Apparent K_m (µM)</th>
<th>Apparent V_max (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>LiCl</td>
<td>1.6</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>2.2</td>
<td>14</td>
<td>0.11</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.0</td>
<td>1.1</td>
<td>0.10</td>
</tr>
<tr>
<td>RbCl</td>
<td>5.0</td>
<td>4.4</td>
<td>0.11</td>
</tr>
<tr>
<td>CsCl</td>
<td>5.3</td>
<td>2.0</td>
<td>0.07</td>
</tr>
</tbody>
</table>

a n. d., not determined.

In the course of purifying the ligase, it was discovered that (NH₄)SO₄ markedly stimulated the DNA joining reaction,⁴ and the data presented in Fig. 2 were obtained in the presence of NH₄⁺. As shown in Table II and Fig. 3, the E. coli DNA ligase is in fact activated by a number of different monovalent cations. Although there was no significant stimulation or inhibition of ligase activity by Na⁺ (0 to 10 mM), considerable activation of the enzyme was observed at very low concentrations of Li⁺, K⁺, NH₄⁺, Rb⁺, and Cs⁺. Furthermore, activation of the enzyme by at least the latter four cations obeyed saturation kinetics. As shown in Table II, the differences in activation by K⁺, NH₄⁺,

or Rb+ ions are due to different $K_m$ values. The maximal velocities observed in the presence of saturating concentrations of these ions were identical, suggesting that they activate the enzyme by a similar mechanism.

Of the monovalent cations shown in Table II, NH$_4^+$ was the most effective. In the presence of a saturating level of DPN and a concentration of 5'-phosphoryl termini approximately equal to the $K_m$ value for this substrate, the $K_m$ for NH$_4^+$ was 1.1 mM. Several substituted primary amines were also tested for their effect on the enzyme (inset, Fig. 3). Methylammonium ion was only partially active as compared to NH$_4^+$, while ethylammonium ion had no effect on activity, suggesting that the activation by NH$_4^+$ is not due to its similarity to organic amines. As shown in Table III (Lines 1 and 2), the NH$_4^+$ activation is manifested as a marked increase in the true $V_{max}$ of the reaction. In these experiments the true $V_{max}$ was determined from a saturation curve for the DNA substrate, d(pA)$_{100}$ d(pT)$_{100}$ in the presence of a saturating concentration of DPN. Under these conditions, 10 mM (NH$_4$)$_2$SO$_4$ increased the true $V_{max}$ 20-fold while producing only a slight increase in the $K_m$ for the DNA. In contrast to its profound activation of the E. coli enzyme, 10 mM (NH$_4$)$_2$SO$_4$ had almost no effect on the activity of the T4 induced DNA ligase.$^3$ Thus, in addition to their different cofactor (29), and polynucleotide specificities (30–34), the two enzymes are also distinguished by their response to NH$_4^+$.

**Kinetics of Partial Reactions**

If ligase-adenylate and DNA-adenylate are true intermediates in the joining reaction, then they should react at rates that are at least equal to that of the over-all reaction. Kinetic parameters for several of the partial reactions catalyzed by the ligase were compared with the value for the joining reaction. To determine which of the partial reactions were affected by monovalent cations, most of the experiments described below were carried out in the presence and absence of NH$_4^+$.

**Ligase-AMP Formation—**When ligase is incubated with DPN, covalent ligase-AMP is formed (2, 3). A detailed kinetic study of this exchange reaction was not possible since the high substrate concentrations ($>$50 µm), necessary for the low specific activity of the available nicotinamide-labeled DPN, were nearly saturating for the enzyme. Nevertheless, as shown in Table III the maximal rates observed for the exchange are significantly greater than the rate of the over-all joining reaction determined in the presence of NH$_4^+$. Clearly then, ligase-AMP can be formed at a rate sufficient to account for its involvement in the over-all reaction. In contrast to the effects of monovalent cations on the over-all reaction, NH$_4^+$ at 20 mM had virtually no effect on the rate of the DPN-NMN exchange reaction. This finding indicates that NH$_4^+$ acts at a step subsequent to the formation of ligase-AMP.

The apparent second order rate constants for the formation and discharge of ligase-AMP were determined by measuring the initial rates of ligase-AMP formation (or discharge by NMN) at extremely low reactant concentrations (approximately 10$^{-6}$

![Fig. 3](https://www.jbc.org/content/251/10/7506/F3.large.jpg)

**Table III**

Kinetic analysis of partial reactions catalyzed by E. coli DNA ligase

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Variable substrate</th>
<th>Fixed substrate</th>
<th>$K_m$ for variable substrate</th>
<th>Turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA joining</td>
<td>d(pA)$<em>{100}$ d(pT)$</em>{100}$</td>
<td>DPN</td>
<td>0.056</td>
<td>0.28</td>
</tr>
<tr>
<td>DPN-NMN exchange</td>
<td>d(pA)$<em>{100}$ d(pT)$</em>{100}$</td>
<td>NMN</td>
<td>0.066</td>
<td>1.4</td>
</tr>
<tr>
<td>DNA-adenylate + AMP</td>
<td>DNA-AMP</td>
<td>DPN</td>
<td>0.011</td>
<td>45–60</td>
</tr>
<tr>
<td></td>
<td>DNA-AMP</td>
<td>DPN</td>
<td>0.028</td>
<td>10</td>
</tr>
</tbody>
</table>

*P. Modrich and I. R. Lehman, unpublished experiments.*
As shown in Fig. 4, the formation of ligase-AMP under these conditions was first order in both enzyme and DPN over the range of concentrations investigated. Similar results were obtained for the discharge reaction (not shown). The second order rate constants calculated from these experiments are given in Table IV. If it is assumed that a rapid equilibrium binding step followed by a slow first order reaction does not occur under these very dilute conditions, then the rate constants shown in Table IV represent the rates of binding of DPN and NMN to free ligase and ligase-AMP, respectively. If a rapid equilibrium is involved, then these rate constants represent lower limits on the rates of binding of these two substrates. It is noteworthy that the second order rate constant for reaction of NMN with ligase-AMP is 10-fold lower than that for reaction of DPN with free ligase.

The ready reversibility of ligase-AMP formation (2, 3) implies that a significant fraction of the free energy of the pyrophosphate bond of DPN is retained in the ligase-AMP intermediate. This finding is not surprising since a phosphoamide bond links the adenylyl group to the e-amino group of a lysine residue (1). We have determined the equilibrium constant for the formation of ligase-AMP, under standard reaction conditions (pH 8 and 30°), by determining the fraction of the enzyme in the adenylylated form as a function of the free (NMN): (DPN) ratio, according to the equation:

$$\frac{1}{[E-AMP]} = \frac{1}{[E]} + \frac{1}{K_{eq} [E]} \frac{[NMN]}{([DPN])}$$

The equilibrium constant determined from the slope of Fig. 5 is 28. This value may not solely reflect the free energy differences between the pyrophosphate bond of DPN and the phosphoamide bond in ligase-AMP, since it may include a term for the neutralization of a proton by the buffer. Since the e-amino group of the lysine residue involved in the phosphoamide linkage may be protonated in the unadenylated enzyme, a proton should then be lost during the adenylylation reaction and the fate of this proton is not known. An equilibrium constant of this magnitude strongly suggests that virtually all the ligase in the cell is in the adenylylated form since the intracellular concentration of DPN in E. coli is about 0.5 mM (35).}

**DNA-adenylate Formation**—DNA-adenylate does not normally accumulate during the DNA ligase reaction (4). However, Harvey et al. (24) have shown that DNA-adenylate can be formed in T4 DNA ligase-catalyzed reactions conducted at 0° and at pH 5.6. Rather than grossly changing the reaction conditions to study the rate of DNA-adenylate synthesis by the E. coli enzyme, we have employed an altered DNA substrate. As shown in the scheme for the mechanism of the ligase reaction...
Fig. 5. Equilibrium constant for ligase-AMP formation. Reaction mixtures (1 ml) contained, in addition to the standard components ("Experimental Procedure"), 0.01 m (NH₄)₂SO₄, 100 mm [γ³²P]DPN, 0.20 μg of ligase, and an amount of MNM to yield the ratio shown on the abscissa. After 5 and 10 min at 30°, 0.2-ml samples were removed and ligase-[³²P]AMP was determined by acid precipitation as described in the legend to Fig. 4. In every instance the 5- and 10-min points agreed within 5%, and consequently the reactions were judged to be at equilibrium. The line shown was determined by least squares analysis. The total enzyme concentration calculated from the inverse of the intercept (see text) was 1.83 nm. This value corresponds to a yield of ligase-AMP of only 0.68 mole of AMP per 74,000 g of protein, and as discussed in the previous paper (6) may reflect some residual adsorption of the enzyme to the siliconized reaction tubes. The equilibrium constant calculated from the slope 1/Kₑq = 1.83 nm, is 28.

(Fig. 1), elimination of AMP from DNA-adenylate presumably requires an adjacent 3'-hydroxyl terminated polynucleotide. Consequently, we have investigated the activity of the DNA ligase on d(pA)₈₁₀·d(pT)₁₄₀·d(pT)₁₄₀ in which the d(pT)₁₄₀ chains are terminated with a 3'-dideoxythymidylate residue. As shown in Table V, incubation of the dideoxy-terminated substrate with ligase and [γ³²P]AMP resulted in the formation of DNA adenylate. As expected, the accumulation of this product was dependent upon enzyme, and 5'-phosphoryl, 3'-dTMP terminated d(pT)₁₄₀ chains d(pT)₁₄₀ chains possessing 5'-phosphoryl and 3'-hydroxyl termini were much less active, and 5'-hydroxyl terminated oligomers were inert. However, the rate of synthesis of DNA-adenylate under these conditions was extremely low, at least 1000-fold lower than the rate of joining of d(pT)₁₄₀ annealed to d(pA)₁₈₀ under the same conditions. In fact, in order to detect adenyllylation of the dideoxy-terminated polymer as shown in Table V, it was necessary to use concentrations of enzyme of the same order of magnitude as the concentration of single strand breaks. If DNA-adenylate is an obligatory intermediate in the DNA ligase reaction, then it follows that a 3'-hydroxyl terminus is essential for adenyllylation of the adjacent 5'-phosphate at the single strand break. It is not clear whether the 3'-hydroxyl group is required merely for normal binding of the enzyme at the single strand break or whether it is essential for some subsequent step in the catalysis. We have found that dideoxy-terminated d(pT)₈₅ chains inhibit the joining of unterminated chains, indicating that the enzyme does bind to single strand breaks containing a dideoxy terminus.

<table>
<thead>
<tr>
<th>d(pT)₁₄₀ termini</th>
<th>Ligase</th>
<th>DNA-adenylate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-P, 3' ddTMP</td>
<td>0</td>
<td>0.034</td>
</tr>
<tr>
<td>5'-OH, 3' ddTMP</td>
<td>0.075</td>
<td>0.029</td>
</tr>
<tr>
<td>5'-OH, 3' ddTMP</td>
<td>0.75</td>
<td>0.036</td>
</tr>
<tr>
<td>5'-P, 3' ddTMP</td>
<td>0.075</td>
<td>0.20</td>
</tr>
<tr>
<td>5'-P, 3' ddTMP</td>
<td>0.75</td>
<td>1.80</td>
</tr>
<tr>
<td>5'-P, 3' ddTMP</td>
<td>0.75</td>
<td>0.062</td>
</tr>
</tbody>
</table>

Release of AMP from DNA-adenylate—When synthetic d(pT)₈₅-adenylate is incubated with the unadenylylated form of the ligase in the presence of poly d(pA), AMP is released from the polymer and a phosphodiester bond is formed in stoichiometric amounts (5). We have investigated the kinetics of this reaction in the presence and absence of NH₄⁺ (Table III, Lines 4 and 5). In these experiments, d(pA)₁₈₀·[³²P]p·p(dT)₁₄₀·d(pT)₁₄₀ was incubated with the enzyme and conversion of the [³²P]AMP moiety to an acid-soluble form was measured. Although the maximal velocity of this reaction is not affected by NH₄⁺, the Kₘ for the substrate is increased. When the rate of the reaction in the absence of NH₄⁺ is compared with the rate of the over-all reaction under identical conditions (Table III, Line 2), it is clear that the rate of release of AMP from DNA-adenylate is faster than the rate of DNA joining. This result is consistent with the involvement of DNA-adenylate in the over-all joining reaction. However, in the presence of NH₄⁺ (conditions optimal for the ligase reaction), the rate of release of AMP from DNA-adenylate is significantly less than the rate of the over-all reaction. Furthermore, DNA-adenylate does not accumulate in DNA-joining reactions in the presence of NH₄⁺. Four explanations may account for the results obtained in the presence of NH₄⁺. DNA-adenylate may not be an obligatory intermediate but rather the product of a side reaction. Secondly, the low rate may reflect a slow conformational change of the unadenylylated ligase required for binding to DNA-adenylate. In the normal course of the ligase reaction, dissociation of ligase from DNA-adenylate may not occur before the latter is converted to products; consequently, experiments in which synthetic DNA-adenylate is incubated with the ligase may introduce a slow binding step that need not occur when the enzyme proceeds through the normal course of catalysis. Finally, the true rate constant for the release of AMP from DNA-adenylate may not have been determined in the presence of NH₄⁺.

* The joining reaction with the d(pA)₁₈₀·d(pT)₁₄₀ substrate measures conversion of a γ-labeled 5'-phosphoryl terminus to a phosphatase resistant form (21). Therefore, the increased rate of conversion of γ³²P to a phosphatase resistant form in the presence of NH₄⁺ could reflect an accumulation of DNA-adenylate rather than an increase in the rate of phosphodiester bond synthesis. However, at least 98% of the phosphatase-resistant γ³²P generated in the presence of NH₄⁺ remained resistant to phosphatase after treatment with 0.5 N HCl at 100° for 30 min. Since the pyrophosphate bond in DNA-adenylate would be hydrolyzed by these vigorous conditions, this possibility has been ruled out.
of NH₄⁺ because another reaction may also occur. Thus, when ligase is incubated with DNA-adenylate, the reaction can proceed to yield a phosphodiester bond and AMP, but the DNA-adenylate may also serve as a substrate for formation of ligase-AMP and DNA containing a single strand break with a 5'-phosphoryl terminus (36). If NH₄⁺ facilitates the dissociation of ligase-AMP from a single strand break, then the turnover number determined for the forward reaction would be too low. Ligase-AMP is inactive in releasing AMP from DNA-adenylate (4, 5), and the formation of free enzyme-adenylate would effectively result in a fraction of the enzyme becoming inactive. We have in fact obtained evidence in support of the latter hypothesis.

As shown in Table VI, when DNA-adenylate is incubated with ligase and NMN in the absence of NH₄⁺, then essentially all of the AMP released from the DNA is recovered as AMP. However, in the presence of 0.02 M NH₄Cl, 20% is recovered as DPN. Since the replacement of NH₄Cl by the same concentration of NaCl did not produce this result, a nonspecific ionic strength effect can be ruled out. This finding therefore suggests that NH₄⁺ facilitates the dissociation of ligase-AMP from DNA. Furthermore, since NH₄⁺ presumably does not affect the equilibrium constant for the over-all reaction, it follows that NH₄⁺ should increase the rate of binding of the adenylylated enzyme to this substrate. To test this possibility the apparent second order rate constants were measured for the release of AMP from ligase-AMP by DNA in the presence and absence of NH₄⁺. As shown in Table VII, 0.02 M NH₄⁺ increased the apparent second order rate constant for this reaction 10-fold. Again, since there was no effect of 0.02 M Na⁺, the rate increase must reflect a specificity for NH₄⁺. These results are consistent with the hypothesis that ammonium ion increases the rate of association and dissociation of ligase-AMP and DNA, and may explain the anomalous results obtained in the kinetic analysis of AMP release from DNA-adenylate. They do not, of course, rule out the other possibilities mentioned above. Furthermore, they do not eliminate involvement of NH₄⁺ in catalytic events subsequent to the binding of ligase-AMP to DNA.

The rate of binding of ligase-AMP to DNA is an extremely fast reaction, particularly in the presence of NH₄⁺. The rate constants shown in Table VII represent only lower limits since they were calculated using the assumption that all the 5'-phosphoryl groups are independent in solution. Control experiments in which 0.02 M NaCl was substituted for NH₄Cl showed that the rate increase was due to the presence of NH₄⁺ rather than to a nonspecific ionic strength effect.

### Table VI

<table>
<thead>
<tr>
<th>Additions to reaction</th>
<th>Released AMP recovered as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
</tr>
<tr>
<td>None</td>
<td>98</td>
</tr>
<tr>
<td>0.02 M NaCl</td>
<td>67</td>
</tr>
<tr>
<td>0.02 M NH₄Cl</td>
<td>81</td>
</tr>
</tbody>
</table>

#### DISCUSSION

Except for the anomalously low turnover number observed for phosphodiester bond formation from DNA-adenylate in the presence of NH₄⁺, our findings are consistent with the mechanism for the DNA ligase reaction described in Fig. 1. The kinetics observed with DNA-adenylate in the presence of NH₄⁺, might at first suggest that it is not an obligatory intermediate in the overall reaction. However, unless an unlikely concerted mechanism is involved, it is reasonable to assume that covalent activation of the single strand break must occur for phosphodiester bond synthesis to proceed. Covalent activation could occur in ways other than by adenylylation of the 5'-phosphate at the single strand break. Thus, activation could occur by adenylylation of the 3'-hydroxyl terminus, however, DNA structures containing a single strand break with an adenylylated 3'-hydroxyl terminus have never been detected (4). Alter native, the formation of a covalent enzyme-DNA intermediate from ligase-AMP and DNA could serve to activate the single strand break. We have used the substrate d(pA)₁₀₀₀-[5'-³²P]d(pT)₁₄₀-dl(dT)₁₀ to assay for a covalent enzyme DNA intermediate in which the protein is bound to the 5'-phosphoryl terminus, but were unable to detect such a species.\(^\text{5}\)

\(^\text{5}\) When d(pA)₁₀₀₀-[5'-³²P]d(pT)₁₄₀-dl(dT)₁₀ was incubated with ligase and DPN under conditions of enzyme excess, some of the

### Table VII

<table>
<thead>
<tr>
<th>NH₄⁺</th>
<th>Apparent second order rate constant</th>
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<tbody>
<tr>
<td></td>
<td>(n^{-1} \text{ min}^{-1})</td>
</tr>
<tr>
<td>-</td>
<td>1.4 ± 0.3 \times 10^6</td>
</tr>
<tr>
<td>+</td>
<td>1.5 ± 0.2 \times 10^8</td>
</tr>
</tbody>
</table>
of these findings, and since the apparently anomalous kinetics observed with DNA-adenylate can be explained plausibly by the ammonium ion-induced dissociation of ligase-AMP from DNA and perhaps by a slow conformational change required for binding of DNA-adenylate, we feel that our results are consistent with the mechanism shown in Fig. 1. The recent demonstration that both ligase-AMP and DNA-adenylate accumulate during reversal of the reaction (36) offers additional support for this mechanism, particularly in regard to the involvement of DNA-adenylate.

The effects of monovalent cations on the ligase reaction are striking. There seems to be little correlation of the effects seen with the unsolvated ionic radii. On the other hand, it is not clear that stimulation of the enzyme by all the cations tested occurs by a common mechanism. At least in the case of $K^+$, $NH_4^+$, and $Rb^+$, in which a common mechanism may be involved, there is a correlation between the similar ionic radii and enzyme activation. The effect of $NH_4^+$ on the reaction probably occurs subsequent to the formation of ligase-AMP, and may involve a conformational change in the protein. However, attempts to detect such a conformational change in ligase-AMP by ultraviolet difference spectroscopy have yielded negative results.

The turnover number for the ligase is surprisingly low. This finding does not reflect a loss of enzymatic activity during the purification since the over-all yield of enzyme is 10 to 15%, and the yields at each step are 70% or better; some activity being discarded in choosing the most active fractions (6). Also, the stoichiometry of the adenylylation reaction (6) and the finding that essentially all of the AMP in the adenylylated enzyme can be released upon incubation with DNA suggests that the ligase preparation is fully active. The most reasonable interpretation is that the enzyme has an inherently low catalytic constant. E. coli appears to contain about 300 DNA ligase molecules per cell (6). A turnover number of 25 min$^{-1}$ at 30°C therefore indicates a capability of sealing 7,500 single strand breaks per min per cell. Assuming that both strands of the E. coli chromosome replicate discontinuously (37, 38), that the time to replicate the chromosome is about 65 min at 30°C (39-41), and that the average length of an "Okazaki fragment" is 1000 nucleotides (37), then about 200 sealing events per min per cell would be sufficient to account for the joining of these intermediates in the discontinuous replication of DNA within a replication period. Thus, a few per cent of the normal amount of ligase should be sufficient for DNA replication. Extracts of the temperature sensitive ligase mutant of E. coli, $\Delta$ig ts7, contain about 1 to 3% of the level of DNA joining activity at permissive temperature as the wild type parent under similar conditions (38, 42). Since the mutant grows normally at permissive temperature (although the rate of joining of Okazaki fragments is clearly depressed), it seems reasonable to conclude that the cell has a relatively limited requirement for ligase-catalyzed joining events, and that this is within the capabilities of the enzyme as determined in vitro.

Acknowledgment—We are very much indebted to Professor William P. Jencks for his helpful criticisms and comments during the course of this work and in the preparation of this manuscript.

REFERENCES