Deoxyribonucleic Acid Ligase

ISOLATION AND PHYSICAL CHARACTERIZATION OF THE HOMOGENEOUS ENZYME FROM ESCHERICHIA COLI

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SUMMARY

DNA ligase of Escherichia coli has been purified to homogeneity as judged by polyacrylamide gel electrophoresis and by analytical ultracentrifugation. The molecular weights of the native, and the denatured and reduced forms of the enzyme are 74,000 ± 3,000, hence, ligase consists of a single polypeptide chain. When the unadenylylated form of the enzyme is incubated with diprophosphopyridine nucleotide, approximately 1 mole of adenosine 5'-monophosphate becomes covalently linked per 74,000 g of protein.

The DNA ligase of Escherichia coli catalyzes the synthesis of phosphodiester bonds at single strand interruptions in duplex DNA, coupled to the cleavage of the pyrophosphate bond of DPN (1–3). Although it has been demonstrated that enzyme-adenylate and DNA-adenylate are formed in the DNA joining reaction (1–5), definitive proof that these two compounds are true kinetic intermediates requires the demonstration that their rates of reaction be as great or greater than the over-all DNA joining rate.

We have therefore purified the E. coli DNA ligase to physical homogeneity and undertaken a kinetic analysis of the reaction which it catalyzes. In this paper we describe a purification procedure which leads to the isolation of physically homogeneous enzyme in good yield. We also describe the physical properties of the pure DNA ligase. In the following paper we present a detailed kinetic analysis of the reaction catalyzed by the pure enzyme. A preliminary report of this work has appeared (6).

EXPERIMENTAL PROCEDURE

Materials

[32P]AMP (50 Ci per mmole) was prepared by the method of Symons (7). [32P]DPN was synthesized chemically from the

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When the DNA ligase was purified from *E. coli* strain B, the streptomycin supernatant fraction obtained from the DNA polymerase purification (13) was used as the source of enzyme. This fraction was kindly provided by Dr. A. Kornberg and Dr. II. Sobell. The *E. coli* K12 ligase overproducing strain LCS1 (14) was generously provided by Dr. Martin Gellert, National Institutes of Health. This strain was grown in 100-liter cultures of yeast extract-phosphate-glucose medium (15) at 37°C with aeration in a New Brunswick fermenter. When the absorbance at 595 nm reached 2.5 to 2.7, the culture was chilled to 4°C with the refrigeration unit of the fermenter. The cells were harvested with a refrigerated Sharples centrifuge and the cell paste was stored at −20°C until ready for use.

**Methods**

**Enzyme Assays**—The DNA ligase was diluted into 0.05 M Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM MgCl₂, 0.01 M (NH₄)₂SO₄, 0.05 mg per ml of BSA. DNA joining activity of the *E. coli* ligase was assayed by d(A-Tₘ) circle formation as previously described (12), except that the enzyme diluent and reaction mixture were made 0.01 M in (NH₄)₂SO₄ (diluted from a 1 M stock solution neutralized to pH 7.5 with NH₄OH). Instead of collection of the exonuclease III-resistant d(A-Tₘ) circles by acid precipitation, 80- to 100-μl aliquots of the exonuclease III digest were spotted on 1.5-cm squares of DEAE-paper (Whatman DES1). The DES1 squares were washed with 0.3 M ammonium formate (pH 7.8) as described by Brutlag and Kornberg (16).

Adenylylation of the ligase was carried out in a buffer consisting of 0.015 M Tris-HCl (pH 8.0), 0.01 M (NH₄)₂SO₄, 4 mM MgCl₂, 1 mM EDTA, and 100 μg per ml of BSA. [³²P]DPN (0.5 to 1 × 10⁵ cpm per pmole) was present in at least a 10-fold excess over the ligase, which was present at concentrations ranging from 0.1 to 100 nM. Incubation was at 30°C in siliconized (Siliclad Clay-Adams) glass tubes. The reaction was terminated by removing samples of appropriate volume and making them 0.2 M in Tris base, 20 mM in EDTA, and 0.2 mg per ml in BSA. The [³²P]-labeled ligase-AMP was precipitated by adding cold tri-chloroacetic acid to a final concentration of 7%. After 5 min at 0°C the precipitate was collected on Millipore filters (HA, 0.45 μ) which were washed four times with 10 ml of cold 1 N HCl.

When [³²P]ligase-AMP was isolated on a preparative scale, the adenylylation reaction was terminated by adding EDTA to a final concentration of 10 mM. The reaction was applied to a siliconized column (30 cm × 0.5 cm²) of Sephadex G-50 (fine) equilibrated with 0.02 M Tris-HCl (pH 8.0), 1 mM EDTA, 0.01 M (NH₄)₂SO₄, 0.5 mM 2-mercaptoethanol, and 0.5 mg per ml of BSA. The ligase-AMP, which was excluded by the gel, was collected into siliconized glass tubes.

**Enzyme activity** was determined by the method of Richardson et al. (17) as modified by Jovin et al. (13), using [³²P]-d(A-Tₘ) copolymer (4000 cpm per pmole) as substrate.

**Polyacrylamide Gel Electrophoresis**—Electrophoresis in gels of 7.5% acrylamide employed two discontinuous systems (19). System A (resolving pH = 8.0 at 0°C): resolving buffer, 0.235 M bis(2-hydroxyethyl) imino-tris(hydroxymethyl)methane (bis-tris), 0.049 M HCl; stacking buffer, 0.055 M cacodylic acid, 0.038 M bis-tris; upper buffer, 0.04 M N-2-hydroxyethylpipperazine-N’-2-ethanesulfonic acid (Hepes), 0.0158 M bis-tris; lower buffer, 0.0025 M bis-tris, 0.05 N HCl. System B (resolving pH = 8.3 at 0°C): resolving buffer, 0.243 M bis-tris, 0.0345 M HCl; stacking buffer, 0.0585 M bis-tris, 0.040 M H₂PO₄; upper buffer, 0.052 M bis-tris, 0.0403 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES); lower buffer, 0.0025 M bis-tris, 0.05 N HCl. Gel length was 7 cm. Electrophoresis was conducted at 4°C at a constant current of 1.5 ma per gel and was terminated when the tracking dye (bromphenol blue) had migrated about 6 cm. Protein was identified by staining with Coomassie brilliant blue (18).

**Sedimentation Analysis**—Analytical centrifugation experiments were performed with an ANH-4 rotor in a Beckman model E ultracentrifuge equipped with schlieren and Rayleigh optics. Prior to sedimentation, the unadenylylated form of the ligase was dialyzed exhaustively at 4°C against 0.02 M potassium phosphate buffer (pH 6.50), 0.01 M NH₄Cl (pH 6.50), 0.20 M KCl, 0.5 mM dithiothreitol.

Velocity sedimentation of the enzyme was carried out in a filled Epon 12-mm double-sector cell with sapphire windows. The sample sector was filled with 0.45 ml of the dialyzed ligase solution (2.7 mg per ml), and the reference sector contained 0.46 ml of the dialysis buffer. Sedimentation was at 56,000 rpm at 20.6°C. Schlieren photographs were taken at intervals of about 10 min on Kodak Spectroscopic IIIG plates. A phase angle of 60°C was used. The sedimentation coefficient was corrected to standard conditions by assuming that s²/(1-φₚ) is invariant, where s is the sedimentation coefficient, φ is solvent viscosity, p is the protein partial specific volume, and ρ the solvent density.

Sedimentation equilibrium analysis was performed as described by Chervenka (20) in a 12-mm double-sector cell with a double-sector capillary synchotonic boundary centerpiece. The sample sector contained about 0.12 ml of dialyzed enzyme at 2.2 mg per ml (corresponding to a column height of 3.17 mm) which was raised off the cell bottom by 0.03 ml of fluorescent oil (FC-43, 3M Co.). The reference sector contained 0.18 ml of dialysis buffer. The cell was centrifuged at 22,000 rpm for 2.0 hours and then at 9,000 rpm for 30 hours at 16.6°C. Rayleigh photographs were taken on Kodak Spectroscopic IIG plates at 25 hours and 30 hours after reduction of speed. Since there was no difference in the number of fringes on the two plates, the run was judged to be at equilibrium. The initial protein concentration, C₀, in units of fringes, was determined by a boundary forming run following the equilibrium run. The molecular weight was determined from the slope of a plot of ln C versus φ according to the equation $M - 2RT/1 - φp = C_d(φ^2)$. A Gaertner comparator was employed for measurements on schlieren and Rayleigh plates. A partial specific volume, φ, of 0.740 at 25°C was calculated on the basis of the amino acid composition (21, 22). For use in calculations, this value was corrected for an assumed temperature dependence of 5 × 10⁻⁴ ml·g⁻¹·degree⁻¹ (23).

**Amino Acid Analysis**—Amino acid analyses were performed on a Beckman model 121 Automatic Amino Acid analyzer. The enzyme was dialyzed exhaustively against 0.01 M potassium phosphate buffer (pH 7.5). Portions of the dialyzed enzyme (about 100 μg) were taken to dryness on a rotary evaporator, a crystal of phenol was added (24), and the residue dissolved in 1.2 ml of 6 N HCl. Hydrolysis was performed at 110°C for 24, 48, and 73 hours. The values for serine and threonine were obtained by extrapolating to zero time of hydrolysis. The values cited for valine and leucine are averages of the 48- and 73-hour determinations. Half-cystine was determined as cysteic acid after performic acid oxidation (25). Tryptophan was estimated from the absorption spectrum of the native enzyme assuming a molar extinction coefficient at 278 nm of 5.55 × 10⁴ (26).
RESULTS

Purification of DNA Ligase

Unless indicated otherwise, all steps were performed at 0-4°C, and centrifugation was at 15,000 × g for 20 to 30 min. The enzyme was routinely concentrated after each step by precipitation with (NH₄)₂SO₄ (0.47 kg per liter, to 70% saturation). Solid (NH₄)₂SO₄ was added over a period of 20 to 30 min with stirring. After stirring for an additional hour, the precipitate was collected by centrifugation. A summary of the purification procedure is given in Table I.

Preparation of Extract—Cells of E. coli strain LC81 (660 g, wet weight) were suspended in 2.5 liters of 0.05 M Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol with the aid of a Waring Blender. The cells were disrupted by two passages through a Manton-Gaulin homogenizer at a pressure of 6500 to 7000 pounds. The extract was clarified by centrifugation and buffer added to adjust the protein concentration to 20 mg per ml (Fraction I).

Streptomycin Fractionation—A freshly prepared 5% aqueous solution of streptomycin sulfate (620 ml) was added to 3.1 liters of extract over a 20-min period with stirring. After stirring for an additional 20 min, the suspension was centrifuged. The supernatant fluid (3.4 liters) was diluted by the addition of 6.8 liters of cold H₂O, and then an additional 3.4 liters of 5% streptomycin sulfate were added. The suspension was stirred for 20 min, and the insoluble material removed by centrifugation in 1-liter batches in a Sorvall Superspeed centrifuge. The supernatant fluid (15.3 liters) was concentrated by (NH₄)₂SO₄ precipitation (Fraction II).

Alumina Gel C₇ Adsorption and Elution—Fraction II was suspended in 270 ml of 0.02 M potassium phosphate (pH 6.5), 2 mM EDTA, and 1 mM 2-mercaptoethanol (Buffer A) to a protein concentration of 50 mg per ml. Two and four-tenths liters of 0.01 M potassium phosphate (pH 7.5)-0.01 M (NH₄)₂SO₄ were added, followed by 810 ml of alumina gel C₇. After stirring for 20 min, the gel was collected by centrifugation. The pellet was suspended in 2.7 liters of 0.03 M potassium phosphate (pH 7.5)-0.01 M (NH₄)₂SO₄, and after stirring for 20 min the gel was collected by centrifugation. The ligase was eluted by suspending the gel in 2.7 liters of 0.4 M potassium phosphate (pH 7.5), 0.01 M (NH₄)₂SO₄, 2 mM EDTA, and 1 mM 2-mercaptoethanol. After stirring for 30 min the alumina gel C₇ was removed by centrifugation, and the eluate concentrated by (NH₄)₂SO₄ precipitation (Fraction III).

DEAE-cellulose Adsorption and Elution—This step removes residual nucleic acid and is required for retention of the enzyme on phosphocellulose. Fraction III was dissolved in Buffer A and the volume adjusted to 215 ml to yield a protein concentration of 20 mg per ml. The solution was dialyzed against 4-liter portions (three changes) of 0.15 M potassium phosphate (pH 7.5), 0.01 M (NH₄)₂SO₄, 2 mM EDTA, and 1 mM 2-mercaptoethanol for a total of 4 hours and then applied at a rate of 300 ml per hour to a DEAE-cellulose column (17.5 cm × 15.4 cm²) equilibrated with the same buffer. The column was washed with buffer (500 ml) and protein that did not adsorb (500 ml) collected (Fraction IV).

Phosphocellulose Chromatography of Adenylylated Enzyme—Fraction IV was dialyzed against 11 liters of Buffer A for 2 hours. Dialyzed enzyme was made 5 mM in MgCl₂ and 50 μM in DPN and placed in a 30°C bath for 10 min, at which time the temperature of the solution was 23°C. After an additional 30-min incubation at 0°C, EDTA was added to a concentration of 0.9 mM. The adenylylated enzyme was dialyzed against 11-liter portions (two changes) of 0.2 M potassium phosphate (pH 6.5), 2 mM EDTA, 1 mM 2-mercaptoethanol, and 2 μM DPN for 2.5 hours, and immediately applied at a rate of 90 ml per hour to a phosphocellulose column (82 cm × 9.2 cm²) equilibrated with the DPN-supplemented buffer. The column was washed with 1.5 liters of this buffer and then eluted with a 3.6-liter linear gradient of potassium phosphate (pH 6.5, 0.02 to 0.2 M) containing 2 mM EDTA, 1 mM 2-mercaptoethanol, and 2 μM DPN. Most of the DNA ligase activity was recovered from the column at the end of the 0.02 M potassium phosphate wash, well separated from the unadsorbed protein. However, a second peak of activity eluting at 0.07 M potassium phosphate along with a major peak of contaminating protein was often observed. Usually (six experiments) the early eluting fraction contained the majority of recovered activity; however, in two experiments activity eluting at 0.07 M potassium phosphate was much more significant, comprising 40% or 90% of recovered activity (see below). Early eluting fractions with a specific activity greater than 300 units per mg were pooled (1,200 ml) and taken to 70% saturation with ultrapure (NH₄)₂SO₄. The (NH₄)₂SO₄ suspension was allowed to stand overnight at 0°C, and the precipitate collected by centrifugation at 15,000 × g for 3 hours (Fraction V).

DEAE-Sephadex Chromatography—Fraction V was dissolved in 28 ml of 0.02 M Tris-HCl (pH 8.0), 2 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.2 M NaCl, and then dialyzed against two 1-liter portions of this buffer for a total of 4 hours. After removal of a small amount of insoluble material by centrifugation, the dialyzed enzyme was applied to a column of DEAE-Sephadex A-50 (28 cm × 2.9 cm²) equilibrated with the dialysis buffer. The column was washed with 160 ml of starting buffer, and then eluted with a 560-ml linear gradient of NaCl (0.2 to 0.5 M) containing 0.02 M Tris-HCl (pH 8.0), 2 mM MgCl₂, and 1 mM 2-mercaptoethanol. The flow rate was 20 ml per hour and 10-ml fractions were collected. Ligase activity eluted as a single peak at an NaCl concentration of 0.25 M. Fractions with a specific

| Fraction | Step | Total protein | Specific activity | Recovery |
|----------|------|---------------|------------------|---------|
| I        | Extract | 62,000 | 7.2 | 100 |
| II       | Ammonium sulfate | 27,000 | 13 | 77 |
| III      | Alumina gel C₇ | 7,400 | 34 | 56 |
| IV       | DEAE-cellulose | 6,500 | 40 | 58 |
| V        | Phosphocellulose (adenylated enzyme) | 150 | 930 | 32 |
| VI       | DEAE-Sephadex | 19 | 4,500 | 19 |
| VII      | Phosphocellulose (unadenylated enzyme) | 7.3 | 7,500 | 12 |

a Protein determined by amino acid analysis.
b Protein determined by absorbance at 280 and 260 nm (28).
activity greater than 2000 units per mg were pooled (47 ml) and precipitated with ultrapure (NH₄)₂SO₄ as in Fraction V (Fraction VI).

Phosphocellulose Chromatography of Unadenylated Enzyme—Fraction VI was dissolved in 5 ml of Buffer A containing 5% glycerol. It was dialyzed against 1 liter of 0.05 M Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM 2-mercaptoethanol, and 5% glycerol for 2 hours. The dialyzed enzyme was made 0.28 M in NMN and 5 mM in MgCl₂ and incubated at 30°C for 3 min. After further incubation at 0°C for 60 min, EDTA was added to a final concentration of 7 mM, and the solution dialyzed against 250-ml portions of Buffer A (two changes) for a total of 2.5 hours. The dialyzed enzyme was applied at a flow rate of 10 ml per hour to a column of phosphocellulose (23 cm x 1.1 cm) equilibrated with Buffer A. After washing with 50 ml of Buffer A, the column was eluted with a 200-ml linear gradient of potassium phosphate (pH 6.5, 0.02 to 0.2 M) containing 2 mM EDTA, 1 mM 2-mercaptoethanol, and 6-ml fractions were collected. The ligase was eluted from the column at a potassium phosphate concentration of 0.06 to 0.07 M. The peak fractions representing about 75% of the recovered activity were pooled (21 ml) and the protein precipitated with ultrapure (NH₄)₂SO₄. The pellet was dissolved in 0.7 ml of 0.15 M potassium phosphate (pH 6.5), 0.01 M (NH₄)₂SO₄, 2 mM EDTA, and 1 mM 2-mercaptoethanol, and then dialyzed against 100-ml portions of this buffer (two changes) for 3 hours. The dialyzed enzyme was made 50% in glycerol and stored at -20°C (Fraction VII).

This procedure has also been used to purify the DNA ligase from the streptomycinsupernatant fluid obtained as a side fraction of the DNA polymerase I purification (13) from E. coli B. Cold water, 0.56 volume, and a fresh 5% solution of streptomycin sulfate, 0.52 volume, were added to the streptomycin supernatant fraction. After removal of the precipitate by centrifugation, the supernatant fluid was concentrated with (NH₄)₂SO₄ as for Fraction II. The remaining steps in the purification were carried out as described above. The specific activity of Fraction VII prepared from E. coli B was identical to the same fraction obtained from LC81 that overproduces ligase.

Except for phosphocellulose chromatography of adenylylated enzyme, all steps of the purification were easily reproducible. As noted above, a significant fraction of the enzyme was occasionally found to elute at 0.07 M potassium phosphate instead of at the end of the 0.02 M wash. In one instance 90% of the recovered activity eluted at 0.07 M potassium phosphate. Despite the fact that the specific activity of the enzyme recovered in this case was only 30% of that expected for this step, it was possible to obtain a ligase preparation of 95% purity by continuing with the purification, and subjecting the enzyme recovered after the final step to rechromatography on DEAE-Sphadex. The following procedure was used. Fraction VII (290,000 units in 11 ml, 7,500 units per mg, protein determined by absorbance at 280 and 260 nm (28)) was dialyzed against 1,000 ml of 0.025 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 5% glycerol for 2 hours. The dialyzed enzyme was made 50 μM in DPN, 4 mM in MgCl₂ and incubated at 30°C for 5 min. EDTA was then added to a final concentration of 5 mM, and the solution dialyzed against 1,000-ml portions (three changes) of 0.02 M Tris HCl (pH 8.0), 2 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.2 M NaCl for a total of 3 hours. The dialyzed enzyme was then applied to a column of DEAE-Sphadex A-50 (22.5 cm x 2.9 cm) and chromatographed as described above. Fractions with a specific activity of 10,000 units per mg (protein determined by absorbance at 280 and 260 nm) were pooled (142,000 units) and concentrated with ultrapure (NH₄)₂SO₄. The enzyme was dialyzed and stored as described for Fraction VII.

DNA ligase purified by this procedure was stable for at least 1 year (<10% loss in DNA joining activity). Furthermore, the purified enzyme was free of detectable exonuclease activity on double stranded DNA in the absence of AMP (29) and contained no detectable exonuclease activity on (dAT₉)ₙ-copolymer (<1 umole/30 min per mg). Fraction VII of the DNA ligase was used for all the studies to be described.

Fractions II and III were stable for at least 3 months, and Fraction V was stable for at least 2 weeks when stored as ammonium sulfate pellet at -20°C. Fractions IV and VI were stable overnight at 0°C, while Fraction I lost 10 to 20% of its activity when stored under these conditions.

Electrophoretic Analysis

DNA ligase was subjected to electrophoretic analysis under both native and denaturing conditions. Results of typical gels are presented in Fig. 1. When reduced and denatured enzyme was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, a single protein band was detected. Furthermore, the identical mobilities of 32P-labeled enzyme-AMP and the single protein band on sodium dodecyl sulfate acrylamide gels indicated that DNA ligase activity was associated with this major protein species (Fig. 2). A single protein zone was also observed after electrophoresis under native conditions at pH 8.0 or 8.3. Since 25 to 40 μg of protein were applied to each gel, these results indicate a purity of greater than 98%.

The mobility of the ligase on sodium dodecyl sulfate gels relative to the mobility of bromophenol blue was 0.49 (S.D. = 0.01, seven experiments). This value was compared with the mobilities of several proteins of known molecular weight including β-galactosidase digested BSA, DNA polymerase I, DNA ligase, aldolase, and lysozyme (18). The apparent molecular weight for the denatured and reduced form of the DNA ligase calculated from this standard curve was 74,000 ± 3,000. The mobilities of the enzymes obtained from E. coli B and from the overproducing K12 strain LC81 were indistinguishable. As judged by this criterion, as well as by their specific activities, the two enzymes appear to be identical. The remainder of this section will be devoted to properties of the ligase obtained from LC81.

Sedimentation Analysis

The weight average molecular weight of the purified native ligase determined by sedimentation equilibrium ultracentrifugation was 77,000 (Fig. 3). The slight deviation from linearity of the ln C versus r² plot may reflect the presence of a high molecular weight impurity (approximately 5%). Alternatively, it could represent a small amount of aggregation. The value of 77,000 is close to that obtained for the denatured enzyme by sodium dodecyl sulfate gel electrophoresis (74,000), indicating that the E. coli DNA ligase is composed of a single polypeptide chain.

The ηωω of the purified enzyme determined by analytical sedimentation at a protein concentration of 2.1 mg per ml (corrected for radial dilution) was 3.91 S. This value agrees reasonably well with the value of 4.2 S obtained for DNA ligase activity by sucrose gradient centrifugation (6). Except for the presence of the minor fast sedimenting contaminant mentioned above (see Fig. 4, top left), a single boundary was observed.
FIG. 1. Polyacrylamide gel electrophoresis of DNA ligase (Fraction VII). Polyacrylamide gel electrophoresis was carried out as described under “Methods.” The two gels on the left were run under native conditions, the center gel in System A, and the gel on the left in System B. The gel on the right was run under denaturing conditions in the presence of sodium dodecyl sulfate. From left to right the protein added was 30 µg, 40 µg, and 25 µg, respectively.

during the entire sedimentation velocity experiment, providing additional evidence that the ligase is homogeneous. The sedimentation coefficient of 3.9 S is lower than expected for a spherical protein of molecular weight 74,000, and suggests that the enzyme may have an asymmetric shape.

Amino Acid Analysis

The amino acid composition of DNA ligase is presented in Table II. The relatively low content of tyrosine may account for the unusual ultraviolet spectrum of the enzyme (Fig. 5), in which the ratio of absorbance at 280 nm to that at 260 nm for the unadenylylated form of ligase was only 1.42. Based on the protein concentration determined from the amino acid analysis and the ultraviolet spectrum, the extinction at 280 nm of a 1 mg per ml solution of the unadenylylated form of the enzyme was 0.72.

Stoichiometry of Ligase-AMP Formation

When the ligase was incubated with [32P]DPN, 0.76 mole of [32P]AMP (S.D. = 0.05, six determinations) was bound per 74,000 g of protein as judged either by acid precipitation or by filtration through Sephadex G-50. Experiments employing radioactive DPN were conducted at dilute enzyme concentrations (0.1 to 100 nM), and under these conditions there was significant adsorption of ligase-AMP to the surface of reaction tubes; this problem was partially overcome by use of siliconized
Fig. 4. Sedimentation velocity centrifugation of DNA ligase. Sedimentation was performed at 56,050 rpm at 20.6°C as described under "Experimental Procedure." Schlieren photographs were taken at the indicated times after reaching two-thirds speed (37,000 rpm). A minor fast sedimenting contaminant, which sediments about twice as fast as the major boundary, is visible in the 45-mm photograph.

Fig. 5. Ultraviolet absorption spectra of ligase and ligase-AMP. Spectra were determined in 0.01 M potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol at room temperature. Ligase-AMP was prepared in a reaction containing 0.02 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, 3 mM MgCl₂, 200 μM DPN, and 1 mg per ml of ligase. After 5 min at 25°C, the reaction was terminated by adding EDTA to a final concentration of 4 mM; the product was then dialyzed exhaustively against 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol.

reaction. The stoichiometry determined by this method is 1.2 to 1.3 moles of AMP/74,000 g. It therefore seems likely that 1 mole of AMP is covalently bound per 74,000 g of protein.

### DISCUSSION

The purification procedure described here for the *E. coli* DNA ligase leads to isolation in good yield of homogeneous enzyme as judged by several criteria. The complexity of the ligase reaction might suggest an oligomeric enzyme. In fact, Zimmerman and Oshinsky (30) found that prolonged dialysis of a partially purified preparation in dilute buffer resulted in the appearance of a low molecular weight form of the enzyme that could still generate enzyme-AMP but did not catalyze the joining reaction, and on the basis of these observations, proposed a subunit structure for the ligase. Our physical studies of the homogeneous enzyme are inconsistent with an oligomeric structure and indicate that it is composed of a single polypeptide chain of molecular weight 74,000. The stoichiometry of the adenylylation reaction is also consistent with a functional unit of this molecular weight. Moreover, the pure enzyme has retained more than 90% of its DNA joining activity during storage for a year without any indication of an altered enzymatic species. Possibly the partially active, low molecular weight form of the ligase observed by Zimmerman and Oshinsky was generated by proteolytic cleavage of the native protein during prolonged dialysis. Mild trypsin treatment of the purified ligase does in fact yield fragments that can still be adenylylated by DPN but which are inactive in the DNA joining reaction (6).
Given the molecular weight of the protein, it is possible to estimate the number of DNA ligase molecules per bacterium by comparing the specific activity of the pure enzyme with that observed in crude extracts. Since crude extracts do not contain inhibitors of DNA joining activity as measured by the d(A-T)$_n$ copolymer assay, such a calculation is probably valid. Based on such an estimate, wild type *E. coli* growing in rich medium contain about 200 to 400 molecules of DNA ligase per cell.

**REFERENCES**

1. Olivera, B. M., Hall, Z. W., Anraku, Y., Chien, J. R., and Lehman, I. R. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 27.
2. Gellert, M., Little, J. W., Oshinsky, C. K., and Zimmerman, S. B. (1968) *Cold Spring Harbor Symp. Quant. Biol.* 33, 21.
3. Richardson, C. C. (1969) *Annu. Rev. Biochem.* 38, 795.
4. Olivera, B. M., Hall, Z. W., and Lehman, I. R. (1968) *Proc. Nat. Acad. Sci. U. S. A.* 61, 237.
5. Hall, Z. W., and Lehman, I. R. (1969) *J. Biol. Chem.* 244, 49.
6. Modrich, P., and Lehman, I. R. (1972) *Fed. Proc.* 31, 441.
7. Symons, R. H. (1966) *Biochem. Biophys. Res. Commun.* 24, 872.
8. Shuster, L., Kafan, N. O., and Voelkel, F. E. (1955) *J. Biol. Chem.* 215, 195.
9. Symons, R. H. (1969) *Biochim. Biophys. Acta* 190, 545.
10. Kornberg, A. (1990) *J. Biol. Chem.* 265, 779.
11. Randerath, K., and Randerath, E. (1967) *Methods Enzymol.* 12A, 323.
12. Modrich, P., and Lehman, I. R. (1970) *J. Biol. Chem.* 245, 3626.
13. Joynt, T. M., Englund, P. T., and Bertsch, L. L. (1969) *J. Biol. Chem.* 244, 296.
14. Gellert, M., and Bullock, M. L. (1970) *Proc. Nat. Acad. Sci. U. S. A.* 67, 1580.
15. Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., and Kornberg, A. (1964) *J. Biol. Chem.* 239, 222.
16. Brutlag, D., and Kornberg, A. (1972) *J. Biol. Chem.* 247, 241.
17. Richardson, C. C., Lehman, I. R., and Kornberg, A. (1964) *J. Biol. Chem.* 239, 251.
18. Weber, K., and Oshinsky, C. K. (1969) *J. Biol. Chem.* 244, 440d.
19. Joynt, T. M. (1973) *Biochemistry* 12, 879.
20. Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, Spino Division, Beckman Instruments, Palo Alto, Calif.
21. Cohn, E. J., and Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, Reinhold, New York.
22. McMeekin, T. L., Groves, M. L., and Hepp, N. J. (1949) *J. Amer. Chem. Soc.* 71, 3208.
23. Bull, H. B., and Bresl, K. (1968) *J. Phys. Chem.* 72, 1817.
24. Sanger, F., and Thompson, E. O. P. (1963) *Biochim. Biophys. Acta* 71, 489.
25. Moore, S. (1963) *J. Biol. Chem.* 238, 235.
26. Wetlauffer, D. B. (1962) *Adv. Protein Chem.* 17, 310.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265.
28. Wardburg, O., and Christiansen, W. (1942) *Biochem. Z.* 310, 384.
29. Modrich, P., Lehman, I. R., and Wang, J. C. (1972) *J. Biol. Chem.* 247, 6370.
30. Zimmerman, S. B., and Oshinsky, C. K. (1969) *J. Biol. Chem.* 244, 4689.
Deoxyribonucleic Acid Ligase: ISOLATION AND PHYSICAL CHARACTERIZATION OF THE HOMOGENEOUS ENZYME FROM ESCHERICHIA COLI
Paul Modrich, Yasuhiro Anraku and I. R. Lehman

J. Biol. Chem. 1973, 248:7495-7501.

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