**The bacteriophage T7 DNA ligase**

The bacteriophage T7 DNA ligase gene was amplified using polymerase chain reaction-based methods and cloned into a T7 promoter-based expression vector. The protein was overexpressed to greater than 15% of total soluble protein and purified to homogeneity, yielding 60–70 mg of protein per liter of bacterial culture. An initial physical and biochemical characterization of the enzyme reveals that it exists as a monomer and can ligate nicked, cohesive, and blunt-ended DNA fragments. Inhibition of the enzyme activity by a nonhydrolyzable ATP analogue was also investigated. The enzyme has been crystallized from methoxypolyethylene glycol.

**The crystals are of the orthorhombic space group P2_12_12 and diffract to 2.6 Å. The unit cell dimensions are a = 66.1 Å, b = 87.6 Å, and c = 78.6 Å, with one monomer in the asymmetric unit (V_m = 2.77 Å³/Da). This is the first member of the DNA ligase family of enzymes to be crystallized.**

DNA ligases catalyze the formation of phosphodiester bonds at single-strand breaks between adjacent 3'-hydroxyl and 5'-phosphate termini in double-stranded DNA (for reviews see Refs. 1–3). Polynucleotide ligases are ubiquitous cell proteins that are required for a number of important cellular processes, including replication of DNA, and the repair of damaged DNA, as evidenced by the number of viruses that have genes encoding their own ligases. Despite their occurrence in all organisms, DNA ligases show a wide diversity of molecular sizes, and properties. DNA ligases can be divided into two broad classes: those requiring NAD⁺ and those requiring ATP. The eucaryotic and virally encoded enzymes all require ATP. The ligases in this class catalyze an exchange reaction between pyrophosphate and the 5'-phosphate group at the single-strand break site. Finally, DNA ligase catalyzes the DNA ligation step with the loss of free AMP. In spite of these similarities between the two classes of enzyme the manner by which the bacterial and eucaryotic proteins become activated is rather different. For eucaryotic ligases, the enzyme-AMP complex is formed after reaction of the enzyme with ATP with the release of free pyrophosphate. The bacterial ligases become adenylated in an unusual reaction, which involves the deavage of NAD⁺ and the release of nicotinamide mononucleotide (2). It has also been reported that the bacterial enzymes, unlike the ATP-dependent enzymes, are stimulated up to 20-fold by monovalent cations, particularly ammonium ions (11).

The ATP-dependent DNA ligases contain only a few areas of sequence homology, the most conserved of these is the KDGXX motif, which has been shown to contain the active site lysine for a number of nucleotidyl transfer enzymes including DNA and RNA ligases (9, 12) and RNA guanylyltransferases (13). The second most conserved motif (SLRFPRFIRIR) is located in the extreme C termini of the proteins, but its function is currently unknown. Greater homology can be shown if the sequence alignments are limited to more restricted sets (14). The most highly conserved sequences are located primarily in the C-terminal region of the protein, with the majority of insertions occurring in the N-terminal end.

Bacteriophage T7 encodes a DNA ligase of molecular mass 41,133 Da based on the gene sequence (5). The enzyme can utilize either ATP or, to a lesser extent, dATP as a cofactor, and catalyzes an exchange reaction between pyrophosphate and either ATP or dATP. The optimal pH range for the enzyme is 7.2–7.7 (2). In common with other DNA ligases, the enzyme also requires a divalent cation for activity. This appears to be fulfilled by Mg²⁺ ions in vivo, although other ions, such as Ca²⁺, can substitute to give reduced activity. While the 60-kDa DNA ligase from bacteriophage T4 has been well characterized biochemically and genetically (2), an extensive study of the smaller T7 enzyme has not been reported. Previous work has revealed that both T4 and T7 DNA ligases are able to join DNA annealed to RNA and, to a slight extent, even RNA annealed to its complementary RNA strand (15). Neither enzyme appears to be capable of ligating single-stranded DNA.

We describe the cloning and overexpression of the T7 DNA ligase gene in Escherichia coli. The gene was placed under the control of a T7 promoter, which allowed us to tightly control the level of gene expression. Strains harboring this plasmid expressed the protein at >15% of soluble cell protein. The enzyme has been purified to near homogeneity, and the physical and biochemical properties of the protein have been evaluated. We
have also crystallized the protein using vapor diffusion methods, and these crystals diffract to 2.6 Å.

EXPERIMENTAL PROCEDURES

Strains and Cloning Vectors—The following strains of E. coli were used as hosts for pET21d (Novagen) constructs: XL1-Blue (supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA, [F'proAB, lacIqZD+M15, Tn10 (etn)]) (16) for the propagation of clones and the preparation of single-stranded DNA for sequencing; GR501 (Hfr, thi-1, ptsI1, lig-251) (17), a conditional lethal DNA ligase (lig) mutant to test if the clones had complementary ligase activity; and B834(DE3) (hsdS gal(lac)lysS857ind1 met-lamD5 lacZD857 T7 gene1) (18,19) for the overexpression of T7 DNA ligase. E. coli strains were grown aerobically in Luria broth or on Luria broth agar plates containing the appropriate antibiotics.

General Techniques—All restriction endonucleases and modification enzymes were used according to the manufacturer’s protocols. All the transformations, DNA isolations, and manipulations were performed essentially as described previously unless otherwise stated (20). Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer. The oligonucleotides were desalted on NAP G200 columns (Pharmacia Biotech Inc.) according to the manufacturer’s instructions.

PCR of the T7 Ligase Gene—Two oligonucleotides based on the T7 genomic sequence were designed: a 5’-primer containing a NcoI site, 5’-GA TAT ACC ATG GTG AAC ACT AAC-3’ and a 3’-primer containing a HindIII site, 5’-GGG TTT TAA GCT TAC ATT TTC TCT TGA GGG-3’. PCR was performed with these primers and genomic bacteriophage T7 DNA (Sigma) using AmpliTaq (Perkin-Elmer) in 100 μl of PCR buffer and cycled as described (21). The PCR reaction was electrophoresed in 1.0% agarose (Life Technologies, Inc.), and the 1-kilobase product was excised from the gel and purified using a Qiagen DNA extraction kit (Qiagen).

Cloning and Overexpression of the T7 lig Gene—The PCR product was purified as described above and cleaved with NcoI and HindIII, ligated into dephosphorylated NcoI/HindIII-cut pET21d (Novagen), and transformed into E. coli XL1-Blue. Positive clones containing the ligase gene (pT7lig) were isolated by restriction analysis and screened for expression by transforming into B834 (DE3). Selected colonies were grown at 37°C in 5 ml of Luria broth, containing ampicillin and chloramphenicol, to an A600 of 0.6, induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside, and grown for a further 3 h. The level of induction was monitored using 10% SDS-polyacrylamide gel electrophoresis.

DNA Sequencing—The dideoxy method (22) was used to confirm the complete nucleotide sequence of the T7 lig gene. Single-stranded template DNA was produced in E. coli XL1-Blue using helper phage M13K07 (Pharmacia) according to the manufacturer’s instructions.

Purification of T7 Ligase—Two 1-liter cultures of Luria broth containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol were inoculated with a 5-ml culture of B834(DE3) [pLysS] (pT7lig) and grown at 37°C until the A600 reached 0.6–0.7. The cultures were induced by the addition of 0.5 mM isopropyl-1-thio-D-galactopyranoside, and grown for a further 3 h. The level of induction was monitored using 10% SDS-polyacrylamide gel electrophoresis.

The abbreviations used are: PCR, polymerase chain reaction; F-ara-ATP, 9-β-arabinofuranosyl-2-fluoroadenosine triphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; AMPPNP, 5'-adenylyl-β,γ-imidodiphosphate.

Fig. 1. Reaction mechanism of DNA ligases. Shown is a schematic diagram of the mechanism of action of T7 DNA ligase. All ATP and NAD+ -dependent ligases appear to join DNA in a similar way but utilize a different nucleotide energy source to catalyze the reaction.
by centrifugation at 20,000 × g, the supernatant was precipitated by the addition of an equal volume of a saturated ammonium sulfate solution. The precipitate was harvested by centrifugation at 20,000 × g, and the pellet was resuspended in a volume of buffer A, which resulted in a solution whose conductivity was less than that of a 100 mM solution of sodium chloride. This was applied to a heparin-Sepharose column (20 ml), equilibrated with 90% buffer A and 10% buffer B (buffer A containing 2 M sodium chloride). After washing the column with a further 2 column volumes of this mixture, the ligase was step-eluted by washing with 40% buffer B. SDS-PAGE confirmed that this peak contained the semi-purified enzyme at high concentrations. The protein was diluted with buffer to reduce the effective salt concentration to 100 mM NaCl, and this was loaded onto a blue-Sepharose column, which had been pre-equilibrated in buffer A. The protein was step-eluted with 50% buffer B. The concentration of NaCl in the peak fractions was reduced to less than 50 mM by dilution with buffer A. This was loaded onto a Q-Sepharose column (40 ml) pre-equilibrated in buffer A. The column was then washed with 80 ml of buffer A before elution of the protein with 30% buffer B. Purity was again monitored by electrophoresing samples on a 10% SDS-polyacrylamide gel. Trace contaminants were removed by gel filtration on a Superdex 200 column.

DNA Ligase Assays—Ligation of DNA fragments with cohesive ends was performed in a final volume of 10 μl containing 0.3 μM of EcoRI/HindIII-digested λ phage DNA, 5 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, and added nucleotide cofactors as indicated. Reactions were incubated at room temperature for 20 min and stopped by the addition of 50 mM EDTA followed by incubation at 65°C for 10 min. Samples were electrophoresed in 1% agarose, and the gel was stained with ethidium bromide.

DNA ligase assay substrate (22-mer) was radiolabeled by incubating 20 μg of the oligonucleotide with 100 μCi of [γ-³²P]ATP (3000 Ci/mmol; Amersham Corp.) and 50 units of T4 polynucleotide kinase for 45 min at 37°C followed by 10 min at 70°C. The unincorporated label was removed by centrifugation through a 5-200 microspin column (Pharmacia). The DNA ligase assay was performed essentially as described previously (23). The complementary 18- and 22-mer oligonucleotides were annealed to single-stranded M13mp19 by incubation at 70°C for 2 min and allowed to cool for 1 h. The annealed DNA was incubated with ligase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM DTT) unless otherwise stated, in the presence of enzyme and nucleotide cofactors as indicated, in a total volume of 10 μl for 15 min at 25°C. The reactions were terminated by the addition of sequencing stopping buffer (Sequenase kit, U.S. Biochemical Corp.) followed by heating at 95°C for 5 min. The ligation products were subjected to electrophoresis on a 15% polyacrylamide gel. The gels were dried before being subjected to autoradiography with Fuji RX x-ray film.

Cloning, Overproduction, and Purification of T7 Ligase—To produce the enzyme in large quantities, it was necessary to amplify by PCR the T7 ligase gene from bacteriophage T7 genomic DNA and subclone it into a T7 promoter-based expression vector. The 5′-PCR primer contained an Ncol site, allowing the PCR product to be cloned into pET21d. The Met-2 codon was changed to Val-2 (GTG) to prevent secondary translation occurring at this position. The 3′-primer contained a HindIII site after the stop codon. The PCR product was subjected to agarose gel electrophoresis, and the 1-kilobase DNA fragment was excised and purified. The DNA was digested with Ncol and HindIII and further gel-purified. This fragment was ligated into dephosphorylated, HindIII-cleaved pET21d, transformed into E. coli XL1-blue and plated onto LB agar plates containing ampicillin. A large number of colonies were found to have the correct DNA insert size upon digestion with Ncol and HindIII. Surprisingly, a majority of the clones were able to complement E. coli GR501, a conditional-lethal DNA ligase mutant that is not viable at 42°C, despite the fact that the T7 ligase gene is under the control of a T7 RNA polymerase promoter, which should not be recognized in this cell line. A low level of basal expression of the T7 ligase gene must be occurring that is sufficient to complement the lig-deficient strain, possibly as a result of read-through from the β-lactamase promoter on the plasmid, which is on the same DNA strand as the coding sequence of the ligase gene.

The T7 DNA ligase gene was fully sequenced, and the sequence agreed with that published previously (5). The resulting active clone was screened for overexpression of the T7 ligase gene by transforming into B834(DE3)[pLysS], inoculating single colonies into 5-ml cultures of LB containing ampicillin and chloramphenicol and growing for several hours. The cells were induced by the addition of isopropyl-1-thio-β-D-galactopyranoside and grown for 3 h. Samples were taken and analyzed by SDS-PAGE. In all cases a large amount of a protein with an apparent molecular mass of approximately 40 kDa accumulated in the induced cells but was absent from uninduced cultures. The crude sonicated cell extract supernatant was precipitated with ammonium sulfate. The protein was then purified using a heparin-Sepharose column followed by blue-Sepharose, Q-Sepharose, and finally gel filtration using a Superdex S-200 column. The protein was greater than 99% pure at this stage as determined by 12% SDS-PAGE (Fig. 2). The final yield of enzyme was typically 60–70 mg/liter using the host strain B834(DE3) compared with 10–15 mg/liter when BL21(DE3) was used as the expression host (26).

Physical Properties of T7 DNA Ligase—T7 ligase has a predicted molecular mass of 41,133 Da based on its amino acid sequence of the ligase gene. The apparent molecular mass of approximately 40 kDa accumu-
analyzed by SDS-PAGE. The substrate specificity of purified T7 ligase was determined using a PhastGel (Pharmacia) gel filtration column (pre-equilibrated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA). Fractions of 1 ml were collected, and peak fractions were analyzed by SDS-PAGE.

sequence. However, during the purification it was observed that the protein eluted in the void volume of a Superdex S-75 gel filtration column, suggesting either that the protein was running anomalously or that it was aggregating to form higher order complexes. To investigate this finding, native T7 ligase was loaded onto a Superdex S-200 column that had been standardized with proteins of known molecular weight. These gel filtration studies were performed in the absence of any ligands in the standard elution buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM DTT, 150 mM NaCl). The protein eluted from the column with an apparent molecular mass of 85–90 kDa (Fig. 3). SDS-PAGE analysis confirmed that this peak had the expected molecular mass for T7 ligase (41 kDa). We examined the effects of a number of metal ions on the protein. Zinc, nickel, cobalt, manganese, and the majority of other heavy metals caused the protein to aggregate (data not shown). In the case of nickel and the other heavy metals this effect occurs very rapidly (minutes) at stoichiometric levels of the compounds. This phenomenon was observed first in crystallization trials, the addition of various metals to solutions containing high concentrations of protein (5–15 mg/ml) caused instant visible precipitation that was not reversible by dialysis. The mechanism by which this aggregation occurs is unclear but should be better understood when the structure of the enzyme has been determined.

Mass spectroscopy and equilibrium sedimentation were employed to determine whether the enzyme exists as a monomer or dimer. According to equilibrium sedimentation, the protein appears to be monomeric with a calculated molecular weight of 40,645 ± 300 (Fig. 4). The molecular mass of native T7 ligase was determined to be 41,132 Da using electrospray mass spectroscopy (Fig. 4), with the adenylated form having a mass of 41,460 Da. Approximately one-third of the purified protein exists in the adenylated form.

The isoelectric point (pI) of T7 ligase has been calculated to be 5.2 based on the amino acid sequence. The isoelectric point of purified T7 ligase was determined using a PhastGel (Pharmacia). The pI was shown to be close to 5.8 (data not shown), but the protein is insoluble below its pI. DNA Substrate Specificity and Catalytic Properties of T7 Ligase—The substrate specificity of purified T7 ligase was tested on a series of natural and synthetic DNA substrates. It was shown previously that T7 DNA ligase, assayed in crude extracts, can ligate oligo(dT)–oligo(A) and oligo(A)–oligo(dT) (15). The purified enzyme is very efficient at ligating DNA containing cohesive ends and nicked sites (Fig. 5). However, it is incapable of performing single-strand DNA ligations in common with both the T4 and E. coli ligases (Fig. 5). T4 DNA ligase has the ability to join blunt-ended DNA fragments (27), and this activity is greatly enhanced by the addition of macro-molecular crowding agents such as polyethylene glycol (28). The addition of such agents allows other ligases, such as those from E. coli, rat liver, and Thermus thermophilus, to perform blunt-end ligations (29). T7 ligase can ligate blunt-ended DNA fragments efficiently in the presence of a wide range of polyethylene glycols but is inactive in the absence of such crowding agents. This activity is optimal at between 20 and 30% of PEG 6000 and the efficiency of the reaction under these conditions is comparable with that of the T4 enzyme (Fig. 6).

It has been shown that T7 ligase requires magnesium and uses ATP, and to a lesser extent dATP, as the cofactor in the DNA joining reaction (2). The $K_m$ for ATP or dATP in the joining reaction is approximately $6 \times 10^{-6}$ M. In the exchange reaction the $K_m$ for ATP is $3 \times 10^{-7}$ M, while that for dATP is 10-fold higher (2). We have examined the catalytic properties of the enzyme using a radiolabeled oligonucleotide assay described previously (23). The DNA substrate consists of two oligonucleotide primers (18-mer and 22-mer), one 5'-labeled with [γ-32P]ATP, annealed adjacently onto single-stranded M13 DNA, creating a single synthetic nick site. We observed that ligation of this nicked substrate and cohesive end DNA fragments by both T7 and T4 ligases occurs at comparable rates, according to the published values (30) of approximately 1,200 Weiss units/mg of protein (data not shown). We observed that both purified T7 and commercial T4 DNA ligases can efficiently ligate DNA in the absence of ATP (Fig. 5). Mass spectroscopy analysis of the purified protein revealed that greater than 30% of the T7 enzyme exists in the adenylated form (Fig. 4). This adduct is very stable to acid and alkali treatments, which has also been reported for other DNA ligases (31). Many reports have shown that AMP can be removed by preincubation DNA ligase with either nicked DNA or PP, (31, 32). However, we were unable to reduce the level of adenylation of the T7 enzyme to less than 10% after these treatments. The addition of PP, caused complete inhibition of ligation by adenylated T7 ligase in the absence of ATP but was less effective on the T4 enzyme under similar conditions (Fig. 5). It has been shown previously that incubating T4 ligase-AMP with PP, results in the release of the adenylate moiety and the appearance of ATP (31). However, it is possible to restore activity of the T7 enzyme by removal of PP, by dialysis even in the presence of activated charcoal. Enzyme treated in this way retained the ability to perform ligations in the absence of ATP, and thus a significant proportion of the enzyme must still be in the adenylated form.

The addition of 5 mM EDTA inhibits completely the formation of the AMP adduct (data not shown). We found that adenylated T7 and T4 ligases can perform the AMP transfer and sealing steps even in the presence of 10 mM EDTA (Fig. 5). This contrasts with reports of an absolute requirement for divalent metal ions in the transfer and sealing reactions of human I, T4, and E. coli ligases (23, 33, 34).

A variety of nucleotides (CTP, dTTP, GTP, NAD+) were unable to substitute for ATP in the ligation reaction and also did not inhibit the adenylated enzyme. They also had no effect on the reaction in the presence of ATP, even at concentrations up to 5 mM (data not shown). However, the ATP analogue, AMPNP, inhibited significantly the ligation of nicked (Fig. 5) and cohesive end DNA (data not shown) by the adenylated T7 enzyme in the absence of ATP. This ATP analogue contains a bridging nitrogen instead of oxygen between the β- and γ-phosphates. It is interesting that this modification makes the nucleotide an inhibitor (Fig. 5), since all DNA ligases characterized to date deave ATP between the α- and β-phosphates.
releasing PPi (Fig. 1). In contrast, adenylated T4 DNA ligase is not inhibited by AMPPNP under similar conditions (Fig. 5), nor does AMPPNP affect the activity of T4 ligase in the presence of ATP. To characterize further the inhibitory effects of AMPPNP on T7 ligase, increasing amounts of the analogue were incubated with the enzyme prior to the addition of DNA substrate. These results showed that a decrease in ligation activity was directly proportional to the concentration of AMPPNP (data not shown). Levels in excess of 10 mM were required to inhibit the enzyme by greater than 90%.

The T7 enzyme was incubated with either [α-32P]ATP or [γ-32P]ATP in ligase buffer as described previously (35). The protein was separated by SDS-PAGE, and autoradiography showed that the enzyme had become selectively labeled with the α- but not the γ-labeled ATP substrate (Fig. 7), in common with other DNA ligases.

Preliminary Crystallographic Characterization—T7 ligase crystals grew as wedge-shaped plates, which appeared after approximately 14 days and reached a maximum size of 0.8 × 0.3 × 0.2 mm (Fig. 8). However, in the majority of the hanging
drops, crystals did not appear even under optimal conditions. To overcome this variability in crystal growth we successfully used microseeding to initiate nucleation in drops that had been pre-equilibrated for 1–2 weeks. Crystals appeared rapidly (hours) and reached full size after 2–4 days. This method was also used to produce large quantities of selenomethionine-substituted crystals. Seeded crystals were superior to unseeded ones and tended to grow larger.

The crystals were only moderately stable on exposure to x-rays but it was possible to collect complete native data to 2.8 Å from a number of crystals using synchrotron radiation (Daresbury, UK; EMBL, Hamburg, Germany; and Brookhaven National Laboratory, Upton, NY) and an image plate detector. The crystals are orthorhombic and belong to the space group P2₁2₁2₁ with unit cell dimensions of $a = 66.1$ Å, $b = 87.6$ Å, $c = 78.6$ Å. Assuming that there is one monomer in the asymmetric unit, these crystals have a calculated $V_m$ of 2.77 Å³/Da (36). Crystal lifetime has been extended further by flash freezing at 100 K in the presence of 25% glycerol, and this has allowed 2.6 Å data to be collected from a single crystal. Selenomethionine-substituted protein has also been crystallized, and data have been collected to 2.8-Å resolution. The selenium positions have been determined using direct methods as implemented in SHELX (37) and used to estimate an initial set of phases. These phases have been used to determine heavy atom positions in a number of other derivatives using difference Fourier techniques. The resulting electron density map is of sufficient quality to reveal secondary structural elements, and model building is under way.

**DISCUSSION**

A number of the larger eucaryotic/viral ATP-dependent ligases have been studied in detail at the genetic and biochemical level. In this report we describe the characterization of bacteriophage T7 DNA ligase, one of the smallest members of this family of enzymes. We have cloned and overexpressed T7 DNA ligase.
DNA ligase in E. coli and purified the protein in large amounts, allowing us to characterize some of its physical and biochemical properties. We have confirmed the previously deduced molecular mass of the native enzyme to be 41,133 Da using mass spectrometry. This analysis also revealed that approximately 30% of the purified enzyme exists in the adenylylated form, in common with a number of other ligases (7). The adenylylated form of the enzyme is remarkably stable, and the adduct is resistant to acid and alkali treatments. The discrepancy in the molecular weight determined by gel filtration and mass spectrometry indicates an irregular shape for the ligase molecule, which has been suggested for other DNA ligases on the basis of analytical gel filtration and sedimentation centrifugation data (38, 39). Mammalian DNA ligase I has been shown to have a markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric structure with a frictional ratio of 1.9 (40) and resembles the markedly asymmetric 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