**Mammalian Deoxyribonucleic Acid Ligase**

**ISOLATION OF AN ACTIVE ENZYME-ADENYLATE COMPLEX***

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**Stefan Söderhäll and Tomas Lindahl**

*From the Department of Chemistry, Karolinska Institute, Stockholm, Sweden*

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**SUMMARY**

An ATP-dependent DNA ligase, partly purified from calf thymus, forms a covalent enzyme-adenylate complex on incubation with [14C]ATP. The isolated complex joins DNA single strand breaks with 3'-hydroxy and 5'-phosphate termini, in the absence of ATP, and the adenylate residue is released during this reaction. On incubation of the ligase-adenylate complex with pyrophosphate, the radioactive moiety is released as ATP. These data indicate that the complex is a reaction intermediate, and that DNA ligase from mammalian cells acts by the same mechanism as microbial DNA ligases.

The NAD-dependent DNA ligase of *Escherichia coli* and the ATP-dependent DNA ligase induced by phage T4 in *E. coli* have been studied extensively, as these enzymes appear to be involved in the replication and repair of DNA (1). Both enzymes act by first forming a covalent enzyme-adenylate complex that subsequently joins single strand breaks in DNA (2-5), with the intermittent formation of a DNA-adenylate complex (3). An ATP-dependent DNA ligase has also been found in extracts of cells from higher organisms (6-12). While the ligase from mammalian cells has been purified in several laboratories (6, 10, 12), active reaction intermediates have not been isolated.

Attempts to do this have been hampered by the lability of the ligase preparations from some sources of mammalian cells (6), and by the small amounts of ligase activity found in higher organisms in comparison with the microbial systems. Thus, the ATP-dependent DNA ligase activity in a cell extract from T4-infected *E. coli* is 500 to 1000 times higher than that in the most active extracts from mammalian tissues (6) or plant cells (8). The reaction mechanism of the mammalian DNA ligase has therefore remained somewhat unclear, in spite of the previous observation of an ATP-dependent pyrophosphate exchange reaction catalyzed by a partly purified enzyme preparation (6).

In the present work, a covalent enzyme-adenylate complex has been isolated after incubation of DNA ligase, partly purified from calf thymus, with [14C]ATP. The complex repairs single strand breaks in double helical DNA in the absence of ATP, with the simultaneous discharge of the adenylate moiety. These results strongly indicate that mammalian DNA ligase acts by the same reaction mechanism as the ligase from T4-infected *E. coli*.

**MATERIALS AND METHODS**

**Enzyme**—DNA ligase was purified from calf thymus by methods described previously (6, 13).

The DNA ligase preparation (45 mg of protein in 6 ml, 1.8 ligase units per mg) was incubated in 0.05 M Tris-HCl, 0.01 M MgCl₂, 10⁻³ M dithiothreitol, 10⁻⁴ M sodium pyrophosphate, pH 7.4, for 15 min at 20°. The reaction was stopped by chilling to 0° and addition of 0.1 volume of 0.5 M EDTA, pH 7.4. The enzyme was then freed from pyrophosphate by gel filtration on Sephadex G-100 (2 x 87 cm) in 0.3 M NaCl, 0.05 M Tris-HCl, 10⁻⁴ M EDTA, 10⁻⁴ M dithiothreitol, pH 7.4. The nonretarded protein fraction was recovered, precipitated with ammonium sulfate (80% saturation), redissolved in 0.1 volume of the column buffer, and dialyzed against the same solvent for 10 hours.

This enzyme preparation (20 mg of protein in 2 ml) was considerably more stable than the DNA ligase preparations previously made from rabbit tissues (6, 13), and retained 50% of its activity after 3 weeks at 0°. The enzyme could be preserved for long time periods (6 months) without loss of activity by rapidly freezing the protein pellet obtained after the final ammonium sulfate precipitation, followed by storage at -70°. The frozen enzyme was thawed, dissolved in cold buffer, and dialyzed as above before use.

**Nucleic Acids**—Nonradioactive and [14C]thymidine labeled DNA (25,000 cpm per µg) from phage T7 were prepared by the method of Richardson (14). Single strand breaks with 5'-phosphate termini were introduced into the DNA to produce 0 to 4% acid-soluble material by incubation of the DNA (200 µg per ml) for 10 min at 25° with pancreatic DNAse I (0.05 to 1 µg per ml) in 0.05 M Tris-HCl, 0.02 M MgCl₂, pH 7.4. The reaction was stopped by addition of EDTA to 0.05 M, heating at 55° for 5 min, and dialysis at 2° against 0.05 M Tris-HCl, 10⁻³ M EDTA, pH 7.4. Denatured nicked DNA was obtained by heating this material at 100° for 10 min, followed by rapid cooling. DNA strand breaks with 5'-OH termini were introduced by treatment with micrococcal nuclease (0.05 µg per ml) for 20 min at 25° in 0.02 M N₂-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-KOH, 0.006 M CaCl₂, pH 8.5, to produce 4% acid-soluble material. The reaction was stopped by addition of EDTA and dialysis as above.
size determinations of denatured [14C]T7 DNA were performed by zone centrifugation in linear 5 to 20% sucrose gradients, containing 0.5 M NaCl, 0.3 M NaOH, 0.01 M EDTA, for 4 hours at 40,000 rpm and 4° in the Spinco SW 50.1 rotor.

Other Materials—[4C]ATP (412 μCi per μmole, uniformly labeled), [γ-3P]ATP (8000 μCi per μmole), and [2-3H]thymidine (90 μCi per μmole) were obtained from NEN Chemicals. Pancreatic DNase (1 x crystall.; micrococcal nuclease, and alkaline phosphatase were obtained from Worthington, and crystalline hexokinase from Boehringer. Sephadex G-100 was purchased from Pharmacia, and (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonanleic acid) from Sigma.

Assay—DNA ligase was measured by the method of Weiss et al. (13) as previously described (13), except that incubations were for 75 min at 20°. With an excess of enzyme, a 2-fold higher amount of the single strand breaks in the extensively nicked ligase substrate could be repaired at 20° than at 37°. One enzyme unit converts 1 μmole of 32P to an alkaline phosphatase-resistant form under the standard assay conditions.

ATP, ADP, AMP, and adenosine and ademine were separated by paper chromatography on Whatman No. 3MM paper in isobutyric acid, H2O, concentrated NH4OH, 0.2 M EDTA (66:33:1:0.5). For determinations of acid-soluble material in nuclease-treated, nonradioactive DNA preparations, aliquots of the DNA solutions were incubated with an equal volume of 0.5% perchloric acid for 5 min at 0°, followed by centrifugation at 10,000 x g for 10 min. The supernatant solutions were recovered, and the concentration and acid-soluble oligonucleotides determined spectrophotometrically. Radioactive DNA solutions were precipitated in the same fashion together with 0.1 mg per ml of denatured calf thymus DNA as carrier, and after centrifugation the radioactivity of an aliquot of the supernatant solution was determined in a scintillation counter in the presence of 5 ml of "Aquaasol" (NEN Chemicals). In experiments with the radioactive enzyme-adenylate complex acid-solubility was measured in the same fashion, except that 0.5 mg per ml of bovine serum albumin was employed as carrier instead of DNA.

RESULTS

Isolation of a Protein-Adenylate Complex—The partly purified calf thymus DNA ligase, 5.5 mg, was incubated with [14C]ATP (20 μmoles, 8 x 10⁶ cpm) in 1 ml of 0.05 M Tris-HCl, pH 7.4, 0.05 M NaCl, 0.01 M MgCl₂, 10⁻³ M K₂HPO₄, 10⁻³ M EDTA, 10⁻³ M dithiothreitol, for 25 min at 20°. The reaction was stopped by chilling to 0° and addition of EDTA to 0.05 M. The material was then chromatographed on Sephadex G-100 (1 x 27 cm) in 0.3 M NaCl, 0.05 M Tris-HCl, pH 7.4, 10⁻³ M EDTA, 10⁻³ M dithiothreitol. Each fraction contained 1 ml.

![Fig. 1. Isolation of ligase-adenylate complex. Partly purified calf thymus DNA ligase, 5.5 mg, was incubated with [14C]ATP (20 μmoles, 8 x 10⁶ cpm) in 1 ml of 0.05 M Tris-HCl, pH 7.4, 0.05 M NaCl, 0.01 M MgCl₂, 10⁻³ M K₂HPO₄, 10⁻³ M EDTA, 10⁻³ M dithiothreitol, for 25 min at 20°. The reaction was stopped by chilling to 0° and addition of EDTA to 0.05 M. The material was then chromatographed on Sephadex G-100 (1 x 27 cm) in 0.3 M NaCl, 0.05 M Tris-HCl, pH 7.4, 10⁻³ M EDTA, 10⁻³ M dithiothreitol. Each fraction contained 1 ml.](http://www.jbc.org/)

Release of the Adenylate Complex by Nicked DNA—The enzyme-adenylate complex (100 μg per ml protein) was incubated with DNA (4 μg per ml, if not otherwise stated) in 0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl₂, 10⁻³ M EDTA, 10⁻³ M dithiothreitol, at 0°. At various times, 0.12-ml aliquots were removed; after precipitation of the acid-insoluble material, the radioactivities of the supernatants were determined. A — A, intact T7 DNA; DNA nicked with pancreatic DNase (4% acid-soluble); DNA, small DNA, 0.8 μg per ml; O O O, same DNA, 4 μg per ml, MgCl₂ excluded from the reaction mixture; V V V, DNA nicked with micrococcal nuclease (4% acid-soluble).

![Fig. 2. Reaction of ligase-adenylate with nicked DNA—The enzyme-adenylate complex (100 μg per ml protein) was incubated with DNA (4 μg per ml, if not otherwise stated) in 0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl₂, 10⁻³ M EDTA, 10⁻³ M dithiothreitol, at 0°. At various times, 0.12-ml aliquots were removed; after precipitation of the acid-insoluble material, the radioactivities of the supernatants were determined. A — A, intact T7 DNA; DNA nicked with pancreatic DNase (4% acid-soluble); DNA, small DNA, 0.8 μg per ml; O O O, same DNA, 4 μg per ml, MgCl₂ excluded from the reaction mixture; V V V, DNA nicked with micrococcal nuclease (4% acid-soluble).](http://www.jbc.org/)
The release of 0.94 $^{14}$C-AMP residues from the complex was determined to be $8 \times 10^{-7}$ M for the calf thymus DNA ligase.

Incubation of an aliquot of the $^{14}$C-labeled protein in 0.1 M HCl for 1 min at 100$^\circ$C converted the radioactive moiety (>85%) to an acid-soluble form. This material was identified as AMP (80% yield). A minor proportion (20%) of the material was also recovered as adenine due to acid-catalyzed depurination. Heating of free $^{14}$ClATP under identical conditions caused <15% conversion to AMP.

**Stability of Complex**—The $^{14}$C-AMP residue remained acid-insoluble (<10% solubilization) after incubation for 10 min in 0.1 M HCl or 60 min in 0.1 M NaOH at 25$^\circ$C. This stability of the complex indicates strongly that the adenylyl residue was covalently bound to a macromolecule. Incubation of the complex for 10 min at 100$^\circ$C in 0.1 M HCl or 1 M NaOH caused the $^{14}$C residue to be released (>85%) in an acid-soluble form, while treatment with 0.1 M HCl for 30 min at 25$^\circ$C or with 0.05 M Tris-HCl, 10$^{-3}$ M EDTA, pH 7.4 for 10 min at 100$^\circ$C caused 30 to 35% conversion to acid-solubility.

Further, incubation with 3 M hydroxylamine at pH 7.75 as described by Gumport and Lehman (16) caused 67% and 85% of the label to be released in 15 and 30 min, respectively, while incubation with 0.2 M hydroxylamine at pH 7 (16) caused <20% release. These results are similar to data previously reported for the enzyme-adenylate complexes of microbial ligases (2-4, 16), and suggest that the adenylyl residue is bound to protein by a phosphoamide bond (16).

**Enzymatic Activity of Complex**—The protein-adenylate complex, which contained 92% acid-insoluble radioactive material, was incubated at 0$^\circ$C with DNA-containing single strand breaks (Fig. 2). Most of the radioactivity (65%) was rapidly released in an acid-soluble form when $\geq 2$ $\mu$g per ml of DNA, pretreated with pancreatic DNase to produce single strand breaks with 5'-phosphate termini, was added to the incubation mixture. In contrast, DNA without single strand breaks, or with single strand breaks containing 5'-hydroxy termini, showed little or no activity (<5% of that of nicked DNA with 5'-phosphate termini) to promote the release of $^{14}$C-label from the complex. Further, if the DNA containing nicks with 5'-phosphate termini was heat-denatured before use, or if MgCl$_2$ was not included in the reaction mixture, no release of $^{14}$C was detected. These data demonstrate that at least 65% of the isolated protein-AMP complex is accounted for as an enzyme that interacts with single strand breaks in bihelical DNA, and the data below suggest that this activity is due to DNA ligase.

The acid-soluble material released by DNA ligase in the reaction with nicked DNA was identified as free AMP (>$>50$%). When the enzyme-adenylate complex was incubated under the same conditions with 10$^{-4}$ M pyrophosphate instead of nicked DNA, the $^{14}$C label was released to $>90%$ in an acid-soluble form, and this material was identified as ATP (85% yield; <3% of either ADP, AMP, adenosine, or adenine). These data show that the formation of an enzyme-adenylate complex that occurs on incubation of mammalian DNA ligase with ATP is a reversible reaction, as expected from our previous results on ligase-catalyzed pyrophosphate exchange (6).

When the ligase-AMP complex was studied in the standard ligase assay, performed in the absence of added ATP, conversion of the 5'-3P residues to an alkaline phosphatase-resistant form could be demonstrated to the extent of 17 picomoles per mg of protein. DNA ligase, not preincubated with $^{14}$CJATP, caused no detectable repair under these conditions (see below, Fig. 3). For each 3P residue that was converted to a phosphatase-resistant form, the release of 0.94 $^{14}$C-AMP residues from the complex was observed (Table I). It, thus, appears that 1 AMP residue was released per repairable single strand interruption. Five picomoles of 3P in the substrate were repaired, when the maximum amount of ATP that could have been present as a contaminant in the preparation of the complex was 1 picomole. Thus, at least 80% of the DNA joining catalyzed by the enzyme-adenylate complex must have occurred without ATP consumption. On incubation of the enzyme-adenylate complex (100 $\mu$g per ml) with nicked $^{14}$CJ7 DNA (2 $\mu$g per ml, <1% acid-soluble) for 10 min at 0$^\circ$C under the solvent conditions given in Fig. 2, followed

![Graph](http://www.jbc.org/)
by phenol extraction and dialysis, it was observed by alkaline sucrose gradient centrifugation that an increase in the sedimentation coefficient of the DNA strands from 24.5 S (5% of the DNA sedimenting as intact strands of T7 DNA) to 33 S (50% of the DNA sedimenting as intact strands of T7 DNA) had been obtained.

**ATP-independent DNA Ligase Activity**—When the calf thymus DNA ligase was prepared as described, except that the incubation with pyrophosphate before the final gel filtration step was omitted, 1.6% of the ligase activity in the standard assay was independent of ATP (Fig. 3). However, after incubation with pyrophosphate, no such ATP-independent activity could be detected (<0.2%). In order to control if the residual activity could be due to ATP contamination, the ligase substrate and the enzyme were separately preincubated in the assay buffer with crystalline hexokinase (1 μg per ml) and glucose (0.1 M) for 10 min at 20°C before mixing. This preincubation did not reduce the amount of ATP-independent ligase activity. It is therefore concluded that the DNA ligase preparation contained a small proportion of the enzyme in the form of a ligase-AMP complex.

**DISCUSSION**

The reported properties of mammalian DNA ligase demonstrate the great similarity between this enzyme and the well-characterized DNA ligases from *E. coli* and phage-infected *E. coli*. These enzymes form a covalent complex of similar chemical stability with AMP in the presence of cofactor, and the adenylyl moiety of the complex is discharged in a Mg2+ requiring reaction during the repair of double stranded DNA with single strand breaks having 5′-phosphate and 3′-hydroxy termini. Consequently, it seems fairly certain that all three enzymes act by the same mechanism. In agreement with this notion, a DNA-adenylate complex has been isolated in low yield (8%) after incubation of the mammalian DNA ligase-adenylate complex with nicked DNA at pH 6.5 for 30 s at 0°C, and the adenylyl residue of this DNA-AMP complex is released on further incubation with DNA ligase.1

A requirement in the assay of mammalian DNA ligase for protein factor(s) present in boiled crude cell extracts has been reported by Spadari et al. (10) for a ligase partly purified from human cells. With the calf thymus enzyme preparations employed here, we have been unable to detect any dependence of

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1 S. Söderhäll, in preparation.

2 S. Söderhäll and T. Lindahl, unpublished results.
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