Communication

Different Substrate Specificities of the Two DNA Ligases of Mammalian Cells*

Janet E. Arrand†, Anne E. Willis, Iain Goldsmith, and Tomas Lindahl
From the Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, United Kingdom

Mammalian cells contain the DNA ligases I and II. These enzymes show different molecular weights and heatabilities, and antibodies against ligase I do not inhibit ligase II. Here, the nonidentical substrate specificities of the enzymes are described. Under standard reaction conditions DNA ligase I, but not ligase II, catalyzes blunt-end joining of DNA, while ligase I1 is both in monomeric form and as a 400-kDa dimer in cell extracts (6, 11). In addition, ligase I is susceptible to proteolysis, and antibodies against ligase I do not inhibit ligase I. This enzyme is induced up to 15-fold together with DNA ligase II are allowed to proceed (41). Furthermore, on subcellular fractionation DNA ligase II appears to be more firmly associated with the chromatin than does ligase I (4, 6).

EXPERIMENTAL PROCEDURES

Enzyme Purification—DNA ligases I and II were purified from calf thymus as described previously (3, 4) with minor modifications. Briefly, 600 g of fresh thymus glands were disrupted at 0 °C in a Waring blender with 3 liters of a buffer containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml each of the protease inhibitors pepstatin, leupeptin, and chymostatin. After centrifugation, nucleic acids were removed by precipitation with 0.5% Triton X-100 and 20% ethanol. Both ligase I and ligase II were present in this fraction, which was applied directly to a hydroxyapatite (HA-Ultrogel, LKB Products) column (2.5 × 18 cm) and eluted with a linear gradient (300 ml) of 0 to 200 mM KHP04, 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol. This procedure separates ligases I and II (see below, Fig. 2). The two peaks of activity were further purified separately as described previously (4), ligase I by gradient chromatography on phosphocellulose and gel filtration on Sephadex G-150, and ligase II by gel filtration only. Active fractions were pooled, 3-fold concentrated by dialysis against column buffers containing 50% glycerol, and stored at −20 °C. In agreement with previous results (4), DNA ligase I preparations by this procedure were approximately 1000-fold purified in 10% yield and had specific activities of 0.01-0.02 unit (12)/mg protein, while DNA ligase II preparations were about 200-fold purified in 15% yield and had specific activities of 0.003-0.005 unit/mg protein. Phase T4 DNA ligase was purchased from New England Biolabs.

Ligase Assays—Reaction mixtures (40 μl) contained 70 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 50 μg/ml nuclease-free bovine serum albumin, poly(dA):oligo(dT) substrates, or DNA, and a limiting amount of DNA ligase (1 × 106 units when not otherwise stated). Incubations were at 16 °C for 1 h. Using a poly(dA):oligo(dT) substrate (3000 cpm), the assay was linearly dependent on enzyme concentrations within the range 0-1.5 × 107 units for either mammalian DNA ligase I or ligase II. Hybrid substrates were prepared by annealing 5'-32P-labeled oligo(dT) 14- to poly(dA). Oligo(T8)-poly(dA) substrates were similarly prepared and used for enzyme assays during purification. The conversion of 5'-32P-labeled phosphodiesters to alkaline phosphatase-resistant diesters was measured (12), Poly(dA), poly(dT), and oligo(T8)-poly(dA) substrates were purchased from P-L Biochemicals. The oligo(T8)-dT was treated with alkaline phosphatase and then radioactively labeled (in the presence of 2 mM KHP04 to inhibit the phosphatase) with γ-[32P]ATP (3000 Ci mmol−1) and T4 polynucleotide kinase.

Nicked circular DNA was prepared by EcoRI cleavage of plasmid pAT153 in the presence of ethidium bromide (14); 0.2 μg of nicked DNA was included in each 20-μl reaction mixture with 1.5 × 105 units (12) of T4 DNA ligase, or mammalian ligase I or II. Reactions were allowed to proceed for 1 h at 14 °C and were terminated by extraction with buffered phenolchloroform (1:1). The DNA solutions were subsequently characterized by agarose gel electrophoresis.

Blunt-ended DNA substrates were prepared by cleavage of pX174 replicative form DNA with HpaI. DNA (0.2 μg) was incubated in a

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† Present address: CRC Mammalian Cell DNA Repair Group, Dept. of Zoology, University of Cambridge, U. K.
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40-μl standard reaction mixture supplemented with polyethylene glycol 6000 (17.5% when not otherwise stated) for 1 h at 37 °C with 1.5 x 10^6 units (when not otherwise stated) of T4 DNA ligase or mammalian DNA ligase I or II.

Determination of 3'-Mononucleotides—Poly(dA).oligo(5'-[32P]dT) and poly(rA).oligo(5'-[32P]dT), treated with 3 x 10^7 units of DNA ligase I or DNA ligase II to obtain maximum joining, were then treated with alkaline phosphatase to remove susceptible residues, heated at 100 °C for 30 min to inactivate the enzyme, dialyzed, and then degraded to 3'-mononucleotides by digestion with micrococcal nuclease (Worthington) and spleen phosphodiesterase (Sigma) (12). *P-Labeled dTMP residues were chromatographed on a Partisil Sax HPLC column (1.5 x 25 cm) using 10 mM potassium phosphate, pH 3.0, to separate 5'-dTMP (elution time 10.8 min) from 3'-dTMP (elution time 12.8 min). Authentic nucleotide markers were purchased from Sigma.

Inhibition of Ligases—Ligase I antibody (0.5 μg, purified from serum by precipitation with ammonium sulfate (4)) was preincubated for 5 min at 4 °C with T4 ligase or mammalian ligase I or II in reaction mixtures. The conversion of nicked circular DNA to a covalently closed form by mammalian ligase I was inhibited, while ligase II and T4 ligase were not affected (Fig. 1). Heat inactivation experiments with the mammalian ligases I and II were performed as described previously (3).

RESULTS AND DISCUSSION

Joining of Hybrid Substrate—T4 DNA ligase effectively joins oligodeoxyribonucleotides hydrogen-bonded to a complementary polyribonucleotide, while *Escherichia coli* DNA ligase is unable to catalyze this reaction (2). Similarly to the *E. coli* enzyme, the ATP-dependent mammalian DNA ligase I does not show detectable activity with a hybrid oligo(dT)-poly(rA) substrate (9), as also observed here (Fig. 2). Thus, reaction mixtures containing a concentration (1.5 x 10^6 units) of DNA ligase I 10 times higher than required for maximal joining of a poly(dA).oligo(dT) substrate yielded no detectable joining (<5%) of an equivalent amount of the poly(rA).oligo(dT) substrate. The lack of detectable activity of ligase I with the hybrid substrate was not due to degradation of this substrate by contaminating mammalian RNase H, since artificial mixtures of our ligase I preparation and small amounts of T4 ligase were as active as the T4 enzyme by itself (data not shown). In contrast to the results obtained with ligase I, the mammalian ligase II joined the hybrid substrate at about 50% of the rate observed with a nicked double-stranded polydeoxyribonucleotide (Fig. 2). The reaction of DNA ligase II with either substrate was directly

FIG. 1. Activity of DNA ligases under standard assay conditions in the presence of antibodies to mammalian ligase I. The conversion of nicked circular (NC) plasmid DNA to covalently closed molecules (CC) was visualized after electrophoretic separation on 1% agarose gels containing 0.5 μg ml^-1 ethidium bromide. Lane 1, no ligase added; lanes 2 and 3, T4 DNA ligase; lanes 4 and 5, mammalian ligase I; lanes 6 and 7, mammalian ligase II. Ligase I antibody was included in reactions where indicated.

FIG. 2. Assays of mammalian DNA ligases I and II with a hybrid substrate. The two calf thymus DNA ligases (from 50 g of tissue, material partly purified by phosphocellulose chromatography) were separated by hydroxyapatite gradient chromatography (3) and column fractions (5-μl aliquots) were assayed directly with poly(dA).oligo(5'-[32P]dT) (●) or poly(rA).oligo(dT) (▲) substrates, as described under "Experimental Procedures." Reactions with the poly(dA).oligo(dT) substrate were also performed in the presence of antibodies to ligase I (○). Dashed line represents A_260.

Antiserum

|     | - | + | - | + | - | + | - |
|-----|---|---|---|---|---|---|---|
| 1   | 2 | 3 | 4 | 5 | 6 | 7 |

Ligation Products

|             | 3.7 kb | 1.2 kb | 0.4 kb |
|-------------|--------|--------|--------|
| NC -        |        |        |        |
| Linear -    |        |        |        |
| CC -        |        |        |        |

FIG. 3. Blunt-end joining by DNA ligases. The enzymes were assayed (as described under "Experimental Procedures") for their ability to join HindII fragments of φX174 replicative form DNA in the presence of 17.5% polyethylene glycol 6000. Reaction products were characterized by agarose gel electrophoresis as in Fig. 1. Lanes 1 and 8, no enzyme added; lanes 2 and 3, T4 DNA ligase; lanes 4 and 5, mammalian ligase I; lanes 6 and 7, mammalian ligase II. Antibody to ligase I was added as indicated.
proportional to time (up to 2 h at 16 °C) and to enzyme concentration (up to \( 3 \times 10^{-7} \) units). Moreover, the joining activities for oligo(dT) bound to either a poly(dA) or a poly(rA) complementary chain cochromatographed during hydroxypatite chromatography (Fig. 2) as well as on further purification of ligase II by gel filtration. Furthermore, the two activities exhibited the same heat lability (50% inactivation in 5 min at 42 °C) and may be ascribed to the same enzyme. As shown earlier, ligase II is unable to join strand interruptions in double-stranded polynucleotides, or single-stranded oligo(dT) molecules (1). By comparison, ligase II must be more than 100-fold more active than ligase I with the poly(rA)-oligo(dT) substrate under our standard assay conditions and a range of related conditions. The presence of a distinct catalytic activity of mammalian ligase II, not found in ligase I, would appear to confirm that the two enzymes represent different gene products.

Formation of 3'-5'-Phosphodiester Bonds—In order to demonstrate that the alkaline phosphatase resistance of radioactive phosphate residues in the polymer substrates treated with mammalian DNA ligase I or ligase II was due to the formation of phosphodiester bonds, the substrates were degraded to mononucleotides with micrococcal nuclease and spleen phosphodiesterase (12). Generation of 3'-5'-phosphodiester bonds between oligo([5'-32P]dT) moieties hydrogen-bonded to poly(dA) or poly(rA) would allow the recovery of [3'-32P]dTMP, while this would not be possible if no ligation had occurred. After incubation of poly(dA)-oligo([5'-32P]dT) with DNA ligase I (1.5 \( \times 10^{-7} \) units) under standard reaction conditions, more than 90% of the phosphate-resistant radioactive material was recovered as 3'-dTMP after analysis by high pressure liquid chromatography. Similarly, incubation of poly(rA)-oligo([5'-32P]dT) with mammalian DNA ligase II (3 \( \times 10^{-7} \) units) allowed the isolation of >90% of the phosphate-resistant radioactive material as 3'-dTMP. These data show that both ligase I and ligase II act as DNA ligases, and that ligase II generates phosphodiester bonds with the poly(rA)-oligo(dT) hybrid substrate.

Blunt-end Joining of DNA—The T4 DNA ligase can join blunt-ended DNA fragments (2), and this activity has often been employed for the construction of recombinant DNA molecules. This function is less efficient than the sealing of single-strand interruptions in DNA, but blunt-end joining can be promoted in reaction mixtures by macromolecular crowding conditions, e.g., by the addition of polyethylene glycol (15). When the mammalian DNA ligases I and II were assayed with a blunt-ended DNA substrate in the presence of 17.5% polyethylene glycol 6000 (Fig. 3), ligase I was able to perform this joining reaction (lane 4), while ligase II showed no detectable activity (lane 6). The activity of ligase I was blocked by antibodies against the enzyme (lane 5) while T4 DNA ligase was not similarly inhibited (lanes 2 and 3). Further, the activity of ligase I was proportional to enzyme concentration (up to 1.5 \( \times 10^{-6} \) units) and approximately linearly dependent on time up to 60 min. Increasing the enzyme concentration 5-fold, decreasing the temperature to 16 °C while increasing the time of incubation, or supplementing the reaction mixture with KCl (50–200 mM) failed to reveal any detectable activity of ligase II with the blunt-ended DNA substrate.

To determine the efficiencies of blunt-end joining at different concentrations of polyethylene glycol 6000, the concentrations of this reagent were varied in reaction mixtures (Fig. 4). Mammalian DNA ligase I showed optimal blunt-end joining at 17.5% polyethylene glycol 6000 (lane 5), with lower but still detectable joining occurring at 12.5% (lane 4) and 22.5% (lane 7) polyethylene glycol. Blunt-end joining by ligase I was not observed after 1 h at 37 °C in the absence of added polyethylene glycol 6000 (lane 2) but could be detected after incubation at 16 °C for 72 h (~30% of maximal joining). We estimate that in the absence of polyethylene glycol, DNA ligase I performs blunt-end joining of DNA 20–50 times less efficiently than sealing of single-strand interruptions. No detectable blunt-end joining catalyzed by DNA ligase II was observed at any polyethylene glycol concentrations (lanes 9–14), either at 37 or 16 °C. We conclude that mammalian DNA ligase I, but not ligase II, is able to join blunt-ended DNA molecules under our standard assay conditions. Zimmerman and Pfeiffer (15) have reported that a partly purified DNA ligase preparation from rat liver nuclei could seal double-strand breaks in DNA, and this activity may now tentatively be assigned to DNA ligase I.

Ligase Heterogeneity—The number of mammalian DNA ligases has been a controversial matter. The existence of ligases I and II as two separate activities (4) has been confirmed by Creissen and Shall (16) and by Chan and Becker (6, 11). However, Teraoka and Tsukada (10) have reported that mammalian cells only seem to contain one DNA ligase, corresponding to ligase I, and these authors and Mezzina et al. (17) have further suggested that different size classes of mammalian ligase might perhaps be ascribed to proteolysis. In contrast, in a more recent publication (7), Teraoka and Tsukada have confirmed our finding that antibodies against calf thymus DNA ligase I do not inhibit ligase II. Yeast cells appear to have a single DNA ligase (18) but this does not necessarily imply a similar situation for mammalian cells; for example, DNA polymerase β has not been found in yeast, although it is widely distributed among higher eukaryotes (19). The availability of separate and specific assay procedures for each of the two mammalian DNA ligase activities, as described here, should help to further clarify the situation. Since only one of the two enzymes may be required for essential replication events, it becomes an interesting possibility that one of the inherited human syndromes associated with retarded strand joining of damaged DNA could be associated with a molecular defect in a DNA ligase (20, 21).

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