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DNA Ligases from Rat Liver. Purification and Partial Characterization of Two Molecular Forms†

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ABSTRACT: The differential ability of mammalian DNA ligases to use oligo(dT)·poly(rA) as a substrate has been used to detect, and thereby extensively purify, two immunologically distinct forms of DNA ligase from rat liver. The activity of DNA ligase I, which is unable to use this template, is uniquely increased during liver regeneration, while that of DNA ligase II remains at a low level. Both enzymes require ATP and Mg^{2+} for activity and form an adenylylated intermediate which is stable and reactive. After SDS–PAGE, such radiolabeled complexes correspond to polypeptides of 130 000 and 80 000 Da for DNA ligase I and to 100 000 Da for DNA ligase II. That these labeled polypeptides do indeed correspond to active polypeptides of two different forms of DNA ligase is shown by the removal of the radiolabeled AMP, only when the intermediate is incubated with an appropriate substrate. In contrast to other eukaryotic DNA ligases, rat liver DNA ligase II has a lower K_m for ATP (1.2×10^{-5} M) than DNA ligase I (6×10^{-5} M). Also, DNA ligase II can use ATPαS as a cofactor in the ligation reaction much more efficiently than DNA ligase I, further discriminating the ATP binding sites of these enzymes. Finally, antibodies raised against the 130 000-Da polypeptide of DNA ligase I specifically recognize this species in an immunoblot and inhibit only the activity of DNA ligase I.

DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 5′-phosphoryl and 3′-hydroxyl termini at single-strand nicks in double-stranded DNA (Lehman, 1974). It is therefore an essential element of the coordinated multienzyme processes of semiconservative DNA replication and DNA excision repair, both of which involve the joining of single-stranded breaks.

In mammalian cells, two forms of DNA ligase have been reported in a number of tissues including calf thymus, rat liver, and human placenta (Soderhall & Lindahl, 1975; Teraoka et al., 1986; Bhat & Grossman, 1986). DNA ligase I is the predominant activity in rapidly growing cells and is generally isolated as a 130-kDa polypeptide. DNA ligase II was first described from calf thymus as a late eluting activity during hydroxyapatite chromatography (Soderhall & Lindahl, 1973). The activity of this enzyme is similar in quiescent and dividing cells and is associated with a 68–80-kDa polypeptide (Teraoka et al., 1986; Arrand et al., 1986; Lindahl et al., 1989). Furthermore, it has been shown that these two enzymic forms are immunologically distinct: antibodies against DNA ligase I do not cross-react with DNA ligase II, and vice versa (Soderhall & Lindahl, 1975; Teraoka & Tsukada, 1986).

Earlier reports on DNA ligase from rat liver showed that the total level of DNA ligase activity increased in conjunction with nuclear DNA synthesis during liver regeneration (Tsukada & Ichimura, 1971). Using a rabbit antiserum raised against calf thymus DNA ligase I, Soderhall (1976) showed that although DNA ligase II (obtained by hydroxyapatite chromatography) was the major activity in normal liver, it was the DNA ligase I component which was uniquely increased during liver regeneration. About the same time, the purification of a DNA ligase from rat liver nuclei was described (Zimmerman & Levin, 1975). When compared with contemporary data, this enzyme exhibited many of the properties

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described for mammalian DNA ligase II. More recently, Zimmerman and Pfeiffer (1983) have shown that this DNA ligase preparation can ligate blunt-ended DNA, which was subsequently shown by Arrand et al. (1986) to be an inherent property of calf thymus DNA ligase I, but not of DNA ligase II. Thus, the existence of DNA ligase II in rat liver was not determined unambiguously.

In addition, there have been several contradictory reports on the existence of two different forms of DNA ligase in mammalian cells, and it has been suggested that the susceptibility of DNA ligase I to proteolysis in vitro could lead to anomalous results (Teraoka & Tsukada, 1982; Mezzina et al., 1987). Furthermore, it was shown that differential elution during hydroxyapatite chromatography is not in itself a sufficient criterion to distinguish two separate enzyme species (Mezzina et al., 1987).

Recently, Arrand et al. (1986) demonstrated that mammalian DNA ligase II, unlike DNA ligase I, can ligate oligo(dT) monomers hydrogen-bonded to poly(rA). Thus, this discriminatory assay provides a useful biochemical criterion for identifying this enzyme during purification. Here, we show that two immunologically and biochemically distinct forms of DNA ligase are present in rat liver, several of their biochemical and physical properties being different of those of DNA ligases previously isolated from other mammalian tissues.

Experimental Procedures

Materials

Chromatographic Media. Phosphocellulose (P11) was obtained from Whatman while HA-Ultrogel, ssDNA-Ultrogel, and Ultrogel AcA 34 gel filtration medium were from IBF Biotechnics (France).

Proteins. Polynucleotide kinase was obtained from NEN Research Products and *Escherichia coli* alkaline phosphatase from Sigma. ^{14}C-methylated protein markers were from Amersham while protein standards for gel filtration and density gradients were from Pharmacia. ^{125}I-Labeled protein A prepared by the Bolton and Hunter reagent (>30 mCi/mg) was from Amersham.

Nucleotides and Oligo- and Polynucleotides. [α-^{32}P]ATP (3000 Ci/mmol), [α-^{35}S]ATP (1000–1500 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from NEN. Poly(dA), poly(rA), $(dT)_{16}$, dephosphooligo$(dT)_{16}$, and dATP were obtained from Pharmacia while ATP was from Boehringer Mannheim.

Chemicals. Phenylmethanesulfonyl fluoride (PMSF),[1] obtained from Sigma, was dissolved in 2-propanol at a concentration of 0.2 M, stored at −20 °C until required, and added to buffers at room temperature. Sodium metabisulfite (Sigma) was prepared as a 1.0 M solution of sodium bisulfite (pH 8.0) and stored at 4 °C. Ammonium sulfate (enzyme grade) was obtained from Schwarz/Mann and prepared as a saturated solution at 20 °C in 50 mM KPO_4, pH 7.6. All chemicals used in polyacrylamide gel electrophoresis were from Bio-Rad. Sucrose (density gradient grade), glycerol (extra pure), 2-mercaptoethanol (2-ME), and Triton X-100 were from Merck.

Methods

DNA Ligase Assay. DNA ligase was assayed by using a modification of the method described by Olivera and Lehman (1967). Dephosphooligo$(dT)_{16}$ was phosphorylated with [γ-^{32}P]ATP by polynucleotide kinase in the presence of 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 5 mM dithiothreitol for 15 min at 37 °C. Unless otherwise stated, each reaction mixture (50 μL) contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol, 250 μg/mL BSA, 1 mM ATP, 0.5 μM [^{32}P]$(dT)_{16}$ [(1.5–2.4) × 10^7 cpm/nmol], 4.5 μM $(dT)_{16}$, and 5 μM poly(dA) or poly(rA). Under these conditions, purified fractions containing only DNA ligase II could utilize the oligo(dT)·poly(rA) substrate with 90–100% of the efficiency of the DNA substrate. The ligase reaction was carried out at 37 °C for an appropriate period (generally between 2 and 15 min), stopped by heating at 80 °C for 2 min, and placed in an ice–water bath, where 1 unit of alkaline phosphatase was added. Following incubation at 80 °C for 10 min, a second unit of phosphatase was added and the incubation repeated, after which 150 μg of total yeast RNA was added as carrier, followed by trichloroacetic acid to a final concentration of 5%. The acid-insoluble material was collected on nitrocellulose filters (Schleicher & Schuell, BA 85) and radioactivity determined by scintillation counting. One unit of DNA ligase activity was defined as the amount that converts 1 nmol of [^{32}P]$(dT)_{16}$ to a phosphatase-resistant form in 1 h. Values are given in units per milligram of protein.

Formation of Radiolabeled DNA Ligase–AMP Complexes. The covalent attachment of [^{32}P]AMP, or [^{35}S]AMP, to DNA ligase was carried out in 25-μL aliquots containing 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 μCi of [α-^{32}P]ATP (or [α-^{35}S]ATP), and 0.5 unit of enzyme. After incubation for 1 h at 25 °C, the reaction was either stopped by the addition of the Laemmli loading buffer (Laemmli, 1970) or placed on ice before incubation with putative ligase substrates for 5 min at 37 °C. Finally, each sample was incubated for 90 s at 90 °C prior to being loaded on an SDS–polyacrylamide gel. These were composed of a 5% stacking gel and a 10% separating gel. Electrophoresis was carried out for 6 h at 25 mA.

Protein Determinations. Protein content was determined by the method of Bradford, using bovine serum albumin as the standard (Bradford, 1976).

Purification of DNA Ligase I. (A) Preparation of the Postmicrosomal Fraction. Regenerating livers were obtained from 5–6-month-old animals, 44 h after partial hepatectomy, by which time regeneration was 50–60% complete by weight (De Recondo et al., 1973).

All the following purification steps were carried out at 4 °C. Following dissection, the liver was diced and placed in 2 volumes of buffer containing 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 0.25 M sucrose, 10 mM $NaHSO_3$, 1 mM PMSF, 1 mM 2-ME, 2 μg/mL leupeptin, and 2 μg/mL pepstatin. After homogenization by three strokes of a serated pestle in a Dounce homogenizer, the resulting material was strained through four layers of gauze cloth and centrifuged for 80 min at 50 000 rpm in a Beckman 50.2 Ti rotor. The nonlipidic supernatant was collected, filtered through gauze cloth, and stored at −80 °C until required (fraction I).

(B) Phosphocellulose Chromatography and Ammonium Sulfate Precipitation. Fraction I was loaded onto a phosphocellulose column (2.5 × 20 cm) equilibrated with 55 mM KPO_4, pH 7.6, in solution A (10 mM $NaHSO_3$, 1 mM PMSF, 1 mM 2-ME, 0.2 mM EDTA, 2 μg/mL leupeptin, and 10% v/v glycerol) at a protein concentration of 30–40 mg/mL of column bed. After being loaded, the column was washed with this buffer until no further protein was detected in the eluate.

[1] Abbreviations: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate.

DNA ligase activity was then eluted stepwise, the bulk of the DNA ligase I activity eluting with 110 mM KPO_4, pH 7.6, in solution A. A further elution with 165 mM KPO_4, pH 7.6, in the same solution removed the remaining DNA ligase I from the column and also eluted the DNA ligase II activity as shown by the appearance of activity with the poly(rA) substrate. Fractions of the first elution step, containing only DNA ligase I activity, were pooled, and a saturated solution of ammonium sulfate was slowly added to a final concentration of 55%. After being stirred for 1 h, the protein precipitate was collected by centrifugation at 10000*g* for 1 h and then resuspended in 10 mM KPO_4, pH 7.6, in solution B (0.4 M NaCl, 10 mM $NaHSO_3$, 5 mM 2-ME, 0.2 mM EDTA, and 10% v/v glycerol) (fraction II).

(*C*) *Hydroxyapatite Chromatography.* Fraction II was dialyzed overnight against 10 mM KPO_4, pH 7.6, in solution B and loaded onto a column of HA–Ultrogel at a protein concentration of 2 mg/mL of column bed. The column was then washed with 3 column volumes of the same buffer to elute unbound proteins, before the DNA ligase activity was eluted with 125 mM KPO_4, pH 7.6, in solution B. Fractions containing ligase activity were pooled, and saturated ammonium sulfate was added to a final concentration of 70% (fraction III).

(*D*) *Gel Filtration.* Gel filtration was carried out using a gel with a broad range of separation (Ultrogel AcA 34) in a column of 1.5 × 75 cm. This column was calibrated with the following marker proteins: ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), BSA (68 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa). Elution of the column was carried out at a flow rate of 6 mL/h.

The ammonium sulfate pellet was resuspended in 50 mM KPO_4, pH 7.6, in solution C (0.8 M NaCl, 10 mM $NaHSO_3$, 10 mM 2-ME, 0.2 mM EDTA, 10% v/v glycerol, and 0.025% v/v Triton X-100) to a final protein concentration of 10–15 mg/mL and loaded directly. DNA ligase I activity eluted as a single peak corresponding to between 200 and 450 kDa. Pooled fractions (fraction IV) were then dialyzed overnight against 20 mM KPO_4 in solution D (10 mM $NaHSO_3$, 5 mM 2-ME, 0.2 mM EDTA, and 20% v/v glycerol).

(*E*) *Single-Stranded DNA–Ultrogel Chromatography.* Fraction IV was loaded onto a column of ssDNA–Ultrogel at a protein concentration of 1 mg/mL of column bed. The column was washed with 3 volumes of 20 mM KPO_4, pH 7.6, in solution D and then with a 0–350 mM linear gradient of NaCl in the same buffer. The DNA ligase activity eluted at an ionic strength equivalent to 150 mM NaCl. Fractions containing DNA ligase activity were pooled (fraction V) and dialyzed overnight against buffer E (50 mM KPO_4, 0.4 M NaCl, 10 mM $NaHSO_3$, 5 mM 2-ME, and 0.2 mM EDTA) containing 20% v/v glycerol.

(*F*) *Density Gradient Sedimentation.* Immediately prior to loading, fraction V was diluted 4-fold with buffer E and concentrated by using a Centricon-30 concentrator (Amicon). This fraction was then loaded onto 10–30% v/v glycerol gradients (11 mL) prepared in buffer E. The glycerol gradients were centrifuged for 40 h at 40000 rpm in a Beckman SW41 rotor. Protein markers (catalase, aldolase, BSA, and chymotrypsinogen A) were run in a parallel gradient.

Following centrifugation, the gradients were fractionated from the bottom of the gradient, and each fraction was tested for DNA ligase activity. Additionally, a 5-μL aliquot was taken from fractions containing ligase activity and incubated with [α-^{32}P]ATP as previously described, and the resulting ligase–[^{32}P]AMP complexes were separated by SDS–PAGE and visualized by autoradiography.

Purification of DNA Ligase II. The purification of DNA ligase II was carried out in essentially the same manner as that described for DNA ligase I. Using the oligo(dT)·poly(rA) assay, it was possible to detect the activity of DNA ligase II at each step of the purification and thus to design a purification scheme which maximized the separation of this activity from DNA ligase I. In addition, because of the lower amounts of the latter enzyme in normal liver, this was the preferred source of DNA ligase II, the livers being obtained from 6–12-month-old WAG rats.

Thus, during phosphocellulose chromatography, DNA ligase II was only detected in the 165 mM KPO_4 eluate and could be separated from the bulk of the DNA ligase I during this step. Similarly, during gel filtration, DNA ligase II eluted before the DNA ligase I activity, corresponding to a molecular mass of greater than 450 kDa. Finally, the complete separation of the two molecular forms of DNA ligase was obtained by density gradient sedimentation. Fraction V, prepared as described for DNA ligase I, was loaded onto 5–20% sucrose gradients (5 mL) prepared in buffer E. The gradients were centrifuged for 16 h at 45000 rpm in a Beckman SW 55 rotor, and protein markers were run in a parallel gradient. Each gradient fraction was assayed for DNA ligase activity using both the poly(dA) and poly(rA) substrates, and fractions containing DNA ligase II were pooled (fraction VI). As previously described, fractions containing DNA ligase activity were incubated with [α-^{32}P]ATP and the resulting ligase–AMP complexes separated by SDS–PAGE and visualized by autoradiography.

Production of Polyclonal Antibodies against DNA Ligase I. Aliquots of fraction V (3 × 5 μL) were incubated with [α-^{32}P]ATP as described above, mixed with 1500–3000 units of the same fraction, and subjected to SDS–PAGE. The position of the 130-kDa DNA ligase I polypeptide in the gel was detected by autoradiography and a strip containing this band cut from the gel. Polypeptides in this strip were then electroeluted into a membrane trap (Biotrap, Schleicher & Schuell), emulsified with complete Freund's adjuvant (1:1), and injected subcutaneously into the back of a rabbit (New Zealand White). Two weeks later, a second injection was carried out in a similar manner; the antigen was emulsified with incomplete Freund's adjuvant (1:1). Four further boosts were given biweekly after which blood was taken from the marginal ear vein and the serum obtained by centrifugation. Further serum was taken after similar boosts as required.

Protein Transfer and Immunoblotting. The electrophoretic transfer of polypeptides to nitrocellulose (Schleicher & Schuell, BA 83) after SDS–PAGE was achieved by using a Bio-Rad Trans Blot apparatus. Transfer was carried out for 6 h at 150 mA at 4 °C according to Towbin et al. (1979). Immunodetection was carried out according to the method of Burnette (1981) except that saturation of the blots was by 5% nonfat milk, and wash buffers contained 0.1% Tween-20.

Results

Purification. We have reported previously the partial purification of a single 130-kDa form of DNA ligase from rat liver (Mezzina et al., 1987). Using the oligo(dT)·poly(rA) assay as a criterion for DNA ligase II, we reinvestigated our system for the existence of this enzyme. Although no activity was detected with this substrate in the postmicrosomal fractions, DNA ligase II activity was detected after phosphocellulose chromatography of fraction I from both normal and regenerating liver. From this result, we were able to show that the observed increase in activity in regenerating rat liver is

Table I: Purification of DNA Ligase I from Regenerating Rat Liver[a]

fraction	volume (mL)	protein (mg)	activity (units)	specific activity (units/mg)	purification (*x*-fold)	yield (%)
(I) postmicrosomal supernatant	250	3900	58500	15	1	100
(II) phosphocellulose ammonium sulfate	5.5	27	20358	754	50	35
(III) hydroxyapatite ammonium sulfate	1.6	17.8	13421	755	50	23
(IV) gel filtration	3.8	3.9	5821	1492	100	10
(V) ssDNA–Ultrogel	0.31	0.21	2725	12976	865	5
(VI) glycerol gradient	0.55	0.03	703	23433	1562	1.2

[a] Activity was measured as described under Methods using oligo(dT)·poly(dA) as substrate.

Table II: Purification of DNA Ligase II from Normal Rat Liver[a]

fraction	volume (mL)	protein (mg)	activity (units)	specific activity (units/mg)	purification (*x*-fold)	yield (%)
(I) postmicrosomal supernatant	85	1700	1568	0.9	1	100
(II) phosphocellulose ammonium sulfate	5.8	20.3	542	27	30	35
(III) hydroxyapatite ammonium sulfate	2.1	9.5	368	107	43	24
(IV) gel filtration	2.9	3.1	339	107	119	22
(V) ssDNA–Ultrogel	1.1	1.1	167	152	169	11
(VI) sucrose gradient	0.55	0.17	48	291	323	3

[a] Activity was measured as described under Methods using oligo(dT)·poly(dA) as substrate.

due only to DNA ligase I (not shown). We therefore used regenerating liver as our source of this enzyme, while the lower amounts of DNA ligase I in normal liver facilitated the purification of DNA ligase II.

Purification of DNA Ligase I from Regenerating Liver. A large increase in the specific activity of DNA ligase is observed after both phosphocellulose and single-stranded Ultrogel chromatography (Table I).

During phosphocellulose chromatography, DNA ligase I was eluted by 110 mM potassium phosphate, although a significant amount was also eluted with DNA ligase II at 165 mM phosphate. In order to ensure an essentially DNA ligase II free first elution step and eventually a high specific activity, we fractionated these two eluates separately. Details of the purification DNA ligase I from the 110 mM eluate are given in Table I. All our experimental data indicate that the form of DNA ligase I found in both eluates is identical.

Although hydroxyapatite chromatography did not lead to a significant improvement of specific activity, the inclusion of this step resulted in a better purification by ssDNA–Ultrogel chromatography, yielding a 1500-fold purification of the enzyme after density gradient sedimentation.

Purification of DNA Ligase II from Normal Rat Liver. The purification of DNA ligase II from normal liver is detailed in Table II. Following phosphocellulose and hydroxyapatite chromatography, gel filtration enabled a further separation of the two enzymes, as shown in Figure 1. Thus, while DNA ligase II eluted first as a single peak of activity with a calculated Stokes radius of 6.4 nm, DNA ligase I was more retarded on the column and eluted as a single broad peak of activity with a calculated Stokes radius of 5.5 nm.

Fractions containing DNA ligase II were further purified by ssDNA–Ultrogel chromatography before density gradient sedimentation was used to complete the separation of the two enzymic forms. As shown in Figure 2, panel A, a single peak of activity (5.8 S) is observed with the oligo(dT)·poly(rA) substrate. When oligo(dT)·poly(dA) was used to measure the ligase activity, two peaks were observed with sedimentation coefficients of 4 and 5.8 S. That the 4S peak is indeed due to DNA ligase I is shown by the sedimentation profile of fraction V of DNA ligase I purified from regenerating rat liver (Figure 2, panel B).

Molecular Mass. Since the two forms of DNA ligase were not purified to homogeneity, it was necessary to label the polypeptides with DNA ligase activity in order to determine

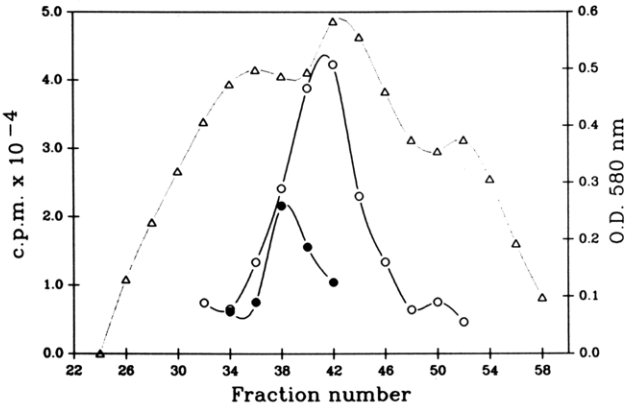

FIGURE 1: Gel filtration of DNA ligases I and II from regenerating rat liver (fraction III, see Methods). Fractions were assayed for DNA ligase activity with oligo(dT)·poly(dA) (○) and oligo(dT)·poly(rA) (●), and protein was determined by the method of Bradford (△).

their relative molecular mass by SDS–PAGE. This was achieved by a scheme involving the first step of the enzyme's reaction mechanism, i.e., the covalent binding of AMP (Lehman, 1974). Using [α-^{32}P]ATP in the adenylylation reaction, it was then possible to visualize the AMP binding polypeptides by autoradiography (Figure 2, panels C and D). The results show that the 4S activity (DNA ligase I) is associated principally with a polypeptide of 130 kDa, although a smaller band of 80 kDa is also evident in many of our preparations. The broad peak of DNA ligase I activity observed in Figure 2A in combination with its substrate specificity for oligo(dT)·poly(dA) (Figure 5) suggests that the 80-kDa polypeptide has both the DNA ligase activity and specificity of the 130-kDa polypeptide. On the other hand, the DNA ligase II activity (5.8 S) is associated with a single polypeptide of 100 kDa (Figure 2C).

Thus, although the molecular weight of DNA ligase I agrees with previously reported data for the calf thymus enzyme, the molecular mass of DNA ligase II is significantly greater than that reported for the corresponding calf thymus enzyme (Teraoka et al., 1986).

Biochemical Characterization. Under standard assay conditions with oligo(dT)·poly(dA), both enzymes have pH optima around pH 7.8, although DNA ligase I exhibits maximal activity over a broader pH range. Mg^{2+} is essential for activity, both enzymes having maximal ligase activity at about 6 mM. Although Mn^{2+} could be substituted for Mg^{2+}

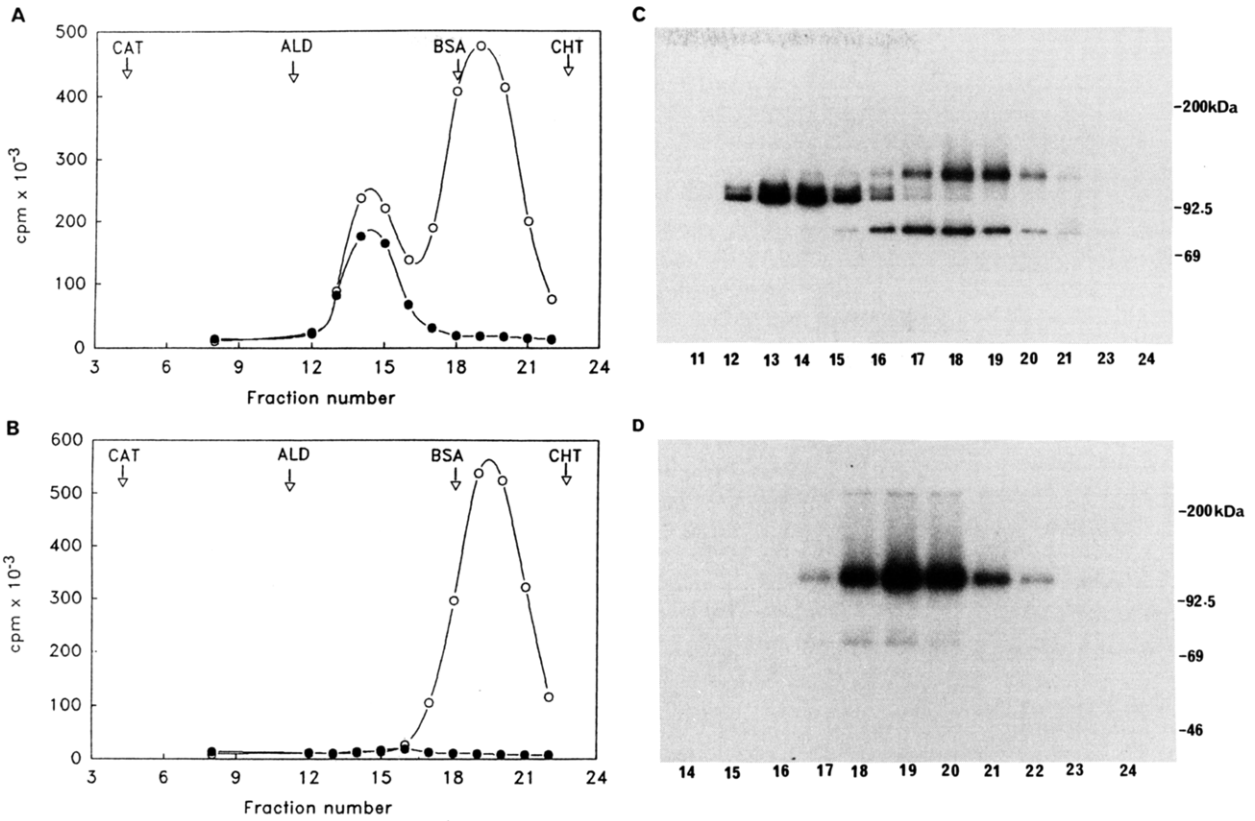

FIGURE 2: Density gradient sedimentation of rat liver DNA ligases I and II. (Panel A) Fraction V from normal rat liver was sedimented in a 5–20% sucrose gradient as described under Methods. Fractions were assayed for DNA ligase activity with oligo(dT)·poly(dA) (○) and oligo(dT)·poly(rA) (●). The protein markers indicated were catalase (11.3 S), aldolase (7.5 S), bovine serum albumin (4.6 S), and chymotrypsinogen A (2.4 S). (Panel B) Fraction V (DNA ligase I–DNA ligase II free) from regenerating rat liver was sedimented in a 10–30% glycerol gradient as described under Methods. Assays and protein markers were as described for panel A. (Panel C) Aliquots (5 μL) of fractions from the density gradient shown in panel A were incubated with [α-^{32}P]ATP and then subjected to SDS–PAGE. Marker proteins run in adjacent lanes and indicated by their molecular weight (×10^{-3}) were myosin, rabbit muscle phosphorylase, bovine serum albumin, and ovalbumin. (Panel D) Aliquots of fractions from the gradient shown in panel B.

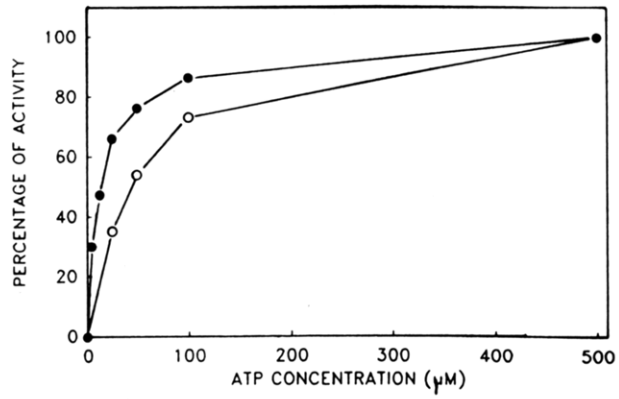

FIGURE 3: Effect of ATP concentration on the activity of DNA ligase I (○) and DNA ligase II (●).

(maximal activity at 1.2 mM is 60% of that at 6 mM Mg^{2+}), no activity is observed with Ca^{2+}. While concentrations of NaCl up to 60 mM stimulate ligase activity by a factor of 1.5, concentrations greater than this are inhibitory to both enzymes.

Cofactor Binding. For both DNA ligases I and II, inclusion of ATP in the reaction mixture is essential for subsequent ligation activity. In our assay, no activity was observed when ATP was omitted, or when NAD^+, the cofactor utilized by *E. coli* DNA ligase, was substituted for ATP (not shown). As shown in Figure 3, however, the two enzyme forms can be differentiated by their affinity for ATP: DNA ligase I has a lower affinity for ATP (K_m = 6 × 10^{-5} M, as determined by a double-reciprocal plot of Lineweaver and Burk) than DNA ligase II (K_m = 1.2 × 10^{-5} M), suggesting a slight

Table III: Use of ATPαS by DNA Ligases I and II

DNA ligase	substrate	cofactor	time of incubation (min)	cpm
I	oligo(dT)·poly(dA)	ATP	20	228 730
		ATPαS	60	10 111
		none	60	5 138
I	oligo(dT)·poly(rA)	ATP	20	4 876
		ATPαS	60	5 437
		none	60	4 865
II	oligo(dT)·poly(dA)	ATP	20	459 810
		ATPαS	60	85 033
		none	60	5 309
II	oligo(dT)·poly(rA)	ATP	20	146 810
		ATPαS	60	52 685
		none	60	4 558

difference in the ATP binding sites of these enzymes. To study this difference further, the effect of various ATP analogues was examined.

Deoxyadenosine triphosphate could not replace ATP in the reaction, and competitively inhibited both enzymes (not shown). However, although both enzymes could use ATPγS as a cofactor (not shown), when the α-S-substituted analogue was used, a striking difference in the ability of the two enzymes to carry out ligation was observed (Table III).

In order to demonstrate that the phosphatase-resistant radioactivity measured in these assays was due to ligation events, the $(dT)_{16}$ substrate and any reaction products were separated in denaturating polyacrylamide gels as previously described by Mezzina et al. (1984). The results (Figure 4) show that the phosphatase resistance assay is indeed a true measure of

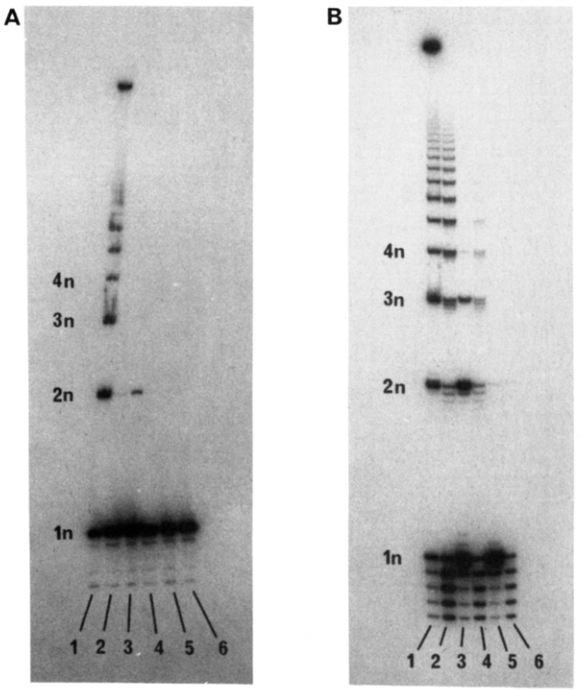

FIGURE 4: Denaturing polyacrylamide gel electrophoresis of oligo(dT) following incubation with DNA ligase. (Panel A) DNA ligase I. (Panel B) DNA ligase II. For both, the cofactor used in each reaction was as follows: lanes 1 and 2, ATP; lanes 3 and 4, ATPαS; lanes 5 and 6, cofactor omitted. For odd lanes, the substrate was oligo(dT)·poly(dA) and for even lanes oligo(dT)·poly(rA).

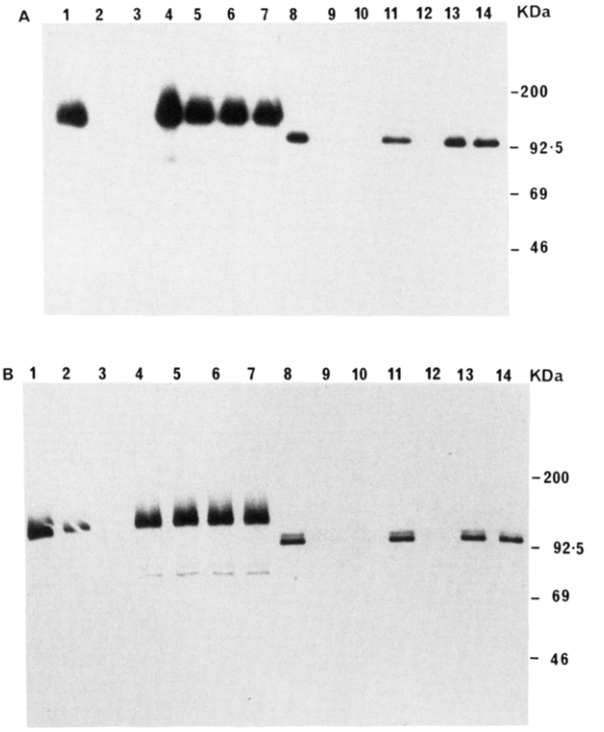

FIGURE 5: Substrate specificity of rat liver DNA ligases as shown by the preferential removal of radioactive AMP from the ligase–AMP complexes. Aliquots of DNA ligase I (lanes 1–7) and DNA ligase II (lanes 8–14) were adenylylated with [α-^{32}P]ATP (panel A) or [α-^{35}S]ATP (panel B) and then incubated for 5 min at 37 °C with (lanes 1 and 8) control, (lanes 2 and 9) 10 mM PP_i, (lanes 3 and 10) 5 μM oligo(dT)·poly(dA), (lanes 4 and 11) 5 μM dephosphooligo(dT)·poly(dA), (lanes 5 and 12) 5 μM oligo(dT)·poly(rA), (lanes 6 and 13) 5 μM dephosphooligo(dT)·poly(rA), and (lanes 7 and 14) 5 μM oligo(dT).

ligation activity, phosphatase-resistant radioactivity being present only in fractions where ligation products are visible in the autoradiogram.

Since no ligated products are observed when the cofactor is omitted from the reaction mixture (Figure 4, panels A and B, lanes 5 and 6), the observed activity is not due to the presence of different levels of ATP nor to differential amounts of ligase–AMP complexes in the two enzyme fractions. Therefore, it is clear that only DNA ligase II can use ATPαS effectively and with both substrates (Figure 4, panel B, lanes 3 and 4). However, following this long period of incubation (60 min), a small amount of ligation is also observed for DNA ligase I. That this is not due to the presence of ATP in the ATPαS preparation is shown in Figure 5. These results also confirm that only DNA ligase II can ligate oligo(dT) when it is annealed to poly(rA).

DNA Ligase–AMP Complex Is a Reactive Intermediate. To determine if the radiolabeled ligase–AMP complexes revealed after SDS–PAGE were in fact due to active DNA ligase polypeptides and not to similarly acting enzymes which cosedimented with the DNA ligase activity, the labeled polypeptides were incubated under various regimes chosen to exploit the known differences in their substrate specificity. Figure 5 shows the results of such experiments. Aliquots of DNA ligases I and II were labeled with either [α-^{32}P]ATP (panel A) or [α-^{35}S]ATP (panel B) and then incubated in the appropriate regime.

Ligase–[^{32}P]AMP. It is clear that both enzymes can form a reaction intermediate when incubated with [α-^{32}P]ATP and that the polypeptides visualized after SDS–PAGE do correspond to DNA ligases I and II. This is confirmed by the disappearance of the ^{32}P-labeled polypeptides when incubated with the oligo(dT)·poly(dA) substrate (panel A, lanes 3 and 10), whereas only the putative DNA ligase II polypeptide disappears when the oligo(dT)·poly(rA) substrate is substituted (panel A, lanes 5 and 12).

No activity is detected when either enzyme is incubated with oligo(dT) in the absence of a complementary strand (panel A, lanes 7 and 14) or when the oligo(dT) lacks a 5′-phosphate to accept the AMP moiety from the ligase–AMP complex (lanes 4, 6, 11, and 13). The addition of inorganic pyrophosphate (PP_i) to the intermediate does, however, result in the loss of the ^{32}P label due to the reversal of the adenylylation reaction (lanes 2 and 9).

Ligase–[^{35}S]AMP. When incubated with [α-^{35}S]ATP (Figure 5, panel B), both DNA ligases I and II can form a ligase–[^{35}S]AMP complex, even though the activity of DNA ligase I was barely detectable with this cofactor (Table III and Figure 4). As with the ligase–[^{32}P]AMP complex, these were found to be reactive intermediates, the label being lost only when the complex was incubated with an appropriate substrate, indicating that the low level of DNA ligase activity observed with ATPαS is indeed due to this cofactor and not to contaminating ATP.

No label was lost when dephosphooligo(dT) was substituted for oligo(dT), nor when oligo(dT) alone was added. In the presence of PP_i, in contrast to the modified DNA ligase II complex, a radioactive polypeptide, corresponding to DNA ligase I, is still observed, indicating that formation of the modified intermediate complex is not easily reversed.

Thus, the data show that both DNA ligases I and II can form reactive ligase–AMP complexes either with ATP or with ATPαS and that the radioactive bands revealed by autoradiography are due to polypeptides with two different DNA ligase activities. In addition, they indicate that although the enzyme's ATP binding sites are dissimilar, their mechanism of action is similar, the second step requiring the transfer of

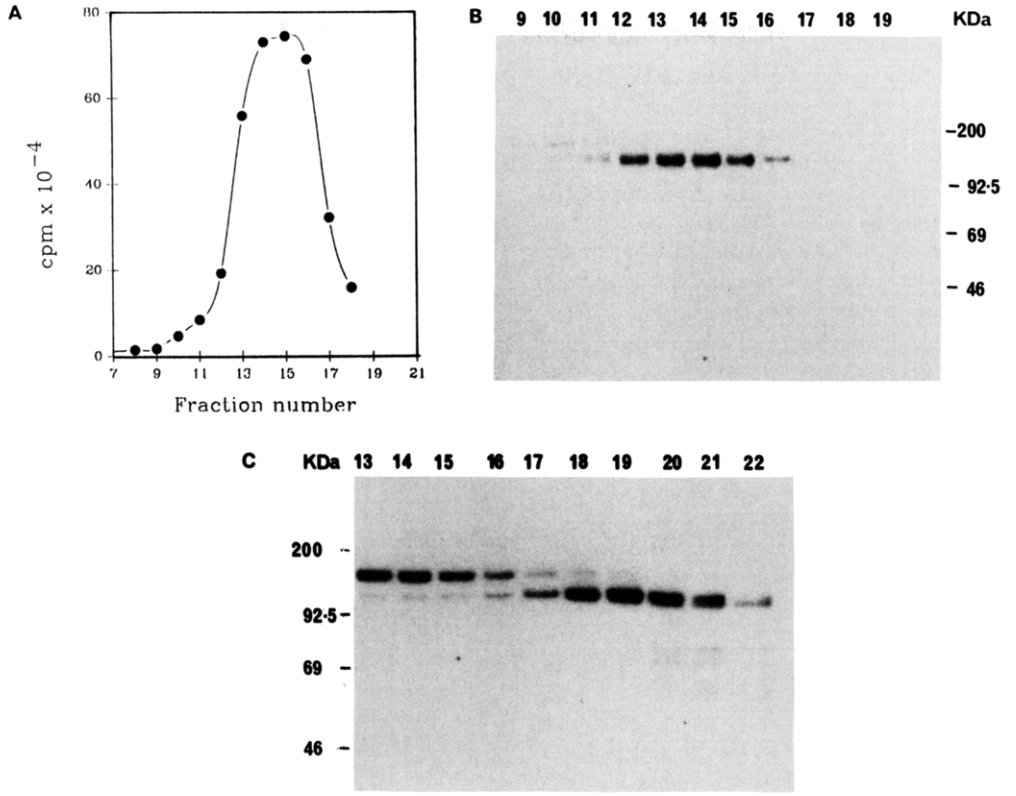

FIGURE 6: Immunoblot analysis of DNA ligase fractions. (Panel A) Glycerol gradient fractionation of DNA ligase I. (Panel B) Immunoblot of the glycerol gradient fractionation shown in panel A. (Panel C) Immunoblot analysis of a sucrose gradient fractionation of DNA ligases I and II (see Figure 2, panel B).

the AMP moiety to a 5′-phosphate, presumably at the site of ligation.

Immunological Detection. DNA ligase I fractions obtained after density gradient sedimentation were subjected to SDS–PAGE; the separated polypeptides were transferred to nitrocellulose and incubated with rabbit antiserum prepared against the 130-kDa polypeptide. Figure 6 shows the activity of DNA ligase I and the corresponding immunoblot for such a gradient (panels A and B).

Where activity corresponding to DNA ligase I is detected in the gradient fractions, a band of 130 kDa is also observed in the corresponding immunoblot. The intensity of the ^{125}I-labeled protein A band also closely follows the peak of DNA ligase I activity, suggesting that the rabbit antiserum does contain antibodies against the 130-kDa polypeptide.

Figure 6 also shows (panel C) that in fractions containing DNA ligase II (Figure 2, panel A), the rabbit antiserum does not recognize the 100-kDa polypeptide, previously shown (Figure 5) to be associated with this enzyme activity. However, a species of high molecular mass is seen in the immunoblot. This band is never AMP labeled, and the intensity of the ^{125}I-labeled protein A labeling does not follow the peak of ligase activity in a density gradient (see Figure 2, panel C). Therefore, we believe it to be due to a contaminating polypeptide which was present in the samples used to prepare antibodies against the 130-kDa polypeptide.

To further demonstrate that the antibodies raised against the 130-kDa polypeptide were specific for DNA ligase I, purified enzyme fractions were preincubated with either preimmune or immune rabbit serum prior to enzyme assay. The results (Figure 7) show that (i) DNA ligase I activity was inhibited by nearly 50% by the antiserum at concentrations which had no inhibitory effect when the preimmune serum was used and (ii) at the same concentration, the antiserum had no effect on the activity of DNA ligase II.

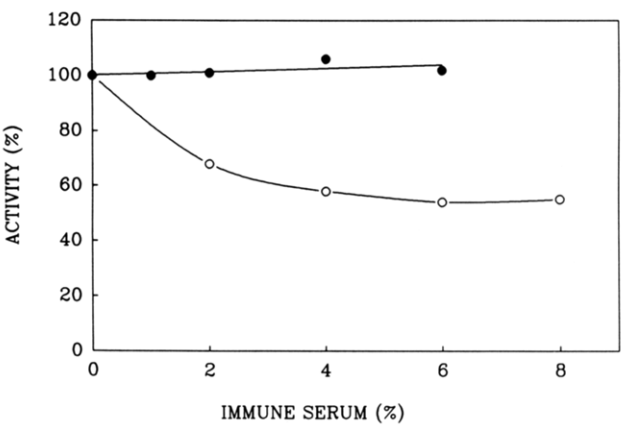

FIGURE 7: Inhibition of DNA ligase I activity by rabbit antiserum directed against the 130-kDa polypeptide. DNA ligase I (○); DNA ligase II (●).

Thus, we conclude that the antiserum is specific for DNA ligase I and that the two molecular forms of DNA ligase we have identified in rat liver are immunologically distinct. Experiments are now underway to perform such an analysis with an antiserum directed against DNA ligase II.

Discussion

The presence of multiple forms of mammalian DNA ligase is now well documented (Teraoka et al., 1986; Lindahl et al., 1989). However, it has not always been clear whether enzymes designated DNA ligase I and II following different purification procedures refer consistently to the same two independent molecular forms of the enzyme. In addition, previous attempts to determine the existence of DNA ligase II in rat liver have produced conflicting results (Soderhall, 1976; Teraoka & Tsukada, 1979; Mezzina et al., 1987). With this in mind, we decided to reappraise the question of rat liver DNA ligase using an apparently specific assay for DNA ligase II [i.e., activity

with oligo(dT)·poly(rA)] as our main criterion for detecting this enzymic species (Arrand et al., 1986).

After verifying that such an activity is present in rat liver, it was then possible to design a purification scheme to maximize its separation from DNA ligase I. However, unlike earlier reports (Soderhall & Lindahl, 1973; Soderhall, 1976), we were unable to separate the two ligase activities by hydroxyapatite chromatography. Likely, this could be explained by some differences in the structure of the hydroxyapatite powder, as in the earlier studies it was not a commercial preparation (T. Lindahl, personal communication).

Furthermore, we were unable to detect a late eluting form of DNA ligase during gel filtration chromatography, as previously described for rat liver by Chan and Becker (1985). Indeed, DNA ligase II eluted before DNA ligase I during gel filtration chromatography and sedimented faster in a density gradient, indicating that the native form of DNA ligase II is larger than that of DNA ligase I. However, when the ligase–AMP complexes were subjected to SDS–PAGE, the polypeptide containing the ATP binding site of DNA ligase II was found to be smaller (100 kDa) than that of the equivalent, predominant polypeptide of DNA ligase I (130 kDa). While this apparent discrepancy may be explained by the reported asymmetric structure of these enzymes, it is interesting to speculate that some other factor may be lost during the SDS denaturation of DNA ligase II, resulting in its lower molecular weight value and explaining the inability to detect DNA ligase II by the activity gel method (Mezzina, Elder, and Rossignol, unpublished results).

DNA ligase I from rat liver satisfies the criteria used to distinguish DNA ligase I in mammalian cells: (i) its level is increased in rapidly dividing cells; (ii) it cannot use oligo(dT)·poly(rA) as a substrate for ligation; and (iii) it can ligate blunt-ended DNA (Elder and Rossignol, unpublished results). However, many of its biochemical characteristics are significantly different from those of the DNA ligase I from calf thymus, although they do correspond to those described previously for a DNA ligase isolated from rat liver nuclei (Zimmerman & Levin, 1976). Thus, rat liver DNA ligase I is inhibited by relatively low concentrations of NaCl, shows a similar dependence for pH and Mg^{2+}, and, under similar reaction conditions, has a similarly high K_m for ATP.

Therefore, although contemporary data suggested that the enzyme isolated by Zimmerman and Levin was DNA ligase II, our results indicate otherwise. More recently, the same laboratory has shown that their enzyme preparation can ligate blunt-ended DNA strands (Zimmerman & Pfeiffer, 1983), a property we have found to be uniquely associated with rat liver DNA ligase I (unpublished), in good agreement with data published for calf thymus DNA ligase I (Arrand et al., 1986).

At 100K, the molecular weight for the DNA ligase II–AMP complex is significantly larger than that reported by Teraoka et al. (1986) for DNA ligase II from calf thymus (68K). Also, at 1.2×10^{-5} M, DNA ligase II from rat liver has a lower K_m for ATP than previously described for this class of enzyme from any source and, uniquely, a higher affinity for ATP than DNA ligase I isolated from the same tissue. It is therefore interesting to speculate that the DNA ligase II described herein has been isolated in a more native form than has previously been achieved for this enzyme. Further corroborating evidence comes from our failure to detect any large difference in the heat lability of these two molecular forms of DNA ligase (unpublished) in contrast to an earlier report (Soderhall, 1976).

The appearance of an AMP-labeled polypeptide at 80 kDa in some fractions of DNA ligase I suggests to us that the proteolytic degradation of the predominant form of DNA ligase may have been one reason for previous contradictory reports on DNA ligases in rat liver. The recent article by Mezzina et al. (1987) which showed that proteolytic degradation is much more prevalent in fractions from normal liver than from regenerating liver indicates that by some extraction procedures the 80-kDa polypeptide may be more readily apparent than in others. However, as we detected this polypeptide species only as a minor component after the final step of purification, we did not evaluate its elution pattern from hydroxyapatite, nor by gel filtration. It is interesting to note that the smaller form of DNA ligase I is much less immunoreactive in an immunoblot. This leads us to speculate that if the 80-kDa form can, under some conditions, be separated from the high molecular weight form of DNA ligase I, it may have altered biochemical properties, or may be mistaken for an immunologically distinct form of the enzyme.

While DNA ligase I is presumably involved in the joining of Okazaki fragments during DNA replication, the role of DNA ligase II in the cell is still not known. Although several authors have speculated on a role for this enzyme in DNA repair, this remains to be elucidated (Chan & Becker, 1985; Creissen & Shall, 1982; Mezzina et al., 1982). Similarly, a physiological role, if any, for the joining of DNA strands annealed to RNA has yet to be demonstrated, although a role in the maintenance of genomic integrity during transcription can be speculated. However, with the various specific biochemical tests now available to differentiate between the activity of DNA ligases I and II (including the specific utilization of ATPαS by DNA ligase II), it should be possible to more precisely define a role for these two enzymes in the cell.

Finally, we propose that confusion over the multiplicity of DNA ligases in this tissue has arisen (i) because of the susceptibility of DNA ligase I to proteolytic degradation during the purification procedure, (ii) due to the apparent high molecular mass of DNA ligase II when subjected to gel filtration, or density gradient sedimentation, and (iii) as a result of several atypical biochemical parameters associated with DNA ligase I from rat liver.

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Characterization of the Structure and Melting of DNAs Containing Backbone Nicks and Gaps†

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ABSTRACT: A DNA molecule containing a gap (a missing phosphate) has been examined and compared to two other molecules of the same sequence, one containing a nick (a phosphorylated gap) and the other a normal duplex containing no break in the backbone. A second gapped sequence was also compared to a normal duplex of the same sequence. The molecules containing nicks or gaps were generated as dumbbell molecules, short helices closed by a loop at each end. The dumbbells were formed by the association of two hairpins with self-complementary dangling 5′-ends. Nuclear magnetic resonance was used to monitor the melting transition and to probe structural differences between molecules. Under the conditions used here no change in stability was observed upon phosphorylation of the gap. Structural changes upon phosphorylation of a gap or closure of a nick were minimal and were localized to the region immediately around the gap or nick. Two transitions can be observed as a gapped or nicked molecule melts, although the resolution of the two transitions varies with the salt concentration. At moderate to high salt (≥30 mM) the molecule melts essentially all at once. At low salt the two transitions occur at temperatures that differ by as much as 15 °C. In addition, comparison with other NMR melting studies indicates that the duplex formed by the overlap of the dangling ends of the hairpins is stabilized relative to a free duplex of the same sequence, probably by stacking onto the hairpin stem.

The development of methods for the synthesis of DNA oligomers in large quantities has made any short sequence accessible to study by physical techniques such as NMR.[1] These short fragments allow one to obtain the resolution needed for detailed NMR structural studies. A variety of systems, including normal duplexes, mismatches, modified bases, and drug complexes, have been studied already. [A comprehensive review of this area has been presented recently by van de Ven and Hilbers (1988).] In the present work we describe studies of DNA oligomers containing a nick (a single broken C3′-phosphate bond) or a gap (a missing phosphate) in the backbone. These nicks and gaps are among the simplest forms of DNA damage that can occur. The generation of a nick is one of the first steps in the repair of a pyrimidine dimer, formed when DNA is exposed to ultraviolet light. In addition, the "sticky end" ligation reactions, crucial to the "cut and paste" of cloning, go through such structures as intermediates. An earlier study of nicked DNA based on sedimentation and viscosity measurements of calf thymus DNA concluded that, in general, nicks do not affect flexibility (Hays & Zimm, 1970). This suggests that nicks do not significantly alter the structure of DNA. The use of NMR to study small oligomers can provide a more detailed description of the DNA structure at the sites of nicks and gaps and characterize the melting

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMT, dimethoxytrityl; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; HPLC, high-performance liquid chromatography; TSP, 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid; EDTA, ethylenediaminetetraacetic acid.

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