TWO DNA LIGASE ACTIVITIES FROM CALF THYMUS

S. Söderhäll and T. Lindahl

Department of Chemistry
Karolinska Institutet
S-104 01 Stockholm 60, Sweden

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SUMMARY. Cell extracts from calf thymus contain two DNA ligase activities, separable by hydroxyapatite chromatography and by gel filtration. Their molecular weights, as estimated from sedimentation coefficients and Stokes radii, are M = 175,000 and M = 85,000, respectively. The two activities both require Mg^{++} and ATP as cofactors, and convert nicked circular DNA molecules to a covalently closed form. The larger of the two ligase activities is more heat-stable than the smaller one, and is also active over a broader pH range.

Extracts from mammalian cells contain ATP-dependent DNA ligase activity (1). We have recently purified a DNA ligase from calf thymus and shown that it acts by the same mechanism as the microbial DNA ligases (2). In the course of enzyme purification, a second, minor peak of ligase activity was regularly observed on hydroxyapatite chromatography. The present report deals with this activity, DNA ligase II, which has clearly different properties from the previously described enzyme (DNA ligase I).

A hydroxyapatite chromatography experiment with an enzyme fraction from calf thymus, 50-fold purified with respect to DNA ligase activity, is shown in Fig. 1. Approximately 85% of the ligase activity eluted early from the column, at 0.09 M phosphate, and this material (DNA ligase I) is identical with the enzyme previously studied (2). A second peak of activity, DNA ligase II, appeared at a salt concentration of 0.28 M phosphate. The two peaks of activity were concentrated separately by precipitation with ammonium sulphate (80% saturation), and further purified by gel filtration on Sephadex G-150 in 0.3 M KCl, 0.05 M Tris-HCl, 10^{-3} M EDTA, and 10^{-3} M dithiothreitol, pH 7.4. A single,

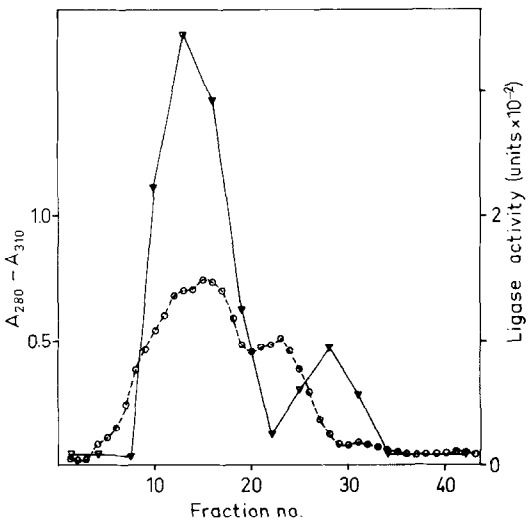
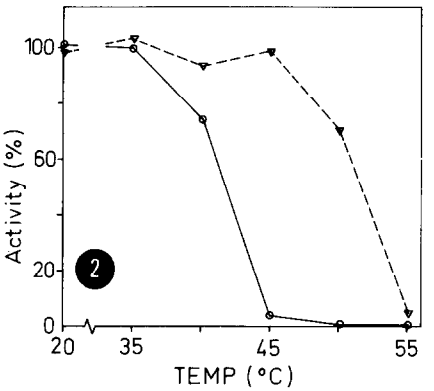


Figure 1. Fractionation of calf thymus DNA ligases by hydroxyapatite chromatography. Calf thymus glands (1 600 g) were homogenized in a Waring Blendor with 5 volumes of 0.1 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH 7.4, and cellular debris was removed by centrifugation. Ligase activity was purified from this cell extract by ammonium sulphate fractionation and phosphocellulose chromatography (1). Active fractions were pooled (570 ml, 1 300 mg protein, 1 500 ligase units (13)), dialyzed against 0.015 M potassium phosphate, pH 7.2, for 12 hours, and applied to a column of hydroxyapatite (4x17 cm). After washing the column with 600 ml of the same buffer, a linear gradient between this buffer and 0.5 M potassium phosphate, pH 7.2, was applied, and 100 ml fractions were collected. No ligase activity was detected in the unadsorbed protein fraction, or could be eluted with 1 M potassium phosphate, pH 7.2, at the end of the experiment. All solvents contained 0.01 M 2-merceptoethanol, and all operations performed at 4°. DNA ligase activity was assayed by the method of Weiss et al. (13). The reaction mixture contained 0.07 M Tris-HCl, pH 7.6, 0.01 M $MgCl_2$, 0.005 M K_2HPO_4, 0.001 M dithiothreitol, 0.0005 M ATP, 3 µg 5'-^{32}P-phosphoryl DNA, and enzyme. Incubations were at 20° for 75 minutes. o---o = protein; ▽——▽ = ligase activity.

symmetrical peak of DNA ligase activity was obtained in each experiment, but the two activities eluted at different K_{av} (3) values. Thus, no detectable interconversion between the two activities could be induced by storage in solution for 48 hours at 4°, or by precipitation with ammonium sulphate. An enzyme-adenylate complex, obtained by incubation of DNA ligase I with [^{14}C] ATP (2), had the same K_{av} value as uncharged ligase I. The column was calibrated with several reference proteins in a separate experiment (4). The K_{av} values and Stokes radii



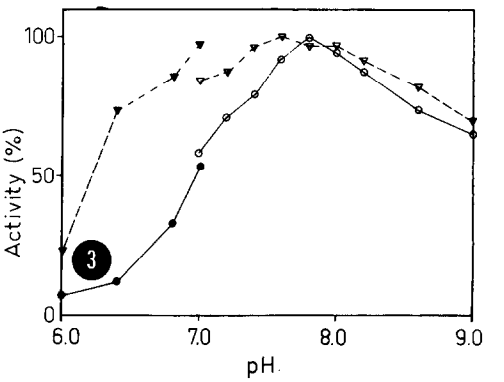


Figure 2. Heat inactivation of thymus DNA ligase activities. Aliquots of solutions of ligase I and ligase II (0.8 mg/ml protein, 2.3 ligase units/ml) in 0.3 M KCl, 0.05 M Tris-HCl, pH 7.4 (at 20°), 0.001 M dithiothreitol, 0.001 M EDTA were heated at the temperatures indicated for 5 minutes. After heating, the solutions were rapidly chilled to 0°, left for 30 minutes, centrifuged to remove precipitated protein, and assayed for DNA ligase activity. ▽---▽ = ligase I activity; o——o = ligase II activity.

Figure 3. pH dependence of thymus DNA ligase activities. Enzyme activity was measured as described in Fig. 1, except that the Tris-HCl buffer was of the pH indicated in the figure. Below pH 7.0, this buffer was replaced with 0.05 M 2-(N-morpholino)ethanesulfonic acid - KOH buffer (filled symbols). ▽---▽ = ligase I activity; o——o = ligase II activity.

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were K_{av} = 0.05, r = 52 Å, for DNA ligase I, and K_{av} = 0.23, r = 44 Å, for DNA ligase II. In the experiments below, a DNA ligase II fraction freed from DNA ligase I by hydroxyapatite chromatography and gel filtration was employed.

The sedimentation coefficients of the two ligase activities were determined by sucrose gradient centrifugation in the presence of catalase, alcohol dehydrogenase, and carbonic anhydrase as references (5), and were S = 8.0 S for DNA ligase I, and S = 4.6 S for DNA ligase II. Together with the Stokes radii, these data give the approximate molecular weights of M = 175,000 for ligase I, and M = 85,000 for ligase II by the Svedberg equation (4). The frictional ratios are f/f_o = 1.4 for ligase I and f/f_o = 1.5 for ligase II, which indicates that both proteins have an asymmetric shape.

Both ligase activities required Mg^{++} and ATP as cofactors. Little or no activity (less than 3% of normal activity) was observed in the standard assay in the absence of either of these compounds. Traces of activity sometimes observed when ATP was excluded could be due to the presence of preformed enzyme-adenylate complexes (2). Neither of the activities could use NAD^+ (10^{-6} - 10^{-3} M) as a cofactor instead of ATP.

The two ligase activities showed a large difference in their sensitivity to heat (Fig. 3). DNA ligase I was stable to prolonged heating at temperatures below 50°, and was inactivated by first order kinetics at 52°, with 50% inactivation occurring in 5 minutes under the conditions given in Fig. 2. In contrast, the DNA ligase II activity was rapidly lost above 40° (Fig. 2). At 42°, inactivation by first order kinetics was observed, with 50% loss of activity after 5 minutes. When an artificial mixture containing equal amounts of the two purified ligase activities was incubated at 45°, 50% of the activity was lost in less than 5 minutes, while the rest of the activity remained practically unchanged for more than 15 minutes. DNA ligase II was more labile than ligase I also with regard to prolonged storage at 2°. When ligase solutions containing 1.5 mg/ml protein were stored in the buffer used for the gel filtration experiments, 50% inactivation was observed after 15-30 days for ligase I and after 2-3 days for ligase II in several different experiments.

The two enzyme activities showed similar but not identical pH dependence (Fig. 3). DNA ligase I has a broad pH optimum between pH 7.4 and 8.0 in Tris-HCl buffer, while ligase II is maximally active at pH 7.8 and less active below pH 7.6. Thus, at pH 6.5-7.0, DNA ligase I is considerably more effective than ligase II. Further, at pH 7.0, ligase I was more active in 2-(N-morpholino)ethanesulfonic acid - KOH buffer than in Tris-HCl buffer, while the reverse was observed for

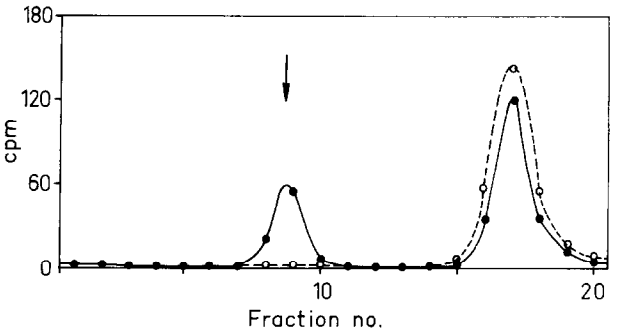

Figure 4. Conversion of hydrogen bonded DNA circles to covalently closed molecules. Nicked PM2[^{32}P]DNA (35 ng) was incubated with 5 µg (0.013 units) DNA ligase II (●——●), or with the same amount of enzyme solution preheated at 47° for 5 minutes (o----o), at 20° for 10 minutes in the reaction mixture given in Fig. 1, supplemented with 0.1 M NaCl. The reaction was stopped by the addition of EDTA to 0.05 M and one volume of water-saturated phenol. After mixing, the aqeous phase was recovered, dialyzed against 0.4 M NaCl, 0.02 M Tris-HCl, 0.01 M EDTA, pH 8.0, and analyzed for the presence of covalently closed DNA circles by alkaline sucrose gradient centrifugation as previously described (6). The arrow in the figure indicates the position of covalently closed circular PM2 DNA, as determined in a separate experiment.

ligase II (Fig. 3). No difference was noted between the activities in their response to the presence of neutral salts in the standard ligase reaction mixture; with 0.1 M and 0.2 M NaCl added, approximately 50% *vs.* 85% inhibition was observed for both.

The identity of the newly detected ligase II activity with a DNA ligase was confirmed by demonstrating the conversion of circular DNA molecules containing single-strand breaks to covalently closed DNA circles by this enzyme. PM2 [^{32}P] DNA (6) was treated with pancreatic DNase to introduce approximately one single-strand break per molecule. The nicked circles were purified by neutral sucrose gradient centrifugation (6), freed from sucrose by dialysis, and incubated with ligase Both ligase I and ligase II could convert DNA circles containing nicks to covalently closed circles, as judged from alkaline sucrose gradient centrifugation experiments. The data obtained with DNA ligase II are shown in Fig. 4. The activity of ligase II in this reaction was destroy

ed by heating at 47° for 5 minutes (Fig. 4), as anticipated from the data in Fig. 2, while ligase I was refractory to such heat treatment.

DISCUSSION

Three possible interpretations of these data may be proposed: a) two different DNA ligases are present in calf thymus, b) ligase II is an active subunit of ligase I, c) the smaller ligase II is an active fragment of ligase I produced by proteolytic cleavage. Further work on the properties, and the subcellular and cellular distribution of these activities should serve to further clarify this point. In view of the large difference in heat stability between the activities, it seems unlikely to us that the smaller, but more heat-labile ligase II could be a monomer or other type of subunit of ligase I. The relative proportions of activities I and II have been the same in several ligase preparations from different batches of thymus, and no interconversion between the two forms has been observed. However, the possibility of a conversion of ligase I to an active fragment, "ligase II", by proteolytic degradation cannot be ruled out at present.

The relation of these two DNA ligase activities from calf thymus to DNA ligases purified from other sources of mammalian cells (1,7,8) remains to be established. The DNA ligase I described here is the enzyme we have used in reaction mechanism studies (2), and also appears to be the same DNA ligase as that purified by Bertazzoni et al. from calf thymus (9). As DNA ligase II requires both Mg^{++} and ATP as cofactors, it is probably not identical with the mammalian enzyme activity studied by Champoux and Dulbecco that untwists superhelical DNA (10).

At least four different DNA polymerizing activities are present in thymus cell extracts, and these enzymes have rather different properties from the three bacterial DNA polymerases (11). Two of these

thymus enzymes are immunologcally related, but nevertheless show different subcellular distribution and response to induction by cell proliferation (12). In view of these results, it would not be surprising if a more complex situation is present in thymus cells than in bacteria also with regard to DNA ligases.

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