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Two distinct DNA ligases from *Drosophila melanogaster* embryos

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Embryos of *Drosophila melanogaster* contain two distinct DNA ligases (DNA ligase I and II). DNA ligase I was eluted at 0.2 M KCl and DNA ligase II at 0.6 M KCl on phosphocellulose column chromatography. The former was rich in early developing embryos and its activity decreased during embryonic development. The latter was found constantly throughout the developing stages of embryos. DNA ligase I existed in a cytoplasmic fraction and DNA ligase II is concentrated in nuclei. Both enzymes ligate 5′-phosphoryl and 3′-hydroxyl groups in oligo(dT) in the presence of poly(dA). DNA ligase II is also able to join oligo(dT)(poly(rA). Both enzymes require ATP and Mg^{2+} for activity. The K_m for ATP is 2.7×10^{-6} M for DNA ligase I, and 3.0×10^{-5} M for DNA ligase II. DNA ligase I requires dithiothreitol and polyvinyl alcohol, but DNA ligase II does not. Both enzymes are inhibited in the presence of *N*-ethylmaleimide. DNA ligase I is active at a low salt concentration (0–30 mM KCl), but DNA ligase II is active at high salt concentrations (50–100 mM). DNA ligase I is more labile than DNA ligase II. The molecular masses of DNA ligase-AMP adducts were determined as 86 and 75 kDa for DNA ligase I, and as 70 (major protein) and 90 kDa (minor protein) for DNA ligase II under denaturing conditions. A sedimentation coefficient of 4.2 S was observed for DNA ligase II. Consequently, *Drosophila* DNA ligase I and II are quite similar to mammalian DNA ligase I and II. *Drosophila* DNA ligase I and a DNA ligase by B.A. Rabin et al. [(1986) J. Biol. Chem. 261, 10637–10645] seem to be the same enzyme.

DNA ligase I; DNA ligase II; Embryonic development; (*Drosophila melanogaster*)

1. INTRODUCTION

DNA ligases (EC 6.5.1.) which catalyze the formation of phosphodiester linkages between DNA chains are required for replication, repair and recombination. The prokaryotic ligases have been characterized in detail and considered as a single species [1–3]. In contrast, it is believed that mammalian cells contain at least two antigenically distinct ligases [1,2,4–9]. The major ligase activity in proliferating cells is due to a high molecular mass protein termed DNA ligase I and the enzyme is considered to play an important role in DNA replication. Whereas, DNA ligase II is the principal activity in resting cells and the molecular mass is smaller, but the role is not clear. These ligases are also distinguishable in a number of properties such as their affinity for ATP [1,4,10], substrate specificity for oligo(dT)-poly(rA) or blunt-end joining of DNA [1,4,11], and the effect of ionic strength [4,9]. In other eukaryotes, such as chicken, sea urchin and amphibia, it is also known that there are more than one DNA ligase present [12–14].

We are interested in the biochemical role of two DNA ligases and whether these are common in most eukaryotes. Is it true that DNA ligase I is

Abbreviations: DTT, dithiothreitol; PVA, polyvinyl alcohol

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mainly involved in DNA replication and DNA ligase II in DNA repair? *Drosophila melanogaster* was chosen for this purpose, because this organism is well characterized biochemically and genetically. This paper describes that *Drosophila melanogaster* embryos contain two distinct DNA ligases, referred to as DNA ligase I and II. DNA ligase I, which was detected at 0.2 M KCl elution on phosphocellulose column chromatography, was the major activity found in the early development of embryos and decreased during embryonic development. DNA ligase II, which eluted at 0.6 M KCl, was found throughout the developmental stages. The properties of the two enzymes were characterized and compared with those of mammalian DNA ligase I and II. Recently, a DNA ligase from *Drosophila melanogaster* embryos has been purified about 2100-fold [15]. This enzyme is considered to be the same as the DNA ligase I which is described here.

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from Amersham; $(dT)_{10}$ was from P-L Biochemicals; poly(dA), poly(rA) DTT, leupeptin, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), cytochrome *c* and alkaline phosphatase were from Sigma; ATP, polynucleotide kinase, lactate dehydrogenase, pyruvate kinase and T_4 DNA ligase were from Boehringer Mannheim GmBH; egg albumin was from Seikagaku Kogyo. P11 and glass filter G/F were obtained from Whatman Ltd. Molecular mass protein standards for gel electrophoresis were purchased from Bio-Rad. All other chemicals were reagent grade.

2.2. Drosophila *embryos*

D. melanogaster (Oregon R) embryos (0–4 h, 0–8 h, 0–16 h post-fertilization) were collected, washed and dechorionated and rapidly frozen in liquid N_2 and stored at -70°C.

2.3. *Assay for DNA ligase*

Preparation of 5′-^{32}P-labeled $(dT)_{10}$ and the assay method were as described [3].

The reaction mixture (40 μl) contained 50 mM Tris-HCl, pH 7.6, 12 mM $MgCl_2$, 6.6 mM DTT, 0.2 mM ATP, 8 μg BSA, 25 mM KCl, 10% PVA, 400 pmol poly(dA) and 400 pmol [5′-^{32}P]$(dT)_{10}$ (concentration in total phosphate) and the enzyme solution. After incubation at 27°C for 2 h, the reaction was terminated by heating at 100°C for 2 min. To the reaction mixture 5 μl of alkaline phosphatase (0.18 units) were added and the mixture was incubated at 65°C for 40 min. After the addition of 50 μl of carrier DNA (2 mg/ml), the mixture was spotted on a Whatman GF/F glass fiber disk (2.4 cm diameter) and the disk was washed twice in cold 5% CCl_3COOH containing 1% $NaPP_i$ for 20 min, successively in cold 5% CCl_3COOH for 20 min twice, in cold ethanol and dried in ether. The radioactivity of the acid-insoluble materials on the disk was counted in a toluene scintillator. One unit of the enzyme is defined as being equivalent to 1 nmol of $^{32}P_i$ resistant to alkaline phosphatase under standard assay conditions. One unit of A_{280} is the quantity of protein which has an absorbance of 1.0 when dissolved in 1 ml of buffer and measured in a 1 cm light path at 280 nm.

2.4. *Formation and detection of DNA ligase-AMP adducts*

Covalent ^{32}P-labelled ligase-AMP adducts were prepared according to the method of Banks and Barker [16]. The labeled proteins were charged on electrophoresis through a 3.5% stacking/10% separating gel containing SDS. The gel was stained with Coomassie brilliant blue and destained in 7% acetic acid. After dehydration, the gel was autoradiographed on a Kodak-Omat RPI 6 film at -80°C.

2.5. *8 M urea-polyacrylamide gel electrophoresis*

The reaction products of [5′-^{32}P]$(dT)_{10}$ with poly(dA) were analyzed by electrophoresis in Tris-borate-EDTA, pH 8.3, on 20% polyacrylamide gel containing 8 M urea [17]. The samples containing 8 M urea, 0.01% of xylene cyanol and bromophenol blue were loaded on a vertical slab gel (14 × 16 × 0.15 cm) at 400 V for 3 h. After the electrophoresis, the gel was autoradiographed.

2.6. *Determination of sedimentation coefficient*

Samples (0.13 ml) were layered onto preformed, linear 15–30% glycerol gradients containing 50 mM Na_2HPO_4-KH_2PO_4, pH 7.7, 200 mM

$(NH_4)_2SO_4$, 1 mM 2-mercaptoethanol, 1 mM EDTA and protease inhibitors. The gradients were centrifuged in a Beckman SW 50.1 rotor at 45 000 rpm for 20 h at 4°C. Five drop fractions were collected from the bottom of tubes and 5 μl aliquots were assayed for DNA ligase activity. BSA was included as internal standard, and pyruvate kinase, lactate dehydrogenase, BSA, egg albumin and cytochrome *c* were run in parallel tubes as standards.

2.7. *Protease inhibitors*

The following freshly prepared protease inhibitors were added to buffers immediately before use: 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 μg/ml leupeptin.

3. RESULTS AND DISCUSSION

All operations were performed at 4°C unless otherwise indicated.

3.1. *Preparation of the whole extract*

Frozen embryos (20 g) were mixed with 50 ml of 30 mM Tris-HCl, pH 7.9, containing 10 mM EDTA, 1 M NaCl and protease inhibitors (buffer A) and 80 g of glass beads (0.1 mm diameter), homogenized with a Waring blender for 2 min/1 min rest/2 min. The mixture was centrifuged at 9000 rpm for 20 min to get the supernatant. The precipitate was washed with the smallest vol. of buffer A three times and the centrifugation was repeated as above. The first and washed supernatants were combined as a whole extract (109 ml).

3.2. *Ammonium sulfate fractionation*

Solid $(NH_4)_2SO_4$ was added to a whole extract of embryos to 70% saturation. The resulting precipitate was collected by centrifugation at 9000 rpm for 15 min and dissolved in 15 mM Na_2HPO_4-KH_2PO_4, pH 7.7, containing 0.1 M KCl, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol and protease inhibitors (buffer B). The solution was dialyzed against buffer B overnight with two changes.

3.3. *Phosphocellulose (P11) column chromatography*

The dialyzed solution (30 ml) was centrifuged at 13 000 rpm for 30 min and the supernatant was put on a phosphocellulose column (2.6 × 18 cm)

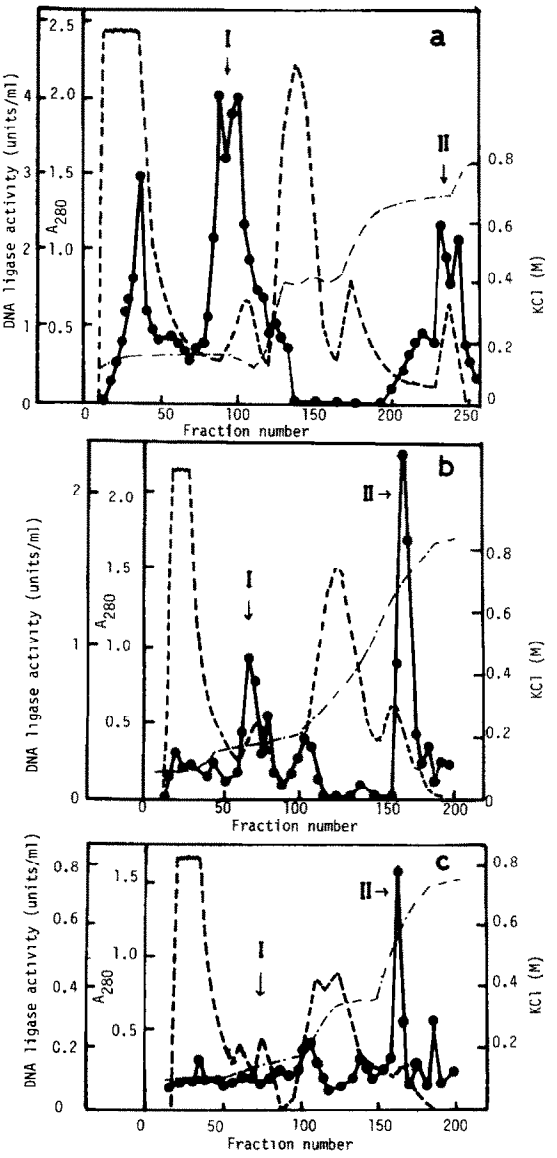

Fig.1. Phosphocellulose column chromatography of the whole extract of *Drosophila* embryos at various developmental stages. (a) 0–4 h embryos; (b) 0–8 h embryos; (c) 0–16 h embryos. The dialyzed enzyme solution extracted from 10 g embryos (a), of which 34 ml was put on a phosphocellulose column (2.6 × 9 cm) equilibrated with buffer B. The column was washed with the same buffer (200 ml), eluted with a linear gradient of 0.1–0.8 M KCl in the same buffer (2 × 130 ml), and washed again with 75 ml of 0.8 M KCl in the same buffer. Fractions of 3 ml each were collected. As (b) and (c) were started from 20 g of embryos, the column scale was 2-fold as described in section 3. 6 ml fractions were collected. (●) DNA ligase activity; (– – –) absorbance at 280 nm; (---) KCl concentration.

equilibrated with buffer B. After washing with 300 ml of the same buffer, the column was eluted with a linear gradient of 0.1–0.8 M KCl in the same buffer (2 × 250 ml), and washed again with 150 ml of 0.8 M KCl in the same buffer. Fig.1 shows elution patterns of P11 column chromatography at various developmental stages of the embryos. DNA ligase activity was detected at 0.2 M, 0.25 M and around 0.6 M KCl, respectively. The enzyme activity at 0.2 M KCl (DNA ligase I) was high in early embryos (0–4 h), but the activity decreased in 8 h embryos and could not be detected in 16 h embryos. In contrast, the 0.6 M KCl eluting activity (DNA ligase II) was found constantly. Although 0.25 M KCl eluting activity was always detected, it was not possible to identify it as a DNA ligase from its characterization.

3.4. *Subcellular distribution of DNA ligase activities*

The subcellular distribution of these activities was investigated in aqueous sucrose solutions. Nuclei from embryos were fractionated essentially according to Fisher et al. [18].

Frozen embryos (50 g) were suspended in 10 vol. of extraction buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 250 mM sucrose and protease inhibitors. The suspended embryos were homogenized with a glass-teflon homogenizer (4 strokes, tight pestle) and filtered through two layers of 120 μm nylon mesh. The filtered crude homogenate was centrifuged at 1000 × g for 10 min. The post nuclear supernatant was removed for cytoplasmic preparation. The crude nuclear pellet was resuspended by gentle vortexing in 5 vol. of extraction buffer. Centrifugation was repeated as above, the supernatant was removed (first wash), and the nuclei were again suspended in 5 vol. of extraction buffer. The nuclear suspension was centrifuged at 1000 × g for 10 min and the supernatant was removed (second wash). Post nuclear supernatant, first wash and second wash supernatants were combined as a cytoplasmic fraction. The purified nuclear pellet was suspended in 15 ml of buffer A and 40 g of glass beads, and homogenized in a Waring blender. The following procedures were the same as for the preparation of the whole extract. The supernatant and the washed solution were combined as a nuclear extract.

After the procedure of $(NH_4)_2SO_4$ precipitation as described for the whole extract, phosphocellulose column chromatography was carried out on the nuclear extract and the cytoplasmic fraction, respectively. P11 column chromatography of the nuclear extract of 16 h embryos showed only 0.6 M KCl eluting activity as a single peak (fig.2). While, 0.2 M KCl eluting activity was detected in a cytoplasmic fraction (not shown). This chromatographic difference and subcellular distribution of DNA ligase I and II from *Drosophila* embryos are similar to mammalian DNA ligase I and II [1,4].

3.5. *Preparation of DNA ligase I and II from* Drosophila *embryos*

To investigate further their properties, both enzymes were separated on P11 column chromatography and concentrated with a YM10 (Amicon) Diaflo ultrafilter. DNA ligase I was found in cytoplasmic fractions, but the best source for preparation of this enzyme was a whole extract from 4 h embryos. The front fraction of 0.2 M KCl eluting activity was usually used as DNA ligase I

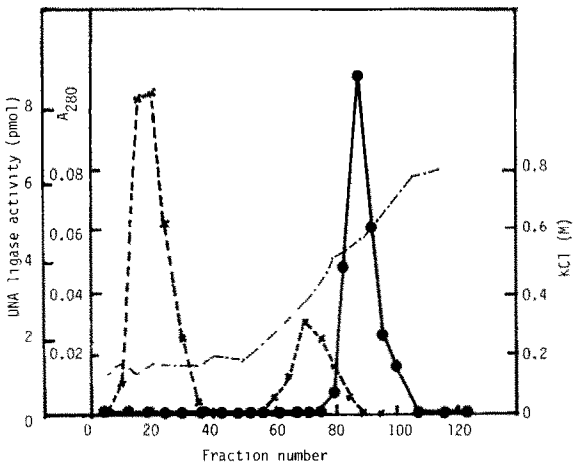

Fig.2. Phosphocellulose column chromatography of the nuclear extract of Drosophila embryos. The dialyzed enzyme solution extracted from nuclei of 0–16 h embryos (20 g), of which 8.5 ml was put on a phosphocellulose column (1.2 × 4.5 cm) equilibrated with buffer B. The column was washed with the same buffer (20 ml), eluted with a linear gradient of 0.1–0.8 M KCl in the same buffer (2 × 25 ml), and washed again with 0.8 M KCl in the same buffer (20 ml). Fractions of 1 ml each were collected. (●) DNA ligase activity; (×) absorbance at 280 nm; (---) KCl concentration.

(table 1a). Although DNA ligase II was detected throughout the embryonic developmental stages, the activity was concentrated in isolated nuclei from 16 h embryos. The concentrated 0.6 M KCl eluting fraction was used as DNA ligase II (table 1b). DNA ligase II was purified about 41-fold. Increase of the activity indicated that most of the nuclease and phosphatase were removed by phosphocellulose column chromatography.

3.6. *Catalytic properties*

As shown in table 2, both DNA ligase I and II require ATP as a cofactor, Mg^{2+} as a divalent cation, and poly(dA) as a complementary strand for oligo(dT). A reducing agent, DTT was necessary for optimal activity of DNA ligase I, but not for DNA ligase II. Treatment with *N*-ethylmaleimide caused complete inhibition of both activities. DNA ligase I was more active in the presence of 10% PVA in the reaction mixtures, but DNA ligase II was inhibited. It is known that nonspecific polymers act as volume excluders [19] and such a crowded solution may well be a more adequate model for intracellular conditions than assay in dilute solutions [20]. Since DNA ligase I was unstable and lost gradually the activity in the

Table 1

Preparation of DNA ligase I and II from *Drosophila* embryos

rocedure	Vol. (ml)	Protein (A_{280}/ml)	Activity (units/ml)	S.A. (units/A_{280})	Yield (%)
a. DNA ligase I from 30 g of 4 h embryos					
(1) Whole extract	240	25.2	–	–	–
(2) $(NH_4)_2SO_4$ (<70%)	105	30.2	–	–	–
(3) Phosphocellulose (0.2 M KCl)					
YM 10 Conc. Front	2.4	19.0	18.65	0.98	–
Back	4.0	21.0	23.65	1.13	–
b. DNA ligase II from 50 g of 16 h embryos					
(1) Nuclear extract	70.0	12.74	–	–	–
(2) $(NH_4)_2SO_4$ (<70%)	28.5	0.58	0.41	0.70 (1)	100
(3) Phosphocellulose (0.6 M KCl)	19.0	0.066	1.48	22.4 (32)	242
(4) Phosphocellulose YM 10 Conc.	2.0	0.53	15.28	28.8 (41)	261

Table 2

Requirements for DNA ligation

Components	DNA ligase I		DNA ligase II	
	Activity (pmol)	Relative activity (%)	Activity (pmol)	Relative activity (%)
Complete	2.89	100	3.45	100
-PVA	1.99	68.9	9.95	288
-DTT	2.37	82.0	8.13	236
-ATP	0.08	2.8	0.05	1.4
-$MgCl_2$	0	0	0	0
-poly(dA)	0.28	9.7	0	0
-enzyme	0	0	0	0
+ *N*-ethylmaleimide (2.5 mM)	0	0	0.09	2.6

storage at 4°C, the presence of PVA seemed to have an effect on stabilizing the reaction conditions. The optimum ligation temperature was at 25°C in the absence of PVA and at 30°C in the presence of PVA for both enzymes. The results indicate that polymer in the reaction mixture increases ligation temperature as known in many DNA ligases [21–23]. The thermostability indicated that DNA ligase I was more labile than DNA ligase II, the former lost 50% of the activity at 32°C after 1 h, but the latter kept the same stability at 37.5°C. This point was different from mammalian DNA ligases, because mammalian DNA ligase II was more heat-labile than DNA ligase I from the same sources.

The affinity for ATP was high for DNA ligase I and low for DNA ligase II, the apparent K_m for ATP was 2.7×10^{-6} M and 3.0×10^{-5} M, respectively. The K_m of DNA ligase II is a 10-times lower value than that of DNA ligase I. The results are quite similar to the K_m of mammalian DNA ligase I and II [1,4].

Effects of monovalent cations are shown in fig.3. Optimum concentrations of KCl presented a striking contrast between two enzymes. DNA ligase I showed high activity at a lower salt concentration (below 30 mM) and DNA ligase II requires a high salt concentration (above 50 mM). In the presence of 25 mM KCl, addition of Na^+ or NH_4^+ showed a similar tendency, i.e. the activity of DNA ligase I was inhibited by further addition of monovalent cations, but that of DNA ligase II was stimulated by Na^+. Ammonium ion has little effect on the stimulation of DNA ligase II.

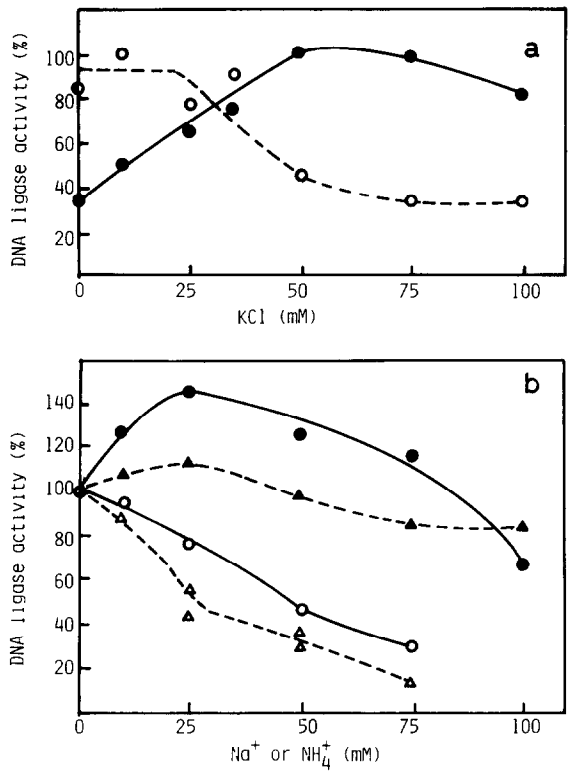

Fig.3. Effects of monovalent cations on *Drosophila* DNA ligase I and II. (a) Effect of KCl concentrations. The activity of DNA ligase I (○) or DNA ligase II (●) was assayed at various concentrations of KCl. (b) Effect of Na^+ or NH_4^+ in the presence of 25 mM KCl. The activity of DNA ligase I (○, △) or DNA ligase II (●, ▲) was assayed at various concentrations of NaCl (○,●) or NH_4Cl (△, ▲) under the conditions described in section 2.

3.7. *Substrate specificity*

Both enzymes ligate 5′-phosphoryl and 3′-hydroxyl group in oligo(dT) in the presence of a complementary strand poly(dA). DNA ligase II also was able to join oligo(dT) chains hydrogen-bonded to poly(rA) at about 5–10% of the rate of joining poly(dA)-oligo(dT) (not shown). This property is the same as for mammalian DNA ligase II [1,4,11]. The time course of the ligation and the products by DNA ligase II are shown in fig.4. The contamination from nucleases seemed to reduce the extent of joining after 30 min.

3.8. *Molecular masses of AMP-adducts*

The molecular masses of DNA ligase I and II were determined as ^{32}P-labeled ligase AMP-adducts on the SDS-polyacrylamide gel electrophoresis. This method is useful in estimating the size directly even in crude extracts [16]. Partially purified DNA ligase I showed two radioactively labeled proteins of M_r 86 000 and 75 000 (fig.5). These values are similar to the results of Rabin et al. [15] of M_r 83 000 and 75 000. DNA ligase II also showed two radioactive proteins, the major band has an M_r of 70 000 and the minor band an M_r of 90 000. Although $(NH_4)_2SO_4$ fractions showed other radioactive proteins with higher M_r values, it is not certain whether there is another high molecular DNA ligase. However, the protein of M_r 75 000 could not be detected in fresh preparation of a $(NH_4)_2SO_4$ fraction of DNA ligase I. This

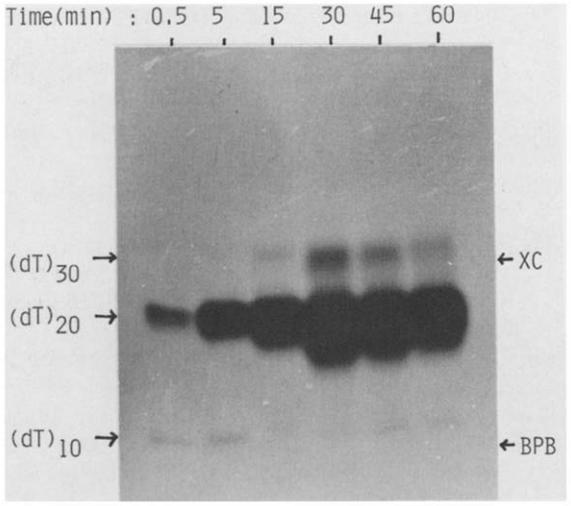

Fig.4. Time course of ligation products of ^{32}P-(dT)^{10}-poly(dA) by DNA ligase II. The reaction products (at 27°C, without PVA and DTT) were analyzed on a 20% polyacrylamide gel containing 8 M urea and autoradiographed.

result suggests that this protein arises from proteolytic cleavage (not shown). It has been reported that the 83 kDa protein of a *Drosophila* DNA ligase can be converted to the 75 and 64 kDa polypeptides by proteolytic cleavage [15]. Mammalian DNA ligase I is considered to be a large enzyme. On prolonged incubation of the crude extract or during enzyme purification, new active forms of the enzyme appear [4,24]. In the case of *Drosophila*, it must be further investigated if the protein of molecular mass 83 or 86 kDa is the original form of DNA ligase I or any larger form can exist. It is known that a protein adenylated with ATP moves slower than the unadenylated protein on a SDS-polyacrylamide gel [25]. As a control experiment, T_4 DNA ligase with a molecular mass of 55 kDa gave 62 kDa in the adenylated form (not shown).

3.9. *Sedimentation coefficient of DNA ligase II*

The sedimentation coefficient of DNA ligase II was determined using 15–30% glycerol gradients.

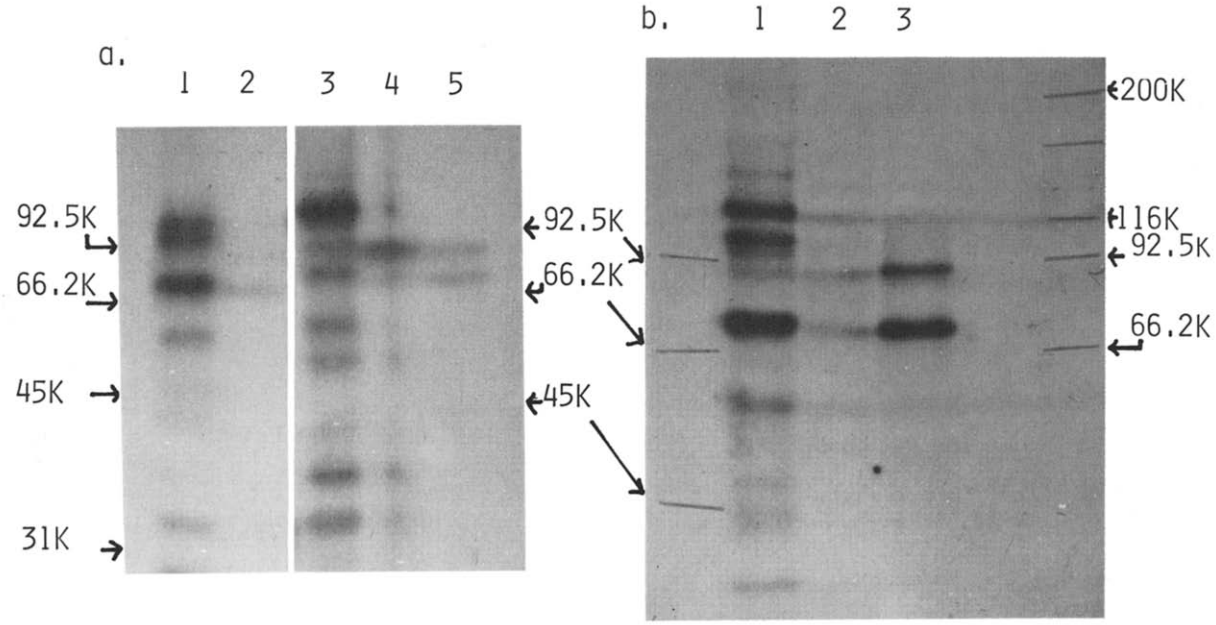

Fig.5. SDS-polyacrylamide gel electrophoresis of DNA ligase-[^{32}P]ATP adducts. A 10% SDS-polyacrylamide gel was prepared and treated as described in section 2. (a) The following enzyme solutions were incubated with [α-^{32}P]ATP on ice. Lanes: 1, $(NH_4)_2SO_4$ fraction of DNA ligase II (0.016 units); 2, DNA ligase II (0.61 units); 3, $(NH_4)_2SO_4$ fraction of DNA ligase I (40 μl); 4, DNA ligase I front (0.74 units); 5, DNA ligase I back (0.94 units). (b) The following enzyme solutions were incubated with [α-^{32}P]ATP on ice (lanes 1, 2) or at 27°C (lane 3). Lanes: 1, $(NH_4)_2SO_4$ fraction of DNA ligase II (0.024 units); 2, DNA ligase II (2.29 units); 3, DNA ligase II (1.14 units). Arrows indicate the migration positions of molecular mass markers, myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase B (92.5 kDa), BSA (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa).

The result gave a single peak of a value of 4.2 S (not shown).

A preliminary result from gel filtration with Sepharose 6B showed two active peaks for DNA ligase II with molecular masses of about 140 and 64 kDa. Mammalian DNA ligase II is considered to be a smaller enzyme [1,4,7,9]. Our results showed that the major DNA ligase II fraction has a smaller molecular mass. However, the larger form of DNA ligase II was also detected in part. It is not clear yet whether this form is the product of aggregation or one of the original forms.

In conclusion, the two distinct DNA ligases I and II in *D. melanogaster* embryos are quite similar to mammalian DNA ligases I and II. The results suggest that there are also two enzymes in lower eukaryotes and they may have different roles in DNA metabolism. Further purification of both enzymes, especially DNA ligase II is in progress to solve immunological relations between these two enzymes and their roles in DNA metabolism.

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