Drosophila topoisomerase I: isolation, purification and characterization

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ABSTRACT

We have purified and characterized topoisomerase I from *Drosophila melanogaster*. The molecular weight of the enzyme is 135,000; 100,000, 90,000, and 65,000 molecular weight products result from degradation of the enzyme. The enzyme relaxes both positive and negative supercoiled DNA. Mg^{++} is not absolutely required, but stimulates the enzymatic activity considerably.

INTRODUCTION

Topoisomerases are the enzymes which convert one circular DNA isomer into another. Two types of topoisomerase are known, topoisomerase I and II (1,2). Topoisomerase I was first isolated from *E. coli* and subsequently from mammalian cells (for review see ref. 3). The enzyme catalyzes a number of reactions: relaxation of supercoiled DNA (4,5), knotting and unknotting of single-stranded circular DNA (6) and formation of a double-stranded circle by two single-stranded complementary DNA rings (7,8).

Eukaryotic topoisomerase I has been reported to have a molecular weight of 60,000-70,000 in several cases (9,10,11). Recently it has been found that topoisomerase I from Hela and wheat germs have molecular weights in 100,000 range (12,13). Following our initial report on purification of topoisomerase I from *Drosophila* (I), we now describe purification of topoisomerase I from *Drosophila melanogaster*. The molecular weight of the enzyme is 135,000. Degradation of the enzyme results in a series of bands on a SDS polyacrylamide gel with molecular weights of 100,000, 90,000, and 65,000 as the main products.

MATERIALS AND METHODS

Topoisomerase activity was assayed by measurement of relaxation of superhelical pBR322 DNA (prepared according to reference [14]). The assay mixture (50 μ l) included 50mM Tris-HCl, pH 7.5, 80mM NaCl, 10mM $MgCl_2$, 0.2mM EDTA, 0.5mM dithiotreitol, 50 μ g/ml of bovine serum albumin, 10 μ g/ml of DNA and

various amounts of enzyme. After incubation at 30°C for 1/2 hr, the reaction was stopped by adding 15 µl of 5% sodium dodecyl sulfate (SDS), 25% glycerol, and 0.25 mg/ml bromophenol blue and the mixture was loaded on 0.7% agarose gel. The gel was electrophoresed at room temperature. The buffer for electrophoresis was TBE (90 mM Tris-Borate, 3 mM NaEDTA, pH 8.3). Staining with ethidium bromide (0.5 µg/ml) was carried out for 30 minutes followed by visualization using short wave UV. The gel was photographed using Polaroid film Type 55 or 57. One unit of enzyme activity is defined as the amount of enzyme which relaxes 0.5 µg of pBR322 DNA under the above assay conditions.

Protein samples were analyzed using SDS polyacrylamide gels as described by Laemmli (15). Silver staining of the gels was performed in some cases (24). Protein concentration was determined using a Biorad protein assay. Isoelectric focusing was performed on 1% agarose gels (reference (16) and FMC, Marine Colloids Division literature). Staining was carried out with Coomassie Blue in 10% methanol, 10% acetic acid for 10-15 minutes and destaining in 0.3% acetic acid.

Phage PM2 DNA was prepared following the published procedure (17). [α -³²P]dATP with a specific activity of 400 Ci/mmol was purchased from New England Nuclear. Nick translation on DNase I-nicked PM2 DNA was carried out using E. coli DNA polymerase in the presence of [α -³²P]dATP and the other deoxynucleotide triphosphates unlabeled (18,19).

Formation of a covalent complex between denatured ³²P-labeled PM2 DNA and Drosophila topoisomerase I was carried out as follows. 2.5 µg of PM2 DNA, ³²P-labelled by nick translation in 10 µl of 10 mM Tris.HCl, pH 8, 0.1 mM EDTA, was denatured in 50 mM NaOH. After incubating at 37°C for 10 minutes, the solution was neutralized by addition of HCl to 50 mM. Tris.HCl, pH 8, was added to a final concentration of 50 mM. The volume of the reaction mixture was adjusted to 100 µl containing 0.4 µg of topoisomerase I, 10 µg gelatin, in 10 mM Tris.HCl, pH 8. The mixture was incubated for 1 hr at 30°C. The sample was made 50 mM in NaOH and further incubated for 1 minute. After neutralization by HCl and addition of Tris.HCl, pH 8.5, to a final concentration of 50 mM, CaCl₂ was added (1.5 mM) and the sample digested overnight at 37°C with staphylococcal nuclease (Worthington), 30 units/ml. The reaction mixture was divided in two and the portions run on a SDS-polyacrylamide gel (5%).

Formation of a covalent complex between fd DNA and Drosophila topoisomerase I was carried out as follows. A reaction mixture (100 µl)

containing 4 μ g fd DNA, 1 μ g of topoisomerase I in 2 mM EDTA, pH 8.0 was incubated at 37°C for 30 minutes. After adding NaOH to a final concentration of 40 mM, incubation was continued for 1 minute. CsCl was added and sedimentation was performed in a Beckman Model E at 40500 rpm for 36 hours using 265 nm wavelength on the absorption scanner.

RESULTS

Purification of the enzyme

Two factors appear to play important roles in isolating high molecular weight topoisomerase I from *Drosophila*. One is speed of the preparation. The total time elapsed from beginning to the end of the preparation described here was approximately 28 hrs. The second factor is the presence of protease inhibitors. Two inhibitors were used in all steps of the purification beginning with cell lysis, phenyl methyl sulfonyl fluoride at 1 mM and sodium bisulfite at 10 mM.

Nuclei were prepared from 6-18 hr *Drosophila melanogaster* embryos as described by Elgin and Hood (20). 100 gms of frozed eggs were divided in two batches and dechorinated with chlorox-water (1:1). The eggs were washed extensively on a nitex filter using tap water. After washing the eggs with 70% ethanol (250 ml) the washing by tap water was continued. All subsequent operations were carried out at 4°C. The dechorinated eggs were suspended in 800 ml of homogenization buffer (0.05 M Tris.HCl, 0.025 M KCl, 0.005 M MgAcetate, 0.35 M sucrose, pH 7.5). Eggs were broken by nitrogen cavitation using 300 lb pressure for 20 minutes in a Parr stainless steel bomb. Hand homogenization was used to break any residual eggs. After passing through an unwoven cloth filter (Miracloth), the nuclei were centrifuged and suspended in 50 ml of lysis buffer (5 mM potassium phosphate, 2 mM $MgCl_2$, 0.5 mM DTT, 1 mM β -mercaptoethanol, pH 7.1). EDTA was added to a final concentration of 4 mM and the suspension stirred for five minutes. An equal volume of 45 mM potassium phosphate, 2 M NaCl, 20% glycerol, 0.5 mM DTT, 1 mM β -mercaptoethanol, pH 7.1 was added. A chromatin gel was formed which became more soluble upon strong stirring with a glass rod for 5 minutes. Dry polyethylene glycol was added to a final concentration of 6%. Magnetic stirring continued until all polyethylene glycol was dissolved. After centrifugation, two volumes of buffer (5 mM potassium phosphate, 10% glycerol, 0.5 mM DTT, 1 mM β -mercaptoethanol, 0.1 mM EDTA) were added to the supernatant and it was directly loaded on a 3.1 x 9 cm column of phosphocellulose which had been

equilibrated with buffer A (15 mM potassium phosphate, pH 7.1, 10% glycerol, 10 mM β -mercaptoethanol, 0.1 mM EDTA) plus 33 mM NaCl. After loading, the column was washed with 100 ml of Buffer A containing 0.33 M NaCl. A sharp gradient (0.5 - 1.5 M NaCl in buffer A, 250 ml of total volume) was applied and 7 ml fractions were collected. Topoisomerase I is eluted in 1 - 1.5 M NaCl (21). The most active fractions were pooled ($\sim$ 35 ml) and loaded directly on a 1 ml hydroxyapatite column which had been equilibrated with buffer A (without EDTA) plus 1 M NaCl. A stepwise elution was carried out with 4 ml aliquots of 0.2, 0.35, 0.6, and 0.8 M potassium phosphate in 10% glycerol, 10 mM β -mercaptoethanol, pH 7.1. The major peak of topoisomerase was eluted in the 0.6 M potassium phosphate fraction. After dialyzing for 3 hrs against 35 mM potassium phosphate, 10 mM β -mercaptoethanol, 50% glycerol, it was stored at -20°C . No change of activity of the enzyme or electrophoretic pattern was observed after storage at -20°C for a few months. The final specific activity of the enzyme was approximately 1×10^7 units/mg assayed under the conditions described. The final activity corresponds to 10% of the original activity present in the egg lysate. 0.3 mg of enzyme was obtained from 100 g of embryos (Table 1).

Sodium dodecyl sulfate polyacrylamide electrophoresis of the enzyme preparation is shown in Fig. 1. There is a distinct band at 135,000 molecular weight which corresponds to the enzyme (see below). Scanning densitometry indicates that the enzyme is at least 70% pure. A number of very minor bands, some of which will be discussed subsequently, are also observed in Fig. 1.

To demonstrate that the 135,000 dalton band corresponds to topoisomerase, the enzyme was eluted from the gel and renatured according to the procedure of Hager and Burgess (22). The data of Fig. 2 clearly demonstrate that the only major band on the denaturing gel which has

Table 1. Purification of topoisomerase 1 from *Drosophila* eggs (100 gms)

Purification Step	Total Activity	Total Protein (mg)	Specific Activity (units/mg)	Yield %
PEG supernatant	3×10^7	480	6×10^4	100
Phosphocellulose column	7×10^6	4.5	1.5×10^6	23
Hydroxylapatite column	3×10^6	0.3	1×10^7	10

Fig. 1. Sodium dodecyl sulfate polyacrylamide electrophoresis of *Drosophila* topoisomerase I. Approximately 1.5 μ g of purified enzyme was precipitated in 10% trichloroacetic acid, dissolved in sample solution and applied to a 10% polyacrylamide gel (lane b). Standard molecular weight markers are myosin (200,000), β galactosidase (116,000), phosphorylase B (94,000), bovine serum albumin (67,000) and ovalbumin (43,000) (from Biorad). Approximately 4 μ g of each protein was used (lane a).

relaxation activity corresponds to that of that of the 135,000 molecular weight. 10% of the input activity was recovered. Further evidence that the major band is topoisomerase I comes from isoelectric focusing under nondenaturing conditions. In Fig. 3(a) the isoelectric focusing pattern is presented. The enzyme was applied in two lanes. One lane was stained and the agarose gel of the adjacent lane was cut into 1/2 cm width pieces. Each piece was crushed and soaked in elution buffer (50 mM Tris, pH 8.0, 0.2 M NaCl, 10 mM MgCl_2 , 10 mM β -mercaptoethanol) overnight. The results of assay of each fraction are shown in Fig. 3(b). The activity (fraction 4) corresponds to the observed band which has an isoelectric point of approximately 7.2. Sodium dodecyl sulfate polyacrylamide electrophoresis of the eluted material followed by silver staining showed a number of degradation products ranging from 60,000–67,000 daltons (data not shown). We believe that most of these bands represent degradation products of the major 135,000 molecular weight protein.

Covalent complex formation between topoisomerase I and ^{32}P -labeled DNA

It has been previously demonstrated that topoisomerase I binds covalently to DNA (23,30). Following extensive digestion of the (labeled)

Fig. 2. Topoisomerase activity eluted from a sodium dodecylsulfate polyacrylamide gel. High molecular weight standard proteins (2 μ g each) (Biorad) were mixed with topoisomerase I and applied to a 10% sodium dodecylsulfate polyacrylamide gel. After staining, individual bands were cut out, eluted and renatured according to Hager and Burgess (22). The final volume of each sample was 2.5 ml. 5 μ l was taken for relaxation activity assay. (1) 200,000 M.W. Band. (2) The segment of the polyacrylamide gel between 200,000 marker and 135,000 band. (3) 135,000 M.W. Band. (4) 94,000 band. (5) 67,000 band. (6) 43,000 band and (7) control DNA. Electrophoresis of the DNA was at 140 volts for 6 hrs.

DNA in the complex by nuclease, the terminal nucleotides are left attached to the enzyme which consequently can be identified on SDS polyacrylamide gels. Phage PM2 DNA was labeled by nick translation by *E.coli* polymerase I in the presence of [α - 32 P]dATP and the other three deoxyribonucleotide triphosphates (19). After denaturing the DNA to single strands, *Drosophila* topoisomerase I was added and the sample was incubated at 30°C for 60 minutes. Sodium hydroxide was then added to denature the enzyme, with the result that a covalent complex was formed. Following neutralization, the complex was digested by staphylococcal nuclease and the sample was loaded on a SDS polyacrylamide gel. The results are shown in Fig. 4. The major bands in Fig.4 correspond to the enzyme products covalently linked to DNA. Besides the 135,000 band, three other major bands corresponding to molecular weights 100,000, 90,000, and 65,000 are observed. We have also carried out silver staining of a similar gel in order to analyze the distribution of different proteins in the mixture. The intensities were quite comparable to those of Fig. 4a (data not shown). We suggest that all the major covalent complexes originated from the 135,000 molecular weight band, which was degraded by protease activity during the

Fig. 3(a). Isoelectric focusing of topoisomerase I. 20 μ g each of myoglobin, carbonic anhydrase and bovine serum albumin (Sigma) with isoelectric points of 8.3, 5.9 and 4.9 respectively were used as standards (a). Approximately 1 μ g of enzyme was dialyzed extensively against 30 mM potassium phosphate, pH 7.1 and applied in lane (b).

Fig. 3(b). Topoisomerase activity from individual segments of agarose gel in lane (b) of Fig. 3(a). Using protein markers as top and bottom of the gel the agarose strip was cut into eight pieces followed by elution and renaturation as described in the text. Lane 4 corresponds to the enzyme band.

procedure. This conclusion is borne out by the results in Fig. 4b where a control experiment was performed. In this case, DNA and enzyme were incubated separately and subjected to the same treatment as in Fig. 4a. The enzyme sample was added to DNA before loading on a sodium dodecyl sulfate polyacrylamide gel. Autoradiography reveals no bands. When a duplicate gel was stained using silver, the pattern shown in Fig. 4b was obtained, showing that under these conditions, the enzyme by itself has been degraded considerably. Some of the characteristic bands of Fig. 4a are observed; in addition, the bands at 100,000, 90,000, and 80,000 show a series of products within each band. The data indicate that the 100,000 M.W. product has the highest intensity relative to the other bands.

Fig. 4(a). Formation of the covalent complex between denatured ^{32}P -labelled PM2 DNA and *Drosophila* topoisomerase I. The sample was prepared as described in Materials and Methods, and run on an SDS gel with 5% acrylamide using 0.075 M sodium phosphate buffer, pH 7, with 0.1% SDS. The resultant autoradiogram is shown in (a). Standard markers which were labeled with ^{125}I are shown in (b) (27). The most prominent band in (b) corresponds to bovine serum albumin.

Fig. 4(b). SDS polyacrylamide gel electrophoresis of PM2 DNA with topoisomerase I in the absence of covalent linkage. 10% acrylamide was used. (a) Biorad high molecular weight standards. (b) Topoisomerase I + DNA control sample incubated as described in the text. The silver staining technique was used.

CsCl banding of the protein-DNA complex gives results consistent with the above. Alkali-treated fdDNA-topoisomerase I complex was subjected to sedimentation. Two peaks are observed, one of free DNA and the other a broad peak due to a DNA-protein complex (Fig. 5). The calculated buoyant density shift of this complex is 24 mg/ml relative to fdDNA. Using this value, the molecular weight of the protein is calculated to be 104,000(23). Mg^{++} effect and relaxation of positive supercoiled DNA

Our study of assay conditions shows that Mg^{++} is not required by the enzyme for DNA relaxation. However, Mg^{++} stimulates relaxation of

Fig. 5. Banding pattern of alkali-treated *Drosophila* topoisomerase I-fdDNA complex in CsCl. (A) free DNA, (B) DNA-enzyme complex.

positively supercoiled DNA (data not shown). When relaxed pBR322 DNA is treated with ethidium bromide and subsequently by topoisomerase I, electrophoresis of the products gives the pattern seen in Fig. 6, indicating that the enzyme is capable of relaxing positive supercoiled DNA (25). The relaxation activity is dependent on the density of positive superhelicity and on concentration of the enzyme. Increasing superhelical density requires more enzyme for relaxation. Two concentrations of ethidium bromide were used. One corresponds to approximately 1/2 of the maximum superhelical density (0.3 $\mu\text{g/ml}$ of ethidium bromide) and the other to full superhelical density (0.6 $\mu\text{g/ml}$ of ethidium bromide) cal-

Fig. 6. Relaxation of positively supercoiled DNA by topoisomerase I. Lanes 1-4 contained 10 $\mu\text{g/ml}$ of pBR 322 DNA with 0.3 $\mu\text{g/ml}$ of ethidium bromide and various amounts of enzyme. Assays were carried out according to the specifications given in Materials and Methods except that Mg^{++} was omitted and the NaCl concentration was 50 mM. 1 μl of diluted enzyme was added to each assay mixture. (1) control DNA (2) 10 units of enzyme (3) 2 units of enzyme (4) 0.4 units of enzyme. Lane 5-8 are identical to 1-4 except that the ethidium bromide concentration was 0.6 $\mu\text{g/ml}$. Electrophoresis was at 40 volts for 18 hrs.

culated on the basis of proper equations (26). Relaxation of DNA in the presence of ethidium bromide followed by its dissociation results in negatively supercoiled DNA. The presence of some positive superhelical DNA in the control DNA (lane 1) is due to the fact that pBR322 DNA was relaxed at 4°C giving rise to some positive superhelix when the DNA was electrophoresed at room temperature.

DISCUSSION

Topoisomerase I activity in *Drosophila* was first detected by Baase and Wang (28); however, no enzyme purification was reported. As pointed out earlier, a number of workers have reported a molecular weight of 60,000 - 70,000 for eukaryotic topoisomerase I. In view of the fact that *E. coli* topoisomerase I has a molecular weight of 110,000, the suggested eukaryotic topoisomerase I molecular weight seemed rather low. Our study of *Drosophila* topoisomerase I, that of Liu and Miller on HeLa topoisomerase I (12), and that of Burgess and his colleagues on wheat topoisomerase I (13) now clearly demonstrate that the molecular weight of eukaryotic topoisomerases I are in the 100,000 dalton range. In this respect, we should point out that our investigation of *Drosophila* topoisomerase I yields a molecular weight of 135,000, with that of the most prominent degradation product being 100,000. It was mentioned earlier that the protein isolated from the isoelectric focusing gel contained a series of bands around 65,000 molecular weight. We can think of two possibilities to explain this degradation. It may be due to considerable amount of heat caused by high voltage (1200 volts, 5 minutes) generated at the end of the isoelectric focusing experiment. Alternatively, a more likely event is degradation of the enzyme during the subsequent renaturation and extraction. Our results clearly show that proteases have not been completely separated from the purified enzyme product.

The isoelectric point of the 67,000 product of topoisomerase I from mouse L cells has been found to be 4.2 (11). Our results for the intact molecule show that topoisomerase I from *Drosophila* has an almost neutral isoelectric point. This implies that there are equal amounts of acidic and basic amino acids in the protein, which may be significant for binding of topoisomerase I to DNA and other proteins. Recently, it has been shown that topoisomerase I and HMG proteins form complexes; this might have interesting implications for transcription (29).

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