DNA Topoisomerase II from Drosophila melanogaster

PURIFICATION AND PHYSICAL CHARACTERIZATION

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A type II DNA topoisomerase has been purified from the nuclei of Drosophila melanogaster 6- to 18-h-old embryos. The enzyme, as assayed by its ability to catenate supercoiled DNA, behaved as a single homogeneous species throughout the procedure and the yield was approximately 0.5 mg of protein/100 g of dechon- ronated embryos. The final product was entirely ATP- dependent and free of topoisomerase I, endonuclease and protease activities. The purified topoisomerase II had a Stokes radius of 69 Å and a sedimentation coefficient ($s_{20,w}$) of 9.2 S, leading to a calculated native molecular weight of approximately 261,000. The protein consists of a single polypeptide of molecular weight 166,000, as determined by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Taken together with the above hydrodynamic studies, the Drosophila enzyme is probably a homodimer, as has been observed for other eukaryotic type II enzymes. Thus, it appears that during the course of evolution the heterologous subunits which comprise bacterial type II topoisomerases have been combined into a single polypeptide chain in eukaryotes.

Topoisomerases are a class of enzymes able to interconvert various topological forms of DNA without altering the primary structure of the DNA. Among the reactions catalyzed by these enzymes are relaxation-supercoiling, knotting-un- knotting and catenation-decatenation of DNA (for reviews, see Refs. 1-5). Mechanistically, topoisomerases fall into two classes. The type I enzymes, such as Escherichia coli $\omega$ protein, are able to relieve torsional constraints in DNA by making a transient single-stranded nick, allowing the rotation of one DNA strand around the other. In contrast, type II topoisomerases alter the topology of DNA by passing one DNA helix through a transient double-stranded break in another helical segment. This was first demonstrated for E. coli DNA gyrase, which can both induce and remove negative supercoils in closed circular duplex DNA using this double-strand passage mechanism (6). Type II and type I topoisomerases are also distinguished by the absolute requirement of the type II enzymes for ATP.

As a consequence of the double-strand passage mechanism, only type II topoisomerases can interlock two or more closed circular duplex DNA molecules and produce a catenane. This ATP-dependent conversion of supercoiled DNA into huge networks of catenated DNA in extracts of Drosophila melano- gastor embryos and Xenopus laevis oocyte nuclei led to the initial discovery of eukaryotic type II topoisomerase activity (7, 8). The catenation reaction requires the presence of a condensing agent, such as histone H1 (7), the trivalent cation spermidine (9-11), or an endogenous factor found in extracts of Drosophila embryos (7). The intramolecular equivalent of catenation, knotting of double-stranded DNA, is also catalyzed by the type II enzymes (12).

The importance of type II topoisomerases in DNA replication and transcription in bacteria is clear (for reviews, see Refs. 2, 3, and 13). The results of genetic and biochemical studies of DNA replication in E. coli demonstrate a crucial role for DNA gyrase (reviewed in Ref. 13). Replication of bacteriophage T4 may also depend upon gyrase or the phage-encoded type II topoisomerase (14, 15). Furthermore, mutations in the DNA topoisomerase I (top) locus, which affect transcription of a number of other genes (16, 17), are often compensated by mutations in DNA gyrase at either the gyr A or gyr B locus (18, 19). These results demonstrate that the supercoiled state of the bacterial DNA is carefully regulated by the opposing actions of E. coli topoisomerase I and gyrase.

The function of type II topoisomerases in eukaryotic cells is less well understood. The results of studies which rely on the sensitivity of type II topoisomerases to inhibitors suggest that these enzymes are important in chromosomal DNA replication and repair (20-23). Other work has led to models for the replication of DNA rings which depend on the action of topoisomerases to segregate daughter circles (24, 25).

To more fully characterize eukaryotic type II topoisomeres and enable further comparisons with the prototypical bacterial enzyme, DNA gyrase, the D. melanogaster topoisomerase II was purified from nuclei of 6- to 18-h-old embryos. The final fraction is completely free of topoisomerase I, endonuclease, and protease activities. Hydrodynamic studies indicate that the native enzyme is a homodimer of the major band observed in SDS-polyacrylamide gels. A similar quaternary structure has also been found for the HeLa cell type II topoisomerase (26). This suggests that the functions of the heterologous subunits in bacterial type II topoisomerases are combined in eukaryotic enzymes. The accompanying paper characterizes the enzyme activity of the Drosophila topoisomerase II and compares it to that of the bacterial DNA gyrase (27).

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1 The abbreviations used are: TX-100, Triton X-100; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
EXPERIMENTAL PROCEDURES

Materials - The chromatographic resin phosphocellulose P-11 was obtained from Bio-Rad Laboratories and Sephadex G-25 from Phamacia. Marker proteins for electrophoresis were obtained from the Sigma Chemical Co. and the Protein Molecular Weight Standards kit. The phosphocellulose column was washed with 0.5 M tris-HCl, pH 7.5, or 0.5 M tris-HCl, pH 8.0, and 50 mM MgCl2, 0.5 M EDTA, 0.5 M dithiothreitol (DTT), 10 mM sodium bisulfite (containing 0.5 M sucrose and 7.5% glycerol). The elution buffer was 0.5 M tris-HCl, pH 7.5, 1 M dithiothreitol, and 0.5 M NaCl.

Proteinase inhibitors - Some samples were prepared in the absence of protease inhibitors to see if the absence of protease inhibitors would affect the results. The protease inhibitors used were phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium fluoride (NaF), and 10 mM sodium molybdate (NaMoO4).

Preparation of Protein Extracts - Samples were homogenized in a Polytron homogenizer, and a portion of the homogenate was removed for determination of protein content. The homogenate was centrifuged at 100,000 g for 1 hour at 4°C. The supernatant was then dialyzed against several changes of 50 mM tris-HCl, pH 7.5, and recovered.

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increasing the collection time, more embryos could be obtained from the same population of flies. Second, the embryo homogenate from 6-18 hr embryos had a lower enzyme activity than that from 1.5 hr embryos. Third, sparging the embryos for a minimum of 4 hr greatly decreased the amount of gelatin protein present. This was otherwise difficult to remove from the topoisomerase II.

At all stages of purification from 1.5-18 hr old embryos, the addition of histone III was required in order to observe catenation activity. In contrast, when histone III was omitted after phosphocellulose chromatography had separated an endogenous contaminating factor from the topoisomerase II. These results indicate that the endogenous factor is present only in early embryos, at a time when the rate of DNA synthesis is most rapid (29).

When Drosophila Kc tissue culture cells were employed as a source of topoisomerase II, the specific activity and chromatin properties observed were similar to those isolated from 1.5-18 hr embryos.

**Purification of Topoisomerase II**

**Preparation and Exclusion of Nuclei** - The first three steps of the purification, as outlined in Fig. 1, comprise a scheme for the isolation of nuclei which was designed to allow rapid and facile handling of large amounts of homogenate. The initial purification step consisted of centrifuging the homogenate at 12,000 g to sediment the nuclei. The nuclei were resuspended in 0.25 M sucrose and then exhaustively extracted with a 0.5 M NaCl solution with 0.02 M EDTA. The specific activity in the nuclear extract (Fraction II) was enriched 15.5-fold with only a minimal loss of activity. The nuclear wash was virtually devoid of enzyme activity (data not shown), demonstrating that the topoisomerase within the nuclei was not removed by this procedure.

The topoisomerase remained soluble during removal of nuclear acids from the nuclear extract by precipitation with polyethylene glycol (PEG) (Fig. 1). As monitored by absorbance at 260 and 260 nm, the contamination by nucleic acids was reduced from greater than 2% to less than 0.1% by enzymatic precipitation using ammonia sulfate precipitation produced a sample (Table I) from which the topoisomerase II was purified and concentrated. This greatly increased the speed and ease of handling of subsequent chromatographic steps.

**Hydroxyapatite Chromatography** - Fractionation of the nuclear extract on hydroxyapatite resulted in a good separation of the topoisomerase from other proteins (Fig. 2A). By first washing the column with buffer containing 100 mM NaCl plus Tris-0.02 M and then with 0.2 M NaCl, a large amount of protein was removed before starting the HAP gradient which was used to elute the topoisomerase. In addition, the 0.2 M NaCl wash was completely devoid of contamination with nucleic acids. The hydroxyapatite step increased the specific activity of the topoisomerase 7-fold.

**Phosphocellulose Chromatography** - Phosphocellulose chromatography of the hydroxyapatite fraction produced a peak of catenation activity which coincided with the major protein peak (Fig. 2B). As shown in Fig. 2, this step removed a predominant 34,000 molecular weight protein which was present in the hydroxyapatite pool (see Fig. 4) and resulted in a 2-fold increase in specific activity.

**Glycerol Gradient Centrifugation** - As a final purification step, the phosphocellulose fraction was sedimented through a glycerol gradient (Fig. 2C). The resulting enzyme activity profile followed exactly the protein profile. This step was especially effective in removing all traces of deoxyribonuclease I from the type II enzyme. The Type I topoisomerase activity, represented by a trailing edge of a small peak routinely observed toward the top of the glycerol gradient, had been removed. The enzyme activity in the gradient shown in Fig. 2 illustrated that the topoisomerase II catenation activity is well separated from contaminating DNA topoisomerase II. In fact, separation of Type I and Type II topoisomerase activity by glycerol gradient centrifugation is complete at every step of purification, including the nuclear extract, the 80% ammonium sulfate fraction, the hydroxyapatite pool (Fig. 2) or the phosphocellulose pool (Fig. 2).

The D. melanogaster topoisomerase II was purified from homogenates of 6- to 18-h-old embryos by the six-step procedure shown in Table I. A single preparation starting with 100 g of embryos yielded nearly 0.5 mg of protein, representing a 6% recovery of enzyme activity. At each step in the purification, a single homogeneous peak of catenation activity was found. The final glycerol gradient pool (Fraction VI) is stable.

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**Table I**

**Purification of topoisomerase II from Drosophila embryos**

Based on 100 g wet weight of dechorionated embryos. Values from four preparations agreed to within ±10%.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Homogenate</td>
<td>ml</td>
<td>mg</td>
<td>%</td>
<td>ml/mg</td>
</tr>
<tr>
<td>II. Nuclear extract</td>
<td>190</td>
<td>230</td>
<td>92</td>
<td>35</td>
</tr>
<tr>
<td>III. 80% (NH₄)₂SO₄, pellet</td>
<td>70</td>
<td>65</td>
<td>45</td>
<td>62</td>
</tr>
<tr>
<td>IV. Hydroxyapatite pool</td>
<td>15</td>
<td>5.5</td>
<td>31</td>
<td>43</td>
</tr>
<tr>
<td>VI. Phosphocellulose pool</td>
<td>6.5</td>
<td>0.77</td>
<td>917</td>
<td>917</td>
</tr>
<tr>
<td>VI. Glycerol gradient pool</td>
<td>0.8</td>
<td>0.44</td>
<td>6</td>
<td>113</td>
</tr>
</tbody>
</table>

*The final yield was 600,000 units at a specific activity of 1.36 × 10⁶ units/mg of protein. One unit is defined as the amount of enzyme required to achieve maximal catenation of 0.3 μg DNA in 1 h utilizing the conditions described under "Experimental Procedures."
for at least one year when stored at 4°C, or if adjusted to 50% glycerol and stored at −20°C. The purified preparation contains no protease or nuclease activities. Moreover, addition of 1000 units of enzyme to the standard assay showed no relaxation in the absence of ATP, indicating that the *Drosophila* type II topoisomerase is completely free from contaminating type I activity.

**Protein Gel Electrophoresis**—The progress of the purification was followed by electrophoresis on SDS-polyacrylamide gels (Fig. 4). Even though 6- to 18-h-old embryos have consumed much of the yolk protein initially present in *Drosophila* eggs, these 45,000–47,000 molecular weight proteins (40) remain as the major species in the homogenate and are prominent in the nuclear extract. Although 60-fold enriched in specific activity, the 80% ammonium sulfate pellet is still a complex mixture of proteins. In contrast, the hydroxyapatite pool clearly shows a 166,000 molecular weight protein identical to the major species in the phosphocellulose and glycerol gradient pools (see below). The increasing prominence of this 166,000 molecular weight protein in successive fractions correlates with the increasing specific activity of the topoisomerase II.

**Molecular Weight and Subunit Structure**—An estimate of the native molecular weight of the enzyme was calculated by combining the results of sedimentation studies and gel filtration chromatography using the method of Siegel and Monty (41). The enzyme (Fraction V) had a sedimentation coefficient of 9.2 S in glycerol gradients containing 1 M NaCl (Fig. 5A). An identical value was observed for the earlier Fractions II, III and IV. In 1 M NaCl, the *Drosophila* topoisomerase II had an observed Stokes radius of 69 Å (Fig. 5B). Combining the sedimentation and gel filtration results leads to a calculated native molecular weight of approximately 261,000 (assuming a partial specific volume of 0.725 cm³/g). The calculated frictional ratio, f/f₀, of 1.6 indicates that the protein has an elongated shape with an axial ratio of about 11 to 1.

Comparison of the calculated native molecular weight (261,000) with the major SDS gel band (166,000) suggests that the native enzyme is a homodimer. The difference between the protomer molecular weights estimated from SDS gels and from hydrodynamic studies may be due to an anomalously low mobility on SDS gels or a partial specific volume different from that assumed.

The sedimentation coefficient of topoisomerase II was unaffected by decreasing the NaCl concentration from 1 to 0.1 M NaCl, but at 50 mM NaCl, the enzyme pelleted. However, the presence of high ionic strength was critical to prevent absorptive losses of topoisomerase during gel filtration. All attempts to lower the NaCl concentration below 1 M, using either Sephacryl or Bio-Gel A resins, led to loss of enzyme activity. Similarly, the HeLa topoisomerase II has been found to give poor recoveries upon gel filtration in 0.2 M potassium phosphate buffer (26).

**Lability of the Topoisomerase II Polypeptide**—Analysis of the polypeptide composition of the enzyme on SDS gels showed a predominant band with a molecular weight of 166,000 (Fig. 4). A cluster of three bands of lesser intensity at 136,000–152,000 molecular weight were also visible. This result was obtained with four independent preparations. The protease inhibitors PMSF and sodium bisulfite were included in all isolations. The addition of 1,10-phenanthroline, ovo- mucoid and soybean trypsin inhibitor to the buffers used from the homogenization step to the preparation of Fraction III caused no alteration in the SDS gel pattern. Furthermore, omission of all protease inhibitors led to an enzyme (Fraction IV) with an SDS gel pattern essentially identical to the normal pattern.
This is further supported by two lines of evidence. First, preliminary mapping data show that the bands share common peptides. Second, antibodies directed against gel-purified preparations of the 152,000 and 143,000 molecular weight polypeptides cross-react with the 166,000 dalton band. Also, when SDS-denatured rapid lysates of \textit{Drosophila} embryos were hybridized with three different anti-topoisomerase II (Fraction VI) antisera, the largest polypeptide which reacted was 166,000 molecular weight. Therefore, the 166,000-dalton band must represent the complete topoisomerase II polypeptide chain and is not a partially digested form of a larger precursor.

Heterogeneity in the SDS gel band pattern as a function of source (42) and freshness of starting material (26) has been noted in the type II topoisomerases from other euakaryotic species. Since added protease inhibitors had no effect on the SDS gel profile, this cleavage may occur \textit{in vivo}, before the homogenization step. When freshly prepared Kc tissue culture cells were used, the relative proportion of the 166,000 molecular weight band was substantially increased (data not shown). Whether this reflects the use of cells which had never been frozen or a source different from embryos remains to be determined. Association of the lower molecular weight bands with the 166,000 molecular weight protein was not disrupted by sedimentation in 1 M NaCl plus 4 M urea, although the sedimentation rate was decreased and the enzyme activity was lost.

**DISCUSSION**

The type II DNA topoisomerase from \textit{D. melanogaster} has been purified from nuclear extracts of 6- to 18-h-old embryos. The presence of a single species of enzyme is indicated, since at every step of the purification procedure, only one homogeneous peak of catenation activity was detected. Other work (43, 44) has identified a single type I topoisomerase in \textit{Drosophila}. Similarly, a single species of type I enzyme and a single species of type II enzyme activity have been found in yeast (42) and HeLa cells (26, 45).

The isolation of \textit{Drosophila} topoisomerase II from embryo and Kc cell nuclei (this work) or Kc cell chromatin (7) demonstrates that the enzyme is located in nuclei. A nuclear localization has also been suggested for type II topoisomerases isolated from other eukaryotic cells (8, 26, 42). Although not eluted from the embryo nuclei during the nuclear washing step, the \textit{Drosophila} topoisomerase II was easily extracted with 0.35 M NaCl.

The \textit{Drosophila} topoisomerase II is composed of a protomer with a molecular weight on SDS-polyacrylamide gels of 166,000. This conclusion is based on several results. 1) The purification of the 166,000-dalton polypeptide coincides exactly with the purification of catenation activity (Table I, Figs. 2 and 4). 2) Interactions between DNA and stoichiometric levels of the purified \textit{Drosophila} protein, which are described in the accompanying paper (27), indicate that enzymatically active topoisomerase II must comprise a substantial portion of the preparation (Fraction VI). The 166,000 molecular weight polypeptide represents at least 70% of Fraction VI (with the remaining 30% being smaller proteolytic products of the topoisomerase) (Fig. 6). 3) Antibodies directed against a gel-purified preparation of this polypeptide inhibit the catalytic relaxation of supercoiled DNA by \textit{Drosophila} topoisomerase II.\textsuperscript{3} Comparable polypeptide molecular weights of 172,000 for the HeLa cell topoisomerase II (26) and

\textsuperscript{3} Shelton, E. R., Osheroff, N., and Brutlag, D. L., unpublished results.
150,000–160,000 for the yeast topoisomerase II (42) have been determined. In addition, the *Drosophila* and HeLa cell (26) enzymes have identical sedimentation coefficients (9.2 S) and similar Stokes radii (69 and 78 Å, respectively), indicating that they may have a common gross physical structure.

Hydrodynamic studies of the *Drosophila* and the HeLa cell (26) type II topoisomerases indicate that both enzymes are homodimers of the major polypeptide seen in SDS gels. Thus, the subunit structures of these two eukaryotic type II topoisomerases differ from the A$_B$$_C$ structure of *E. coli* gyrase (13) and the three nonidentical subunits of the T4 type II topoisomerase (14, 15). The eukaryotic type II topoisomerases may therefore be another instance of a single eukaryotic polypeptide which combines the functions of two or more related bacterial proteins. Two such cases are the enzymes which catalyze the first steps in pyrimidine and fatty acid synthesis (see Refs. 46 and 47 for reviews).

As described in the following paper, the purified *Drosophila* topoisomerase II has been employed to study the double-strand DNA passage reaction by analyzing the relaxation of supercoiled molecules (27). In addition, the anti-*Drosophila* topoisomerase II antibodies, which are discussed above, are currently being employed to further probe the evolutionary relationships between the eukaryotic and the bacterial type II topoisomerases.

**Note Added in Proof**—Similar results concerning the purification and physical characterization of the *Drosophila* topoisomerase II have been found by M. Sander and T.-S. Hsieh (J. Biol. Chem. 258, 8421–8428).

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