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Journal Title: Biochemistry
Volume: Volume 26, Number 19
Publisher: American Chemical Society | 1987-09, Pages 6195-6203
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1021/bi00393a036
Permanent URL: http://pid.emory.edu/ark:/25593/czgv6

Final published version: http://pubs.acs.org/doi/abs/10.1021/bi00393a036

Accessed October 23, 2018 7:45 AM EDT
Purification and Characterization of a Type I DNA Topoisomerase from Calf Thymus Mitochondria

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Received January 9, 1987; Revised Manuscript Received May 12, 1987

ABSTRACT: A type I topoisomerase has been purified more than 4000-fold from calf thymus mitochondria. The enzyme is membrane associated and is effectively solubilized by 1% Triton X-100 treatment of purified mitochondrial inner membranes. This ATP-independent enzyme relaxes positively and negatively supercoiled DNA with \( \Delta L = 1 \). At low ionic strength, the native enzyme appears to be a monomer (sedimentation coefficient of 4.3 S and Stokes radius of 34 Å), but it can form a weakly associated dimer at higher salt concentrations (sedimentation coefficient of 7.0 S and Stokes radius of 47.5 Å). The mitochondrial type I topoisomerase is distinguishable from the nuclear enzyme by its (1) pH profile, (2) thermal stability, (3) response to dimethyl sulfoxide and Berenil, and (4) molecular weight. The mitochondrial enzyme is inhibited by elevated concentrations of the bacterial DNA gyrase inhibitor novobiocin, but not nalidixic or oxolinic acids. Sensitivity to N-ethylmaleimide indicates the importance of cysteine for catalytic activity. It is estimated that there are at least five copies of topoisomerase I per mammalian mitochondrion or a minimum of one to two per mitochondrial genome. In a manner similar to that observed with leukemia (nuclear and mitochondrial), calf thymus (nuclear), and HeLa (nuclear) cell type I topoisomerase, the calf thymus mitochondrial enzyme is inhibited by physiological concentrations of ATP.

Mammalian mitochondrial DNA (mtDNA) is a circular molecule of approximately 16,500 base pairs (bp) (Anderson et al., 1981, 1982; Bibb et al., 1981) whose mode of replication is characterized by the occurrence of displacement loop DNA (Arنبerg et al., 1971; Kasamatsu et al., 1971). Replication of the closed circular mitochondrial genome would introduce topological stress which should require the action of a swivel, e.g., to relieve strain generated by the unwinding of parental circular strands in front of the replication fork during daughter strand elongation. This function could be fulfilled by either a type I or a type II DNA topoisomerase capable of relaxing positively supercoiled DNA. Indirect evidence for the presence of a DNA gyrase-like (type II) topoisomerase and its involvement in mtDNA replication has been obtained in rat liver mitochondrial (Castora & Simpson, 1979; Castora et al., 1983). Direct evidence for a type I topoisomerase has been obtained in rat liver (Castora et al., 1982) and human leukemia (Castora et al., 1985) cells. A type I topoisomerase has been isolated from rat liver (Fairfield et al., 1979, 1985), Xenopus laevis (Brun et al., 1981), and human leukemia (Castora & Lazarus, 1984; Castora & Kelly, 1986) mitochondria. These latter studies have been hampered, however, by the lability and limited yield of purified protein which has been obtainable. In order to circumvent this problem so that further characterization of the biochemical and biophysical properties of this important enzyme may be obtained, we have begun to isolate large amounts of mitochondrial topoisomerase from calf thymus glands. We report here the purification and partial characterization of a type I DNA topoisomerase from calf thymus mitochondria.

EXPERIMENTAL PROCEDURES

Materials

Chromatography. Phosphocellulose was from Bio-Rad; phenylagarose was from Sigma; single-stranded DNA-agarose was from Bethesda Research Laboratories, Inc. Sephadex was from Pharmacia.

Nucleic Acids. Supercoiled pBR322 was prepared as previously described (Castora & Kelly, 1986). Relaxed closed-circular pBR322 DNA was prepared by treatment of supercoiled pBR322 with purified topoisomerase. The sample was deproteinized by phenol extraction, and the DNA was recovered by ethanol precipitation. Positively supercoiled pBR322 was prepared by intercalation of ethidium bromide (5 \( \mu \)M) into relaxed DNA. Unique topoisomers were obtained by separating products of a preparative topoisomerase relaxation assay by agarose gel electrophoresis. Individual topoisomers were recovered from the gel by electroelution into dialysis sacks. Nick-translated pBR322 was prepared according to standard procedures (Maniatis et al., 1982) using \([\alpha^{32P}]\text{dATP} (400 \text{Ci/mm})\). Activated salmon sperm DNA was prepared by DNase I digestion to 2% solubility.

Enzymes. Escherichia coli DNA polymerase I was obtained from Bethesda Research Laboratories, Inc. DNA polymerase \( \gamma \) was obtained from Worthington Biochemicals. Nuclear type I topoisomerase was purified from calf thymus cells according to published procedures (Schmitt et al., 1984).

1 Abbreviations: bp, base pair(s); DMSO, dimethyl sulfoxide; D-mtDNA, displacement loop mitochondrial DNA; H-strand, heavy strand of mtDNA; L-strand, light strand of mtDNA; MIB, mitochondrial isolation buffer; NEM, N-ethylmaleimide; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; ssDNA, single-stranded DNA; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; Pipes, piperaazine-N,N'-bis(2-ethanesulfonic acid); DTT, dithiothreitol.

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Tissues. Calf thymus glands were obtained fresh from a local abattoir.

Radioisotopes. [α-32P]dATP (400 Ci/mmole) was obtained from New England Nuclear. Thymidine 5′-triphosphate, methyl-3H labeled (65 Ci/mmole), was obtained from ICN Radiochemicals.

Inhibitors. Phenylmethanesulfonyl fluoride (PMSF), leupeptin, coumermycin, novobiocin, Berenil, oxolinic acid, nalidixic acid, and N-ethylmaleimide were from Sigma. Pepstatin was from Boehringer Mannheim.

Reagents. Acrylamide, digitonin, ATP, poly(ethylene glycol), 2-mercaptoethanol, sodium dodecyl sulfate, bis(acrylamide), Temed, ammonium persulfate, molecular weight markers, and salmon sperm DNA were from Sigma. Protein was determined by biuret analysis (Gornall et al., 1949).

Methods

Isolation of Mitochondria. All procedures were performed at 4 °C. Fresh calf thymus (2.5 kg) was trimmed of fat and connective tissue, weighed, and passed through a meat grinder. The ground tissue was suspended in 6 volumes (v/w) of mitochondria. The supernatant was recentrifuged at 10000g for 35 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 8500g for 35 min to pellet mitochondria. These were washed once with MIB and recentrifuged. These pellets represent the crude mitochondria. The crude mitochondria were suspended to a final volume of 312 mL with MIB, and 13-mL samples were applied to a two-step sucrose gradient composed of 13 mL each of 25% sucrose and 42.5% sucrose in MIB. The gradients were centrifuged for 19 h at 150000g at 4 °C in an SW50.1 rotor.

Column Chromatography. The diluted PEG supernatant was loaded directly onto a phosphocellulose column (7.1 × 1.8 cm) equilibrated with 50 mM KP, (pH 7.0)/solution A/0.33 M NaCl/2% PEG. The column was washed with 3 volumes of equilibration buffer and then developed with 50 mL each of a two-step gradient: step I was 50 mM KP, (pH 7.0)/solution A/0.8 M NaCl; step II was 50 mM KP, (pH 7.0)/solution A/1.8 M NaCl. The mitochondrial topoisomerase I eluted in step I.

Active fractions were combined and loaded onto a phenylagarose column (2.5 × 0.7 cm) equilibrated in step I phosphocellulose eluant buffer. The column was washed with 5 volumes of step I buffer. The mitochondrial topoisomerase elutes with the load and early wash fractions.

Phenylagarose active fractions (26.2 mL) were combined and dialyzed against 0.1 M KCl/40 mM Tris-HCl (pH 7.5)/solution A for several hours, and the dialysate (23.5 mL) was loaded onto a single-stranded DNA-agarose column (2.0 × 0.7 cm) equilibrated in dialysis buffer. The column was washed with 5 bed volumes of the dialysis buffer and developed with 5-mL steps containing 0.3 and 0.6 M KCl/40 mM Tris (pH 7.5)/solution A, respectively. The enzyme eluted in the 0.3 M KCl step (4 mL).

The purified enzyme was concentrated 2–3-fold in an Amicon concentrating apparatus and stored at −20 °C.

Molecular Weight Determinations. (i) Gel Permeation Chromatography. A Sephadex G-150 column (45 cm³) was prepared and equilibrated either in assay cocktail/8% glycerol or in assay cocktail containing 0.4 M KCl/1% glycerol. The void volume was determined by using Blue Dextran 2000. The column was calibrated with the following marker proteins (molecular weights in parentheses): aldolase (150000), bovine serum albumin (68000), ovalbumin (45000), and cytochrome c (13700). Aliquots of the concentrated ssDNA-agarose fraction or the combined phenylagarose fraction were used. Assays were performed as stated below.

(ii) Glycerol Gradient Centrifugation. Linear gradients of 10–30% glycerol in either assay cocktail or assay cocktail/0.4 M KCl were prepared; 200-μL aliquots of either the concentrated ssDNA-agarose fraction or the combined phenylagarose fraction were dialyzed into the appropriate buffers in 8% glycerol and loaded onto the gradients. A second set of gradients was prepared exactly as above, and the following marker proteins (molecular weights in parentheses) were applied: catalase (232000), aldolase (150000), bovine serum albumin (68000), and cytochrome c (13700). Gradients were centrifuged for 19 h at 150000g at 4 °C in an SW50.1 rotor.

(iii) SDS–Polyacrylamide Gel Electrophoresis. Aliquots from the concentrated ssDNA–agarose fraction and earlier fractions were precipitated with 10% trichloroacetic acid (TCA). Precipitates were washed once with acetone, suspended in SDS sample buffer, boiled 3 min, and electrophoresed through 10% SDS–polyacrylamide slab gels ac-
Whatman DE8 1 filter disks which were counted in a Tri-Carb
At appropriate time intervals, aliquots were adsorbed to
samples were treated with Triton X-100 (1%) prior to assay.
were performed essentially as described (Bertazzoni et al.,
pg/mL ethidium bromide and photographing under ultraviolet
Minaxi 4000 table-top liquid scintillation counter.
were incubated in assay cocktail in the absence of substrate at dif-
cubated in assay cocktail in the absence of substrate at dif-
30 min.
Although we have determined that optimal topoisomerase
activity of the purified enzyme occurs at 120 mM potassium
ion concentration (see below), we routinely assayed column
fractions, the activity was severely lowered or undetectable.
Temperature Experiments. In one set of experiments, to-
poisomerase activity was assessed by incubation of the com-
plete assay mix at different temperatures ranging from 0 to
65 °C. In a second set of experiments, the enzyme was incub-
culated assay cocktail in the absence of substrate at dif-
ferent temperatures (0-55 °C). After various predetermined
time intervals, plasmid DNA was added (30 pg/mL), and the
samples were incubated 30 min at 30 °C.
Linking Number Experiment. A purified unique topoisomer-
as was incubated under standard assay conditions with and
without mitochondrial topoisomerase. After incubation at 30
°C for 1 h, the samples were electrophoresed. Negatively
supercoiled and topoisomerase-relaxed pBR322 were included
as controls. The DNA was transferred to nitrocellulose
(Southern, 1975) and hybridized to nick-translated pBR322,
and the products were visualized by autoradiography.
Electrophoresis. Generally, all assay products were sepa-
rated by electrophoresis on 0.7% agarose in a buffer of 50 mM
Tris–borate (pH 8.3)/10 mM EDTA. The voltage gradient
was 2 V/cm. DNA bands were visualized by staining in 1
μg/mL ethidium bromide and photographing under ultraviolet
illumination using Polaroid 55 film.
DNA Polymerase Assays. The DNA polymerase α assays
were performed essentially as described (Bertazzoni et al.,
1977) using activated salmon sperm DNA (180 μg/mL) with
50 μM dATP, dCTP, and dGTP and 9.2 μM dTTP containing
150 nM [3H]dTTP (2.2 × 10⁴ dpm). The mitochondrial
samples were treated with Triton X-100 (1%) prior to assay.
At appropriate time intervals, aliquots were adsorbed to
Whatman DE81 filter disks which were counted in a Tri-Carb
Minaxi 4000 table-top liquid scintillation counter.

The DNA polymerase γ assays were essentially those de-
scribed by Bertazzoni et al. (1977). The DNA substrate was
poly(rA)/(dT)₁₀ (50 μg/mL) with 50 μM dATP, dCTP, and
dGTP and radiolabeled dTTP as described above. Aliquots
were removed, worked up, and counted as above.

The γ-polymerase assay is essentially specific, but the α-
polymerase assay allows some expression of γ-polymerase
activity. To measure the extent of γ-polymerase contributing
to the observed α-polymerase activity, we determined the
activity of 2.1 × 10⁴ units/mL of commercially obtained po-
ymerase under both α- and γ-polymerase assay conditions. To
compare these enzymes using two different substrates, the
incorporation of label into activated salmon sperm DNA was
related to poly(rA)/(dT)₁₀ by consideration of the base com-
position of salmon sperm DNA, i.e., A (29.7%), T (29.1%),
G (20.8%), and C (20.4%).

Spectrophotometric Assays. All samples were previously
with 1% Triton X-100, except those assayed for amine
oxidase which were treated with digitonin. Each marker en-
zyme was assayed according to published procedures: glu-
cose-6-phosphate dehydrogenase (extramitochondrial cyto-
plasm) (Langdon, 1966); monoamine oxidase (mitochondrial
outer membrane) (Cohen et al., 1972); sulfite oxidase (mi-
tochondrial intermembrane space (Taber et al., 1954); succinic
dehydrogenase (mitochondrial inner membrane) (Tsou et al.,
1956).

Electron Microscopy. Mitochondria and mitoplasts were
fixed with 3% glutaraldehyde in 40 mM Pipes (pH 7.4)/100
mM sucrose/50 mM KCl/10 mM MgCl₂, and pelleted. The
pellets were washed once in buffer without glutaraldehyde,
twice with 50 mM cacodylate, pH 7.4, once with cacodylate
containing 1% OsO₄, and once again with cacodylate. The
pellets were dehydrated with successive washes with 70% and
95% ethanol and then propylene oxide. Pellets were infiltrated
with Araldite overnight. After thin sectioning and staining
with 2.5% uranyl acetate/0.4% lead citrate, we viewed the
samples in a Zeiss EM 10CA transmission electron microscope.

RESULTS

Purification of Mitochondria. The amount of enzymatic
activity associated with mitochondrial topoisomerases has been
estimated to be only 1% of the total cellular activity in am-
phibian (Brun et al., 1981) and human (Castora & Lazarus,
1984) cells. It is, therefore, imperative that highly purified
mitochondria are used for subsequent enzyme isolations. We
have monitored the purity achieved during our isolation pro-
cedure by both biochemical and morphological techniques. A
number of marker enzymes have been assayed, and those
associated with mitochondrial matrix or inner membrane have
shown the expected behavior during the purification (see Table
I). The possibility that small nuclear fragments have pelleted
with the mitochondrial fraction and are thereby contributing
contaminating nuclear topoisomerase was addressed by

Table I: Assays of Selected Marker Enzymes during Purification of Calf Thymus Mitochondria

<table>
<thead>
<tr>
<th>Fraction</th>
<th>glucose-6-phosphate dehydrogenase (units)</th>
<th>amine oxidase (units)</th>
<th>sulfite oxidase (units)</th>
<th>succinic dehydrogenase (units)</th>
<th>DNA polymerase α (units)</th>
<th>γ (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow-spin pellet</td>
<td>4200</td>
<td>NT</td>
<td>800</td>
<td>NT</td>
<td>10⁴</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>Fast-spin pellet</td>
<td>3700</td>
<td>NT</td>
<td>850</td>
<td>NT</td>
<td>7.7 × 10⁴</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>Crude mitochondria</td>
<td>8.3</td>
<td>3650</td>
<td>1.34</td>
<td>5300</td>
<td>1800</td>
<td>1200</td>
</tr>
<tr>
<td>Mitoplast</td>
<td>1.1</td>
<td>2050</td>
<td>1.20</td>
<td>2600</td>
<td>2300</td>
<td>8000</td>
</tr>
<tr>
<td>PEG supernatant</td>
<td>0.3</td>
<td>670</td>
<td>ND</td>
<td>3500</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*One unit of activity is AOD₄⁰₀ of 2.07/min. *One unit of activity is AOD₄⁰₀ of 0.001/min. *One unit of activity is AOD₄⁰₀ of 1.0/min. *One unit of activity is AOD₄⁰₀ of 0.001/min. *One unit of activity incorporates 1.0 mmol of total nucleotides in 1 h at 35 °C. /NT, not tested. /ND, not detectable.
banding the mitochondria in a sucrose step gradient. The greater density of the nuclear fragments causes them to pellet while the mitochondria are suspended in the sucrose. For example, the cytoplasmic marker glucose-6-phosphate dehydrogenase has undergone a 4000-fold reduction when purified mitochondria are recovered from the two-step sucrose gradient. This indicates the success of the procedure in removing contaminating extramitochondrial enzyme activities. Furthermore, the enzyme has undergone an 8000-fold decrease when mitoplasts are recovered from digitonin-treated mitochondria, which shows that stripping the mitochondrial outer membrane further removes cytoplasmic activities which might have been adhering to its surface. Similarly, as one would expect, amine oxidase (an outer membrane marker) and sulfite oxidase (an intermembrane space marker) undergo decreases in activity when digitonin-treated mitoplasts are prepared. Greater than 80% of the amine oxidase is removed by the preparation of mitoplasts, and this is reduced to nondetectable levels when the supernatant from the PEG-treated membranes is assayed. In a like manner, the sulfite oxidase in reduced to undetectable amounts when mitoplast preparations are assayed.

Finally, the mitochondrial DNA polymerase is the DNA polymerase γ which occurs in the matrix of the organelle. The DNA polymerase α is an extramitochondrial enzyme with similar chromatographic properties and, by inference, similar electrostatic properties which might affect its affinity for mitochondria or mitochondrial membranes. We therefore monitored DNA polymerase α and γ activities throughout purification of mitochondria. We used published procedures to assay the uptake of [3H]dTTP under conditions which discriminate between these activities (Bertazzoni et al., 1977). In particular, the substrates were activated salmon sperm DNA (for DNA polymerase α assays) and synthetic poly(rA)/(dT)~10~ (for DNA polymerase γ assays). Because the γ-polymerase will still have slight activity using the activated DNA, we normalized this by measuring polymerase units under α-polymerase assay conditions using commercial DNA polymerase γ. As shown in Table I, the calf thymus α-polymerase activity decreased dramatically from the crude mitochondrial fraction to the preparation of purified mitochondria. This residual α activity was reduced to nondetectable levels after outer mitochondrial membranes were stripped with digitonin. On the other hand, DNA polymerase γ levels followed the expected trend in activity. For example, the amount of activity in crude mitochondria is high because of the occurrence of substantial amounts of enzyme in the cytoplasm; however, much of this is eliminated during purification as is evidenced by the lower amount detected in pure mitochondria. There is a stimulation of γ activity as the outer membrane is stripped off and mitoplasts are formed. This is possibly due to some soluble inhibitor that is present in the intact organelle and which is removed when mitoplasts are prepared. Such a phenomenon has been observed during isolation of polymerase from rat liver mitochondria (Meyer & Simpson, 1970). Since the γ-polymerase is a matrix enzyme, it is not expected to be found with the inner mitochondrial membrane fraction, and this is the case as indicated by assays of the PEG supernatant.

Although these values varied from preparation to preparation, the topoisomerase activity studied was not considered of mitochondrial origin unless the polymerase γ/polymerase α ratio was between 20 and 30 to 1. This ratio is comparable to levels reported for mitochondrial enzymes from other systems (Bertazzoni et al., 1977; Scovassi et al., 1979).

In addition to the marker enzyme analysis, an assessment of the morphological condition of the mitochondria was made by electron microscopy. As clearly seen in Figure 1A, the purified mitochondria show the characteristic, convoluted, electron-dense, inner membrane aspect enclosed by a second outer membrane. On the other hand, the digitonin-treated mitoplasts are seen as larger, less electron-dense structures whose oval or spherical shape results from removal of the outer membrane (Figure 1B).

By these various biochemical and morphological criteria, the topoisomerase purified in this study was judged to be a mitochondrial enzyme. As described later, comparison of several properties of purified nuclear and mitochondrial type I topoisomerases indicates that these enzymes differ in their responses sufficiently to support this identification of their uniqueness and separate origins of location.

Early Stages of Purification. A flow diagram, shown in Figure 2, describes the early stages of the purification. In many of our earlier attempts to purify the mitochondrial topoisomerase, we had isolated pure mitoplasts and then lysed them with a nonionic detergent, Triton X-100. In pilot ex-
Table II: Purification of Topoisomerase I from Calf Mitochondria

<table>
<thead>
<tr>
<th>step</th>
<th>fraction</th>
<th>volume (mL)</th>
<th>total protein (mg)</th>
<th>total sp act. (units/mg)</th>
<th>purification (x-fold)</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>crude mitochondria</td>
<td>144</td>
<td>6250</td>
<td>5760&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>pure mitochondria</td>
<td>60</td>
<td>2916</td>
<td>5760</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>mitoplast</td>
<td>80</td>
<td>1476</td>
<td>5760</td>
<td>3.9</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>diluted poly(ethylene glycol) supernatant</td>
<td>360</td>
<td>499</td>
<td>5760</td>
<td>12.0</td>
<td>13</td>
</tr>
<tr>
<td>V</td>
<td>phosphocellulose</td>
<td>35</td>
<td>4.1</td>
<td>1890</td>
<td>460</td>
<td>500</td>
</tr>
<tr>
<td>VI</td>
<td>phenylagarose wash</td>
<td>26</td>
<td>2.0</td>
<td>1061</td>
<td>540</td>
<td>590</td>
</tr>
<tr>
<td>VII</td>
<td>single-stranded DNA-agarose</td>
<td>4</td>
<td>0.34</td>
<td>256</td>
<td>760</td>
<td>830</td>
</tr>
</tbody>
</table>

<sup>a</sup>The starting material was 2.5 kg of freshly obtained calf thymus glands. Enzyme activity was determined by relaxation assay and analyzed by gel electrophoresis. <sup>a</sup>Due to the presence of nuclease, it was impossible to obtain an accurate measure of topoisomerase activity in early stages of the purification. We have therefore used the value obtained for diluted PEG supernatant as our starting number of enzyme units.

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**Figure 2:** Flow diagram of the early fractionation steps prior to chromatography. The detailed stepwise procedure is described under Experimental Procedures.

---

**Figure 3:** Topoisomerase dilution assay used to determine the total activity in the 3-fold-diluted PEG supernatant. Lane a, control pBR322; lanes b-e, 1 µl of various serial dilutions of the PEG supernatant added to the assays; lane e, neat; lane c, 1:4; lane c, 1:16; lane e, 1:64. The bands labeled I, II, III, and IV are supercoiled, open, completely relaxed, and partially relaxed circular DNA, respectively.

---

**Figure 4:** Phosphocellulose column chromatography. Protein concentration is shown by closed circles. The protein in the load and wash was measured by biuret analysis while the eluted protein concentration was determined by the Bradford dye assay; topoisomerase relaxation activity is shown by the dashed line, and the sodium chloride step gradient is shown by the solid line.

---

**Assay** is shown in Figure 3. Such a dilution assay allows determination of the starting level of topoisomerase activity, 5.76 x 10<sup>6</sup> units. The largest single purification, approximately 40-fold, was achieved in the first chromatographic step using phosphocellulose. A profile of protein and enzymatic activity from the phosphocellulose column is shown in Figure 4. Approximately 99% of the mitochondrial protein passes through the column. The adsorbed enzyme is eluted as a sharp peak in one step with 0.8 M NaCl. By assuming a starting activity of 5.76 x 10<sup>6</sup> units which remains constant through steps I-IV, there is a final recovery of 4% of the total enzyme activity and an overall 830-fold purification of the enzyme relative to crude mitochondria. This actually represents a greater than 4000-fold purification on the basis of whole cell
The results of topoisomerase action on such a substrate are d). The presented in Figure 6. Lane B shows that the amount of capable of completely relaxing highly supercoiled pBR322, of dye) and then the dye were removed, the protein. The enzyme prepared in this way from the was treated with ethidium (to introduce positive supercoils) and care must be taken to maintain a reducing environment by supercoiled pBR322. Lane C contains relaxed enzyme has an apparent molecular weight of 145D. mitochondrial enzyme resembles the other eukaryotic topoisomerase in its ability to relax positively supercoiled DNA. To test that this increase in mobility was in fact due to topoisomerase action, the enzyme aliquot was first denatured by heating for 10 min at 55°C. This heat-inactivated aliquot was unable to produce negatively supercoiled DNA (lane E).

**Molecular Weight Determinations.** The native molecular weight of the mitochondrial topoisomerase was assessed by both gel permeation chromatography and glycerol gradient centrifugation performed at two ionic strengths, 0.4 and 0.06 M KCl; 0.4 M KCl was chosen because the recovery of active enzyme, particularly after gel filtration, was greatest under high-salt conditions. The 0.06 M KCl was used because it resembles the conditions under which the topoisomerase is normally assayed (see Methods). Even though losses of enzymatic activity were significant, if large amounts of enzyme were applied to the column under these low-salt conditions, peaks of topoisomerase activity could be observed. In glycerol gradients containing 0.4 M KCl, the enzyme (fraction VII) had a biphasic sedimentation profile with a major species (Topom) with a sedimentation coefficient of 4.3 S and a minor species (Topom) sedimenting at 7.0 S. In gradients containing 0.06 M KCl, the only detectable species sedimented at 4.3 S (Topom).

In a similar fashion, gel permeation chromatography in the presence of 0.4 M KCl indicated a topoisomerase with a Stokes radius of 47.5 Å (Topom), with a minor species with a Stokes radius of 34.0 Å (Topom). On a Sephadex G-150 column in 0.06 M KCl, the topoisomerase had an apparent Stokes radius of 34.0 Å (Topom).

Combining these sedimentation and gel filtration results allows the determination of the molecular weight of the native enzyme by the method of Siegel and Monty (1966), if one assumes a partial specific volume of 0.725 cm3/g. The native enzyme has an apparent molecular weight of 145,000 at high ionic strength, but at low salt concentration it appears as a 64,000-dalton protein. This suggests that the enzyme may be dimerizing at high salt concentrations.

To assess purity and to determine the denatured molecular weight of the purified enzyme, aliquots of the enzyme preparation at various stages of purification were run on SDS–polyacrylamide gels. The bands on the gel were visualized by...
silver staining (Merril et al., 1981). There is substantial reduction in the number of polypeptides observed when the membranes are prepared from the mitoplasts and also when the PEG supernatant is fractionated on phosphocellulose. Although the phenylagarose column does not remove many proteins, it does seem to remove some factor (protease?) that destabilizes the topoisomerase. Ultimately, the most purified fraction from ssDNA-agarose is seen to contain two major bands of molecular weights 78 000 and 63 000 which copurify throughout the procedure. In several preparations, the relative proportions of these bands have varied, although the 63 000-dalton polypeptide has been the most prominent.

It has been reported that the calf thymus nuclear enzyme can be isolated as a set of multiple bands between 70 000 and 100 000 daltons (Schmitt et al., 1984). In particular, the four major species had molecular weights of 100 000, 89 000, 82 000, and 75 000 with a minor species of 70 000. Reproducing their isolation procedure, we also observe this set of polypeptides in our most purified nuclear enzyme preparation. These nuclear bands do not correspond to the polypeptides we observe in our mitochondrial preparation, which argues against the possibility that the mitochondrial topoisomerase is a nonspecific proteolytic fragment of a larger nuclear contaminant.

Ionic, Temperature, and pH Effects of the Mitochondrial and Nuclear Enzymes. In order to discern similarities or differences in the properties of the nuclear and mitochondrial type I topoisomerases from calf thymus, we have begun to compare certain physical and biochemical characteristics of these enzymes. For example, the molecular weight data discussed above indicate a distinguishing aspect of the mitochondrial enzyme. In addition, we examined the assay temperature, monovalent cation and pH profiles, and thermal stability for both purified enzymes. Optimal activity was observed at 120 mM K⁺. The profiles for the two enzymes essentially overlap except for some small differences at low K⁺ concentrations (30–60 mM). The K⁺ requirement could be met equally as effectively by NH₄⁺, but Na⁺ did not substitute as well for the K⁺ (data not shown). There was no absolute requirement for divalent cations, although the presence of 10 mM Mg²⁺ did stimulate activity in both enzymes 5–10-fold. Ca²⁺ was equally effective as Mg²⁺, but Mn²⁺ did not substitute well for the Mg²⁺ (data not shown).

The pH profiles of the two enzymes differ. The pH optimum for both is pH 7.0 in Tris buffer, but the mitochondrial enzyme has a much narrower pH range over which it is active. There is approximately a 50% loss of activity as the pH is raised or lowered by 1 unit. This is not quite the case with the nuclear enzyme, particularly at the acidic pH values, where, for example, the nuclear topoisomerase maintains 90% and 70% of its activity at pH 6 and 5, respectively, while the mitochondrial enzyme exhibits only 50% and 25%.

Comparison of the temperature profiles for the two enzymes indicates that the optimal assay temperature is found at 30 °C. The profiles are similar, with the nuclear enzyme showing somewhat more activity at room temperature and 4 °C than does the mitochondrial enzyme. Whereas the mitochondrial enzyme was quickly inactivated by preincubation (in the absence of DNA) at 45 °C for 10 min, the nuclear enzyme exhibited much more thermal stability at this temperature, maintaining 80% activity after 60 min. However, both topoisomerases were inactivated within 10 min at 55 °C.

Number of Topoisomerase Molecules in a Mammalian Mitochondrion. On the basis of yields of purified protein from a number of enzyme preparations, we can recover an average of 110 µg of topoisomerase per kilogram of fresh calf thymus. By assuming a native molecular weight for the mitochondrial enzyme of 145 000 (TopoD), we can estimate the number of topoisomerase molecules in the mammalian mitochondrion. For example, in mouse liver cells, there are approximately 8.7 × 10⁶ mitochondria per gram of tissue (Shelton et al., 1953). Assuming a comparable number of mitochondria per gram of calf thymus, it can be calculated that a mammalian mitochondrion possesses about five topoisomerase molecules. The estimates for the number of mtDNA molecules occurring in a single rat liver mitochondrion vary from 5 to 10 (Albright et al., 1985). Therefore, it appears that there may be one-half to one TopoD molecule per genome. If the molecular weight of the enzyme is assumed to be 64 000 (TopoM), there will be one to two TopoM molecules per genome. This is a minimum figure since determination of the amount of topoisomerase in the earliest fractions, e.g., pure mitochondria or PEG supernatant, was not possible due to the nucleases present.

Drug Studies. The mitochondrial topoisomerases from rat liver have been reported to be sensitive to the presence of dimethyl sulfoxide (DMSO). Addition of DMSO seems to interfere with strand sealing since production of nicked DNA and of linear DNA is observed when rat mitochondrial topoisomerase I (Fairfield et al., 1979) and topoisomerase II (F. J. Castora and M. V. Simpson, unpublished results), respectively, are treated with DMSO. Figure 7A shows the results of running topoisomerase assay products on an agarose gel containing 1 µg/ml ethidium bromide. Under these conditions, relaxed form I, which migrates rapidly, is readily separated from nicked form II pBR322. The results were the following.

(1) Nuclear topoisomerase is uninhibited, and possibly even stimulated, by up to 5% DMSO (lanes c and d); at higher concentrations, the enzyme becomes inhibited, 20% DMSO causing approximately 50% inhibition (lane f).

(2) Inhibition of nuclear topoisomerase is not accompanied by an increase in nicked circular DNA (cf. lanes f and a).

(3) The mitochondrial topoisomerase is inhibited by low concentrations of DMSO, 5% decreasing relaxation by about 60% (lane j), with essentially total loss of activity at 20% DMSO (lane l).

(4) The mitochondrial enzyme produces an increase in nicked DNA as relaxation is decreased (cf. lanes i–l and g).

The results with the mitochondrial enzyme suggest that an enzyme–DNA intermediate may be accumulating under the influence of DMSO. This intermediate may be unable to resell the enzyme-bridged DNA break which ultimately leads to the observation of nicked DNA. The absence of open circular DNA in the nuclear assays, even those that show inhibition by DMSO, suggests that the drug–topoisomerase interaction does not lead to trapping or disruption of intermediates but, in fact, may limit binding of the topoisomerase to DNA.

Another drug which has been reported to differentiate between nuclear and mitochondrial topoisomerases in rat is the trypanocidal drug Berenil (Fairfield et al., 1979). Figure 7B shows the effects of this drug on both nuclear and mitochondrial calf thymus topoisomerase. As seen in these densitometric traces, the nuclear enzyme is essentially unaffected by 5–50 µM Berenil. On the other hand, an equivalent level of mitochondrial enzyme is reduced 37% at 5 µM Berenil and 63% at 50 µM Berenil.

Several inhibitors of the bacterial type II topoisomerase (DNA gyrase) were tested for effects on the mitochondrial enzyme. Nalidixic acid and oxolinic acids, at concentrations up
increasing amounts of ATP on the ability of the calf thymus mitochondrial topoisomerase to relax negatively supercoiled DNA. The results (Table III), obtained in the absence of inorganic phosphate, indicate that the sensitivity to ATP inhibition of the mitochondrial enzyme (50% inhibition, ~1.0 mM ATP, with essentially complete inhibition by 5 mM ATP) is approximately equivalent to that reported for the leukemia mitochondrial enzyme (Castora & Kelly, 1986). ATP inhibition of nuclear and mitochondrial type I topoisomerases appears to be a general phenomenon, observable with enzymes from normal or transformed cells.

**DISCUSSION**

A type I DNA topoisomerase has been purified from calf thymus mitochondria. Earlier studies with human mitochondria (Castora & Lazarus, 1984; Castora et al., 1985; Castora & Kelly, 1986) relied on solubilizing the enzyme from intact mitoplasts with a nonionic detergent, but the present study reports the isolation of the calf thymus enzyme from an inner mitochondrial membrane fraction. This change in procedure has resulted in the greater than 4000-fold purification of relatively large amounts of topoisomerase with improved characteristics of stability and homogeneity which have allowed considerable characterization of several biochemical and biophysical properties.

Because of the occurrence of large amounts of topoisomerase outside the mitochondrion, we have attempted to isolate organelles that are as pure as possible. We have prepared clean mitochondria by differential centrifugation and then further eliminated contaminating nuclear or cytoplasmic activities by banding in sucrose and subsequently stripping away the outer mitochondrial membrane with digitonin. The mitoplasts behave morphologically and biochemically as expected. Comparison of our subcellular fractionation procedure and criteria of purity to others in the recent literature (Bertazzoni et al., 1977; Scovassi et al., 1979; Wernette & Kaguni, 1986) indicates that our enzyme is, indeed, located in the mitochondrion.

The enzyme was shown to be capable of relaxing positive as well as negative supercoils in circular DNA. Thus, it has the potential to act as a "swivel" during mammalian mtDNA replication. At present, the in vivo role of the topoisomerase in mitochondrial biogenesis is not firmly established, although inhibitor studies in the rat liver system suggest an involvement of the type I topoisomerase in mtDNA replication and transcription (Fairfield et al., 1985). Our calculations suggest that each mitochondrial genome may have at least one or two topoisomerases allotted to it in order to fulfill these necessary roles.

Much like the type I enzymes from HeLa (Castora & Kelly, 1986; Low & Holden, 1985), human leukemia (Castora & Kelly, 1986), and calf thymus nuclei (Castora & Kelly, 1986) and leukemia cell mitochondria (Castora & Kelly, 1986), the calf mitochondrial topoisomerase was inhibited by physiological

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**Table III: Effects of Drugs and ATP on the Mitochondrial Topoisomerase**

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Inhibition*</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berenil</td>
<td>63</td>
<td>50 μg/mL (10 μM)</td>
</tr>
<tr>
<td>nalidixic acid</td>
<td>0</td>
<td>400 μg/mL (1.6 mM)</td>
</tr>
<tr>
<td>oxolinic acid</td>
<td>0</td>
<td>400 μg/mL (1.6 mM)</td>
</tr>
<tr>
<td>novobiocin</td>
<td>75</td>
<td>200 μg/mL (300 μM)</td>
</tr>
<tr>
<td>DMSO</td>
<td>&gt;95</td>
<td>20% (v/v)</td>
</tr>
<tr>
<td>ATP</td>
<td>50</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

*100% enzyme activity refers to the amount of enzyme necessary to relax all the input DNA in the standard assay.
concentrations of ATP. In fact, a 50% reduction in relaxation was effected by addition of approximately 1.0 mM ATP which is in good agreement with the inhibition by ATP of the leukemia mitochondrial topoisomerase (Castora & Kelly, 1986). More detailed kinetic and mechanistic studies are under way with the purified calf thymus enzymes from both nuclei and mitochonidia in order to elucidate the role played by ATP in regulating topoisomerase activities in the cell. Whenever enzymatic activities occur in different compartments of the cell, e.g., the nucleus and the mitochondrion, it is always of interest to compare various biochemical and biophysical properties of the enzymes in order to determine what similar or distinguishing features they may possess. A detailed analysis of such relationships between the well-characterized nuclear topoisomerase I, which represents about 99% of the total cellular activity, and this new mitochondrial topoisomerase I is already under way. We have found significant differences in thermal stability and molecular weight between the nuclear and mitochondrial topoisomerases, with minor differences in their pH profile and assay temperature. The enzymes also differ in their response to DMSO and Berenil. Part of this comparison will assess immunological aspects of the enzymes and will ultimately require specific antimitochondrial topoisomerase antibodies. The preparation of mitochondrial topoisomerase which we report here yields sufficient quantities of protein to allow antibody production. However, we have initiated such studies using gift antibodies raised against the nuclear type I topoisomerase. Preliminary results with these antibodies indicate that the mitochondrial topoisomerase exhibits a sensitivity approximately half that of the nuclear enzyme (data not shown).

Whether the above differences in physical, biochemical, or immunological behavior are due to differences in molecular weight or to differences in amino acid sequence and epitope structure is a matter of continued research. Although we have attempted to detect a high molecular weight species by denaturing gel electrophoresis of the TopoD peak fractions from gel filtration or glycerol gradient centrifugation in high salt (0.4 M KCl), the only observable bands have been the 78,000- and 63,000-dalton polypeptides. At this point, we have not attempted to detect a high molecular weight species by denaturing gel electrophoresis of the purified enzyme from both nuclei and mitochondria in order to elucidate the role played by ATP in regulating topoisomerase activities in the cell.

ACKNOWLEDGMENTS

We thank Dr. Leroy Liu for his generous gift of antibodies and his helpful discussions. We also thank Philip Rutledge for his help with our electron microscopic analysis.

REFERENCES


