development of this methodology and James J. Crute for useful suggestions.

Registry No. DNA polymerase, 9012-90-2.

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Articles

Escherichia coli DNA Topoisomerase III: Purification and Characterization of a New Type I Enzyme†

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ABSTRACT: A new topoisomerase capable of relaxing negatively supercoiled DNA in *Escherichia coli* has been identified during chromatography on novobiocin–Sepharose. A simple and reproducible purification procedure is described to obtain this enzyme, called topoisomerase III (topo III), in a homogeneous form. The protein is a single polypeptide with a molecular weight of 74000 ± 2000 and is a type I topoisomerase, changing the linking number of DNA circles in steps of one. It is present in deletion strains lacking the *top*A gene and further differs from the well-studied topoisomerase I (ω protein; Eco topo I) in (1) its requirement for K^+ in addition to Mg^{2+} to exhibit optimal activity and (2) its affinity to novobiocin–Sepharose. Positively supercoiled DNA is not relaxed during exposure to the enzyme. Topo III has no ATPase activity, and ATP does not show any discernible effect on the reduction of superhelical turns. The purified topoisomerase has no supercoiling activity and is unaffected by high concentrations of oxolinic acid and novobiocin in the relaxing reaction. Single-stranded DNA and spermidine strongly inhibit the topoisomerase activity.

DNA supercoiling, now recognized as a crucial factor in bacterial gene expression and in prokaryotic chromosome replication (Smith, 1981; Gellert, 1981a,b), is controlled by a ubiquitous and varied class of multifunctional enzymes known as topoisomerases (Wang & Liu, 1979). A change in the linking number (Lk) of closed circular DNA molecules by these enzymes is mediated through transient single-strand breaks (ΔLk = 1; type I enzymes, with no ATP requirement) or transient double-strand breaks (ΔLk = 2; ATP-dependent type II topoisomerases) followed by a strand-passing event (Liu et al., 1980). Hitherto, in *Escherichia coli*, three distinct and well-defined topoisomerase activities have been described. *E. coli* topoisomerase I, familiar in the literature as ω protein, is a 110000-dalton polypeptide (Wang, 1973) encoded by the *top*A gene mapped at 28 min near *cys*B (Sternglanz et al., 1981; Trucksis & Depew, 1981). It is a type I enzyme (Brown & Cozzarelli, 1979) that relaxes negatively supercoiled DNA efficiently but is totally inactive on overwound DNA (Wang, 1971). This enzyme appears to be dispensable for the growth of *E. coli*, but deletion strains lacking *top*A accumulate compensatory gyrase mutations, leading to reduced supercoiling in vivo (DiNardo et al., 1982; Pruss et al., 1982). These secondary mutations seem to modulate DNA gyrase by reducing its content and altering its catalytic properties (Gellert et al., 1982), thus suggesting an important role of topo I in regulation of supercoiling.

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DNA gyrase (topoisomerase II), the best characterized type II enzyme, is essential for viability, DNA replication, and almost every chromosomal function in *E. coli* (Cozzarelli, 1980; Gellert, 1981a,b; Mirkin & Shmerling, 1982; Filutowicz & Jonczyk, 1983). It is unique among topoisomerases in its ability to introduce negative supercoils into DNA at the expense of ATP hydrolysis, and it also catalyzes a weak relaxing activity in the absence of ATP. Gyrase is a tetrameric enzyme composed of two *gyr*A (105 kDa)[1] and two *gyr*B (95 kDa) subunits that are respectively the targets of nalidixic or oxolinic acid and novobiocin or coumermycin (Cozzarelli, 1980). In the reactions catalyzed by DNA gyrase, *gyr*A and *gyr*B proteins contribute respectively to the nicking-closing and ATP-binding functions, and the two separate unlinked genes encoding these subunits seem to be finely regulated according to the cell's need to adjust the critical DNA supercoiling level (Gellert et al., 1982; Menzel & Gellert, 1983). The second type II topoisomerase in *E. coli*, referred to as topo II′, is related to DNA gyrase (Brown et al., 1979; Gellert et al., 1979). It is made up of the *gyr*A subunit and a 50-kDa fragment of the *gyr*B protein. Relaxation of both positive and negative supercoils is unique to this enzyme among *E. coli* topoisomerases. However, topo II′ lacks the supercoiling activity of gyrase and shows no apparent interaction with ATP or novobiocin.

During the course of DNA gyrase purification on a novobiocin–Sepharose affinity column, our attempts to elute bound *gyr*B subunit with ATP proved unsuccessful. Later, in similar studies, Staudenbauer & Orr (1981) reported an identical observation and showed that desorption of *gyr*B from the affinity matrix actually required a denaturant like 5 M urea. However, starting with a cell-free extract of *E. coli*, we were able to detect a topoisomerase activity capable of relaxing negatively supercoiled DNA in the ATP-eluted fractions from novobiocin–Sepharose. Inability of novobiocin and oxolinic acid to curtail this "new activity", which had reaction requirements different from those of topo I,[1] encouraged us to further characterize this enzyme.

Cozzarelli's group has identified a new *E. coli* topoisomerase that they have designated as topo III[1] (Pastorcic, 1982; Dean et al., 1982). From the summary of topo III properties reported recently by Dean et al. (1982), it is clear that this type I enzyme of 75 kDa is unrelated to ω protein and is insensitive to inhibitors of DNA gyrase. The *E. coli* topoisomerase we report in this paper seems to be identical with topo III in its molecular size and enzymatic characteristics. Therefore, we would like to retain the nomenclature employed earlier and use the term topo III to describe the enzyme characterized in this simultaneous investigation. We report here the purification and distinguishing features of this new type I topoisomerase from *E. coli*.

Materials and Methods

Growth of E. coli Cells. Cultures (100 L) of *E. coli* H560 (*pol*A$^-$ *end*A$^-$) in LB medium were routinely grown to late exponential phase in a fermentor. The cells were collected by centrifugation, suspended in 50 mM Tris-HCl (pH 7.5)/2 mM β-mercaptoethanol/1 mM EDTA/10% sucrose, and kept frozen at −70 °C. *E. coli* DM700 [Δ(*top*A *cys*B)], kindly provided by Dr. R. Sternglanz, was grown similarly in the presence of cysteine at 40 μg/mL (Sternglanz et al., 1981).

Chemicals. Sepharose 4B, novobiocin, ATP, and lysozyme were purchased from Sigma Chemical Co. The other products used were single-stranded calf thymus DNA–agarose (BRL), polyacrylamide gel electrophoresis reagents (Bio-Rad), agarose (FMC Corp.), and aquacide (Calbiochem). Double-stranded DNA–cellulose was prepared according to Alberts & Herrick (1971).

Preparation of Novobiocin–Sepharose. Epoxy-activated Sepharose was prepared by reacting Sepharose 4B CL-200 with bis(oxirane) (diglicydyl ether from Sigma) as described by Sundberg & Porath (1974). The activated Sepharose is also available commercially from Pharmacia. Mixing of epoxy-activated Sepharose with novobiocin and subsequent washing protocols to get novobiocin–Sepharose were carried out as detailed by Staudenbauer & Orr (1981).

Enzymes. DNA gyrase was purified by modification of a published procedure (Staudenbauer & Orr, 1981). Eco topo I (ω protein) was a gift from Dr. J. C. Wang (Harvard University). Preparation of eukaryotic nuclear extract, rich in DNA untwisting enzyme, has been described (Lockshon & Morris, 1983).

Plasmid DNA. Plasmid pBR322 was grown in *E. coli* RRI and purified by CsCl/ethidium bromide equilibrium centrifugation (Maniatis et al., 1982). To prepare single topoisomers of pUC8 (Vieira & Messing, 1982), the plasmid was partially relaxed with nuclear extract. The topoisomers were separated in a horizontal 2% agarose gel containing chloroquine phosphate at 1 μg/mL. A marker lane was stained with ethidium bromide and used to locate the bands in the rest of the unstained gel. These were excised and embedded in a slab of 1.5% Sea Plaque agarose and run in the second dimension to resolve the minor positive supercoils present in the relaxed preparation (Lockshon & Morris, 1983). The single band visualized after ethidium bromide staining was removed; after the agarose was melted at 65 °C, the DNA was purified on an Elutip-d column (Schleicher & Schuell) followed by ethanol precipitation.

Topoisomerase Assay. Relaxation of negative supercoils was assayed in a standard reaction mixture (30–50 μL) containing 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 4 mM magnesium acetate, 80 mM KCl, BSA at 50 μg/mL, 0.2% dimethyl sulfoxide (v/v), and 0.4 μg of native pBR322 at 37 °C. The reaction was stopped with 5 μL of a solution containing 4% sarkosyl/1% EDTA. The samples were mixed with bromophenol blue (0.25 mg/mL), which was dissolved in 1% molten agarose and loaded onto a 1.3% horizontal agarose gel (0.6 × 14.5 × 18.8 cm) in 50 mM Tris–phosphate and 1 mM EDTA (pH 7.5). After electrophoresis at 80 V for 14 h, the gels were stained with ethidium bromide (2 μg/mL) and photographed under UV illumination. The relaxation of positively supercoiled DNA was examined by employing fully relaxed pBR322 (0.5 μg) and adding ethidium bromide (0.1–0.3 μg/mL) to the reaction mixture (Jawaherian et al., 1982). Prior to electrophoresis, the samples were extracted with butanol to remove the intercalating agent (Brown et al., 1979).

ATPase Assay. [γ-^{32}P]ATP from New England Nuclear (1000 mCi/μmol) was purified on a DEAE HCO_3^- column to remove contaminating labeled inorganic phosphate (Palmer & Avruch, 1981). The reaction components (20-μL total volume) were the same as in the topoisomerase assay except for reduced concentrations of Mg^{2+} (2 mM) and KCl (50 mM), added 0.8 mM [γ-^{32}P]ATP (1 μCi), and omitted

[1] Abbreviations: topo III, topoisomerase III from *E. coli*; ELISA, enzyme-linked immunoadsorbent assay; PEG, poly(ethylene glycol); Eco topo I, *E. coli* topoisomerase I; PEI–cellulose, poly(ethylenimine)–cellulose; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; ATPase, adenosinetriphosphatase; kDa, kilodaltons; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

plasmid DNA. After incubation for 40 min at 37 °C, the reaction mixture was diluted 5-fold with water, and 2-μL aliquots were spotted on PEI–cellulose thin-layer strips (VWR Scientific), which were previously spotted with a mixture of 5 mM ATP and ADP markers. The chromatogram was developed in 1.0 M formic acid/0.5 M LiCl. ^{32}P-Labeled standards and reaction products were visualized by autoradiography. Radioactive areas were cut out of the chromatograms and quantitated by liquid scintillation counting.

Distribution of Enzyme Activity on Electrophoretic Gels. Nondenaturing polyacrylamide gel electrophoresis of purified topoisomerase (10 μg) in tube gels of 8% polyacrylamide (pH 8.3) was performed at 4 °C according to Davis (1964). Protein samples were loaded after a prerun of 1 h. The gel was sectioned into 1-cm pieces and homogenized in 200 μL of 50 mM Tris-HCl (pH 7.5)/5 mM β-mercaptoethanol/BSA (100 μg/mL). After overnight extraction, the gel pieces were removed by centrifugation, and the supernatants were assayed for enzyme activity. A gel run in parallel was stained with Coomassie blue G-250 (Reisner et al., 1975) for rapid detection of the protein band.

Miscellaneous Procedures. SDS gel electrophoresis was carried out by the procedure of Laemli (1970). Protein concentration was determined by the modified method of Bradford (Read & Northcote, 1981). Monoclonal antibodies against the purified topo III from *E. coli* H_{560} were obtained by the procedure developed by Kohler & Milstein (1975) for producing hybrid cell lines that synthesize antibodies. Briefly, the steps involved were immunization of male BALB/C mice with purified enzyme, detection of antibodies in mouse serum by ELISA and immunoblots, and fusion of splenic lymphocytes with the SP2/0 nonsecretor line by standard procedures (Oi & Herzenberg, 1980; Kennett et al., 1978), followed by cloning and subcloning of stable hybrid cell lines.

Results

Purification of E. coli Topo III. All steps were carried out at 0–4 °C, and the purification was completed in less than 4 days; centrifugation routinely was at 13 000 rpm in GSA Sorvall rotor. Buffers employed were KPEG [30 mM potassium phosphate (pH 7.6)/2 mM β-mercaptoethanol/1 mM EDTA/9% ethylene glycol (v/v)] in steps 1 and 2 and TELS [20 mM Tris-HCl (pH 7.6)/2 mM β-mercaptoethanol/1 mM EDTA/0.1 M NaCl] in steps 3 and 4.

(*Step 1*) *Cell Lysis and* $(NH_4)_2SO_4$ *Precipitation.* Frozen cells (250 g) of *E. coli* H_{560} were thawed in a water bath at room temperature and chilled to 0 °C. The cell suspension was adjusted to 0.3 M NaCl and lysozyme (0.3 mg/mL) added (Bouche et al., 1975). After a gentle mixing followed by incubation for 1 h at 0 °C, the extract was made 5 mM in magnesium acetate and centrifuged for 40 min. Precooled solid $(NH_4)_2SO_4$ was added in small amounts to the cell-free extract to achieve 80% saturation, and the suspension was stirred for 40 min. The precipitate obtained after 20-min centrifugation was dissolved in KPEG buffer and dialyzed overnight.

(*Step 2*) *Affinity Chromatography on Novobiocin–Sepharose.* The affinity matrix (60-mL bed volume) was equilibrated with KPEG buffer. In view of the concentrated and viscous nature of the dialyzed fraction from step 1, passage of it through novobiocin–Sepharose in a column proved to be slow and cumbersome, even with the aid of a peristaltic pump. Therefore, the diluted $(NH_4)_2SO_4$ fraction (2-fold with KPEG buffer) was added to a slurry of equilibrated affinity matrix in nalgene centrifugation bottles and gently mixed by the tilting action of a gyratory mixer for 4 h in the cold. This batchwise procedure has worked well in achieving a higher yield and is helpful in removing most of the unbound proteins by decantation, followed by 1 or 2 washings of Sepharose before packing it into a column. Washing of the column with KPEG buffer was continued until the effluent had an A_{280} of less than 0.05. A slow elution with 5 mM ATP in KPEG buffer (2.5 bed volumes) was performed to desorb topo III along with at least five other proteins (as evidenced by SDS gel electrophoresis). This ATP eluate was dialyzed against TELS buffer (without 0.1 M NaCl) to remove ATP and concentrated in aquacide IIa. Regeneration of novobiocin–Sepharose was achieved by washing with 5 M urea and equilibrating buffer for further use.

(*Step 3*) *Chromatography on Single-Stranded DNA–Agarose.* The ATP eluate from step 2 was adjusted to 0.1 M NaCl and applied to a single-stranded DNA–agarose column (25-mL bed volume) equilibrated with TELS buffer. Subsequently, washings with equilibrating buffer (2–3 bed volumes) and 0.7 M NaCl in TELS were performed until the absorbance at 280 nm was zero. The enzyme was eluted with 3 M NaCl in TELS buffer. Removal of salt and concentration of protein could be simultaneously achieved by dialysis of this fraction against TELS buffer (without 0.1 M NaCl) containing 5% PEG (PEG-6000 from Dow Chemical Co.).

(*Step 4*) *Chromatography on Double-Stranded DNA–Cellulose.* Further purification of topo III from step 3 was often necessary to remove a minor contaminating protein of 98 kDa (less than 10% of total as judged by SDS gel electrophoresis). For this purpose, enzyme from step 3 in TELS buffer was loaded onto an equilibrated double-stranded DNA–cellulose column (8–10-mL bed volume), followed by washing with TELS buffer and then with 0.4 M NaCl in the same buffer. The topoisomerase activity was recovered by elution with 1.5 M NaCl. After dialysis and concentration as described in step 3, the enzyme fraction was made 30% in glycerol (w/v) and stored at −20 °C. Topo III activity remained stable for 6 months under these conditions. The enzyme seemed to be more stable in the presence of the PEG used in concentration steps, but for the purpose of polyacrylamide gel electrophoresis and protein estimation (Lowry et al., 1951), other methods of concentration (aquacide, TCA precipitation, etc.) are recommended in view of the precipitation of PEG in acidic solutions. A typical yield of 180 μg of purified enzyme from 250 g of *E. coli* cells resulted. The progress of topo III purification was not quantitated due to the presence of many relaxing activities in the $(NH_4)_2SO_4$ fraction and a weak DNase activity recovered in the novobiocin–Sepharose step. The homogeneous enzyme from step 4 was highly active, with 10–20 ng showing detectable relaxing activity in the assay employed.

Purity. Electrophoresis of the purified enzyme in presence of 0.1% SDS and 5% mercaptoethanol (Laemli, 1970) revealed a single protein species (Figure 1b) with an estimated molecular weight of 74 000 ± 2000. This preparation migrated as a single staining band (relative mobility to the tracking dye = 0.46) on nondenaturing gels (pH 8.3) and was coincident with the topoisomerase activity (data not shown, see Materials and Methods for experimental details). The protein fraction from single-stranded DNA–agarose (step 3) was often homogeneous, and further purification on DNA–cellulose was not necessary. A minor protein band corresponding to 63 kDa was sometimes observed in eluates from single-stranded DNA–agarose, and it copurified with the prominent 74-kDa band on DNA–cellulose (step 4). This minor species was also present in ATP eluates of step 2 and is recognized by an anti

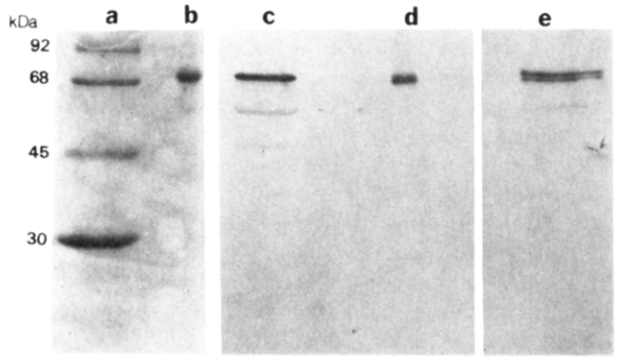

FIGURE 1: SDS gel electrophoresis of purified topoisomerase and immunological evidence for the existence of topo III in *E. coli* DM700. Following electrophoresis in a SDS slab gel (11% acrylamide), the resolved proteins were transferred electrophoretically on to a nitrocellulose sheet (Towbin et al., 1979). The portion of nitrocellulose with lanes a and b was stained with 0.1% amido black and rapidly destained (Burnette, 1981): (a) molecular weight standards of phosphorylase *b*, BSA, ovalbumin, and carbonic anhydrase in the order of decreasing size; (b) purified topo III (5 μg). Transfer of proteins onto nitrocellulose was complete, and no other bands were detected in separate gels stained with Coomassie blue. For demonstrating the presence of topo III antigen in *E. coli* DM700, the cell-free extract from this ω-deletion strain was subjected to purification on novobiocin–Sepharose as described under Results. SDS gel electrophoresis of ATP eluates (20 μg of protein; step 2) from *E. coli* H_{560} and DM700 along with purified enzyme (1 μg) were carried out in panels c–e and blotted. Immunological detection of topo III on the nitrocellulose blot was performed by standard procedures (Towbin et al., 1979; Tsang et al., 1983). The blot was incubated with culture supernatant from a hybridoma raised against topo III from *E. coli* H_{560} (Materials and Methods). Color reaction with 4-chloronaphthol followed exposure of the nitrocellulose strip to antimouse IgG labeled with horseradish peroxidase and further washings. Protein samples in this panel were (c) ATP eluate from *E. coli* H_{560}, (d) purified topo III (*E. coli* H_{560}), and (e) ATP eluate from *E. coli* DM700.

topo III monoclonal antibody (Figure 1c); therefore, it may be a product generated by proteolysis during purification.

Topo III is an enzyme. The molar ratio of protein to DNA molecules relaxed in a standard assay was calculated to be 1:24, thus, demonstrating the catalytic action of the topoisomerase (Kung & Wang, 1977).

Ionic Requirements. No topoisomerase activity was evident in the absence of Mg^{2+}; even in the presence of 150 mM K^+ (Figure 2k). Under the most suitable ionic conditions (2–4 mM Mg^{2+}, 1 mM EDTA) for the activity of Eco topo I (Figure 2b) (Wang, 1971, 1974; Carlson & Wang, 1974), topo III showed very little relaxation (Figure 2c). Addition of KCl in the range 10–150 mM led to a dramatic enhancement of enzyme action; the optimal ionic condition being 4 mM Mg^{2+} and 100 mM KCl (Figure 2d–i). Among the various monovalent cations tested (K^+, Na^+, Li^+, and NH_4^+, at 80 mM), with constant 4 mM Mg^{2+}, K^+ addition resulted in maximum activation, the others being less than 30% effective compared to K^+ (data not shown). High Mg^{2+} (20 mM) alone marginally stimulated the reaction (Figure 2j). Topo III exhibited higher activity in reactions incubated at a temperature range of 40–50 °C compared to the typical 37 °C employed in all our experiments. At 50 °C, we found that topo III was active with 3 mM Mg^{2+} alone and the enzyme showed processive kinetic behavior.

Effect of Gyrase Inhibitors and Other Compounds. Oxolinic acid (Figure 3j,k) and coumarin antibiotics (novobiocin, Figure 3f–h; coumermycin, Figure 3i) exerted no deleterious effect even at 10–200-fold higher concentrations than those required to inhibit *E. coli* DNA gyrase (Sugino et al., 1977; Gellert et al., 1976). Single-stranded DNA (Figure 3d) is a potent inhibitor of topo III. Addition of ATP and *N*-ethylmaleimide (lanes l and c, respectively, of Figure 3) did not show any effect. Spermidine (Figure 3e) curtailed the relaxing activity to a significant extent.

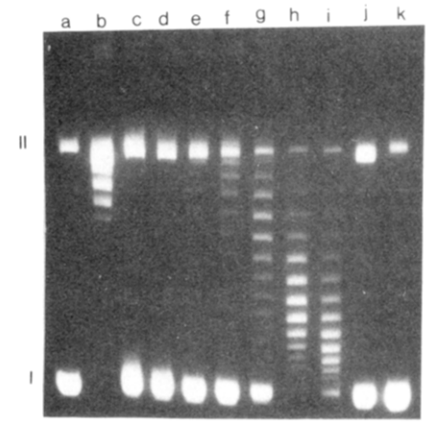

FIGURE 2: Potassium ion requirement for optimal topo III activity. Removal of negative supercoils was measured in a reaction mixture containing 4 mM Mg^{2+} and 1 mM EDTA (except lanes j and k). KCl was added at 10, 25, 50, 75, 100, and 150 mM to the assay mixtures (lanes d–i, respectively), and none was added to c. Lane j had 20 mM Mg^{2+} alone, and lane k had 150 mM KCl alone. No enzyme was added to lane a. Plasmid BR322 DNA (0.9 μg) was incubated with 50 ng of Eco topo I (b) or 0.15 μg of topo III (c–k) at 37 °C for 30 min. Note that in the substrate employed (lane a), DNA moving in the region marked II is a mixture of nicked circles and supercoiled dimer species. Details of agarose gel electrophoresis are described under Materials and Methods.

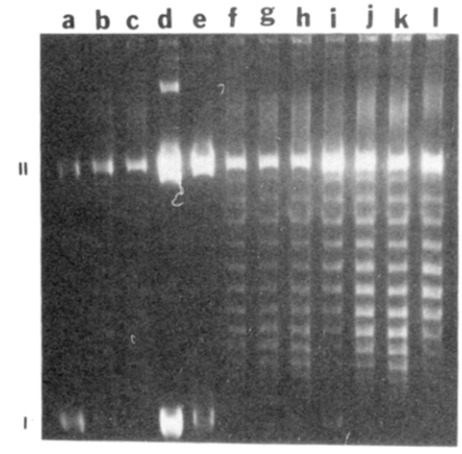

FIGURE 3: Effect of miscellaneous compounds on activity. Topoisomerase reactions were conducted under standard assay conditions (4 mM Mg^{2+}, 80 mM KCl) with 0.2 μg of topo III as described under Materials and Methods: (a) control DNA; (b) complete reaction. Compounds added to the complete reaction were (c) *N*-ethylmaleimide (10 mM) and no dithiothreitol added, (d) fd single-strand DNA circles (60 ng), (e) spermidine (1.5 mM), (f) novobiocin (NOV) (10 μM), (g) NOV (150 μM), (h) NOV (450 μM), (i) coumermycin (50 μg/mL), (j) oxolinic acid (160 μM), (k) oxolinic acid (640 μM), and (l) ATP (1 mM). The extra band near the top of gel in lane d represents the added fd DNA.

Other Catalytic Activities. Topo III, even at high protein concentrations, was totally inert on positively supercoiled DNA. The ethidium bromide present in the reaction mixture to induce positive supercoils (Materials and Methods) did not seem to be inhibitory, since the enzyme was able to relax negatively supercoiled DNA in presence of intercalator at the same concentration (data not shown). No supercoiling activity was detected with added ATP and relaxed DNA as substrate in standard ionic medium or under gyrase assay conditions (Sugino et al., 1977).

ATPase. Binding of topo III to novobiocin–Sepharose and its selective elution with ATP prompted us to check the as-

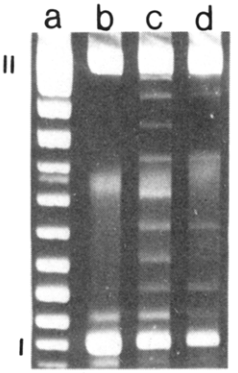

FIGURE 4: *E. coli* topo III relaxes DNA rings in steps of one. By the details described under Materials and Methods, a topoisomer with a unique linking number was isolated from the relaxed plasmid population shown in lane a. Incubation of this topoisomer with topo III (standard reaction mixture) or *E. coli* DNA gyrase in a reaction medium containing 6 mM Mg^{2+}/30 mM KCl/1 mM spermidine was carried out. (a) pUC 8 DNA with polydisperse linking number; (b) purified topoisomer, no enzyme added; (c) reaction with topo III; (d) DNA gyrase reaction.

sociation of ATPase activity with this protein. However, the purified enzyme did not catalyze ATP hydrolysis [less than 0.2 fmol of ATP hydrolyzed min^{-1} (μg of enzyme)$^{-1}$] either by itself or in the presence of (1) single-stranded DNA or native calf thymus DNA or (2) relaxed or supercoiled plasmid DNA.

Topo III Is a Type I Topoisomerase. The unit change in linking number is the best operational distinction between the two classes of topoisomerases. Type II topoisomerases such as DNA gyrase act by making a transient double-strand scission followed by passage of another segment of the double helix through the break, thus changing the linking number in steps of two. Type I enzymes change the linking number in increments of one by means of transient single-strand breaks. We have characterized topo III in this respect by incubating it with a single topoisomer of pUC8 (Figure 4b). It is clear that the product of the topo III reaction (Figure 4c) contains all the topoisomers found in the population from which the unique topoisomer was purified (Figure 4a). Note that when the same single topoisomer was treated with DNA gyrase (Figure 4d), a series of less supercoiled topoisomers, which differ in linking number by increments of two, were produced. From the pattern of bands generated by topo III, we conclude that it is a type I topoisomerase. The conclusion reached from this experiment is clear, in spite of the presence of a contaminating second topoisomer in minor amounts (Figure 4b).

Evidence for the Existence of Topo III in an ω Protein Deficient E. coli Strain. Since ω protein is a 110-kDa entity and is similar to topo III in several properties (e.g., type I mechanism and insensitivity to DNA gyrase inhibitors), it is conceivable that proteolysis of ω protein in cell-free extracts could possibly generate the smaller topo III. To test this possibility, we used *E. coli* DM700, a strain that lacks topo I by the virtue of a *top*A gene deletion (Sternglanz et al., 1981). Two approaches were followed to verify the separate identity of topo III. First, we obtained immunological evidence for the presence of topo III in the DM700 strain (Figure 1c–e). Cell-free extracts from *E. coli* H_{560} (wild-type strain) and DM700 were partially purified on novobiocin–Sepharose (step 2), and the resulting protein fractions were analyzed by the procedures described in the legend to Figure 1. It is clear from the immunoblot that anti topo III monoclonal antibodies recognized an antigen in the ATP eluate from DM700 (a pair of polypeptides of 77 and 75 kDa; Figure 1e) comigrating with the purified H_{560} enzyme (Figure 1d) and the corresponding protein in the ATP eluate of *E. coli* H_{560} (Figure 1c).

Table I: Eco Topo I Does Not Cross-React with Anti Topo III Antibodies[a]

antigen tested	amount of antigen coated to PVC plates (ng)	ELISA (A_{405})
purified topo III from *E. coli* H_{560}	20	0.523
purified topo III from *E. coli* DM700 Δ(*top*A *cys*B)	20	0.493
	40	0.827
Eco topo I	50	0.000
	200	0.000

[a] ELISAs were performed in antigen-coated polystyrene microtiter plates by standard procedures (Carroll & Stollar, 1982). Incubations with a mixture of monoclonal antibodies specific to topo III were followed by exposure to antimouse Ig-alkaline phosphatase conjugate and color reaction. The yellow color generated was measured by reading the plates with a Dynatech micro-ELISA reader.

Further proof was derived by the complete purification of topo III from the DM700 strain. The cell-free extract from this ω-deficient strain was carried through all the purification steps in a manner identical with that adopted for the isolation of topo III from *E. coli* H_{560}. The protein fraction from the purification step 3 indeed contained a topoisomerase activity having similar properties described for topo III with respect to the K^+ ion requirement and inhibition by single-stranded DNA. On SDS gels, the DM700 preparation yielded a major 75-kDa protein band (data not shown). In the above two experiments involving *E. coli* DM700, the bacterial population used was devoid of any contamination; this was checked by testing the cysteine requirement of the DM700 strain (Sternglanz et al., 1981). The data presented in Table I, showing the failure of Eco topo I to cross-react with monoclonal antibodies directed against topo III, provides an additional piece of evidence strongly suggesting the nonhomology and dissimilarity between these two enzymes with similar function in *E. coli*. Interaction of topo III from DM700 with the antibodies, observed in ELISA (Table I), is consistent with the findings of the immunoblot (Figure 1e).

Lack of Eco Topo I Binding to Novobiocin–Sepharose. Since a broad $(NH_4)_2SO_4$ fraction from *E. coli* H_{560} was employed in routine purification, chances of topo I (ω protein) binding to novobiocin–Sepharose and its further purification in steps 3 and 4 were considered likely, particularly in view of the routine usage of single-stranded DNA–agarose and DNA–cellulose in isolation of Eco topo I. This possibility was examined by applying 1 μg of purified Eco topo I to novobiocin–Sepharose (2 mL) in KPEG buffer (step 2, see purification procedure). Topo I was completely excluded from this affinity matrix, and no enzyme activity was recovered in the ATP elution procedure used to desorb topo III.

Discussion

Studies reported in this paper provide strong evidence for the presence of a new type I topoisomerase in *E. coli*, thus making a significant addition to the list of three enzymes (DNA gyrase, topo I, and topo II′) already known in this organism. A simple and rapid isolation procedure has been devised to purify this enzyme to an apparent homogeneous state. Topo III activity was found to reside in a 74-kDa species; its tendency to undergo degradation to 63 kDa and

Table II: Reduction of Superhelical Turns by *E. coli* Topoisomerases: A Comparison[a]

property	topo I (ω)	topo II (gyrase)	topo II′	topo III
subunits, M_r (in kDa)	monomer, 110	*gyr*A, 105 *gyr*B, 95	*gyr*A, 105 *gyr*B fragment, 50	monomer, 75
classification (type)	I	II	II	I
ability to remove positive supercoils	no	yes, only in the presence of β,γ-imido-ATP (Gellert et al., 1981)	yes	no
removal of negative supercoils–effect of various compounds				
optical ionic medium (kinetic behavior)	2–3 mM Mg^{2+}, 0 mM KCl (processive); 2–3 mM Mg^{2+}, 50 mM KCl (distributive)	7.5 mM Mg^{2+}, 25 mM KCl, 2 mM sperimidine (?)	same as gyrase (processive?)	4 mM Mg^{2+}, 80 mM KCl (distributive)
ATP	NE	I	NE	NE
novobiocin	NE	S[b]	NE	NE
oxolinic acid	NE	I	I	NE
single-stranded DNA	I	NE	NE	I
spermidine	I	S	S	I
N-ethylmaleimide	NE	I	I	NE

[a] Abbreviations: NE, no effect; S, stimulation; I, inhibition. [b] We have observed that novobiocin (>80 μM) causes a marginal stimulation of relaxation by DNA gyrase.

smaller bands was noted. It is not known whether the degraded forms retain enzymatic activity. Affinity chromatography on novobiocin–Sepharose together with ATP elution, during which the identity of the topo III came to light, are the key factors in the purification protocol employed. The nature of the interaction of topo III with immobilized novobiocin is not clear; the decreased binding of enzyme to the affinity column in the presence of 0.15–0.30 M salt suggested a weak affinity of this protein to novobiocin–Sepharose, compared to the tight binding observed for *gyr*B subunit (Staudenbauer & Orr, 1981). The failure of the antibiotic (0.45 mM) to inhibit the topoisomerase and the lack of ATPase activity in topo III protein are also of note.

Despite the two unrelated isolation protocols employed by us and the research group led by Dr. Cozzarelli (Pastorcic, 1982) to purify topo III, the enzymatic characteristics recorded are in complete agreement. Further, our preparation and one kindly provided by Dr. Cozzarelli were compared by the immunological criteria described in the legend for Figure 1 and were found to be identical (data not shown). Our finding of topo III in a deletion strain and the insensitivity of the enzyme to ATP and DNA gyrase inhibitors are consistent with the report of Dean et al. (1982).

The requirement for Mg^{2+} is universally exhibited by all prokaryotic topoisomerases. Eco topo I, a typical representative of this category, is optimally active with 2–3 mM Mg^{2+} ions alone. At low ratios of enzyme to substrate, the reaction is processive under these conditions, generating a fraction of almost completely relaxed circles along with a portion of the substrate untouched; addition of monovalent cations in the range of 20–50 mM causes the enzyme to follow distributive kinetics, wherein a gradual reduction of superhelical turns in the whole plasmid population results (Wang, 1973; Liu & Wang, 1979). The presence of monovalent cations at higher concentrations leads to inhibition of topo I activity (Wang, 1971, 1974; Burrington & Morgan, 1976). At the suboptimal temperature of 37 °C we employed, topo III reaction was weak and apparently processive in the presence of only Mg^{2+}. Addition of K^+ ions to this assay medium resulted in enhanced relaxation with distributive kinetics. Inhibition of topo III activity was evident at K^+ concentrations higher than 100 mM. Thus, apart from the marked stimulation of the topo III reaction by K^+ ions in our assay conditions, the influence of monovalent cations on the kinetic course of the reaction for both ω protein and topo III seems to be similar.

From the properties investigated, it is clear that several distinguishing features exist between the known topoisomerases and topo III in *E. coli* (Table II). Topo III seems to be present at a roughly equivalent level to ω protein (yield of 100 μg of protein from 100 g of cells; Carlson & Wang, 1974) in *E. coli*. Further, topo I and topo III share several commonalities in terms of enzymatic characteristics and catalytic mechanism. They are similar in that (a) their action on negatively supercoiled DNA does not require ATP and neither reacts with positively supercoiled DNA, thus showing an identical sense of superhelicity, (b) the inhibitory effect of spermidine on topo I (our unpublished results) is true for topo III as well, and (c) the insensitivity to sulfhydryl blockers is a common property (Wang, 1981). Inhibition of topo I by single-stranded DNA (Wang, 1971) and its type I mechanism are the two other parallel catalytic characteristics shared by topo III. Inhibition by single-stranded DNA and the preference for a negatively supercoiled substrate in the topo I reaction have led to the suggestion that the enzyme requires single-stranded regions of DNA for activity, the transient existence of such regions being favored by negative but not positive supercoiling (Gellert, 1981a). Supportive of this postulate is the correlation found between the specific strand cleavage pattern induced by topo I of *Haemophilus gallinarium* and the un-base-paired sites cleavable by S_1 nuclease on negatively supercoiled substrate (Shishido et al., 1983). Topo III interacts with single-stranded DNA to form a complex, and the addition of a denaturant to this ensemble results in the cleavage of the DNA, leaving the enzyme attached to the broken end (Dean et al., 1982); the properties of such a complex have been studied in detail for ω protein (Depew et al., 1978). These results suggest that topo I and topo III might have similar DNA recognition patterns associated with their type I reaction mechanisms. However, it is pertinent that the type II topoisomerases from *Drosophila melanogaster* (Osheroff et al., 1983) and the T4 phage enzyme (Kreuzer & Jongeneel, 1983) are also inhibited by single-stranded DNA, unlike DNA gyrase.

We have presented unambiguous immunological evidence for the existence of topo III in a ω protein deficient strain of *E. coli*. Enzyme activity similar to topo III in *E. coli* DM700 was also demonstrated by actual protein purification. These data rule out the possibility of topo III being a product of topo I proteolysis. Topo I shows no antigenic cross-reactivity with topo III and no binding to novobiocin–Sepharose. These

features further distinguish the two type I enzymes in *E. coli*. Topo III differs from DNA gyrase in having no supercoiling activity, lacking inhibition by oxolinic acid/novobiocin, and having a type I mechanism. Sensitivity to oxolinic acid and relaxing activity on positively supercoiled DNA by topo II′ are the properties not shared by the new enzyme.

On the basis of its in vitro properties, topo III can be expected to participate along with topo I in regulating supercoiling of the *E. coli* chromosome. It would be interesting to understand the necessity for the apparently redundant activities of topo III and ω protein in the cell. By virtue of the apparent binding sites for ATP and novobiocin associated with topo III, the enzyme could have an additional function in vivo besides a simple role in relaxing supercoils. Definition of the essentiality of topo III and its role in vivo await further studies.

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Registry No. Topo III, 80449-01-0; spermidine, 124-20-9.

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Further Studies on Calf Thymus DNA Polymerase δ Purified to Homogeneity by a New Procedure†

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ABSTRACT: DNA polymerase δ from calf thymus has been purified to apparent homogeneity by a new procedure which utilizes hydrophobic interaction chromatography with phenyl-Sepharose at an early step to separate most of the calcium-dependent protease activity from DNA polymerases δ and α. The purified enzyme migrates as a single protein band on polyacrylamide gel electrophoresis under nondenaturing conditions. The sedimentation coefficient of the enzyme is 7.9 S, and the Stokes radius is 53 Å. A molecular weight of 173K has been calculated for the native enzyme. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the homogeneous enzyme reveals two polypeptides of 125 and 48 kDa. This subunit structure differs from that of DNA polymerase δ prepared by our previous procedure, which was composed of subunits of 60 and 49 kDa [Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 26*, 83–96], suggesting that the 60-kDa polypeptide may have been derived from the 125-kDa polypeptide during enzyme purification, possibly as the result of cleavage of an unusually sensitive peptide bond. DNA polymerse δ is separated from DNA polymerase α by hydrophobic interaction chromatography on phenyl-Sepharose; DNA polymerase δ is eluted at pH 7.2 and DNA polymerase α at pH 8.5. DNA polymerase δ can also be separated from DNA polymerase α by chromatography on hydroxylapatite; DNA polymerase α binds to hydroxylapatite in the presence of 0.5 M KCl, whereas DNA polymerase δ is eluted at 90 mM KCl. DNA polymerase δ is associated with a 3′- to 5′-exonuclease activity, but it is devoid of endonuclease or 5′- to 3′-exonuclease activities. The polymerase activity of DNA polymerase δ is inhibited by aphidicolin, as is the 3′- to 5′-exonuclease activity when the substrate is double-stranded DNA. Aphidicolin does not inhibit exonuclease activity on single-stranded DNA. The coordinated inhibition of both polymerase and exonuclease activities of DNA polymerase δ by aphidicolin is consistent with our observation that both activities reside on the same protein molecule. The present findings are also consistent with the suggestion that the binding of aphidicolin to the enzyme requires the formation of a template-primer–DNA polymerase complex [Huberman, J. A. (1981) *Cell (Cambridge, Mass.) 23*, 647–648].

During the past few years, significant progress has been made on the purification and characterization of high molecular weight species of DNA polymerase from higher eukaryotes (Kornberg, 1980, 1982; Fry, 1982). DNA polymerase α and δ species have been purified to apparent homogeneity from several sources; however, the molecular weights of the native enzymes as well as their subunit composition remain controversial.

A high molecular weight (120K–180K), catalytically active polypeptide has been identified in DNA polymerase α preparations purified from *Drosophila* (Villani et al., 1980; Kaguni et al., 1983) and rat liver (Mechali et al., 1980), whereas no large polypeptide has been found in DNA polymerase α preparation purified from KB cells, rather the peak of polymerase activity on polyacrylamide gel electrophoresis under nondenaturing conditions is found to be associated with a quartet of polypeptides with assigned masses of 70, 65, 59 and 55 kDa[1] (Fisher & Korn, 1977; Filpula et al., 1982). Both high molecular weight and low molecular weight polypeptides have been found to contain polymerase activity in DNA polymerase α species from calf thymus (Holmes et al., 1976; Grummt et al., 1979; Hubscher et al., 1981; Grosse & Krause, 1982) and mouse myeloma (Chen et al., 1979; Karawya & Wilson, 1982).

DNA polymerase δ from rabbit bone marrow has been reported to be a single polypeptide of 122 kDa (Goscin & Byrnes, 1982), whereas we have found that DNA polymerase δ from calf thymus is composed of two subunits of 49 and 60 kDa (Lee et al., 1981). Furthermore, mouse myeloma DNA polymerase α_1, which, like DNA polymerase δ, has an associated 3′- to 5′-exonuclease activity, is composed of subunits of 48 and 52 kDa (Chen et al., 1979).

Proteolysis during enzyme purification has been suggested as a possible explanation for the lack of a large polypeptide in several α polymerases (Banks et al., 1980; Spanos et al., 1981; Grosse & Krauss, 1981). We have been concerned about the possibility that proteolysis during purification of DNA polymerase δ from calf thymus might be responsible for the absence of a high molecular weight subunit in this po-

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[1] Abbreviations: kDa, kilodalton; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

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