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Topoisomerase I from Chicken Erythrocytes: Purification, Characterization, and Detection by a Deoxyribonucleic Acid Binding Assay†

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ABSTRACT: We have purified a deoxyribonucleic acid topoisomerase to near homogeneity from the nuclei of mature chicken erythrocytes. The enzyme relaxes supercoiled DNA in the absence of ATP or Mg^{2+}. It is unable to resolve topologically knotted circular duplex DNA. These properties resemble those of type I eukaryotic topoisomerases capable of breaking and rejoining one strand of duplex DNA at a time. The sedimentation value of the protein is 4.4 S. The molecular weight of the reduced, denatured protein is 100K. After elution from sodium dodecyl sulfate ($NaDodSO_4$) gels and renaturation, topoisomerase activity is found in the band at 100K and in minor bands at 95K, 78K, and 73K. The minor bands are likely to be proteolytic fragments since the M_r 100K protein is cleaved by trypsin to fragments of similar or even smaller size with retention of activity. At KCl concentrations suboptimal for the 100K form, the trypsin cleaved form is severalfold more active than the 100K form. Single-stranded DNA, but not duplex DNA or RNA, inhibits DNA relaxing activity, presumably by forming a covalent complex at the enzyme active site. Preincubation of the enzyme with single-stranded DNA leads to the depletion, in $NaDodSO_4$–polyacrylamide gels, of protein bands corresponding to the 100K topoisomerase, its putative proteolytic fragments, and its tryptic fragments. The reaction which leads to band depletion requires active topoisomerase and conditions where single-stranded DNA inhibits relaxing activity. The band depletion technique provides a convenient assay for the polynucleotide binding activity of topoisomerases and possibly other proteins. The function of the enzyme in the inactive nuclei of mature chicken erythrocytes is unclear. The estimated content of chicken erythrocyte topoisomerase per unit DNA is comparable to that in nuclei active in replication and transcription.

Topoisomerases are enzymes which can interconvert different topological isomers of circular duplex DNA by catalyzing the concerted breaking and rejoining of DNA backbone bonds [for reviews see Champoux (1978a,b), Gellert (1981), Wang & Liu (1979), and Cozzarelli (1980)]. Two types of topoisomerases can be formally distinguished by the mechanism of their breakage–reunion reaction (Liu et al., 1980). Type I topoisomerases act on one strand of duplex DNA at a time while type II enzymes act simultaneously on both strands. In eukaryotes, known type I enzymes do not require ATP (Champoux, 1978a,b; Gellert, 1981; Wang & Liu, 1979; Cozzarelli, 1980) while known type II enzymes do (Liu et al., 1980; Baldi et al., 1980; Hsieh & Brutlag, 1980). Additionally, the two types can be operationally distinguished by the ability of type II but not type I enzymes to produce or resolve catenated forms of covalently closed circular DNA and to remove knots from duplex DNA (Liu et al., 1980, 1981; Baldi et al., 1980; Hsieh & Brutlag, 1980; Mizuuchi et al., 1980; Kreuzer & Cozzarelli, 1980).

Historically, the major type I topoisomerase activity in eukaryotic cells was referred to as the DNA nicking–closing enzyme (also known as untwisting enzyme and relaxing enzyme). The enzyme relaxes supercoiled DNA in the absence of added cofactors and is found in nuclei. The enzyme has been highly purified from several sources (Keller, 1975; Champoux & McConaughy, 1976; Tang, 1978). The reduced, denatured protein consists of a single polypeptide chain of molecular weight (M_r)[1] 60 000–70 000 (60K–70K).

More recently, a type I enzyme designated topoisomerase I has been purified from HeLa cells (Liu & Miller, 1981), wheat germ (Dynan et al., 1981), and *Drosophila* embryos (Wang et al., 1980). The reduced denatured protein is a single polypeptide chain of M_r 100K (HeLa), 111K (wheat germ), or 110K (*Drosophila*). In one of these cases (Liu & Miller, 1981), it has been shown that prevention of proteolysis during isolation and purification of topoisomerase accounts for the finding of higher molecular weight protein. We cannot con-

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[1] Abbreviations: M_r, relative molecular weight; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperzine-*N'*-2-ethanesulfonic acid.

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clude, however, that the ≥100K topoisomerase is the major type I topoisomerase in all cells and that the M_r 60K–70K topoisomerase arises from artifactual proteolysis. Alternative explanations involving in vivo processing of the protein, multiple topoisomerase genes, multiple splicing patterns of a single transcript, or multiple translation products are also possible.

Additional evidence that the ≥100K topoisomerase can be isolated from a wide variety of eukaryotic cells is required before any generalizations can be made. Since the ≥100K enzyme has been isolated from undifferentiated embryonic cells and neoplastic cells, we decided to purify and characterize the type I topoisomerase from differentiated cells. We chose chicken erythrocytes as a source of differentiated cells. Erythrocytes exist as a near homogeneous cell type in chicken blood (Ringertz & Bolund, 1974). They retain their nuclei, unlike mammalian erythrocytes, and possess topoisomerase activity (Bina-Stein et al., 1976). Finally, they offer the potential of minimizing proteolysis artifacts since they are low in protease activity compared to other differentiated cell types (Destree et al., 1975).

Contrary to a recent report of a M_r 62K type I topoisomerase in chicken erythrocytes (Pulleyblank & Ellison, 1982), we find that topoisomerase activity is associated with a major 100K polypeptide and several minor polypeptides of lower molecular weight. The M_r 100K polypeptide is cleaved by trypsin to M_r 64K–68K polypeptides with retention of activity, suggesting that the topoisomerases of M_r <100K in our preparation are the result of proteolysis.

In the course of examining the binding of the enzyme to DNA, we developed a technique which we call "band depletion". The technique is useful for the identification of bands on $NaDodSO_4$–polyacrylamide gels that contain proteins which, like topoisomerases, form a stable complex with polynucleotides.

Experimental Procedures

PM2 phage were grown and purified (Espejo & Canelo, 1968; Espejo et al., 1969), and supercoiled DNA isolated by buoyant CsCl density gradient centrifugation as described (Radloff et al., 1967). P4 DNA was a gift from Leroy Liu, Johns Hopkins University. Chicken erythrocytes were obtained fresh from Springville Laboratories of Roswell Park Memorial Institute, Springville, NY. Topoisomerase relaxation of supercoiled DNA was assayed fluorometrically as described (Kowalski, 1979) with the following modifications. Prior to assay, samples were diluted in 1.0 M KCl, 0.01 M Tris-HCl (pH 7.8), 0.5 mM Na_2EDTA, and 0.005% Triton X-100, and 2 μL of diluted sample was added to an 18-μL reaction mixture containing 1 μg of PM2 DNA in 0.1 M NaCl, 10 mM Hepes (pH 7.8), 0.5 mM Na_2EDTA, and bovine serum albumin (200 μg/mL). The phosphodiesterase solution (Kowalski, 1979) was supplemented with 0.5 mM magnesium acetate. One unit of topoisomerase activity converts 0.1 μg of PM2 DNA to the relaxed form in 10 min at 37 °C. Rapid screening for activity in column fractions was performed by using a fluorescence spot test (Kowalski, 1980). Protein determination was carried out with a fluorescamine assay (Udenfriend et al., 1972). RNA was isolated from mouse liver according to Cox (1968) and was a gift from Nancy Kuhn and William Held, Roswell Park Memorial Institute.

Buffer designations are as follows: citrate buffer is 0.015 M sodium citrate (pH 7.2) and 0.15 M NaCl; STM buffer is 10 mM Tris-HCl (pH 7.1), 3 mM $MgCl_2$, 10 mM NaCl, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Buffer A is 20 mM potassium phosphate (pH 7.0), 0.5 mM PMSF, 1.0 mM Na_2EDTA, and 0.5 mM dithioerythritol (DTE). Since PMSF is hydrolyzed upon storage in aqueous solution at neutral pH ($t_{1/2}$ = 100 min) (Gold, 1967), it was added from a 50 mM stock solution in 2-propanol to the aqueous solutions just prior to their use. Buffer B is 5 mM Tris-HCl, pH 7.4, 35 mM KH_2PO_4, pH 7.5, 0.25 mM DTE, 10 mM EDTA, 5% glycerol, and 0.005% Triton X-100.

Enzyme Purification. All procedures were carried out at 0–4 °C. Chicken blood was cleared of plasma and buffy coat by several washings with 0.9% NaCl followed by centrifugation (2000 rpm in a PR-C) and aspiration of the supernatant (Springville Laboratories). The cell pellet (red cells) was washed once with two pellet volumes of citrate buffer and centrifuged at 5800*g* (6000 rpm, GSA rotor) for 10 min. Nuclei were isolated by the following procedure described by Olins et al. (1976). The cells were lysed by resuspension in two pellet volumes of STM buffer plus 0.5% Nonidet P-40. The resuspended material was stirred gently at 4 °C for 10 min and centrifuged at 5800*g* (10 min) to pellet the nuclei. The resuspension and centrifugation were repeated 3 more times, resulting in an almost white nuclear pellet. The nuclear pellet was then resuspended in buffer A to a volume equal to 10× the pellet volume. An equal volume of buffer A plus 2.0 M KCl was added to lyse the nuclei. The material was then sonicated in order to reduce the viscosity and centrifuged for 15 min at 5800*g* to yield the crude nuclear lysate.

The nuclear lysate was made 0.02% polymin P (Bethesda Research Laboratories) in order to remove DNA. After 30 min, the mixture was centrifuged at 6000 rpm (5800*g*, GSA rotor) in order to pellet the DNA. The supernatant is fraction I.

Fraction I was loaded onto a 2.5 × 10 cm column of hydroxylapatite (Bio-Gel HTP) equilibrated with 20 mM potassium phosphate (pH 7.0), 1 M KCl, 0.1 mM PMSF, and 0.5 mM DTE. After sample loading, the column was washed with 2 column volumes of this same buffer followed by 2 column volumes of 0.2 M potassium phosphate (pH 7.0), 50 mM KCl, 0.5 mM PMSF, and 0.5 mM DTE. The column was developed with a 0.2–1.0 M potassium phosphate (pH 7.0) gradient containing 50 mM KCl, 0.5 mM PMSF and 0.5 mM DTE. Topoisomerase activity eluted between 0.4 and 0.55 M phosphate. The fractions of highest activity were pooled to yield fraction II.

Fraction II was diluted to lower the phosphate concentration to 0.2 M and applied to a 0.8 × 10 cm column of Matrix Gel Blue A (Amicon). The enzyme was eluted in a stepwise fashion with 2 column volumes of potassium phosphate buffer at the following concentrations: 0.2 M, 0.2 M plus 25% ethylene glycol, 0.45 M, 0.6 M, and 0.8 M. All buffers were at pH 7.0 and contained 50 mM KCl, 0.5 mM PMSF, 0.5 mM DTE, 1 mM EDTA, and 0.005% Triton X-100. Triton X-100 stabilizes the activity (Kowalski, 1979) possibly by minimizing loss of protein due to surface adsorption (Kowalski et al., 1976). The 0.45 M fractions were pooled to yield fraction III.

Polyacrylamide Gel Electrophoresis. Electrophoresis of reduced, denaturated protein in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was run on a vertical slab gel system consisting of a 4.5% stacking gel and a 7.5% separation gel as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250.

Reaction of Topoisomerase with Single-Stranded DNA. Reaction with single-stranded DNA was carried out under solution conditions described by Been & Champoux (1980) but with different DNAs. A solution of λDNA (280 μg/mL) was diluted 10 times into 5 mM Tris-HCl, pH 7.4, 35 mM

Table I: Purification of Topoisomerase from an Extract of Chicken Erythrocyte Nuclei[a]

no.	fraction	vol (mL)	act.[b] (units) ($\times 10^{-7}$)	protein (mg)	sp act. (units) ($\times 10^{-5}$/mg)	act. recovered (%)
I	polymin P supernatant	1200	41.0	540	7.6	100
II	hydroxylapatite eluate	200	1.6	9.3	17.2	3.9
III	Matrex Gel Blue A eluate	20	0.6	0.6	84.0	1.5

[a] Prepared from 5.5×10^{11} nuclei obtained from 260 mL of chicken blood. [b] One unit of activity converts 0.1 μg of PM2 DNA I to the relaxed form in 10 min at 37 °C.

KH_2PO_4, pH 7.5, 0.25 mM DTE, 10 mM EDTA, 5% glycerol, and 0.005% Triton X-100 (buffer B). Single-stranded λDNA substrate was prepared by heating this solution at 100 °C for 2 min followed by quick cooling in an ice–water bath. Single-stranded, salmon sperm DNA substrate was prepared by heat denaturation (100 °C, 30 min) of the native DNA (Sigma Chemicals) in H_2O. The denatured DNA was stored as a 0.5 mg/mL solution in 0.05 M ammonium acetate (pH 5.0). The pH of the DNA solution was adjusted to 7.0 prior to use. Topoisomerase was incubated with the indicated weight ratio of either single-stranded λDNA or single-stranded salmon sperm DNA in buffer B for 30 min (or the indicated times) at 37 °C (or the indicated temperature). The reaction was terminated by addition of 15% final concentration of trichloroacetic acid (for gel electrophoresis of protein).

Limited Proteolysis of Topoisomerase I. Digestions were carried out in buffer B (see above) in a final volume of 350 μL. A freshly prepared solution of bovine trypsin was added to the reaction mixture of a trypsin to topoisomerase ratio of 1:42 (w/w) and incubated at 37 °C for the indicated times. Reactions were stopped by adding a 10-fold weight excess of soybean trypsin inhibitor (Kunitz) over trypsin and placing at 4 °C for 10 min.

Determination of Sedimentation Value. Sucrose density gradient centrifugation was carried out in a 5–20% (4.5 mL) sucrose gradient made up in 0.1 M potassium phosphate (pH 7.0), 0.1 M KCl, 0.5 mM PMSF, 0.5 mM DTE, and 0.005% Triton X-100. Gradients were centrifuged for 25 h at 39K rpm in a Beckman SW50.1 rotor at a constant temperature of 1 °C. Following centrifugation the gradients were dripped and assayed for topoisomerase activity. A sample of fraction III material was loaded onto a cold 5–20% gradient in a volume of 150 μL. A second gradient was run which contained the following proteins for gradient calibration: rabbit λ-globulin (7.0 S, M_r 150K), bovine serum albumin (4.4 S, M_r 68K), and soybean trypsin inhibitor (2.3 S, M_r 20K). Sedimentation values were determined as described by Martin & Ames (1961).

Results

Purification of a Type I Topoisomerase. The enzyme was purified (see Experimental Procedures) as summarized in Table I. The activity binds to hydroxylapatite in 1.0 M KCl and elutes between 0.4 and 0.55 M potassium phosphate (fraction II). The topoisomerase activity eluted from Matrix Gel Blue A (Cibacron Blue F3GA coupled to agarose) at a potassium phosphate concentration of 0.45 M (fraction III). While the activity in fractions I and III is stable at 4 °C for at least 1 month, the activity in fraction II is relatively unstable.

The low recovery of activity in fraction III is misleading and is likely the result of stimulation of fraction I activity by a substance(s) not present in fraction III. Addition of an equal volume of heat-inactivated fraction I (100 °C for 10 min to inactivate topoisomerase activity) to fraction III stimulates the activity in fraction III by 5-fold. The stimulatory substance is nondialyzable (M_r ~6000 cutoff). Since fraction I may contain residual polymin P which is a heat-stable, nondialyzable substance, we tested the effect of various concentrations of polymin P on the activity. Polymin P from 10^{-8}% to 10^{-5}% in the assay stimulates the DNA relaxing activity of fraction III by 10-fold but shows no effect on the activity of fraction I. These same concentrations of polymin P do not further stimulate the activity in a 1:1 mixture of fractions I and III. Thus removal of polymin P and/or other stimulatory factors reduces activity 5–10-fold. We estimate that the recovery of fraction III activity may be actually 7.5–15% instead of 1.5%, taking into account this reduction.

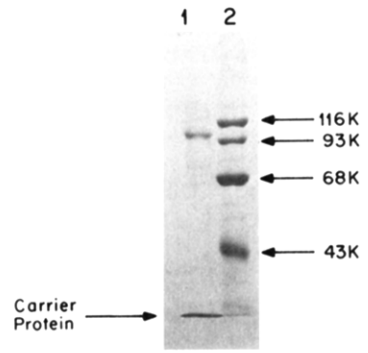

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified topoisomerase. (Lane 1) 2.4 μg of fraction III protein. (Lane 2) Protein standards: *Escherichia coli* β-galactosidase, M_r 116K; rabbit muscle phosphorylase *a*, M_r 93K; bovine serum albumin, M_r 68K; hen egg ovalbumin, M_r 43K. Soybean trypsin inhibitor (Kunitz) was present as a carrier in the trichloroacetic acid precipitation of fraction III protein.

The activity of the topoisomerase isolated from nuclei of chicken erythrocytes resembles that of type I topoisomerases isolated and purified from nuclei of other sources (Liu & Miller, 1981; Dynan et al., 1981; Wang et al., 1980) in that ATP is not required for the relaxation of supercoiled DNA. In addition the enzyme does not require Mg^{2+} and is active in 0.5 mM EDTA. The enzyme is unable to remove knots from topologically knotted circular duplex DNA under conditions where it relaxes supercoiled DNA (see Experimental Procedures) or under conditions where extracts containing ATP-dependent type II topoisomerase activity do remove the knots (Liu et al., 1981). These results suggest that the topoisomerase from chicken erythrocyte nuclei is a type I enzyme.

Purity, Molecular Weight, and Sedimentation Value of the Protein. When fraction III is subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions, a single protein band of M_r 100K predominates (Figure 1). Faint protein bands are also present at M_r 95K, 78K, 73K, and 66K. In different preparations, the intensities of the latter bands relative to the M_r 100K band were always low and variable.

Table II: Effects of Preincubation of Topoisomerase with DNA or Whole Cell RNA on Enzyme Activity[a]

nucleic acid	preincubation temperature (°C)	activity remaining (% of original)[b]
none	37	95
denatured λDNA	37	14
denatured λDNA	0	111
native λDNA	37	97
RNA	37	93
RNA	0	89

[a] Fraction III material was preincubated for 30 min at a 2:1 weight ratio of nucleic acid to protein and then assayed for enzyme activity. DNA and RNA were prepared as described under Experimental Procedures. [b] Activity is expressed as percent of original DNA relaxing activity without preincubation.

In order to determine which of these proteins possess topoisomerase activity, we cut out protein bands from $NaDodSO_4$–polyacrylamide gels and eluted and renatured the protein according to the procedure of Hager & Burgess (1980). All of the protein bands observed in the purified fraction exhibit enzyme activity with the exception of the faint 66K band. In contrast, extracts from gel slices which contained either no detectable protein, or proteins known to be contaminants (as those found in partially purified fractions of topoisomerase), contained no detectable activity. Thus, topoisomerase activity is associated with multiple polypeptides, the most abundant of which has a molecular weight of 100 000. The origin of these multiple forms is discussed in a later section.

The topoisomerase sediments as a single symmetrical peak of activity in a 5–20% sucrose gradient. The sedimentation coefficient of 4.4 S is the same as that for bovine serum albumin, a globular protein of M_r 68K. Protein isolated from the peak fraction is M_r 100K on $NaDodSO_4$ gel electrophoresis. A globular protein of M_r 100K would have a sedimentation coefficient of 5.7 S (Martin & Ames, 1961). Thus, the topoisomerase does not sediment like a globular protein.

Single-Stranded DNA Inhibits Topoisomerase Activity and Depletes Bands Corresponding to Topoisomerase Protein from $NaDodSO_4$–Polyacrylamide Gels. In addition to relaxing supercoiled DNA, type I topoisomerase cleaves and forms a covalent complex with single-stranded DNA under nondenaturing conditions (Been & Champoux, 1980; Prell & Vosberg, 1980; Halligan et al., 1982). The DNA binds to the enzyme active site and inhibits DNA relaxing activity (Been & Champoux, 1980). To confirm the association of type I topoisomerase activity with M_r 100K and lower polypeptides, we wanted to determine whether these polypeptides also bind to single-stranded DNA.

Using inhibition of DNA relaxing activity as an index of complex formation, we tested the effects of single-stranded DNA under various conditions. In addition, we examined the effects of double-stranded DNA, which does not form a stable complex with type I topoisomerase, and whole cell RNA, which had not previously been tested. As shown in Table II, preincubation of the enzyme at 37 °C with single-stranded DNA results in 86% loss of relaxing activity while preincubation at 37 °C with no addition has no effect. Inhibition is time dependent since without preincubation, single-stranded DNA has no effect on relaxing activity. Inhibition is temperature dependent since preincubation at 0 °C with single-stranded DNA shows no loss of activity (Table II). Preincubation at 37 °C with double-stranded DNA or whole cell RNA has no significant effect on activity (Table II). These results show that inhibition of the chicken enzyme is specific

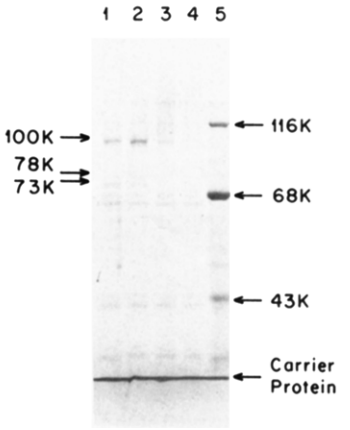

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of partially purified topoisomerase after reaction with single-stranded DNA. The molecular weight values of protein bands which react with denatured salmon sperm DNA are shown on the left. (Lane 1) 1.0 μg of protein incubated at 37 °C for 45 min minus DNA. (Lane 2) 1.0 μg of protein reacted at 0 °C for 45 min with 2.0 μg of denatured DNA. (Lane 3) Same as lane 2 except reacted at 24 °C. (Lane 4) Same as lane 2 except reacted at 37 °C. (Lane 5) Protein standards: *E. coli* β-galactosidase, M_r 116K; bovine serum albumin, M_r 68K; hen ovalbumin, M_r 43K. Carrier protein is soybean trypsin inhibitor.

for single-stranded DNA under conditions where another type I eukaryotic topoisomerase (Been & Champoux, 1980) is inhibited by and forms a covalent complex with single-stranded DNA.

We used $NaDodSO_4$–polyacrylamide gel electrophoresis in order to identify which polypeptides bind to single-stranded DNA. After reacting the enzyme with single-stranded DNA, we boiled the mixture in $NaDodSO_4$ to denature the protein and to dissociate any noncovalent complexes. The mixture was then subjected to gel electrophoresis. Depending on the size of the DNA molecules, a DNA–protein complex is expected to either migrate more slowly than the free protein during gel electrophoresis or not enter the gel at all. As a result, bands in the gel containing proteins which, like type I topoisomerase, form a $NaDodSO_4$ heat-stable complex with single-stranded DNA, will be selectively depleted from the pattern of protein bands seen in the absence of DNA. A partially purified preparation of chicken erythrocyte topoisomerase is shown in Figure 2 (lane 1). After treatment with single-stranded DNA at 37 °C (lane 4), a major band at M_r 100K and minor bands at M_r 95K, 78K, and 73K are removed from the pattern while seven other bands remain. The molecular weight values of the polypeptides in all of the depleted bands correspond precisely to the molecular weight values for polypeptides found in the more highly purified topoisomerase preparation shown in Figure 1 except for a trace band at 66K. In addition, the depleted bands correpsond to the protein bands which contain topoisomerase activity when eluted and renatured from $NaDodSO_4$–polyacrylamide gels. Thus, band depletion is specific for topoisomerase protein in our preparations.

Several additional experiments were performed to further characterize the reaction which leads to band depletion. The reaction requires active topoisomerase since inactivation of DNA relaxing activity by either denaturation of the protein with $NaDodSO_4$ or by reaction with *N*-ethylmaleimide prior to treatment with single-stranded DNA prevents band depletion. Also, substitution of whole cell RNA for single-stranded DNA results in no band depletion. Finally, the reaction is temperature dependent since compared to the band depletion seen after preincubation with single-stranded DNA

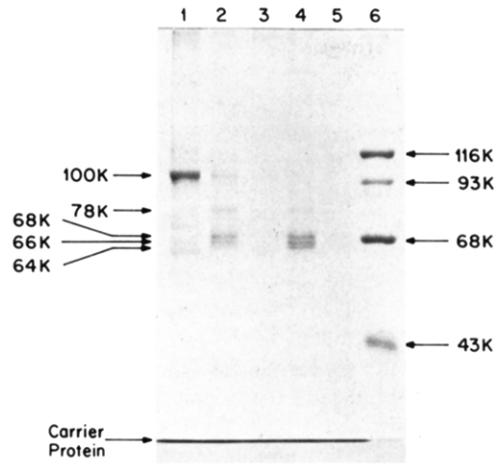

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of digestion products generated by treatment of the topoisomerase with trypsin. Proteolysis was carried out as described under Experimental Procedures. The molecular weights of topoisomerase and the major digestion products are shown on the left. (Lane 1) 4.2 μg of topoisomerase. (Lane 2) 4.2 μg of topoisomerase which was treated with 100 ng of trypsin for 5 min at 37 °C. (Lane 3) Same as lane 2 except the digestion products were reacted with 12 μg of single-stranded salmon sperm DNA at 37 °C for 30 min prior to electrophoresis. (Lane 4) Same as lane 2 except incubation time was 15 min. (Lane 5) Same as lane 4 except digestion products were reacted with 12 μg of single-stranded salmon sperm DNA at 37 °C for 30 min prior to electrophoresis. (Lane 6) Protein standards: *E. coli* β-galactosidase, M_r 116K; rabbit muscle phosphorylase *a*, M_r 93K; bovine serum albumin, M_r 68K; hen egg ovalbumin, M_r 43K.

at 37 °C (Figure 2, lane 4), band depletion is more limited at 24 °C (lane 3) and does not occur at 0 °C (lane 2). In conclusion, the requirements for the band depletion reaction parallel those for inhibition of DNA relaxing activity (Table II).

Limited Proteolysis of Chicken Erythrocyte Topoisomerase. In addition to the abundant 100K topoisomerase, we have also isolated minor polypeptides of M_r 95K, 78K, and 73K which possess topoisomerase activity. One possible source of these minor polypeptides is via proteolytic degradation of 100K topoisomerase. In order to determine if the 100K protein from chicken erythrocytes is sensitive to proteolysis, we investigated the effect of trypsin on the molecular size of the chicken enzyme.

After trypsin treatment, major polypeptides of M_r 68K, 66K, and 64K and a minor polypeptide of 78K appeared at the expense of the M_r 100K and 95K polypeptides (Figure 3). In addition, this limited proteolysis of the 100K protein resulted in no loss of DNA relaxing activity. In fact, under our assay conditions, activity increased 1.5–2-fold. Thus, the 100K topoisomerase is sensitive to proteolysis and is digested in a limited fashion with retention of activity. Similar results have been shown by Liu & Miller (1981) for the enzyme from HeLa cells; however, no data on which of the peptides were active were presented. As shown in Figure 3, the parent M_r 100K protein and four proteolysis products, M_r 78K, 68K, 66K, and 64K, are depleted upon preincubation with single-stranded DNA. These results show that all of these species bind to single-stranded DNA and are likely to contain topoisomerase active sites. Since no bands greater than M_r 20K remain after band depletion, regions of the topoisomerase molecule separated from the active region by protease cleavage and denaturation must be <20K in molecular weight. These results demonstrate that the M_r 100K topoisomerase from chicken erythrocyte nuclei can be cleaved in vitro resulting in polypeptides which retain activity and are similar or even

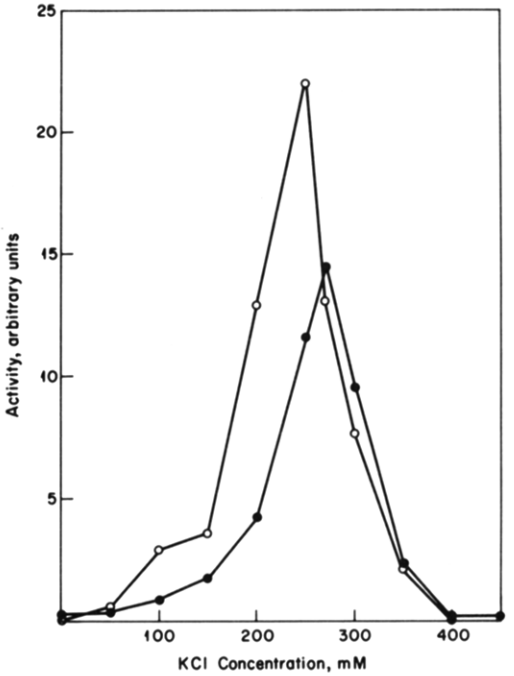

FIGURE 4: DNA relaxing activity as a function of KCl concentration for the 100K topoisomerase (●) and the trypsin-cleaved enzyme (○). Various dilutions of the 100K topoisomerase stock solution (300 units/μL, 30 ng of protein/μL) were assayed for DNA relaxing activity (Kowalski, 1979) at 37 °C in reaction mixtures containing 10 mM Hepes (pH 7.8), 0.5 mM Na_2EDTA, and the indicated concentrations of KCl. The same enzyme stock solution was treated with trypsin (see Experimental Procedures) and assayed in an identical fashion. Values of activity were computed from dilutions which resulted in quantities of relaxed DNA in the linear range of the assay (0.1–0.6 μg of relaxed DNA). These values were normalized for the fold dilution of the enzyme stock solution. An arbitrary unit of activity corresonds to 10 μg of DNA relaxed per μL of enzyme stock solution. In our standard assay mixtures which contain both 100 mM KCl and 100 mM NaCl (see Experimental Procedures), the 100K topoisomerase stock solution would exhibit 3 arbitrary units of activity on this scale.

smaller in size than the multiple molecular weight forms which copurify with the M_r 100K topoisomerase in our preparations.

Dependence of the Activity of the 100K and Trypsin-Cleaved Forms of Topoisomerase on KCl Concentration. The retention of topoisomerase activity despite the protease cleavage of the protein molecule prompted us to compare some of the properties of the M_r 100K and cleaved forms. Any difference(s) that results from cleavage of the enzyme may give a clue as to the role of that portion of the molecule which is cut by trypsin.

Both forms of topoisomerase are inhibited by *N*-ethylmaleimide and single-stranded DNA and are band depleted on $NaDodSO_4$ gels. One discernible difference between the two forms is that the cleaved form exhibits a 1.5–2.0-fold greater activity than the M_r 100K form, under our standard assay conditions. Cleavage of the enzyme could affect its conformation and/or structure, resulting in increased or decreased activity. Since binding affinity can affect activity, we examined the dependence of DNA relaxation on KCl concentration for the two forms.

Our results show that the M_r 100K form of the chicken topoisomerase has an optimal activity at 270 mM KCl (Figure 4). This salt concentration is higher than the values of 150–200 mM found for most type I enzymes from various eukaryotes (Keller, 1975; Tang, 1978; Baase & Wang, 1974; Pulleyblank & Morgan, 1975; Vosberg et al., 1975; McConaughy et al., 1981); however, values below (Dynan et al., 1981; Bauer et al., 1977) and above (Eskin & Morgan, 1978)

these concentrations have also been reported. In contrast to the M_r 100K form, the cleaved forms of the enzyme are more active at lower KCl concentrations, with optimal activity at 250 mM KCl (Figure 4). In addition, the optimal activity of the cleaved forms is 1.5-fold greater than that for the 100K topoisomerase.

These results show that trypsin cleavage of the topoisomerase alters the ionic strength dependence of the enzymatic activity. This shift in the salt profile accounts for the activation of topoisomerase upon trypsin cleavage when assayed under our standard conditions (200 mM salt). The increase in activity observed upon cleavage of the topoisomerase could be the result of a change in the catalytic constant of the enzyme, an alteration in its binding efficiency to DNA, or both. At this time, we are unable to distinguish between these possibilities.

Discussion

We have purified a DNA topoisomerase to near homogeneity from nuclei of mature chicken erythrocytes. The catalytic properties resemble those of type I eukaryotic topoisomerases capable of breaking and rejoining one strand of duplex DNA at a time. The enzyme does not require ATP or Mg^{2+} to relax supercoiled DNA. In addition, it does not perform reactions characteristic of type II topoisomerases such as unknotting of knotted duplex DNA and catenation of covalently closed circular DNA (B. Sahai, unpublished results). Finally, the enzyme is inhibited by single-stranded DNA with formation of an $NaDodSO_4$ heat-stable complex.

The enzyme also resembles some type I eukaryotic topoisomerases in physical and biochemical properties. The molecular weight of the reduced, denatured protein is 100K. This value is identical with that for topoisomerase I from HeLa cells (Liu & Miller, 1981) and similar to those for type I topoisomerases from *Drosophila* early embryos (Wang et al., 1980) and wheat germ (Dynan et al., 1981). Since mature chicken erythrocytes are differentiated cells while these other cell types are undifferentiated, our results show that M_r 100K topoisomerase can also be isolated from a differentiated cell type.

In addition to the major protein of 100K, our preparation also contains minor proteins of M_r 95K, 78K, and 73K, all of which possess topoisomerase activity. Since the M_r 100K topoisomerase can be cleaved by trypsin, into fragments in molecular weight range 64K–78K with retention of activity, we attribute the presence of the minor proteins in the preparation to the proteolytic digestion of the 100K protein during isolation and purification. Pulleyblank & Ellison (1982) isolated a M_r 62K type I topoisomerase from chicken erythrocytes. They reported that larger forms could not be detected when crude or purified samples were analyzed by sedimentation velocity on sucrose gradients. However, it may not be possible to detect larger forms by this method since we find that the 100K form cosediments with a globular protein of M_r 68K. Taken together, our findings raise the possibility that the 62K protein (Pulleyblank & Ellison, 1982) is an active proteolytic fragment of the M_r 100K topoisomerase. Whether the 100K enzyme itself is a fragment of a larger protein and whether the proteolysis has any biological significance remain to be determined.

$NaDodSO_4$–polyacrylamide gel electrophoresis provides a convenient method to identify proteins which bind tightly to polynucleotides. Utilizing the known ability of type I topoisomerases to form a covalent complex with single-stranded DNA (Been & Champoux, 1980; Prell & Vosberg, 1980; Halligan et al., 1982), we found that protein bands depleted by single-stranded DNA corresponded to the bands containing topoisomerase activity. This need not be generally true of all topoisomerase preparations since other proteins may form high-affinity complexes with DNA under the same conditions. In all cases described here, the proportion of the band depleted was high, indicating that nearly all protein molecules in a given band are active in binding single-stranded DNA.

We have also utilized the band depletion technique to probe for the possible interaction of topoisomerase with whole cell RNA. RNA contains considerable single-strand character, and in addition type I topoisomerase activity is associated with ribonucleoprotein particles (Tsubota et al., 1979). The failure of whole cell RNA to deplete the topoisomerase-containing bands under conditions where single-stranded DNA shows near complete removal indicates that topoisomerase does not form a covalent complex with whole cell RNA under these conditions. It is possible, however, that a subfraction of whole cell RNA present in limiting amounts does form a complex and the resulting band depletion is undetectable in our assay.

A number of biological functions have been proposed for type I DNA topoisomerase. It may function as a swivel in DNA replication (Wang, 1971; Champoux & Dulbecco, 1972) and transcription (Bauer et al., 1977; Wang, 1973), relieving the torsional strain generated when strands separate by reducing their linking number. It may regulate transcription from some promoters in prokaryotes by altering DNA supercoiling (Sternglanz et al., 1981; Trucksis & Depew, 1981). It may function in DNA recombination by catalyzing the linkage between complementary strands (Champoux, 1977; Kirkegaard & Wang, 1978) or the breakage–reunion events during integration (Been & Champoux, 1980; Halligan et al., 1982; Champoux, 1978a,b; Kikuchi & Nash, 1979; Sternglanz et al., 1981). Finally, the enzyme may be involved in modulating the DNA linkage during the condensation–decondensation of chromatin (Baase & Wang, 1974; Bauer et al., 1977; Vosberg & Vinograd, 1976). Since none of these processes are known to occur in mature chicken erythrocytes, we can make no correlation between the presence of topoisomerase I and biological function. It is possible that topoisomerase I is required during the maturation process and that its presence in the mature erythrocyte is vestigial. We estimate that there is 0.7–6 fg of topoisomerase protein/pg of DNA in chicken erythroyctes.[2] This ratio is similar to that calculated from the data of Champoux & McConaughy (1976) for the type I topoisomerase from rat liver (1–8 fg/pg) and that calculated from the data of Liu & Miller (1981) for topoisomerase I from HeLa cells (4–8 fg/pg). Thus, the estimated content of topoisomerase per unit DNA in the inactive nuclei of mature chicken erythrocytes is similar to that in nuclei active in transcription and replication.

Added in Proof

Javaherian et al. (1982) have recently isolated a M_r 135000 type I topoisomerase from *Drosophila*. A M_r 100000 form

[2] We have obtained 0.6 mg of near homogeneous topoisomerase from 5.5×10^{11} nuclei containing at least 1.7 pg of DNA/nucleus (Ringertz & Bolund, 1974). We estimate that the recovery of activity is 7.5–15%, taking into account the removal of a stimulatory substance during purification (see Results). To simplify further calculations, we will use an average of 11%. If the purified enzyme is 100% active and enzyme inactivated during purification does not copurify with it, then the percent recovery of activity will reflect the recovery of topoisomerase protein. In this case, we calculate that there is 5.9 fg of topoisomerase protein/pg of DNA. If, on the other hand, all of the topoisomerase protein is recovered but the percent recovery of activity reflects the degree of inactivation of the purified enzyme, then we calculate that there is 0.65 fg of topoisomerase protein/pg of DNA.

of the enzyme results from degradation.

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