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Calcium-Promoted DNA Cleavage by Eukaryotic Topoisomerase II: Trapping the Covalent Enzyme–DNA Complex in an Active Form†

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Received September 4, 1986; Revised Manuscript Received February 18, 1987

ABSTRACT: The effects of calcium ions on interactions between *Drosophila melanogaster* topoisomerase II and DNA were assessed. Although the divalent cation could not support DNA strand passage, it was able to promote high levels of enzyme-mediated DNA cleavage. Moreover, sites of cleavage on plasmid pBR322 generated in calcium-promoted reactions were similar to those obtained in the presence of magnesium. When calcium-containing enzyme–DNA mixtures were treated with ethylenediaminetetraacetic acid, cleaved nucleic acids could be generated in the absence of sodium dodecyl sulfate (SDS) or other denaturing detergents. The product of this SDS-independent calcium-promoted reaction was a covalent topoisomerase II–DNA complex. Enzyme molecules trapped in such complexes were found to be kinetically competent. Therefore, calcium should be a valuable tool for studying the enzymology of topoisomerase II mediated DNA cleavage.

Eukaryotic type II topoisomerases alter the topology of DNA by passing an intact helix of DNA through a transient, enzyme-bound, double-stranded break made in a second helix (Wang, 1982, 1985; Vosberg, 1985). The mechanics of the double-stranded DNA passage reaction necessitate that the enzyme be able to cleave and rejoin the nucleic acid backbone in a concerted fashion.

Since the cleavage/religation cycle is central to the function of topoisomerase II, an assay has been developed that allows the DNA cleavage reaction to be studied in vitro (Sander & Hsieh, 1983; Liu et al., 1983). In this assay, enzyme-mediated DNA cleavage is induced by the rapid addition of a denaturing detergent, such as sodium dodecyl sulfate (SDS),[1] to mixtures

†This work was supported by National Institutes of Health Grant GM-33944.

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of eukaryotic topoisomerase II and DNA. This reaction does not require the presence of a nucleoside triphosphate cofactor (Sander & Hsieh, 1983; Liu et al., 1983), but it is absolutely dependent on the presence of a divalent cation (Sander & Hsieh, 1983; Pommier et al., 1984; Osheroff, 1985).[2] The product of SDS-induced cleavage is a topoisomerase II–DNA complex in which the enzyme is covalently linked to the 5′ termini of the cleaved nucleic acid (Sander & Hsieh, 1983; Liu et al., 1983). Unfortunately, the detergent procedure employed to trap this complex disrupts the enzymes's three-dimensional structure and destroys its catalytic activity. This denaturation has precluded a detailed mechanistic or conformational analysis of topoisomerase II mediated DNA cleavage.

In order to more fully characterize the DNA cleavage reaction, a novel procedure has been developed that generates high levels of topoisomerase II mediated DNA cleavage, even in the complete absence of a denaturing detergent. This was accomplished by employing calcium, rather than magnesium, to promote enzyme activity. The product of the calcium-promoted reaction is a covalent topoisomerase II–DNA complex. Unlike enzyme–DNA complexes trapped by the addition of protein denaturants to reaction mixtures, topoisomerase II molecules trapped within these calcium-promoted covalent complexes are catalytically active.

Experimental Procedures

Drosophila melanogaster DNA topoisomerase II was purified from the nuclei of Kc tissue culture cells or 6–18-h-old embryos by the procedure of Shelton et al. (1983). The isolated enzyme was stored at −20 °C in 15 mM sodium phosphate, pH 7.1, 700 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 35% glycerol. Negatively supercoiled bacterial plasmid pBR322 (Bolivar et al., 1977) DNA was isolated from *Escherichia coli* DH1 (Hanahan, 1983) by a Triton X-100 lysis procedure followed by double banding in cesium chloride–ethidium bromide gradients (Manitatis et al., 1982). Analytical reagent-grade $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, and EDTA were obtained from Fisher; ethidium bromide and Tris were from Sigma; SDS and proteinase K were from E. Merck Biochemicals; restriction endonuclease *Bam*HI and T4 polynucleotide kinase were from New England Biolabs; and bovine serum albumin (nuclease free) was from BRL. All other chemicals were analytical reagent grade.

Agarose Gel Electrophoresis and Quantitation of Reaction Products. The results of assays described below were assessed by agarose gel electrophoresis. After reactions were completed, products were mixed with loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole FF, and 10 mM Tris–HCl, pH 7.9) and applied to 1.0% agarose (MCB) gels. Unless stated otherwise, samples (0.3 μg of DNA) were subjected to electrophoresis in 4 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm until the bromophenol blue had traveled approximately 12.5 cm (3–4 h). Gels were stained for 30 min in an aqueous solution of ethidium bromide (1 μg/mL). DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak 23A and 12 filters using Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Biomed Instruments Model SL-504-XL scanning densitometer. Under the conditions employed, the intensity of the negative was directly proportional to the amount of DNA present.

Catalytic Relaxation of Supercoiled DNA by Topoisomerase II. The steady-state procedure of Osheroff et al. (1983) was employed. Assays contained 1.5 nM (10 ng) topoisomerase II and 10 nM (0.6 μg) negatively supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer (10 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin) which contained 5 mM $MgCl_2$ and 1 mM ATP. Reactions were performed at 30 °C for 6 min and were stopped by the addition of 2.5 μL of loading buffer which contained 100 mM EDTA and 0.5% SDS. Samples were heated to 75 °C for 2 min, and reaction products were electrophoretically resolved and quantitated as described above. When magnesium was replaced by other divalent cations, their concentrations were 5 mM.

Binding of Topoisomerase II to DNA. Binding mixtures consisted of 40 nM enzyme and 5 nM supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer which contained 0–25 mM $MgCl_2$. Samples were incubated at 30 °C for 6 min. The results of binding assays were determined by an agarose gel electrophoretic technique, as previously described (Osheroff, 1986).

Cleavage of DNA by Topoisomerase II. Unless stated otherwise, reactions employed 100 nM enzyme and 5 nM supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer which contained 5 mM $MgCl_2$. Incubation was at 30 °C for 6 min. For detergent-terminated reactions, 2 μL of 10% SDS was added, followed by 1 μL of 250 mM EDTA. For chelator-terminated reactions, enzyme–DNA mixtures were treated with 2 μL of 250 mM EDTA. In some cases, samples were heated to 75 °C for 2 min and/or treated with 2 μL of 0.5–10% SDS following the addition of chelating agent. Neither the addition of SDS nor the heating step affected the levels of chelator-terminated DNA cleavage. For all reactions, 2 μL of 0.8 mg/mL proteinase K was added, and the mixture was incubated at 37 °C for 30 min to digest the topoisomerase II. Loading buffer (2.5 μL) was added, and samples were heated for 2 min at 75 °C. Reaction products were resolved by electrophoresis and quantitated as described above. Unless stated otherwise, when divalent cations other than magnesium were employed, their concentrations were 5 mM.

When sites of topoisomerase II mediated DNA cleavage were mapped, samples were extracted with phenol, precipitated with ethanol, and digested with restriction endonuclease *Bam*HI prior to electrophoresis on agarose gels in 100 mM Tris–borate, pH 8.3, and 2 mM EDTA. DNA fragments were transferred to a GeneScreenPlus (New England Nuclear) hybridization membrane and probed with the single-stranded oligonucleotide pGATCCTCTACGCCGGACGCATCG-TGG-OH which had been labeled at its 5′ terminus with [^{32}P]phosphate using T4 polynucleotide kinase (Southern, 1975; Maniatis et al., 1982). This 26-mer corresponds to base sequence 376–401 in the plasmid pBR322 and abuts the unique *Bam*HI cleavage site (Sutcliffe, 1978). DNA fragments which contained the DNA sequence complementary to the oligonucleotide probe were visualized by autoradiography employing Kodak XAR film and a Du Pont Lightning Plus screen.

Results

Substitution of Magnesium by Other Divalent Cations. D. melanogaster topoisomerase II absolutely requires the presence of a divalent cation for catalytic activity and displays optimal rates of reaction at a magnesium concentration of 5–10 mM

[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] N. Osheroff, unpublished results.

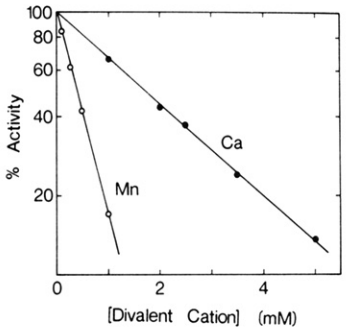

FIGURE 1: Inhibition of magnesium-promoted topoisomerase II catalyzed DNA relaxation by calcium and manganese. Assays contained 5 mM $MgCl_2$ and are described under Experimental Procedures. The concentrations of $CaCl_2$ (●) and $MnCl_2$ (○) are shown in the figure.

(Hsieh & Brutlag, 1980; Osheroff et al., 1983). Therefore, the effects of several divalent cations on the enzyme's ability to relax superhelical DNA twists were determined. No relaxation activity was observed when magnesium was replaced by a number of divalent cations,[3] including cadmium, calcium (Osheroff et al., 1983), copper, manganese (Osheroff et al., 1983), mercury, or zinc. All of the above cations did, however, inhibit the magnesium-promoted DNA relaxation activity of *Drosophila* topoisomerase II. Inhibition plots for calcium and manganese are shown in Figure 1. Fifty percent inhibition was observed at 1.7 mM calcium and 0.4 mM manganese, respectively. Although plots were not generated for the other divalent cations listed above, they all inhibited DNA relaxation by at least 90% at concentrations of 2.5 mM.

Calcium-Promoted DNA Cleavage of Topoisomerse II. Considering the requirement for a divalent cation in DNA cleavage reactions (Sander & Hsieh, 1983; Pommier et al., 1984; Osheroff, 1985),[2] the ability of the above cations to promote enzyme-mediated DNA cleavage in the absence of magnesium was determined.

In these experiments, cleaved DNA products were generated by the addition of high concentrations of SDS to mixtures of *Drosophila* topoisomerase II and supercoiled plasmid molecules. Although the detailed mechanism of DNA cleavage has not yet been established, two alternatives have been proposed (Sander & Hsieh, 1983; Liu et al., 1983): (1) SDS induces enzyme-mediated DNA breakage to take place within an existing precleavage (i.e., "cleavable") topoisomerase II–DNA complex, or (2) SDS rapidly denatures (and thereby traps) a complex in which the nucleic acid has already been cleaved by the enzyme. Irrespective of mechanism, this in vitro reaction is believed to reflect the physiological cleavage of the DNA by topoisomerase II (Udvardy et al., 1985; Yang et al., 1985; Rowe et al., 1986) and results in the formation of a covalent enzyme–cleaved DNA complex (Sander & Hsieh, 1983; Liu et al., 1983). Following digestion of the complexed enzyme with proteinase K, double-stranded cleavage can be visualized by monitoring the conversion of supercoiled circular nucleic acids to linear molecules (Figure 2, lane 3). Single-stranded DNA cleavage, which is also mediated by the enzyme, results in the conversion of supercoiled molecules to nicked circles (lane 3).

In order to observe topoisomerase II mediated DNA cleavage (when magnesium is employed as the divalent cation), the protein denaturant employed must act in a rapid fashion.

[3] Cobalt and iron were also employed, but were found to degrade pBR322 DNA in an enzyme-independent reaction. Accordingly, these divalent cations were not used in subsequent studies.

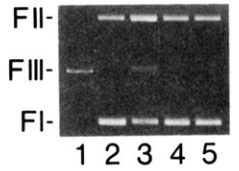

FIGURE 2: Requirement for a denaturing detergent in magnesium-promoted topoisomerase II mediated DNA cleavage reactions. Assays contained 100 nM enzyme, 5 nM plasmid molecules, and 5 mM $MgCl_2$. All other conditions are described under Experimental Procedures. The positions of form I (FI), fully supercoiled DNA; form II (FII), nicked circular plasmid molecules; and form III (FIII), linear molecules, are shown. Lanes 1 and 2, DNA standards; lane 3, DNA cleavage was terminated by the addition of SDS (1% final concentrations) followed by digestion with proteinase K; lane 4, DNA cleavage was terminated by heating to 75 °C for 2 min followed by proteinase K digestion; lane 5, DNA cleavage was terminated by proteinase K digestion.

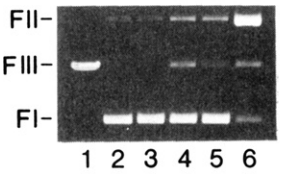

FIGURE 3: Effect of divalent cations on the SDS-terminated cleavage of pBR322 DNA by topoisomerase II. Assays contained 100 nM enzyme and 5 nM negatively supercoiled PBR322 plasmid molecules and are described under Experimental Procedures. The positions of form I, form II, and form III DNAs are as shown in Figure 2. Assays contained only those divalent cations indicated. Lanes 1 and 2, DNA standards; lane 3, cleavage assays were carried out in the absence of a divalent cation; lane 4, cleavage assays were carried out in the presence of 5 mM $MgCl_2$; lane 5, cleavage assays were carried out in the presence of 5 mM $MnCl_2$; lane 6, cleavage assays were carried out in the presence of 5 mM $CaCl_2$.

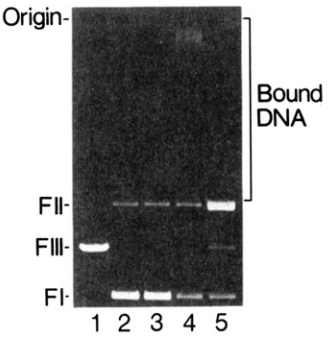

FIGURE 4: Characterization of DNA products generated in SDS-terminated calcium-promoted DNA cleavage reactions. Assays contained 50 nM enzyme, 5 nM negatively supercoiled pBR322 DNA, and 5 mM $CaCl_2$ and are described under Experimental Procedures. The positions of form I, form II, and form III DNAs are shown as in Figure 2. The position of enzyme-bound DNA, which migrates between the origin and the form II DNA band (Osheroff, 1986) is also indicated. Lanes 1 and 2, DNA standards; lane 3, cleavage was carried out in the absence of topoisomerase II; lane 4, reaction mixtures were heated to 80 °C but were not treated with proteinase K following clevage; lane 5, reaction mixtures were treated with proteinase K following cleavage.

When relatively slow acting denaturants, such as heat (Figure 2, lane 4) or proteinase K (lane 5), are substituted for SDS, virtually no DNA cleavage products were generated.

No cleavage was observed when cadmium, copper, mercury, or zinc was employed. However, despite the fact that neither calcium nor manganese could support DNA relaxation, both were found to promote DNA cleavage (Figure 3, see Table II). When magnesium was replaced by calcium ions, high levels of double-stranded and single-stranded DNA cleavage were observed (Figure 3, lane 6). Manganese supported DNA cleavage (lane 5), but with about half the efficiency of either

Table I: Inhibition of Calcium-Promoted Double-Stranded DNA Cleavage by Anti-Topoisomerase II Antiserum[a]

antiserum	relative double-stranded DNA cleavage (%)
none	100
anti-topoisomerase II, 1:50 dilution	79
anti-topoisomerase II, 1:25 dilution	53
anti-topoisomerase II, 1:10 dilution	26
preimmune, 1:10 dilution	89

[a] Reactions contained 5 mM $CaCl_2$ and a 5-fold molar excess of topoisomerase II (25 nM) over supercoiled pBR322 DNA (5 nM). All other conditions are described under Experimental Procedures.

Table II: Effect of Divalent Cations on Topoisomerase II Mediated Double-Stranded DNA Cleavage[a]

divalent cation	SDS-terminated DNA cleavage[b] (%)	EDTA-terminated DNA cleavage[c] (%)	EDTA-terminated cleavage/SDS-terminated cleavage
none	0.0	0.0	
magnesium	19.8	1.0	0.05
calcium	21.0	24.7	1.18
manganese	8.9	4.2	0.47

[a] Reactions contained 5 mM divalent cation and a 20-fold molar excess of topoisomerase II (100 nM) over supercoiled pBR322 DNA (5 nM). All other conditions are described under Experimental Procedures. The values displayed in this table represent the averages of three independent experiments. [b] DNA cleavage was terminated by the addition of SDS (1% final concentration) followed by EDTA (10 mM final concentration) and digestion with proteinase K. [c] DNA cleavage was terminated by the addition of EDTA (25 mM final concentration) followed by the addition of SDS (0.05–1.0% final concentration) and digestion with Proteinase K. Identical results were obtained when the detergent step was omitted.

magnesium (lane 4) or calcium (lane 6).

Several lines of evidence indicate that DNA cleavage reactions promoted by calcium and manganese are mediated by topoisomerase II. First, no cleavage was observed in the absence of the enzyme (Figure 4, lane 3). Second, similar cleavage results were obtained by using four independent preparations of topoisomerase II (two from embryos and two from tissue culture cells). Third, all of the linear and nicked plasmid molecules generated in calcium-promoted reactions were covalently bound to topoisomerase II. Reaction products had to be treated with proteinase K in order to release the resulting linear and nicked DNAs, even after incubation in 1% SDS (final concentration) at 80 °C (Figure 4, lanes 4 and 5). Similar results were found for reactions carried out in the presence of manganese ions. As discussed above, the formation of a covalent enzyme–DNA complex is a hallmark of topoisomerase II mediated cleavage reactions (Sander & Hsieh, 1983; Liu et al., 1983). Fourth, calcium-promoted DNA cleavage reactions were inhibited by rabbit antiserum (Shelton et al., 1983; Berrios et al., 1985) directed against *Drosophila* topoisomerase II (Table I). Finally, similar levels of DNA cleavage were generated with seven independent lots of calcium chloride. This finding makes it highly unlikely that the enzyme-mediated production of cleaved DNA was due to the presence of a contaminant in the calcium preparations.

Calcium-Promoted DNA Cleavage in the Absence of a Denaturing Detergent. As demonstrated in Figure 3, magnesium-promoted topoisomerase II mediated DNA cleavage products are trapped only in the presence of a rapidly acting protein denaturant such as SDS (i.e., SDS-terminated reaction). Moreover, observed levels of DNA cleavage have been shown to drop precipitously if the divalent cation is chelated

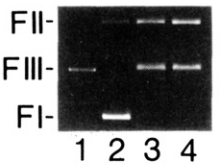

FIGURE 5: EDTA-terminated calcium-promoted DNA cleavage in the absence of a denaturing detergent. Assays contained 75 nM enzyme, 5 nM pBR322 plasmid DNA, and 5 mM $CaCl_2$ and are described under Experimental Procedures. The positions of form I, form II, and form III DNAs are shown as in Figure 2. Lanes 1 and 2, DNA standards; lane 3, EDTA-terminated reactions were denatured with SDS (1% final concentration) and digested with proteinase K; lane 4, EDTA-terminated reactions were digested with proteinase K in the absence of SDS.

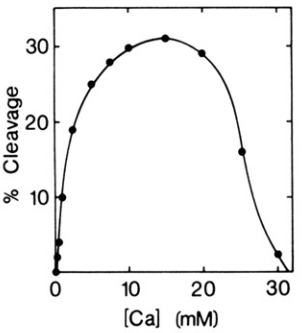

FIGURE 6: Effect of calcium concentration on the double-stranded cleavage of negatively supercoiled pBR322 DNA by topoisomerase II. Assays contained 75 nM enzyme and 5 nM plasmid molecules and were carried out under the EDTA-terminated cleavage conditions described under Experimental Procedures. No denaturing detergent was employed in these assays.

by EDTA prior to the addition of SDS (i.e., EDTA-terminated reaction) (Sander & Hsieh, 1983). As quantitated in Table II, levels of magnesium-promoted DNA cleavage generated in SDS-terminated reactions were approximately 20-fold higher than those generated in EDTA-terminated experiments.

A very different result was found for calcium- and manganese-promoted reactions (Table II). In both cases, substantial amounts of cleaved DNA were generated when EDTA was added prior to the detergent. In fact, levels of cleavage products trapped in calcium-promoted EDTA-terminated reactions were nearly 20% higher than in those reactions which were terminated by the addition of SDS.

Since calcium was able to promote high levels of DNA cleavage in EDTA-terminated reactions when SDS was added after the chelating agent, experiments were carried out in order to determine whether the detergent was required for these reactions. To this end, identical samples were treated with EDTA, followed by the addition of either 1% SDS (final concentration) or an equivalent volume of water. Levels of cleaved DNA were the same for both samples (Figure 5). This demonstrates that calcium can promote efficient topoisomerase II mediated DNA cleavage, even in the complete absence of a denaturing detergent. Moreover, calcium has the ability to promote EDTA-terminated reactions over a wide range of divalent cation concentrations (Figure 6).

Effect of Calcium on the Selection of DNA Cleavage Sites by Topoisomerase II. The effect of calcium on the enzyme's selection of cleavage sites in pBR322 DNA was characterized. As seen in Figure 7, DNA cleavage patterns produced by magnesium-promoted SDS-terminated reactions (lane 1) were nearly identical with those produced in the presence of calcium (lane 2). A similar result was found when calcium-promoted DNA cleavage was terminated with EDTA (lane 3). Therefore, the chemical nature of the divalent cation and the

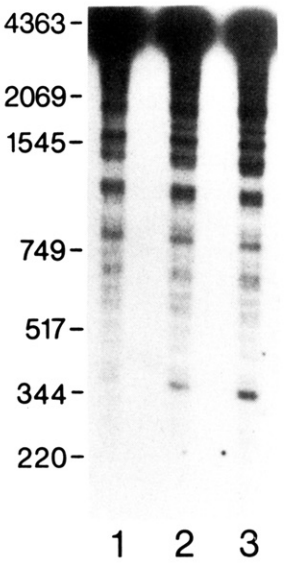

FIGURE 7: Effect of calcium on the selection of cleavage sites in negatively supercoiled pBR322 DNA by topoismerase II. Assays contained 150 nM enzyme and 7.5 nM plasmid molecules. Details of the experiment are given under Experimental Procedures. The relative migrations of linear pBR322 DNA fragments are shown. Fragment lengths are given in base pairs, with 4363 base pairs representing the full-length linear *Bam*HI-cleaved pBR322 molecules. Lane 1, SDS-terminated magnesium-promoted DNA cleavage; lane 2, SDS-terminated calcium-promoted DNA cleavage; lane 3, EDTA-terminated calcium-promoted DNA cleavage.

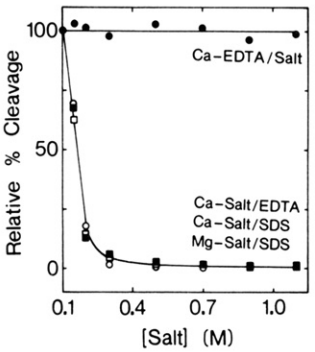

FIGURE 8: Salt reversal of the double-stranded topoisomerase II mediated DNA cleavage reaction. Assays contained 75 nM enzyme, 5 nM plasmid molecules, and 5 mM divalent cation. Details are given under Experimental Procedures. Initial enzyme–DNA incubations were at 100 mM ionic strength. Salt levels were raised by the addition of concentrated NaCl solutions. Salt was added to magnesium-promoted reactions prior to the addition of SDS [Mg–Salt/SDS (■)]. Salt was added to calcium-promoted reactions prior to the addition of either SDS [Ca–Salt/SDS (□)] or EDTA [Ca–Salt/EDTA (○)]. EDTA was added to calcium-promoted reactions prior to the addition of salt [Ca–EDTA/Salt (●)].

method of reaction termination have little influence on the site selectivity of *Drosophila* topoisomerase II.

Salt Reversibility of the Calcium-Promoted DNA Cleavage Reaction. The magnesium-promoted DNA cleavage reaction is readily reversed by the addition of salt (Liu et al., 1983). If mixtures containing *Drosophila* topoisomerase II and supercoiled pBR322 plasmid DNA are incubated at 100 mM ionic strength, treated with increasing concentrations of salt, and allowed to reincubate prior to the addition of SDS, levels of DNA cleavage drop by 50% at a final ionic strength of approximately 160 mM (Figure 8). Identical results were found for calcium-promoted reactions when salt concentrations were increased prior to treatment with SDS or EDTA (Figure 8). This demonstrates that the calcium-promoted DNA cleavage is reversible as long as the divalent cation is present.

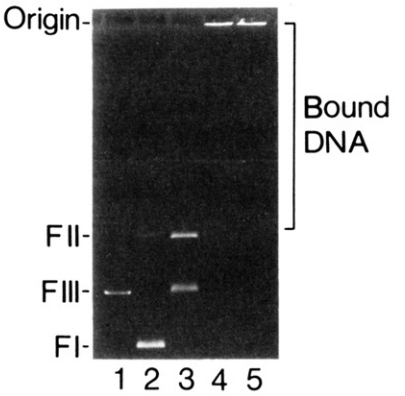

FIGURE 9: Covalent nature of the topoisomerase II–DNA complex trapped by EDTA termination of calcium-promoted DNA cleavage reactions. Assays contained 75 nM topoisomerase II, 5 nM pBR322 plasmid DNA, and 5 mM $CaCl_2$. Details are given under Experimental Procedures. Initial enzyme–DNA interactions were at 100 mM ionic strength. Following EDTA treatment, the salt concentration was raised to 1.1 M by the addition of NaCl. The positions of form I, form II, form III, and enzyme-bound DNAs are shown as in Figure 4. Lanes 1 and 2, DNA standards; lane 3, the topoisomerase II–DNA complex was digested with proteinase K; lane 4, the enzyme–DNA complex was treated with no protein denaturants; lane 5, the enzyme–DNA complex was heated to 75 °C for 2 min.

However, when calcium ions were chelated with EDTA before adding salt to reaction mixtures, DNA cleavage was completely unaffected by ionic strength, even at final salt concentrations which exceeded 1 M (Figure 8). Thus, removal of the divalent cation from calcium-promoted samples appears to freeze the equilibrium of the topoisomerase II mediated DNA cleavage reaction and renders it totally irreversible.

Trapping a Calcium-Promoted Covalent Topoisomerase II–DNA Complex. The product of the calcium-promoted SDS-terminated DNA cleavage reaction is a covalent topoisomerase II–DNA complex (see Figure 4). However, it is not known whether this covalent complex exists prior to topoisomerase II denaturation or is induced to form as a result of the detergent treatment. This question was addressed by characterizing the products of EDTA-terminated calcium-promoted reactions which were carried out in the absence of either SDS or proteinase K (Figure 9). Following EDTA treatment of reaction mixtures, topoisomerase II remained tightly bound to plasmid molecules, even at ionic strengths which exceeded 1 M (lane 4). Furthermore, enzyme–DNA binding within salt-treated samples was not affected by heating at 75 °C for 2 min (lane 5), a denaturing process that does not generate DNA cleavage (see Figure 2). In order to release complexed plasmids, samples had to be digested with proteinase K (Figure 9, lane 3). Quantitatively, the DNA molecules released by this treatment were found to contain double- or single-stranded breaks. Therefore, on the basis of these findings, it is concluded that the topoisomerase II–DNA complex trapped by EDTA treatment of calcium-promoted reactions is covalent in nature. By comparison, noncovalent topoisomerase II–DNA complexes (formed in the absence of a nucleoside triphosphate cofactor) showed greater than 90% dissociation in 0.5 M salt (Osheroff, 1986) and were completely disrupted by heating at 45 °C for 2 min.

Enzyme Activity within the Chelator-Trapped Calcium-Promoted Covalent Topoisomerase II–DNA Complex. The activity of covalently trapped enzyme molecules was evaluated by the experiment shown in Figure 10. Following incubation of *Drosophila* topoisomerase II and DNA with calcium, the divalent cation was chelated by the addition of excess EDTA or [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. Magne-

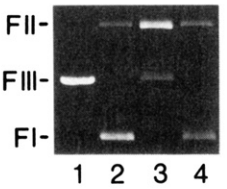

FIGURE 10: Enzymatic activity of topoisomerase II trapped in covalent enzyme–DNA complexes as a result of EDTA-terminated calcium-promoted reactions. Assays contained 100 nM enzyme, 5 nM plasmid molecules, and 5 mM $CaCl_2$ and were carried out as described under Experimental Procedures. The positions of form I, form II, and form III DNAs are shown as in Figure 2. Lanes 1 and 2, DNA standards; lane 3, calcium-containing enzyme–DNA mixtures were treated with EDTA and proteinase K; lane 4, calcium-containing enzyme–DNA mixtures were treated with 10 mM EDTA, incubated for 3 min at 30 °C with 10 mM $MgCl_2$, treated with excess chelator, and digested with proteinase K.

sium ions were then added to the reaction mixture. After a brief incubation, the cleaved and nicked DNAs generated in the presence of calcium (lane 3) were reconverted to their originally supercoiled form (lane 4). This demonstrates that topoisomerase II which is trapped in the calcium-promoted covalent enzyme–DNA complex is kinetically competent.

Discussion

Calcium, manganese, and/or cobalt have been found to support catalytic DNA strand passage by topoisomerase II from HeLa cell (Miller et al., 1981), yeast (Goto & Wang, 1982; Goto et al., 1984), and rabbit carcinoma (Riou et al., 1985), but levels of activity were greatly reduced as compared to magnesium-promoted reactions. None of the divalent cations employed in the present study,[3] including cadmium, calcium, copper, manganese, mercury, or zinc, were able to support DNA relaxation by *Drosophila* topoisomerase II. However, calcium (and to a lesser extent, manganese) was able to promote efficient enzyme-mediated DNA cleavage. Moreover, when calcium-containing reactions were terminated by the addition of EDTA, DNA cleavage products were isolated in the complete absence of a denaturing detergent. This allowed the resulting covalent topoisomerase II–DNA complex to be isolated in an active form.

Recent reports indicate that calcium ions stimulate the SDS-terminated DNA cleavage reactions mediated by eukaryotic topoisomerase I (Andersen et al., 1985) and *E. coli* gyrase.[4,5] While EDTA-terminated reactions were not carried out with these enzymes, the above results suggest that this divalent cation may be able to trap covalent enzyme–DNA complexes with a variety of type I and type II topoisomerases.

Given that (1) DNA cleavage sites are similar in magnesium- and calcium-promoted reactions and (2) magnesium can activate DNA-bound topoisomerase II which is trapped in calcium-promoted reactions, it is likely that the mechanism of enzyme-mediated DNA cleavage is the same with either cation. Calcium-promoted DNA cleavage reactions, however, do generate higher levels of single-stranded DNA breakage than are found in magnesium-containing reactions. Although the physiological implications of this finding are not known, it may be an indication that the type II enzyme cleaves DNA by making two coordinated single-stranded breaks, rather than one concerted double-stranded break in the nucleic acid backbone.

The cleavage state of the DNA trapped in calcium-promoted covalent topoisomerase II–DNA complexes has not been rigorously determined. It is not known whether the enzyme-bound DNA is actually "cleaved" or is "cleavable" and is broken only in the presence of protein denaturants. However, several lines of evidence favor the "cleaved" interpretation. First, topoisomerase II breaks and rejoins the nucleic acid backbone repeatedly during the course of its catalytic DNA strand passage reaction (Wang, 1982, 1985; Vosberg, 1985). Thus, the enzyme must be able to cleave DNA in the complete absence of a protein denaturant. Second, extensive mechanistic studies have shown that type I topoisomerases can cleave DNA in the absence of a protein denaturant and that the nucleic acid molecules trapped within active covalent enzyme–DNA complexes are cleaved (Been & Champoux, 1981; Tse-Dinh et al., 1983; Tse-Dinh, 1986). Third, treatment of EDTA-trapped calcium-promoted topoisomerase II–DNA complexes with proteinase K releases only cleaved nucleic acids. As demonstrated above, protease digestion of topoisomerase II does not itself induce DNA cleavage to take place.

The DNA cleavage reaction mediated by eukaryotic topoisomerase II has been the subject of intense recent study. It has been employed to define the enzyme's sites of interaction on DNA and chromatin (Sander & Hsieh, 1985; Udvardy et al., 1985; Yang et al., 1985; Rowe et al., 1986). Furthermore, several major classes of clinically important antineoplastic agents appear to act by interfering with the cleavage/religation reactions of mammalian topoisomerase II (Yang et al., 1985; Ross, 1985; Zwelling, 1985). All previous studies on DNA cleavage have required the addition of SDS (or other protein denaturants) to reaction mixtures in order to generate cleaved DNA products or trap covalent enzyme–DNA complexes. Unfortunately, this procedure suffers from a major drawback, as the detergent denatures topoisomerase II and destroys its enzymatic activity. This has precluded a detailed mechanistic or conformational analysis of enzyme-mediated DNA cleavage. By using calcium ions, it is now possible to trap high levels of the covalent topoisomerase II–DNA complex under conditions which do not require denaturation of the enzyme. This makes calcium a valuable tool for studying the enzymology of topoisomerase II mediated DNA cleavage.

Acknowledgments

We thank Dr. C. A. Guyer for many stimulating discussions and suggestions, L. Smith and P. Ackerman for their assistance in preparing topoisomerase II, and S. Heaver and D. Sullins for the conscientious preparation of the manuscript.

Registry No. Calcium, 7440-70-2; manganese, 7439-96-5; magnesium, 7439-95-4; DNA topoisomerase, 80449-01-0.

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Thermodynamic Characterization of Interactions between Ornithine Transcarbamylase Leader Peptide and Phospholipid Bilayer Membranes†

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ABSTRACT: The interactions of the targeting sequence of the mitochondrial enzyme ornithine transcarbamylase with phospholipid bilayers of different molecular compositions have been studied by high-sensitivity heating and cooling differential scanning calorimetry, high-sensitivity isothermal titration calorimetry, fluorescence spectroscopy, and electron microscopy. These studies indicate that the leader peptide interacts strongly with dipalmitoylphosphatidylcholine (DPPC) bilayer membranes containing small mole percents of the anionic phospholipids dipalmitoylphosphatidylglycerol (DPPG) or brain phosphatidylserine (brain PS) but not with pure phosphatidylcholines. For the first time, the energetics of the leader peptide–membrane interaction have been measured directly by using calorimetric techniques. At 20 °C, the association of the peptide with the membrane is exothermic and characterized by an association constant of 2.3×10^6 M^{-1} in the case of phosphatidylglycerol-containing and 0.35×10^6 M^{-1} in the case of phosphatidylserine-containing phospholipid bilayers. In both cases, the enthalpy of association is −60 kcal/mol of peptide. Additional experiments using fluorescence techniques suggest that the peptide does not penetrate deeply into the hydrophobic core of the membrane. The addition of the leader peptide to DPPC/DPPG (5:1) or DPPC/brain PS (5:1) small sonicated vesicles results in vesicle fusion. The fusion process is dependent on peptide concentration and is maximal at the phase transition temperature of the vesicles and minimal at temperatures below the phase transition.

Mitochondria contain approximately 200 proteins of which about 90% are synthesized in the cytosol and then transported across one or both mitochondrial membranes to their functional sites [for a review, see Hay et al. (1984), Wickner and Lodish (1985), and Rapoport (1986)]. However, the manner in which the newly synthesized proteins interact with the mitochondrial membranes to facilitate this translocation is not completely understood. Recent studies (Horwich et al., 1985a,b; Hurt et al., 1985; Ono & Tuboi, 1986) have demonstrated that most proteins of the inner membrane, matrix, and intermembrane space are synthesized in precursor form with a 15–30 amino acid N-terminal extension referred to as a target or leader sequence. This presequence not only targets the newly synthesized protein to the mitochondria but also is essential for its subsequent transfer into the organelle. After translocation, the leader sequence is cleaved by a divalent cation-dependent protease, and the mature protein assumes its role in mitochondrial function (Hay et al., 1984; Rapoport, 1986). Several workers (Ito et al., 1985; Gillespie et al., 1985) have shown

† Supported by Grants GM-37911 and BRSG S07-RR07041 from the National Institutes of Health.
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