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Role of the Divalent Cation in Topoisomerase II Mediated Reactions†

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ABSTRACT: The effects of magnesium ions on interactions between *Drosophila melanogaster* topoisomerase II and its substrates were assessed by a number of kinetic and binding assays. Results indicated that the divalent cation plays two distinct functions in promoting enzyme–substrate interactions. One class of magnesium ions participates directly in enzyme-mediated DNA cleavage reactions. A second class of magnesium ions participates directly in topoisomerase II mediated ATPase reactions and functions by providing the enzyme with a magnesium–ATP substrate. In contrast, the divalent cation did not affect the quaternary structure of the enzyme, was not required for the site-specific binding of topoisomerase II to DNA, and did not affect the enzyme's ability to discern the topological state of its nucleic acid substrate.

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). This double-stranded DNA passage reaction proceeds catalytically at the expense of ATP hydrolysis and absolutely requires the presence of divalent magnesium ions (Wang, 1982, 1985; Vosberg, 1985).

Although many of the relationships between eukaryotic topoisomerase II and its substrates have been characterized, very little is known about the enzyme's requirement for magnesium. Many functions for the divalent cation are possible, including roles in enzyme subunit–subunit interactions, recognition and binding of nucleic acids by topoisomerase II, DNA cleavage reactions, DNA strand passage, ATP hydrolysis, and/or enzyme turnover. One previous study concluded that the enzyme's need for a divalent cation went beyond the possible requirement for a magnesium–ATP substrate (Osheroff et al., 1983). Other studies presented evidence which implicated magnesium in reaction steps which occurred during or before DNA cleavage (Sander & Hsieh, 1983; Liu et al., 1983; Pommier et al., 1984). Unfortunately, while the above work confirmed that magnesium was required for interactions between topoisomerase II and its substrates (i.e., DNA and ATP), no specific role for the divalent cation could be ascribed.

In order to more fully define the requirement of *Drosophila melanogaster* topoisomerase II for magnesium, the effects of this divalent cation on the interactions between the enzyme and its substrates were assessed by a number of kinetic and binding assays. Results of the present work indicate that magnesium plays two distinct functions in topoisomerase II–substrate interactions. One class of magnesium ions participates directly in enzyme-mediated DNA cleavage reactions. A second class of magnesium ions promotes topoisomerase II mediated ATPase activity by fulfilling the requirement for a magnesium–ATP substrate.

A preliminary account of some of this work has appeared (Osheroff, 1985).

Experimental Procedures

D. 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). 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 (Maniatis et al., 1982). Analytical reagent-grade $MgCl_2 \cdot 6H_2O$ was obtained from Fisher; ethidium bromide and tris(hydroxymethyl)-aminomethane (Tris)[1] were from Sigma; SDS and proteinase K were from E. Merck Biochemicals; bovine serum albumin (nuclease free) was from BRL; and adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol) and ACS aqueous counting scintillant were from Amersham. All other chemicals were analytical reagent grade.

Agarose Gel Electrophoresis and Quantitation of Reaction Products. With the exception of ATPase experiments, 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. Samples (0.3 μg of DNA) were subjected to electrophoresis in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm. Gels were stained for 30 min in an aqueous solution of ethidium bromide (1 μg/mL). DNA bands were visualized by transillumination

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

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with ultraviolet light (300 nm) and photographed through Kodak 23A and 12 filters using Polaroid type 665 positive/negative film. The amount of DNA in bands 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) topoiosomerase 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 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.

Nonturnover Relaxation of Supercoiled DNA by Stoichiometric Amounts of Topoisomerase II. Assays employed 40 nM enzyme and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer containing 5 mM $MgCl_2$ and 1 mM adenyl-5′-yl imidodiphosphate. Reactions were at 30 °C for 6 min and were terminated by the addition of 2 μL of 250 mM EDTA followed by 1 μL of 10% SDS. Loading buffer (2.5 μL) was added, and samples were heated to 75 °C for 2 min prior to electrophoresis.

Binding of Topoisomerase II to DNA. Binding mixtures consisted of 40 nM enzyme and 5 nM negatively 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. Loading buffer (2.5 μL) was added, and samples were subjected to electrophoresis as described above. DNA binding was determined by monitoring the shift in the electrophoretic mobility of bound nucleic acids compared to free substrates (Osheroff, 1986).

Cleavage of DNA by Topoisomerase II. Reactions employed 100 nM enzyme and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer. The concentration of magnesium was varied from 0 to 15 mM. DNA cleavage was induced by the addition of 2 μL of 10% SDS, followed by 1 μL of 250 mM EDTA. Two microliters of 0.8 mg/mL proteinase K was added, and assay mixtures were 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 prior to electrophoresis.

ATPase Activity. ATPase assays were carried out as previously described (Osheroff et al., 1983). DNA-stimulated reactions contained 7.5 nM topoisomerase II and 250 nM negatively supercoiled pBR322 DNA in a total of 20 μL of relaxation buffer which contained 1 mM [γ-^{32}P]ATP (0.1 Ci/mmol). DNA-independent reactions contained 75 nM topoisomerase II in a total of 20 μL of relaxation buffer which lacked EDTA and contained 1 mM [γ-^{32}P]ATP (0.1 Ci/mmol). ATP hydrolysis was carried out at 30 °C. In all cases, samples (2.5 μL) were removed at 2- or 4-min intervals up to 16 min, spotted onto thin-layer cellulose plates which were impregnated with poly(ethylenimine) (Polygram CEL 300 PEI, Brinkmann), and chromatographed in freshly prepared 0.4 M NH_4HCO_3. Reaction products were visualized by autoradiography with Kodak XAR film. Radioactive areas corresponding to liberated [^{32}P]orthophosphate were cut out of the chromatograms, and the amount of ATP hydrolysis was quantitated by using a Beckman LS-7500 liquid scintillation counter and ACS aqueous counting scintillant. When divalent cations were present, their concentrations were 5 mM.

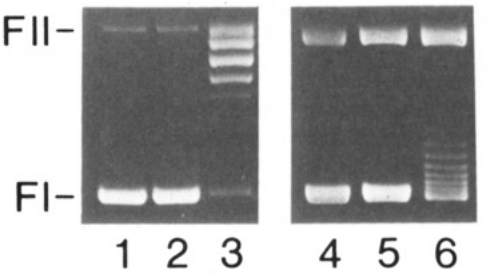

FIGURE 1: Requirement of magnesium ions for catalytic (lanes 1–3) and nonturnover stoichiometric (lanes 4–6) relaxation of DNA by *D. melanogaster* topoisomerase II. Reaction conditions and agarose gel electrophoresis are described under Experimental Procedures. The positions of form I (FI) (fully supercoiled DNA) and form II (FII) (nicked circular plasmid molecules) are shown. Partially relaxed DNA topoisomers migrate intermediate to the form I and form II bands. Lanes 1 and 4, DNA standards; lanes 2 and 5, DNA relaxation reactions carried out in the absence of magnesium; lanes 3 and 6, DNA relaxation reactions carried out in the presence of 5 mM $MgCl_2$.

Velocity Sedimentation. Topoisomerase II (5 μg) along with protein standards (catalase, fibrinogen, ovalbumin, and cytochrome *c*) in a total of 200 μL was layered onto preformed linear 15–40% glycerol gradients as previously described (Shelton et al., 1983). Gradients (11.25 mL) contained 15 mM sodium phosphate, pH 7.1, 350 mM NaCl, and 0.25 mM EDTA in the presence or absence of 5 mM $MgCl_2$. Sedimentation was in a Beckman SW41 rotor at 250000*g* for 66 h at 4 °C. Fifty 0.23-mL fractions were collected from the top of each gradient. Protein standards were located by their absorbance at 410 nm (catalase and cytochrome *c*) or 280 nm (fibrinogen and ovalbumin). Topoisomerase II was located by the catalytic DNA relaxation assay described above. Sedimentation coefficients were normalized to values at 20 °C in water ($s^0_{20,w}$).

RESULTS

Requirement for Magnesium Ions in the DNA Relaxation Reaction. Topoisomerase II from *D. melanogaster* absolutely requires magnesium ions for its catalytic function (Hsieh & Brutlag, 1980), with optimal rates of activity being generated at magnesium concentrations of 5–10 mM (Osheroff et al., 1983). The enzyme's need for a divalent cation in catalytic DNA relaxation assays is demonstrated in Figure 1. Relaxed DNA topoisomers were generated in the presence (lane 3) but not in the absence of magnesium (lane 2).

As a first step toward characterizing the requirement of topoisomerase II for a divalent cation, the effect of magnesium on nonturnover DNA relaxation reactions was studied. In this experiment, stoichiometric, rather than catalytic, levels of topoisomerase II were employed, and the ATP cofactor was replaced with adenyl-5′-yl-imidodiphosphate, a nonhydrolyzable ATP analogue which induces DNA strand passage but will not support turnover of the *Drosophila* enzyme (Osheroff et al., 1983; Osheroff, 1986).[2] The results of this study (Figure 1, lanes 4–6) indicate that DNA strand passage does not take place in the absence of a divalent cation, even

[2] The bacterial plasmid pBR322 contains about 30 negative superhelical twists when isolated from *E. coli* (Liu et al., 1979). Since the topoisomerase II:plasmid ratio employed in this experiment was 8:1 and type II topoisomerases remove two supercoils per DNA strand passage event, if every molecule of enzyme was bound to pBR322 and carried out a single event, the DNA would be approximately 50% relaxed.

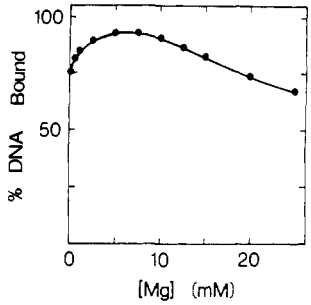

FIGURE 2: Effect of magnesium concentration on the binding of topoisomerase II to negatively supercoiled pBR322 DNA. Assays contained 40 nM enzyme and 5 nM plasmid molecules and are described under Experimental Procedures.

under conditions which do not require the recycling of topoisomerase II. While this finding does not rule out a role for magnesium in the process of enzyme regeneration, it demonstrates that the divalent cation is needed for at least one event which takes place prior to ATP hydrolysis and enzyme turnover. Therefore, the involvement of magnesium ions in enzyme subunit–subunit interactions, DNA recognition and binding events, and DNA cleavage reactions was determined.

Effect of Magnesium on the Quaternary Structure of Topoisomerase II. In the absence of a divalent cation, eukaryotic topoisomerase II exists in solution as a homodimer (Miller et al., 1981; Sander & Hsieh, 1983; Shelton et al., 1983; Goto et al., 1984; Halligan et al., 1985). In order to assess the effects of magnesium on the quaternary structure of topoisomerase II, sedimentation velocity experiments were carried out in the presence and absence of the divalent cation. Under the conditions employed, the enzyme's sedimentation coefficient, 9.25 S (Shelton et al., 1983), did not change in the presence of magnesium. Therefore, the magnesium cofactor does not induce a change in the quaternary structure of topoisomerase II.

Effect of Magnesium on the Binding of Topoisomerase II to DNA. The first step in the relaxation of negatively supercoiled DNA by topoisomerase II is the binding of nucleic acid substrates by the enzyme. The effect of magnesium concentration on this process is shown in Figure 2. In the absence of the divalent cation, 75% of the DNA was bound to the *Drosophila* enzyme. Topoisomerase II–DNA interactions were mildly stimulated by the addition of low levels of magnesium, such that under optimal conditions (5–7.5 mM magnesium), DNA binding rose to 93%. At higher cation levels (>7.5 mM), some inhibition of binding was observed. On the basis of experiments that examined the effect of sodium chloride on topoisomerase II–DNA interactions (Osheroff, 1986), this decrease in binding appears to reflect a general ionic strength effect. Therefore, while topoisomerase II–DNA interactions are affected by the presence and concentration of magnesium ions, the divalent cation clearly is not required for the binding of DNA by the enzyme.

Previous studies demonstrated that in the presence of 5 mM magnesium ions, *Drosophila* topoisomerase II was able to discern the topological state of DNA, binding negatively supercoiled substrates 4–8 times more effectively than it did nicked circular molecules (Osheroff & Brutlag, 1983; Osheroff, 1986). In competitive binding assays which contained equimolar amounts of supercoiled and nicked plasmid molecules, the ratio of supercoiled to nicked DNA bound (approximately 4.6:1) varied less than 7% over a magnesium range of 0–25 mM (Table I). Thus, the ability of the enzyme to discern the topological state of its nucleic acid substrate does not require the presence of a divalent cation.

Table I: Effect of Magnesium Ions on the Recognition of DNA Topology by Topoisomerase II[a]

[magnesium] (mM)	% supercoiled DNA bound/% nicked DNA bound	[magnesium] (mM)	% supercoiled DNA bound/% nicked DNA bound
0	4.7	12.5	4.6
2.5	4.6	15.0	4.8
5.0	4.5	20.0	4.6
7.5	4.5	25.0	4.8
10.0	4.6		

[a] Competitive binding experiments contained equimolar amounts of negatively supercoiled and nicked circular pBR322 DNA and were carried out as described under Experimental Procedures.

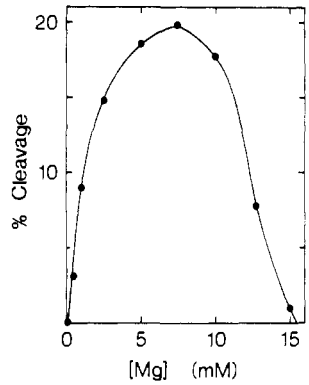

FIGURE 3: Effect of magnesium concentration on the cleavage of negatively supercoiled pBR322 DNA by topoisomerase II. Assays contained 100 nM enzyme and 5 nM plasmid molecules and are described under Experimental Procedures.

Requirement for Magnesium in DNA Cleavage Reactions. Since magnesium is not necessary for DNA binding, its role in double-stranded DNA cleavage [the next step in the DNA relaxation pathway (Wang, 1982, 1985; Vosberg, 1985)] was examined. 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, 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 DNA cleavage can be monitored by the conversion of supercoiled circular nucleic acids to linear molecules (Sander & Hsieh, 1983; Liu et al., 1983).

As demonstrated in Figure 3, *Drosophila* topoisomerase II absolutely requires a divalent cation for its cleavage reaction. Maximal DNA cleavage was observed at magnesium concentrations of 5–10 mM, the same range that was found to yield optimal rates of DNA relaxation (Osheroff et al., 1983). This strongly implies that the enzyme's overall requirement for magnesium reflects its need for the cation in DNA cleavage reactions.

Role of Magnesium in the Topoisomerase II Mediated ATPase Reaction. ATP is essential to the catalytic function of eukaryotic type II topoisomerases (Wang, 1982, 1985; Vosberg, 1985). Binding of the nucleoside triphosphate induces a double-stranded DNA passage event, and ATP hydrolysis is required for enzyme turnover (Osheroff et al., 1983; Osheroff, 1986). Therefore, the effect of magnesium ions on the time course of ATP hydrolysis by *Drosophila* topoisomerase II was examined.

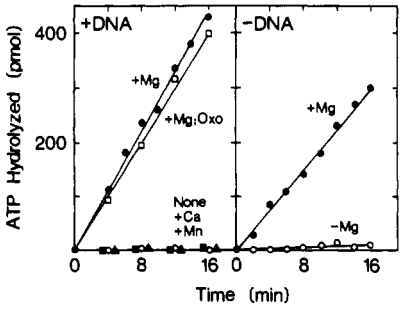

FIGURE 4: Magnesium dependence of the ATPase activity of topoisomerase II. Reaction conditions are given under Experimental Procedures. The left panel (+DNA) shows the time course of the DNA-stimulated ATPase reaction. Assays contained 7.5 nM topoisomerase II and 250 nM negatively supercoiled pBR322 plasmid molecules. Only those divalent cations indicated were included in reactions. Assays were carried out in the presence of 5 mM $MgCl_2$ (●), 5 mM $MgCl_2$ and 2.5 mM oxolinic acid (Oxo) (□), 5 mM $CaCl_2$ (■), and 5 mM $MnCl_2$ (▲) or in the absence of a divalent cation (○). The right panel (–DNA) shows the time course of the DNA-independent ATPase reaction. Assays contained 75 nM topoisomerase II and lacked DNA. Reactions were carried out in the presence (●) or absence (○) of 5 mM $MgCl_2$.

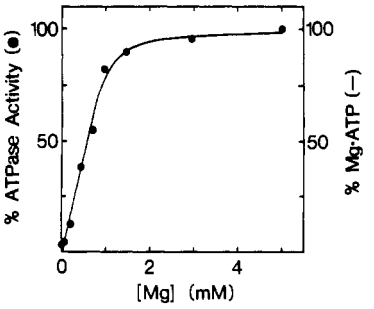

FIGURE 5: Effect of magnesium concentration on the DNA-independent ATPase activity of topoisomerase II. Assay conditions are given under Experimental Procedures. The closed circles indicate the relative rate of ATP hydrolysis by topoisomerase II. The solid line represents the theoretical curve for the binding of 1 mM ATP by magnesium ions (i.e., percent ATP bound) assuming a K_D value of 50 μM (Phillips, 1966).

As shown in Figure 4 (left panel), a high rate of DNA-stimulated ATP hydrolysis was generated in the presence of 5 mM magnesium, but no ATPase activity was observed in the absence of a divalent cation. While this implies that magnesium participates directly in the interaction between the enzyme and ATP, other alternatives must be considered. Since ATP hydrolysis is stimulated 15–20-fold by the presence of negatively supercoiled DNA (Osheroff et al., 1983), it is possible that DNA cleavage (which requires a divalent cation) and/or strand passage may be prerequisites for ATP hydrolysis. These latter two possibilities were eliminated by the following experiments.

First, the effect of oxolinic acid on DNA-stimulated magnesium-promoted ATPase reactions was examined. This compound is a potent inhibitor of type II topoisomerases which allows DNA cleavage, but blocks DNA strand passsage (Wang, 1983, 1985; Vosberg, 1985). At a concentration (2.5 mM) which was approximately 2-fold higher than its K_i value (Osheroff et al., 1983), oxolinic acid showed little ability to inhibit ATP hydrolysis (Figure 4, left panel). Therefore, ATPase activity is not linked to DNA strand passage events. Second, magnesium ions were replaced in reaction mixtures with either calcium or manganese ions. Both of these divalent cations promote enzyme-mediated DNA cleavage reactions, but neither will support DNA strand passage (Osheroff et al., 1983; Osheroff & Zechiedrich, 1987). As seen in Figure 4 (left panel), neither divalent cation promoted ATPase activity. Therefore, the ability to cleave DNA does not correlate with the ability to hydrolyze ATP.

To further demonstrate a direct role for magnesium in the topoisomerase II mediated hydrolysis of ATP, DNA-independent ATPase reactions were carried out. Since reaction rates are considerably slower in the absence of DNA (Osheroff, 1983), enzyme concentrations were increased 10-fold in these experiments. As shown in Figure 4 (right panel), DNA-independent ATPase activity was highly dependent on the presence of a divalent cation. The reaction rate was increased more than 30-fold when 5 mM magnesium was included in assay mixtures. Thus, magnesium ions must function directly in enzyme–ATP interactions.

It is not known whether the requirement for a divalent cation in topoisomerase II mediated ATPase reactions reflects the need for a magnesium-complexed enzyme or a magnesium–ATP substrate. To resolve these two possibilities, the effect of magnesium concentration on the rate of DNA-independent ATP hydrolysis was determined (Figure 5). The rationale for this experiment is as follows. If the role of the divalent cation is to provide the enzyme with a magnesium–ATP substrate, then the ability of the divalent cation to stimulate the rate of ATP hydrolysis should be directly proportional to its ability to complex with the nucleoside triphosphate. Under the assay conditions employed (0.1 M ionic strength, pH 7.9, 30 °C, and 1 mM ATP), the dissociation constant for magnesium–ATP is 50 μM (Phillips, 1966). As seen in Figure 5, the curve which represents the stimulation of ATPase activity by increasing levels of magnesium (closed circles) can be superimposed on the theoretical curve which represents the complexing of ATP by magnesium (assuming a K_D value of 50 μM) (solid line). It therefore appears likely that the function of the divalent cation in topoisomerase II mediated ATPase reactions is to present the enzyme with a magnesium–ATP substrate.

Discussion

Magnesium is essential for the catalytic activity of eukaryotic topoisomerase II (Wang, 1982, 1985; Vosberg, 1985). The results of the present study indicate that the divalent cation performs two distinct functions for the type II enzyme from *D. melanogaster*. One class of magnesium ions participates directly in topoisomerase II mediated DNA cleavage reactions. On the basis of concentration dependence experiments, levels of divalent cation which yield optimal DNA cleavage are similar to those required to produce maximal DNA relaxation (Osheroff et al., 1983). Since magnesium ions [and other cations which promote DNA cleavage (Osheroff & Zechiedrich, 1987)] readily bind to nucleic acids (Saenger, 1984), it is not known whether this class of ions acts by complexing with topoisomerase II or with its DNA substrate. A second class of magnesium ions is required for enzyme-mediated ATP hydrolysis reactions. This class of divalent cations appears to fulfill the enzyme's need for a magnesium–ATP substrate.

Previous reports on prokaryotic and eukaryotic type II topoisomerases demonstrated that enzyme-mediated DNA cleavage was blocked when magnesium ions were chelated or left out of reaction mixtures (Sugino et al., 1977; Higgins & Cozzarelli, 1982; Sander & Hsieh, 1983; Pommier et al., 1984). However, no direct role for the cation in cleavage reactions could be ascribed, as (1) all of the above studies were carried out in the presence of ATP and/or DNA binding agents (Shen & Pernet, 1985), (2) studies on eukaryotic topoisomerase II did not examine the effects of magnesium on enzyme–DNA interactions which took place prior to cleavage, and (3) the large effect (approximately 3-fold stimulation) of

magnesium on the binding of *E. coli* gyrase to DNA (Higgins & Cozzarelli, 1982) obscured the possible participation of divalent cations in later interactions.

Although the presence of magnesium stimulates physical interactions between *D. melanogaster* topoisomerase II and its nucleic acid substrate by about 20%, it is not required for protein–DNA binding. In addition, the divalent cation is not involved in the recognition of DNA topology, has little effect on the selection of DNA cleavage sites by topoisomerase II (Osheroff & Zechiedrich, 1987), and does not affect the quaternary structure of the homodimeric *Drosophila* type II enzyme. Therefore, regarding its use of a divalent cation in enzyme–nucleic acid interactions, topoisomerase II appears to be similar to the type II restriction endonucleases. These latter homodimeric enzymes do not require magnesium to maintain subunit interactions, bind DNA, or recognize their specific DNA cleavage sites, but do require a divalent cation in order to catalyze DNA cleavage (Modrich, 1982; Modrich & Roberts, 1982).

Topoisomerase II is a ubiquitous enzyme that is essential to the viability of eukaryotic cells (Goto & Wang, 1984; DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985). It is involved in many aspects of DNA metabolism and is required for chromosome segregation (Wang, 1982, 1985; Vosberg, 1985). Clearly, before the in vivo functions of the enzyme can be fully delineated, its in vitro activities must be well characterized. Determining the role of the divalent cation in topoisomerase II–substrate interactions represents a fundamental part of this characterization.

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Registry No. ATPase, 9000-83-3; DNA topoisomerase, 80449-01-0; Mg, 7439-95-4.

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