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## Metal Ion Interactions in the DNA Cleavage/Ligation Active Site of Human Topoisomerase II $\alpha$ <sup>†</sup>

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### Abstract

Human topoisomerase II $\alpha$  utilizes a two-metal-ion mechanism for DNA cleavage. One of the metal ions ( $M_1^{2+}$ ) is believed to make a critical interaction with the 3'-bridging atom of the scissile phosphate, while the other ( $M_2^{2+}$ ) is believed to interact with a non-bridging oxygen of the scissile phosphate. Based on structural and mutagenesis studies of prokaryotic nucleic acid enzymes, it has been proposed that the active site divalent metal ions interact with type II topoisomerases through a series of conserved acidic amino acid residues. The homologous residues in human topoisomerase II $\alpha$  are E461, D541, D543, and D545. To address the validity of these assignments and to delineate interactions between individual amino acids and  $M_1^{2+}$  and  $M_2^{2+}$ , we individually mutated each of these acidic amino acid residues in topoisomerase II $\alpha$  to either cysteine or alanine. Mutant enzymes displayed a marked loss of catalytic and DNA cleavage activity as well as a reduced affinity for divalent metal ions. Additional experiments determined the ability of wild-type and mutant topoisomerase II $\alpha$  enzymes to cleave an oligonucleotide substrate that contained a sulfur atom in place of the 3'-bridging oxygen of the scissile phosphate in the presence of  $Mg^{2+}$ ,  $Mn^{2+}$ , or  $Ca^{2+}$ . Based on the results of these studies, we conclude that the four acidic amino acid residues interact with metal ions in the DNA cleavage/ligation active site of topoisomerase II $\alpha$ . Furthermore, we propose that  $M_1^{2+}$  interacts with E461, D543, and D545 and  $M_2^{2+}$  interacts with E461 and D541.

Fundamental nuclear processes, such as replication and recombination, often generate tangles and knots in DNA (1–3). If these topological alterations in the genetic material are not successfully resolved, the ability of chromosomes to segregate during mitosis will be severely impeded (2–5).

DNA tangles and knots are removed by type II topoisomerases (2,4,6–9). These essential enzymes act by generating transient double-stranded breaks in DNA and passing an intact double helix through the break (4,10). Like many enzymes that break or rejoin nucleic acids, type II topoisomerases require divalent metal ions as cofactors for catalysis (11). Magnesium

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#### SUPPORTING INFORMATION AVAILABLE

Data and parameters for the kinetic analysis are available online in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

appears to be the physiological metal ion used by these enzymes (11). Recent studies indicate that human topoisomerase II $\alpha$  and II $\beta$  both utilize a two-metal-ion mechanism for DNA cleavage (12,13). This mechanism is similar to those proposed for the scission reaction of DNA gyrase (a bacterial type II topoisomerase) (14) as well as the polymerization reactions of primases and some DNA polymerases (15–17).

During the DNA cleavage reaction of topoisomerase II $\alpha$  and II $\beta$ , one divalent metal ion (metal ion 1) makes an important interaction with the 3'-bridging oxygen atom of the scissile phosphate (12,13). This interaction accelerates rates of enzyme-mediated DNA scission, most likely by stabilizing the leaving group (12–14). A second metal ion (metal ion 2) appears to contact a non-bridging atom of the scissile phosphate in the active site of topoisomerase II $\beta$  (13). This interaction plays a significant role in DNA cleavage mediated by the  $\beta$  isoform and greatly stimulates scission. As proposed previously, metal ion 2 is believed to stabilize the DNA transition state and/or help deprotonate the active site tyrosine (12–14,18). Although topoisomerase II $\alpha$  has an absolute requirement for metal ion 2 (12), the role of this divalent cation in its DNA cleavage reaction is unclear. It is not definitive as to whether it interacts with a non-bridging oxygen in the active site of topoisomerase II $\alpha$ . However, if the interaction exists, it does not affect rates of DNA cleavage.

Type IA topoisomerases, type II topoisomerases, and primases share a conserved region known as the *TOPRIM* domain (19,20). This region has been implicated in coordinating divalent metal ions that are used during nucleic acid cleavage, ligation, and polymerization reactions (14, 19–21). Based on structural studies of *Escherichia coli* topoisomerase III, primase (dnaG), and DNA gyrase, together with mutagenesis studies of DNA gyrase, it has been proposed that the divalent metal ions in the active sites of type II topoisomerases interact with the protein through a series of conserved acidic amino acid residues (Figure 1) (14,18,19,22). The homologous residues in human topoisomerase II $\alpha$  are E461, D541, D543, and D545 (23). These assignments are consistent with a recent crystallographic structure of the catalytic core of yeast topoisomerase II in a non-covalent complex with DNA (24). However, this latter structure contained only one divalent metal ion and the active site tyrosine residue was located too far from the scissile bond to support DNA cleavage. Thus, specific interactions between individual amino acids and the two divalent metal ions in the DNA cleavage/ligation active site of type II topoisomerases have yet to be fully elucidated.

In an effort to more completely define interactions between divalent metal ions and type II topoisomerases, we carried out a series of kinetic and mutagenesis studies with topoisomerase II $\alpha$ . Results confirm an important role for E461, D541, D543, and D545 in DNA cleavage mediated by the human enzyme and delineate contacts between individual amino acid residues and metal ions 1 and 2.

## EXPERIMENTAL PROCEDURES

### Enzymes

Wild-type and mutant human topoisomerase II $\alpha$  proteins were expressed in *Saccharomyces cerevisiae* JEL1 $\Delta$ top1 cells and purified as described previously (25–27). Individual point mutations in human topoisomerase II $\alpha$  were generated using the PCR-based Lightning Mutagenesis Kit (Stratagene). D541, D543, D545, and E461 were individually mutated to either Cys or Ala in the inducible overexpression YEpWOB6 plasmid. Primer sequences were as follows: D545C forward 5' – GGAAGATAATGATTATGACAGATCAGGACCAATGTGGTTCACATC – 3'; reverse 5' – GATGTGGGAACACATTGGTCCTGATCTGTCATAATCATTATCTTCC – 3'; D545A forward 5' – GGAAGATAATGATTATGACAGATCAGGACCAAGCTGGTTCACATC – 3'; reverse

5' – GATGTGGGAACCAGCTTGGTCCTGATCTGTCATAATCATTATCTTCC – 3';  
 D543C forward 5' –  
 GGAAGATAATGATTATGACAGATCAGTGCCAAGATGGTTCACATC – 3'; reverse  
 5' – GATGTGGGAACCATCTTGGCACTGATCTGTCATAATCATTATCTTCC – 3';  
 D543A forward 5' –  
 GGAAGATAAGATTATGACAGATCAGGCCAAGATGGTTCACATC – 3'; reverse  
 5' – GATGTGGGAACCATCTTGGCCTGATCTGTCATAATCATTATCTTCC – 3';  
 D541C forward 5' –  
 GGAAGATAATGATTATGACATGTCAGGACCAAGATGGTTCACATC – 3'; reverse  
 5' – GATGTGGGAACCATCTTGGTCCTGACATGTCATAATCATTATCTTCC – 3';  
 D541A forward 5' –  
 GGAAGATAATGATTATGACAGCTCAGGACCAAGATGGTTCACATC – 3'; reverse  
 5' – GATGTGGGAACCATCTTGGTCCTGAGCTGTCATAA TCATTATCTTCC – 3';  
 E461C forward 5' –  
 GAGTGTACGCTTATCCTGACTTGTGGAGATTCAGCCAAAACCTTGGCTG – 3';  
 reverse 5' –  
 CAGCCAAAGTTTTGGCTGAATCTCCACAAGTCAGGATAAGCGTACACTC – 3'; and  
 E461A forward 5' –  
 GAGTGTACGCTTATCCTGACTGCGGGAGATTCAGCCAAAACCTTGGCTG – 3';  
 reverse 5' –  
 CAGCCAAAGTTTTGGCTGAATCTCCCGCAGTCAGGATAAGCGTACACTC – 3'. In  
 all cases, mutant topoisomerase II $\alpha$  genes were isolated and sequenced before being  
 transformed into JEL1 $\Delta$ top1 yeast cells. The purity of mutant enzymes was assessed by  
 polyacrylamide gel electrophoresis. Proteins were visualized with coomassie or silver stains  
 (data not shown). Enzymes were >95% homogeneous. Contamination with yeast  
 topoisomerase II was too low to quantify (<0.2%), as determined by western blot analysis using  
 antibodies directed against the yeast type II enzyme.

### Preparation of Oligonucleotides

A 50 bp duplex oligonucleotide was designed using a previously identified topoisomerase II cleavage site from pBR322 (28). Wild-type oligonucleotide sequences were generated using an Applied Biosystems DNA synthesizer. The 50-mer top and bottom sequences were 5'-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3' and 5'-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCAA-3', respectively. The arrow denotes the point of scission by topoisomerase II. The top strand was composed of two shorter sequences that produced a nick at the location of the scissile bond.

DNA containing a single 3'-bridging phosphorothiolate linkage was synthesized as described previously (29). The location of the phosphorothiolate was at the normal scissile bond on the bottom strand. Substrates containing a racemic phosphorothioate in place of the non-bridging scissile bond oxygens of the bottom strand were synthesized by Operon.

### Radioactive Labeling of Oligonucleotides

[ $\gamma$ -<sup>32</sup>P]ATP (~5000 Ci/mmol) was obtained from ICN. Single-stranded oligonucleotides were labeled on their 5'-termini using T4 polynucleotide kinase (New England Biolabs). Following labeling and gel purification, complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

### DNA Cleavage of Oligonucleotide Substrates

DNA cleavage assays were carried out by the procedure of Deweese et al. (29). DNA cleavage reactions with wild-type or mutant human topoisomerase II $\alpha$  proteins contained 200 nM enzyme and 100 nM double-stranded oligonucleotide in a total of 10  $\mu$ L of DNA cleavage

buffer [10 mM Tris-HCl, pH 7.9, 135 mM KCl, 0.1 mM EDTA, and 2.5% glycerol]. Unless otherwise noted, the concentration of the divalent cation added to reaction mixtures was 5 mM. In some cases, the concentration of divalent cation ( $\text{MgCl}_2$ ,  $\text{MnCl}_2$ , or  $\text{CaCl}_2$ ) was varied and/or combinations of the cations were used. Experiments that monitored DNA cleavage over a range that included divalent cation concentrations below 1 mM utilized cleavage buffer that lacked EDTA. Reactions were initiated by the addition of enzyme and were incubated for 0 to 30 min at 37 °C. DNA cleavage products were trapped by the addition of 2  $\mu\text{L}$  of 10% SDS followed by 2  $\mu\text{L}$  of 250 mM NaEDTA, pH 8.0. Proteinase K (2  $\mu\text{L}$  of 0.8 mg/mL) was added to digest the enzyme. Cleavage products were resolved by electrophoresis in a 14% denaturing polyacrylamide gel. To inhibit oxidation of cleaved oligonucleotides containing 3'-terminal –SH moieties and the formation of multimers in the gel, 100 mM DTT was added to the sample loading buffer. DNA cleavage products were visualized and quantified using a Bio-Rad Molecular Imager.

Pre-equilibrium DNA cleavage reactions were monitored for 0.5 s to 3 s using a KinTek model RQF-3 chemical quench flow apparatus (Austin, TX). Cleavage was initiated by rapidly mixing two independent solutions. The first contained a noncovalent complex formed between human topoisomerase II $\alpha$  and  $^{32}\text{P}$ -labeled oligonucleotide in cleavage buffer that lacked divalent cation. The second solution contained cleavage buffer in which the concentration of  $\text{MgCl}_2$  was 2 times higher than normal (concentration of  $\text{MgCl}_2$  in the final reaction mixture ranged from 0.5–10 mM). The two solutions were mixed at 37 °C, and DNA cleavage was quenched with 1% SDS (v/v final concentration). Products were processed and analyzed as described above.

Pre-steady-state data were fitted using the program DynaFit (30). Best fits were obtained for plots of time courses versus metal ion concentration using least-squares regression by Levenberg-Marquardt methods. The full analysis is included in Supporting Information.

### Topoisomerase II-mediated Relaxation of Plasmid DNA

DNA relaxation assays were performed according to the procedure of McClendon et al. (31). Reaction mixtures contained 0.5–20 nM wild-type or 20 nM mutant human topoisomerase II $\alpha$  proteins, 1 mM ATP, and 5 nM negatively supercoiled pBR322 in a total of 20  $\mu\text{L}$  of 10 mM Tris-HCl, pH 7.9, 175 mM KCl, 5 mM  $\text{MgCl}_2$ , 0.1 mM NaEDTA, and 2.5% glycerol. Mixtures were incubated at 37 °C for 0–20 min and stopped by the addition of 3  $\mu\text{L}$  of 0.5% SDS and 77 mM EDTA. Samples were mixed with 2  $\mu\text{L}$  of agarose gel loading buffer [60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue (w/v), and 0.5% xylene cyanol FF (w/v)] and subjected to electrophoresis in 1% agarose gels in 100 mM Tris-borate, pH 8.3, and 2 mM EDTA. Gels were stained for 30 min with 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide. DNA bands were visualized by UV light and were quantified using an Alpha Innotech digital imaging system (San Leandro, CA). DNA relaxation was monitored by the loss of the initial supercoiled substrate.

### Topoisomerase II-mediated Cleavage of Plasmid DNA

Plasmid DNA cleavage reactions were performed using the procedure of Fortune and Osheroff (32). Reaction mixtures contained 200 nM wild-type or mutant human topoisomerase II proteins and 5 nM negatively supercoiled pBR322 DNA in 20  $\mu\text{L}$  of DNA cleavage buffer containing 5 mM  $\text{MgCl}_2$  and 100  $\mu\text{M}$  etoposide or amsacrine. DNA cleavage mixtures were incubated for 6 min at 37 °C. DNA cleavage complexes were trapped by adding 2  $\mu\text{L}$  of 5% SDS followed by 2  $\mu\text{L}$  of 250 mM  $\text{Na}_2\text{EDTA}$  (pH 8.0). Proteinase K was added (2  $\mu\text{L}$  of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 37 °C to digest topoisomerase II. Samples were mixed with 2  $\mu\text{L}$  of agarose gel loading buffer, heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH

8.3) and 2 mM EDTA containing 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide. Double-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized and quantified as described above.

## RESULTS AND DISCUSSION

### Use of $\text{Mg}^{2+}$ During Topoisomerase II-mediated DNA Cleavage

As discussed earlier, human topoisomerase II $\alpha$  utilizes a two-metal-ion mechanism for its DNA scission reaction. Based on the relative abilities of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  to support cleavage of wild-type oligonucleotides and substrates containing 3'-bridging phosphorothiolates, it was proposed that metal ion 1 bound to topoisomerase II $\alpha$  with an affinity that was  $\sim 10$ -fold higher than that of metal ion 2 (12). However, the interaction of the physiological metal ion,  $\text{Mg}^{2+}$ , with topoisomerase II $\alpha$  has not been explored in detail. Therefore, a kinetic approach was employed to further define the parameters of  $\text{Mg}^{2+}$  utilization by topoisomerase II $\alpha$ .

As a first step, the cleavage of a wild-type substrate was determined over a  $\text{Mg}^{2+}$  concentration range of 1  $\mu\text{M}$  to 10 mM (Figure 2). To simplify the analysis and increase baseline levels of scission, we used a substrate that contained a nick at the scissile bond of the unlabeled strand (12). Previous work has demonstrated that the presence of the nick enhances both the rate and level of DNA cleavage on the opposite strand (12,33).

Optimal DNA cleavage was observed at  $\sim 2.5$ – $5.0$  mM  $\text{Mg}^{2+}$ . The decrease in enzyme activity at higher metal ion concentrations has been observed for other type II topoisomerases and most likely represents an ionic strength effect (36).

At the low end of the  $\text{Mg}^{2+}$  concentration range, the dependence of the metal ion appeared to be biphasic in nature. This biphasic metal ion concentration dependence suggests that there are two  $\text{Mg}^{2+}$  sites in the DNA cleavage/ligation domain of topoisomerase II $\alpha$  and that both need to be filled in order to support scission. The initial (*i.e.*, high affinity) phase appears to be completely filled somewhere between  $\sim 100$ – $250$   $\mu\text{M}$   $\text{Mg}^{2+}$ , implying an apparent  $K_D$  value for this site that probably is less than 100  $\mu\text{M}$ . Based on the results of previous studies (12, 13), we propose that this high affinity interaction represents the binding of  $\text{Mg}^{2+}$  to the site of metal ion 1. One-half maximal DNA cleavage activity was observed at  $\sim 1.0$ – $1.5$  mM  $\text{Mg}^{2+}$ . This finding suggests that  $\text{Mg}^{2+}$  binds to the site of metal ion 2 with an apparent  $K_D$  value that is in this latter concentration range. By comparison, the concentration of free  $\text{Mg}^{2+}$  within the cell is estimated to be  $\sim 0.5$ – $1.5$  mM (34).

To extend the above experiments, the intrinsic  $K_D$  value of human topoisomerase II $\alpha$  for  $\text{Mg}^{2+}$  was determined by analyzing the metal ion dependence of the pre-equilibrium kinetics of enzyme-mediated DNA cleavage. Experiments utilized a  $\text{Mg}^{2+}$  concentration range of 0.5–10 mM. Since both metal ions 1 and 2 must be present in order to support DNA cleavage, we started at a concentration at which the first metal ion binding site was likely to be saturated. Thus, data from the kinetic analysis reflect primarily the interaction of  $\text{Mg}^{2+}$  with the second, or low affinity, metal ion binding site. In addition, since the substrate used for these studies contained a nick at the scissile bond on the unlabeled strand, the enzyme can cleave only the scissile bond on the labeled strand. As a result, the overall equation describing the reaction being monitored is reduced to the following:



Plots of percent DNA cleavage versus cation concentration were fitted to a single-exponential equation to generate apparent rate constants for the reaction at each concentration. Apparent rate constants were then plotted versus cation concentration (Figure 3) and analyzed using the program DynaFit (30).

The analysis was performed using a series of values for the forward cleavage ( $k_2$ ) and reverse ligation ( $k_{-2}$ ) reaction rates. In all cases, best fits were obtained at  $k_{-2}/k_2$  ratios in the range of ~40–500, implying that topoisomerase II $\alpha$  ligates DNA considerably faster than it cleaves the double helix. This finding is consistent with the fact that the human type II enzyme maintains very low equilibrium levels of covalent enzyme-cleaved DNA complexes (E•DNA\*•Metal) (4,10,33,35).

Finally, over a wide range of  $k_1$  and  $k_{-1}$ , best fits always yielded a  $k_{-1}/k_1$  ratio (*i.e.*,  $K_D$  value) of ~1.2 mM. This apparent  $K_D$  for the binding of Mg<sup>2+</sup> to the second metal ion site is similar to the value obtained in the equilibrium titration experiment discussed above.

### Amino Acid Residues Involved in Metal Ion Coordination

Based on crystallographic and mutagenesis studies with bacterial topoisomerases and primase, it has been proposed that divalent metal ions are coordinated to human topoisomerase II $\alpha$  through a series of acidic amino acid residues (see Figure 1) (12,14,18–22). Metal ion 1 is postulated to interact with E461, D543, and D545, while metal ion 2 is postulated to interact with E461 and D541 (12). These residues are highly conserved among the type IA and type IIA families of topoisomerases (19,21,23).

To address the validity of these assignments in human topoisomerase II $\alpha$ , the four acidic amino acids were individually mutated to either Ala (to remove functional groups) or Cys (to potentially enhance interactions with thiophilic divalent metal ions). Each of the mutant enzymes was purified and characterized. As seen in Figure 4, the overall catalytic activity of the mutant enzymes, assessed by the ability to relax negatively supercoiled plasmid DNA in the presence of 5 mM Mg<sup>2+</sup>, was dramatically reduced as compared to that of the wild-type enzyme. Even the most active of the mutant enzymes, hTop2 $\alpha$ D541C, required 10– to 20–fold higher enzyme concentrations than wild-type topoisomerase II $\alpha$  to achieve similar rates of DNA relaxation. The least active mutant enzyme, hTop2 $\alpha$ E461A, required >40–fold higher concentrations.

In order to examine the roles of the acidic amino acids in coordinating divalent metal ions during the scission reaction, the ability of the mutant enzymes to cleave negatively supercoiled DNA in the presence of 5 mM Mg<sup>2+</sup> was determined. Equilibrium levels of cleavage generated by the mutant enzymes were too low to quantify reliably. Therefore, cleavage experiments were carried out in the presence of either 100  $\mu$ M etoposide or amsacrine. Both of these anticancer drugs are potent topoisomerase II poisons that increase levels of enzyme-mediated DNA scission by interfering with the ability of topoisomerase II to ligate cleaved molecules. Once again, all of the mutant enzymes displayed a level of activity that was considerably lower than that of wild-type topoisomerase II $\alpha$  (Figure 5). Together with the DNA relaxation results, these findings provide strong evidence that E461, D541, D543, and D545 play important roles in catalysis mediated by human topoisomerase II $\alpha$ .

In addition to the requirement for divalent metal ions in the DNA cleavage/ligation active site of type II topoisomerases, these enzymes also utilize a divalent metal to coordinate the high energy ATP cofactor in a separate ATPase active site (36). To ensure that the mutations affected metal ion coordination only in the cleavage/ligation active site, the ability of two mutant enzymes to hydrolyze ATP in absence of DNA was determined using a thin layer chromatography technique (37). hTop2 $\alpha$ E461C and hTop2 $\alpha$ D543C were chosen for this study

because they represent one of the least active and one of the most active mutants, respectively. Despite the fact that both mutant enzymes displayed a relaxation activity that was less than 5% that of wild-type, rates of ATP hydrolysis were ~70% and 200% that of wild-type topoisomerase II $\alpha$  (data not shown). Since the effects of the mutations on ATPase activity did not reflect their effects on DNA relaxation, it is concluded that the primary defect in the enzymes is their inability to cleave DNA efficiently.

### Interactions Between Acidic Amino Acid Residues and Metal Ions 1 and 2

In an effort to delineate interactions between specific amino acid residues and metal ions 1 and 2, the ability of wild-type topoisomerase II $\alpha$  and the mutant enzymes to cleave a series of oligonucleotides was assessed. Three different substrates were employed for these experiments. All had the same nucleotide sequence. However, one contained a wild-type 3'-bridging oxygen at the scissile bond, while the other two substituted a sulfur atom at either the non-bridging (phosphorothioate) or 3'-bridging (phosphorothiolate) position. In addition to the different substrates, DNA cleavage was determined in the presence of 5 mM Mg<sup>2+</sup>, Mn<sup>2+</sup>, or Ca<sup>2+</sup>. Within this series, Mn<sup>2+</sup> is the "softest," or most thiophilic metal, and Mg<sup>2+</sup> and Ca<sup>2+</sup> are harder, or less thiophilic (38–40). Soft metal ions often prefer sulfur over oxygen as an inner-sphere ligand, while hard metals usually coordinate more readily with oxygen (16,38–43). If there is a direct interaction between the metal ion and a scissile phosphate atom (or a sulfur-containing amino acid) that facilitates catalysis, relative levels or rates of scission with substrates containing a sulfur atom in place of the oxygen should increase in the presence of soft (thiophilic) metals (12,16,41–43). Conversely, less cleavage should be generated in reactions that contain hard metals (12,16,41–43).

As seen in Figure 6 (Top), compared to wild-type human topoisomerase II $\alpha$ , levels of cleavage of the wild-type substrate were dramatically reduced for all of the mutant enzymes. Mutations at D545 and E461 resulted in enzymes that displayed almost no cleavage activity, while marginal levels of cleavage were observed with the D543 and D541 mutant enzymes. It is notable that hTop2 $\alpha$ D541C and hTop2 $\alpha$ D543C, which among the D->C mutations displayed the greatest ability to cleave DNA in the presence of Mg<sup>2+</sup>, also displayed the greatest ability to relax plasmid DNA in the presence of Mg<sup>2+</sup>. However, a similar correlation was not observed among the D->A mutations.

In all cases, poor activity was seen with the substrate that contained the non-bridging phosphorothioate (Figure 6 Middle). This is consistent with the previous finding that this substrate generally is cleaved more poorly than wild-type oligonucleotides.

Unlike the other substrates, oligonucleotides that contain a 3'-bridging phosphorothiolate cannot be ligated appreciably by topoisomerase II $\alpha$  (29). As a result, much higher levels of cleavage complexes accumulate with this substrate. This property provides a more sensitive assay to determine the ability of a given enzyme to cleave DNA.

As observed with the wild-type and non-bridging phosphorothioate oligonucleotides, hTop2 $\alpha$ E461C and hTop2 $\alpha$ E461A displayed virtually no ability to cleave the 3'-bridging phosphorothiolate substrate (Figures 6 Bottom, 7, and 8). This finding is consistent with the proposal that E461 plays an important role in coordinating both metal ions 1 and 2. However, in contrast to the E461 mutants, modest to high levels of DNA scission were generated by most of the aspartic acid mutants (Figures 6 Bottom, 7, and 8). In all cases, higher levels and rates of cleavage were observed in the D->C than in the D->A mutants when Mn<sup>2+</sup> was employed as the divalent metal ion. Since Mn<sup>2+</sup> is a thiophilic metal ion, this finding implies a direct interaction between divalent cations and D541, D543, and D545.

hTop2 $\alpha$ D545C and hTop2 $\alpha$ D545A cleaved the 3'-bridging phosphorothiolate, but did so only in the presence of Mn<sup>2+</sup>. The fact that the DNA cleavage activity of the D545 mutants can be rescued only in the presence of a thiophilic metal ion and an S–P scissile bond strongly suggests that this residue coordinates metal ion 1, as predicted by the two-metal ion model for topoisomerase II $\alpha$ . As determined by the metal ion concentration required to yield 1/2 maximal DNA cleavage, hTop2 $\alpha$ D545C and hTop2 $\alpha$ D545A displayed reduced affinities for Mn<sup>2+</sup> [apparent K<sub>D</sub>  $\approx$  2.5 mM for each mutant as compared to  $\sim$ 14  $\mu$ M for the wild-type enzyme (Figure 7)] and decreased rates of cleavage [rate  $\approx$  1000-fold lower than that of the wild-type enzyme (Figure 8)].

hTop2 $\alpha$ D543C and hTop2 $\alpha$ D543A cleaved the phosphorothiolate substrate in the presence of all three metal ions (Mg<sup>2+</sup>, Mn<sup>2+</sup>, or Ca<sup>2+</sup>). However, the highest levels of scission always were observed with Mn<sup>2+</sup>, the most thiophilic metal ion of the three. Once again, these data support the proposed interaction of D543 with metal ion 1. As above, hTop2 $\alpha$ D543C and hTop2 $\alpha$ D543A displayed decreased affinities for Mn<sup>2+</sup> [apparent K<sub>D</sub>  $\approx$  20  $\mu$ M and 140  $\mu$ M, respectively (Figure 7)] and decreased rates of cleavage [rates  $\approx$  2- and 40-fold lower than that of the wild-type enzyme, respectively (Figure 8)].

The interpretation of results with hTop2 $\alpha$ D541C and hTop2 $\alpha$ D541A is less straightforward. The two-metal-ion mechanism predicts that D541 interacts with metal ion 2. The finding that hTop2 $\alpha$ D541A cannot be rescued more than a few percent by any of the divalent metal ions (Figures 6 Bottom, 7, and 8) is consistent with this postulate. hTop2 $\alpha$ D541C is, however, rescued by Mn<sup>2+</sup>, but not by the other two divalent cations. As above, the affinity of Mn<sup>2+</sup> (apparent K<sub>D</sub>  $\approx$  34  $\mu$ M, Figure 7) and rates of DNA cleavage (rate  $\approx$  10-fold lower than that of the wild-type enzyme, Figure 8) were reduced as compared to topoisomerase II $\alpha$ . The fact that the cleavage activity of the 3'-bridging S–P bond is seen only in the presence of a thiophilic metal ion could imply that D541 coordinates with metal ion 1 rather than 2. However, if this latter interpretation were correct, it is likely that hTop2 $\alpha$ D541A would also support DNA cleavage in the presence of Mn<sup>2+</sup>, as observed for hTop2 $\alpha$ D545A and hTop2 $\alpha$ D543A. Therefore, an alternative and more likely explanation for the Mn<sup>2+</sup>-specific activity of hTop2 $\alpha$ D541C is that D541 binds metal ion 2, and the –SH group of the cysteine (which is not present in the D->A mutation) rather than the S–P scissile bond coordinates the metal ion. Further experimentation will be necessary to resolve this issue more fully.

### Use of Two Metal Ions for DNA Cleavage by Human Topoisomerase II $\alpha$

Topoisomerase II $\alpha$  utilizes two metal ions to support its DNA cleavage activity. This was demonstrated by experiments that compared levels of DNA cleavage monitored in the presence of Ca<sup>2+</sup>, Mn<sup>2+</sup>, or a combination of the two divalent metal ions (12). Near saturating concentrations of Ca<sup>2+</sup> were paired with sub-saturating concentrations of Mn<sup>2+</sup>. Rates of scission of a 3'-bridging phosphorothiolate substrate generated by wild-type topoisomerase II $\alpha$  in the presence of both cations were >10-fold higher than predicted by the arithmetic sum of cleavage seen when either individual metal ion was employed (12).

Similar experiments were carried out with two mutant enzymes, hTop2 $\alpha$ D543C and hTop2 $\alpha$ D541C. These two enzymes were chosen because both supported high levels of DNA scission with the 3'-bridging phosphorothiolate in the presence of Mn<sup>2+</sup>. Results are shown in Figure 9. In both cases, there was a greater than predicted effect of the two metal ions on DNA cleavage. Therefore, it appears that the mutant enzymes still employ a two-metal-ion mechanism for topoisomerase II-mediated DNA cleavage.

It is notable that the two-metal-ion enhancement of DNA scission with hTop2 $\alpha$ D543C and hTop2 $\alpha$ D541C was not as great as observed with the wild-type enzyme. This finding is consistent with the fact that neither mutant enzyme is able to utilize Ca<sup>2+</sup> nearly as well as



wild-type topoisomerase II $\alpha$ . Consequently, the effect of the two metal ions, while present, is reduced.

### **A Model for the Use of Divalent Metal Ions for DNA Cleavage Mediated by Human Type II Topoisomerases**

Based on previous structural, mutagenesis, and kinetic studies, it has been proposed that human type II topoisomerases utilize a two-metal ion mechanism for their DNA cleavage reaction (Figure 10) (12–14). In this model, there is an important interaction between metal ion 1 and the 3'-bridging atom of the scissile phosphate that accelerates rates of enzyme-mediated DNA cleavage. There also appears to be an interaction between metal ion 2 and a non-bridging oxygen of the scissile phosphate. This interaction plays a significant role in DNA cleavage mediated by topoisomerase II $\beta$  (13). Although topoisomerase II $\alpha$  has an absolute requirement for metal ion 2, the proposed interaction with the non-bridging oxygen has only a modest effect on rates of DNA cleavage.

Previous studies have predicted an interaction between several acidic amino acid residues and the metal ions in type II topoisomerases. Mutagenesis studies have confirmed the importance of some of these residues in the bacterial type II enzyme, DNA gyrase, and in human topoisomerase II $\beta$  (14,22). However, the importance of the homologous residues in human topoisomerase II $\alpha$  (E461, D541, D543, and D545) had not been demonstrated. In addition, while interactions between specific amino acid residues and metal ions 1 and 2 have been proposed, assignments have not been validated by enzymological studies for any type II enzyme.

Experiments were carried out that monitored DNA relaxation and cleavage (levels, rates, and metal ion concentration dependence) by mutant enzymes in which four acidic amino acid residues were individually converted to either a cysteine or an alanine. Results of these studies confirm the importance of E461, D541, D543, and D545 for the activity of human topoisomerase II $\alpha$  and strongly suggest that each plays a role in metal ion binding. Furthermore, we propose that D543 and D545 play an important role in coordinating metal ion 1, that D541 plays an important role in coordinating metal ion 2, and that E461 plays an important role in coordinating both metal ions. The present study refines the two-metal-ion model for human topoisomerase II and provides a better-developed platform for additional studies regarding the role of metal ions in topoisomerase II-mediated processes.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **Acknowledgments**

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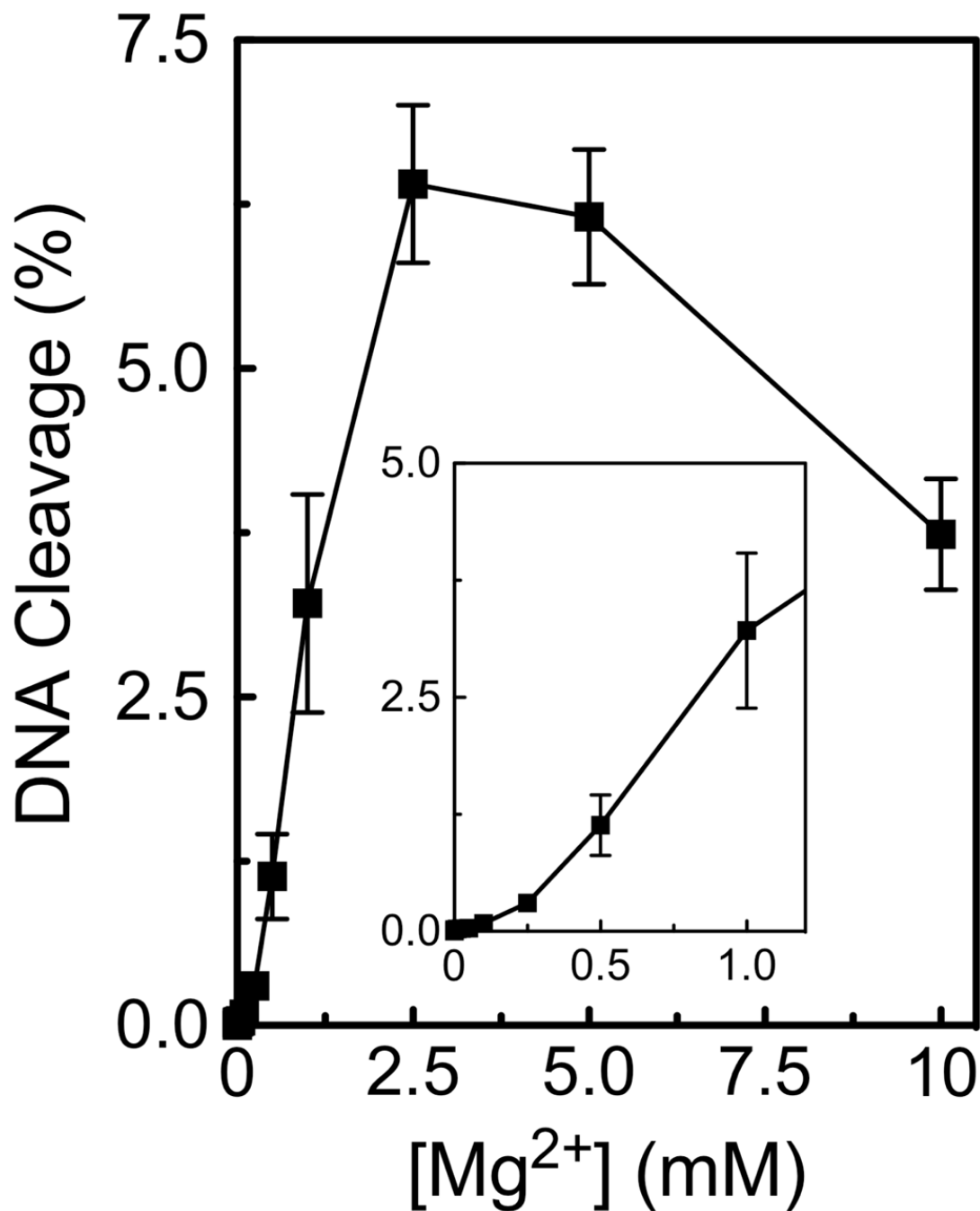
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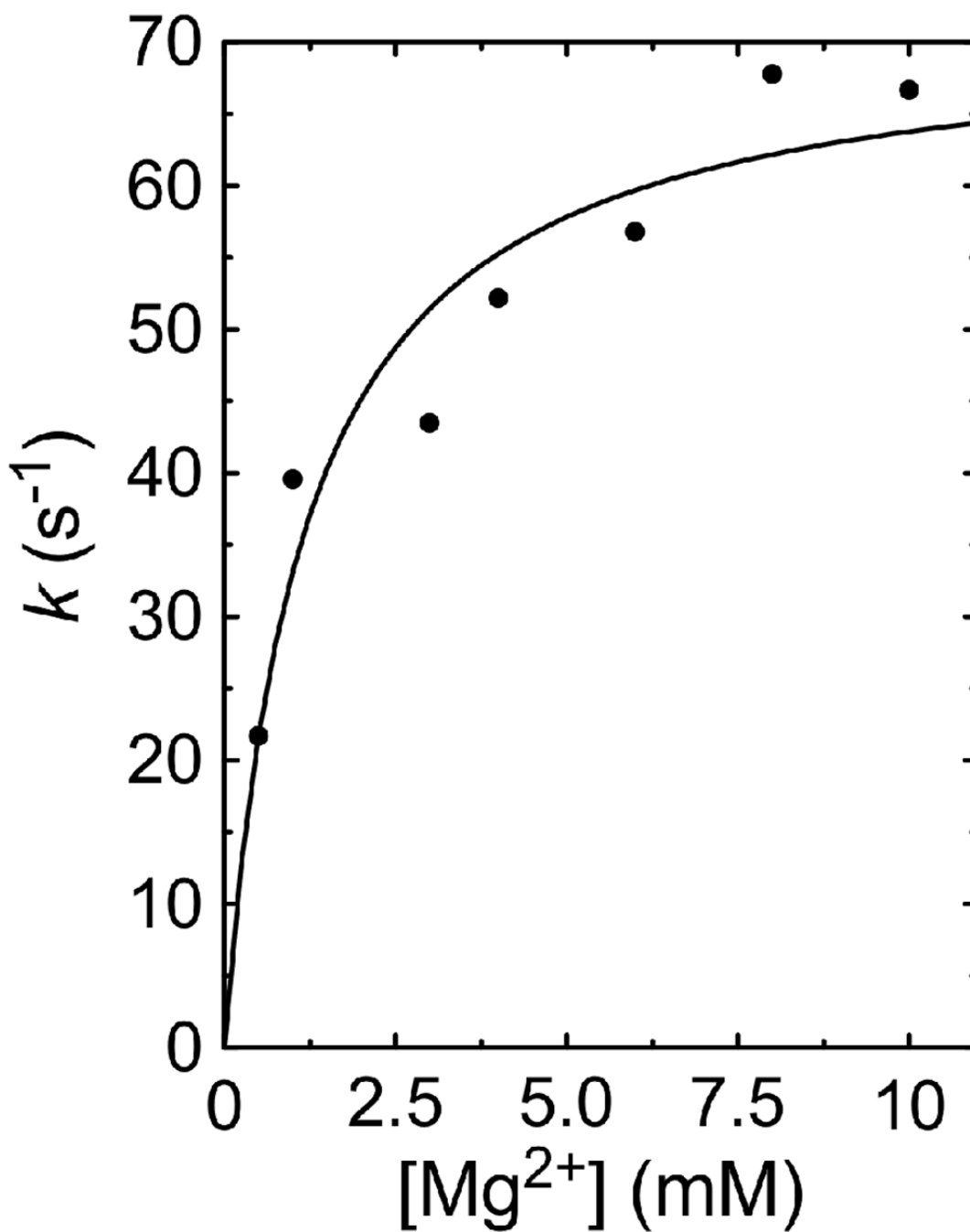
HsTop2a	459	LTEGD	538	IMTDQDQDGS
HsTop2b	475	LTEGD	554	IMTDQDQDGS
ScTop2	447	LTEGD	523	IMTDQDHDGS
EcGyrB	442	LVEGD	495	IMTDADVDGS
EcParE	416	LVEGD	487	ILADADSGL
EcTop3	5	IAEKP	100	HAGDPDREGQ
EcDnaG	262	VVEGY	305	GCYDGDGRAGR

**Figure 1.**

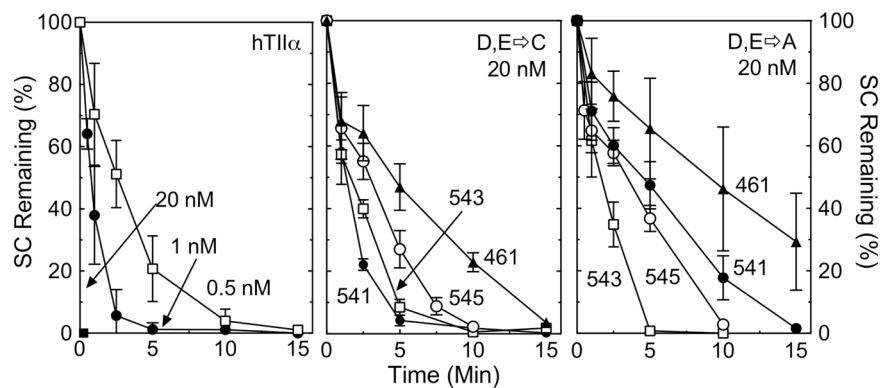
Amino acids postulated to coordinate divalent metal ions in the active site of nucleic acid enzymes. Sequence alignments for enzymes that contain a TOPRIM domain, including human topoisomerase II $\alpha$  (HsTop2a), topoisomerase II $\beta$  (HsTop2b), *S. cerevisiae* topoisomerase II (ScTop2), *E. coli* Gyr B subunit of DNA gyrase (EcGyrB), *E. coli* Par E subunit of topoisomerase IV (EcParE), *E. coli* topoisomerase III (EcTop3), and *E. coli* DnaG (EcDnaG) are shown. Conserved acidic amino acid residues that are proposed to bind divalent metal ions are highlighted. Sequences are from Refs. (19,23).



**Figure 2.** Divalent metal ion concentration dependence of topoisomerase II-mediated DNA cleavage. Oligonucleotide cleavage reactions mediated by human topoisomerase II $\alpha$  were performed over 30 min in the presence of 1  $\mu$ M to 10 mM Mg<sup>2+</sup>. Reactions utilized a wild-type substrate that contained a nick at the scissile bond on the opposite (unlabeled) strand. The inset shows DNA cleavage levels at concentrations up to 1 mM Mg<sup>2+</sup>. Error bars represent the standard deviation of three independent experiments.

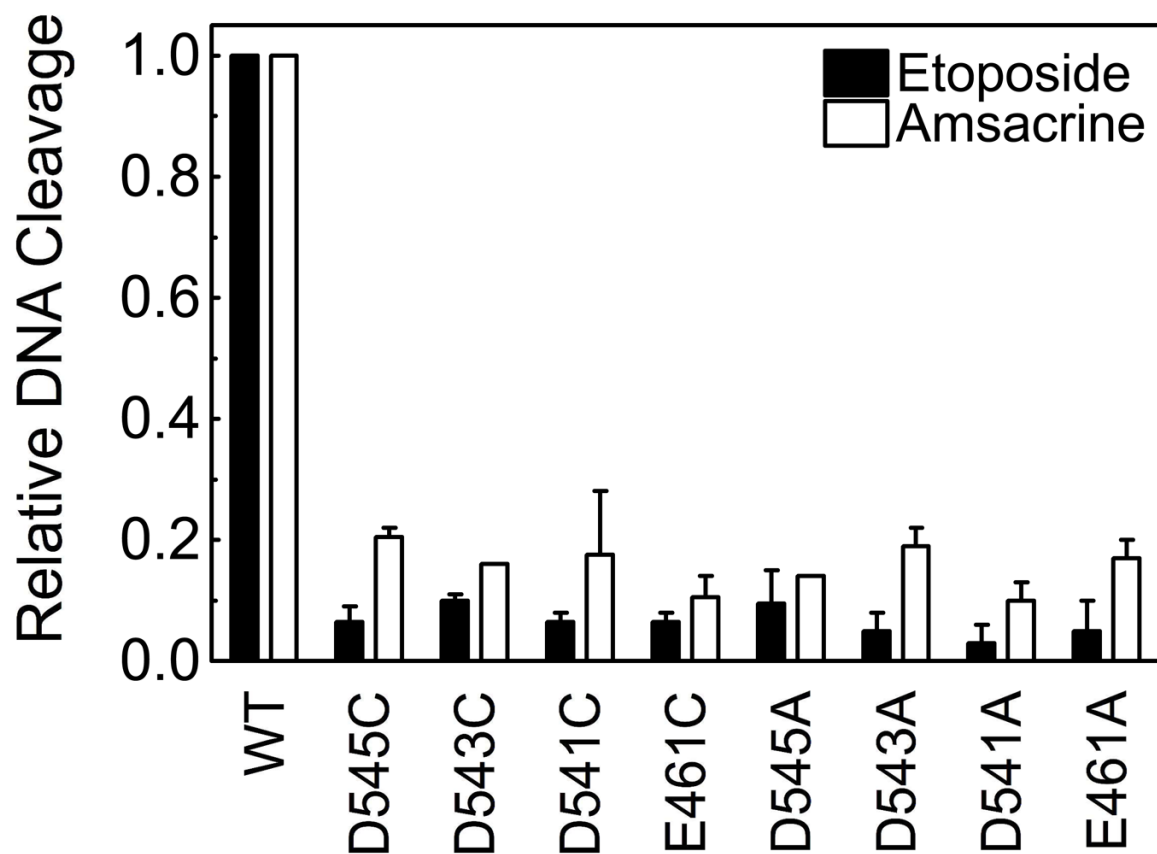


**Figure 3.** Kinetic analysis of metal ion dependence during topoisomerase II-mediated DNA cleavage. Pre-equilibrium oligonucleotide cleavage reactions mediated by human topoisomerase II $\alpha$  were monitored over a time course of 5 ms to 3 s in the presence of 0.5 to 10 mM  $\text{Mg}^{2+}$ . Reactions utilized a wild-type substrate that contained a nick at the scissile bond on the opposite (unlabeled) strand. Data points represent single exponential rate constants obtained from each reaction plotted versus metal ion concentration. A best fit curve is shown that was generated using Dynafit (30) with the equation shown in the text.



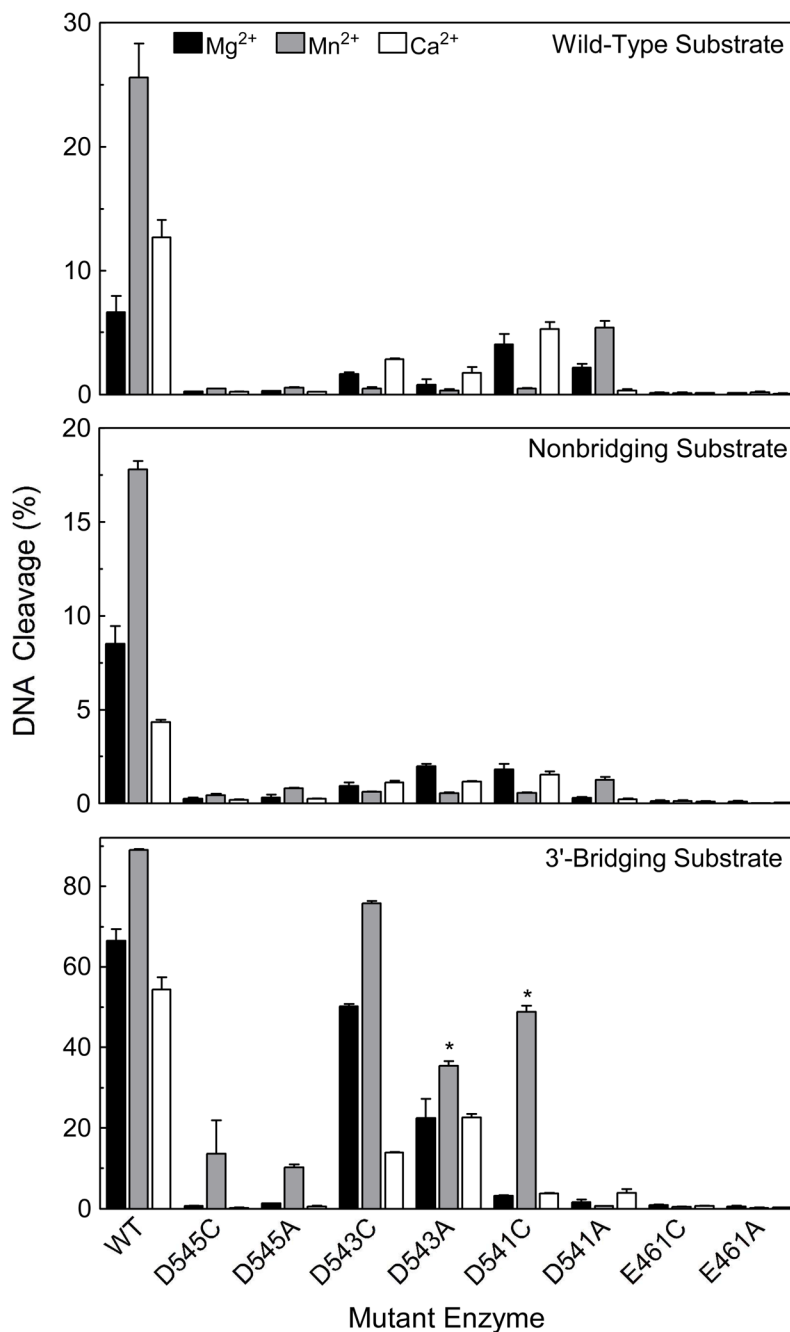
**Figure 4.**

DNA relaxation activity of mutant enzymes. DNA relaxation assays were carried out using negatively supercoiled pBR322 and wild-type or mutant topoisomerase II $\alpha$  enzymes in the presence of 5 mM Mg $^{2+}$ . Left panel, the plot shows the loss of supercoiled plasmid in the presence of wild-type topoisomerase II $\alpha$  at 0.5, 1, or 20 nM. Middle panel, the plot shows the loss of supercoiled plasmid in the presence of the D,E->C mutant enzymes at 20 nM. Right panel, the plot shows the loss of supercoiled plasmid in the presence of the D,E->A mutant enzymes at 20 nM. Error bars represent the standard error of the mean of at least two independent experiments.



**Figure 5.** Plasmid DNA cleavage mediated by mutant enzymes. Cleavage assays were performed using negatively supercoiled pBR322 and wild-type or mutant topoisomerase II $\alpha$  enzymes in the presence of 100  $\mu$ M etoposide (closed bars) or 100  $\mu$ M amsacrine (open bars). Results for wild-type (WT) and each of the mutant enzymes are shown. Data are plotted relative to the WT enzyme. Error bars represent the standard error of the mean of two independent experiments.

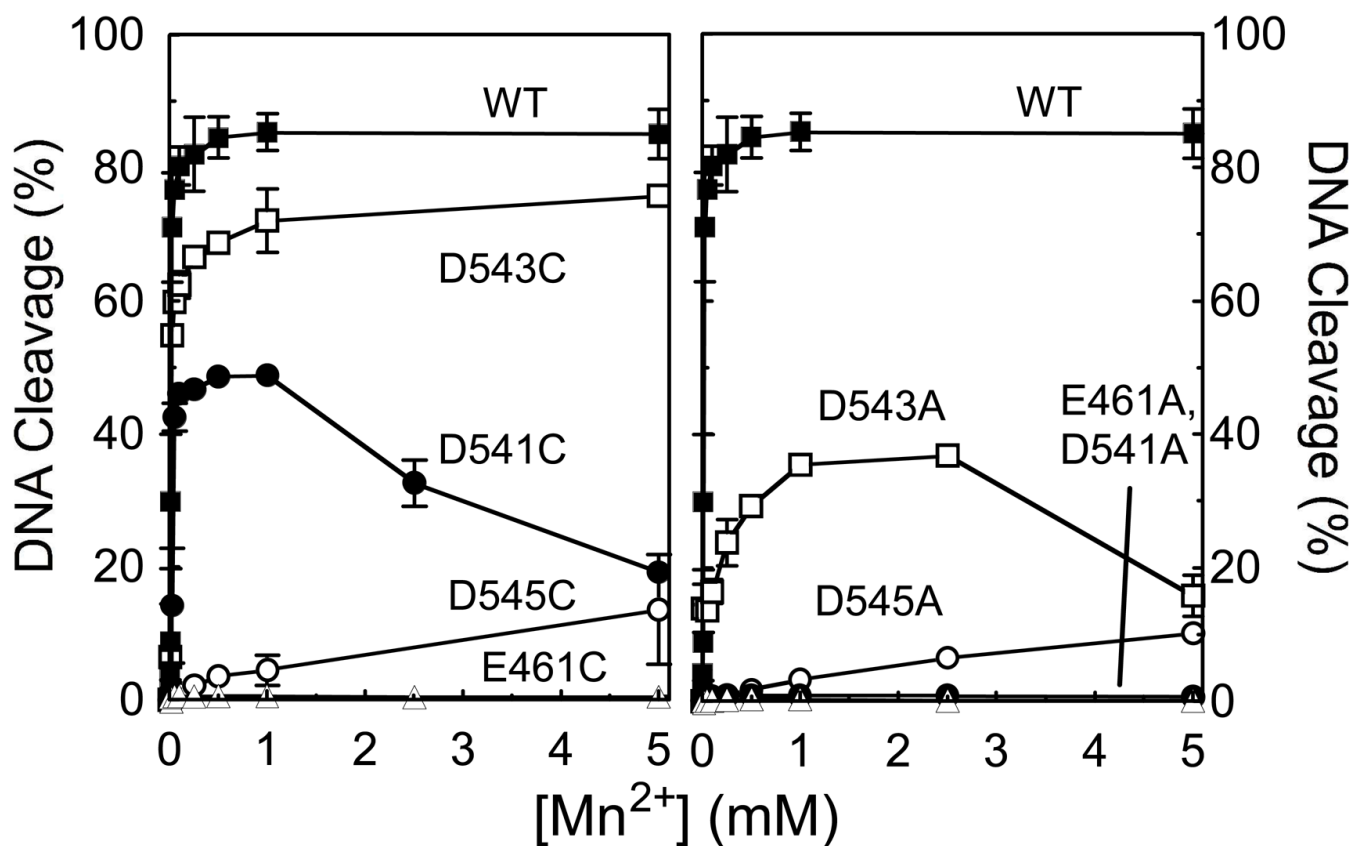




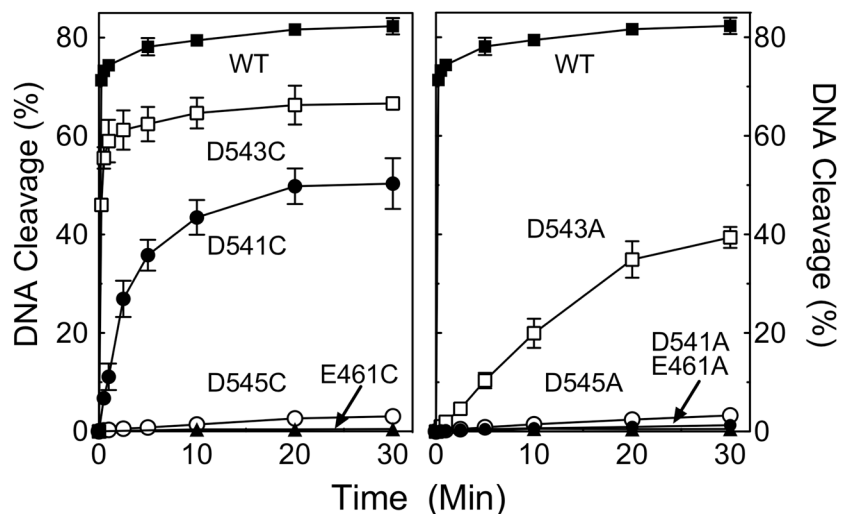
**Figure 6.**

Oligonucleotide DNA cleavage mediated by mutant enzymes. All substrates contained a nick at the scissile bond on the opposite (unlabeled) strand. Data were generated with a wild-type (top panel), nonbridging phosphorothioate (middle panel), or 3'-bridging phosphorothioate (bottom panel) substrate. Oligonucleotides were cleaved in the presence of 5 mM Mg<sup>2+</sup> (black bars), Mn<sup>2+</sup> (gray bars), or Ca<sup>2+</sup> (open bars) with wild-type human topoisomerase II $\alpha$  (WT), or mutant enzymes that contained D545C, D545A, D543C, D543A, D541C, D541A, E461C, or E461A. DNA cleavage data for the D543A and D541C enzymes (denoted by asterisks) in the bottom panel (3'-bridging phosphorothioate substrate) were generated in the presence of 1 mM Mn<sup>2+</sup>. In these cases, optimal levels of DNA scission were observed at 1 mM rather

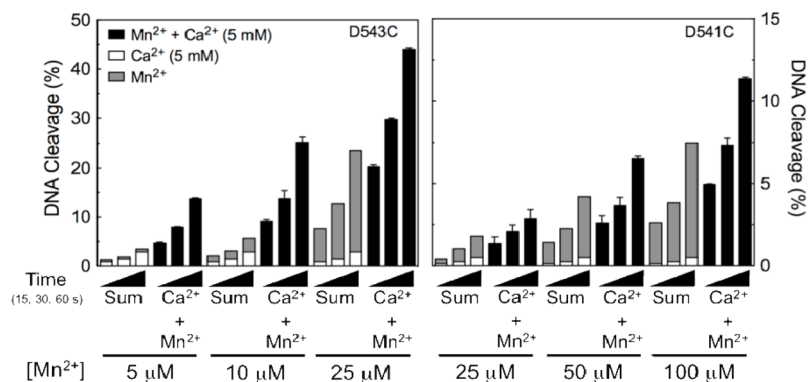
than 5 mM metal ion (see Figure 7). Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.



**Figure 7.** Mn<sup>2+</sup> concentration dependence of topoisomerase II-mediated DNA cleavage of a 3'-bridging phosphorothiolate substrate. The oligonucleotide contained a nick at the scissile bond on the opposite (unlabeled) strand. Reactions with wild-type or mutant human topoisomerase II $\alpha$  enzymes were carried out in the presence of 1  $\mu$ M to 10 mM Mn<sup>2+</sup>. Left panel, DNA cleavage mediated by wild-type (WT, closed squares) or D,E->C mutant enzymes (E461C, closed triangles; D541C, closed circles; D543C, open squares; D545C, open circles). Right panel, cleavage mediated by wild-type (WT, closed squares) or D,E->A mutant enzymes (E461A, closed triangles; D541A, closed circles; D543A, open squares; D545A, open circles). Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.

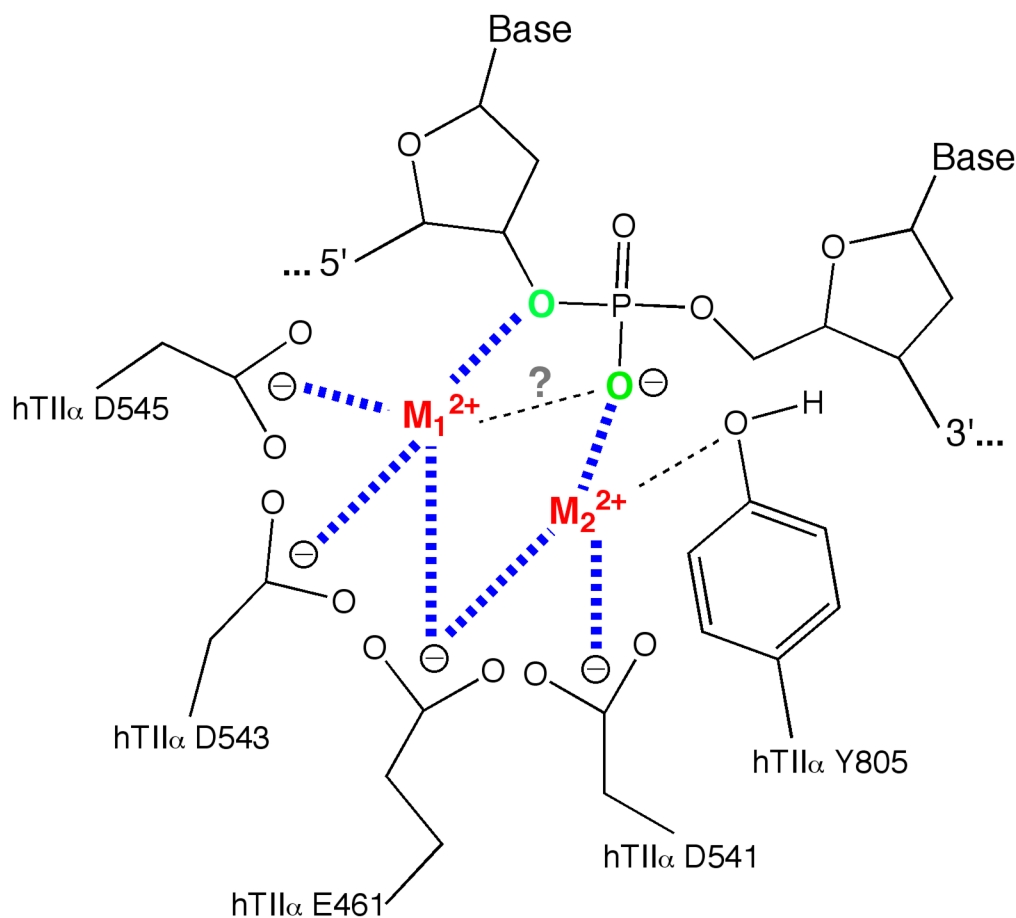


**Figure 8.** Time course for topoisomerase II-mediated cleavage of a 3'-bridging phosphorothiolate substrate in the presence of  $Mn^{2+}$ . The oligonucleotide contained a nick at the scissile bond on the opposite (unlabeled) strand. Reactions with wild-type or mutant human topoisomerase II $\alpha$  enzymes were carried out in the presence of 1 mM  $Mn^{2+}$  over a time course from 15 s to 30 min. Left panel, DNA cleavage mediated by wild-type (WT, closed squares) or D,E->C mutant enzymes (E461C, closed triangles; D541C, closed circles; D543C, open squares; D545C, open circles). Right panel, cleavage mediated by wild-type (WT, closed squares) or D,E->A mutant enzymes (E461A, closed triangles; D541A, closed circles; D543A, open squares; D545A, open circles). Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.



**Figure 9.**

Cleavage of a 3'-bridging phosphorothiolate oligonucleotide substrate by topoisomerase II $\alpha$  mutant enzymes in the presence of divalent metal ion combinations. The oligonucleotide contained a nick at the scissile bond on the opposite (unlabeled) strand. DNA cleavage is shown for hTop2 $\alpha$ D543C (left panel) and hTop2 $\alpha$ D541C (right panel). Reactions were carried out for 15, 30 or 60 s in the presence of 5 mM Ca<sup>2+</sup> alone (open bars), 5–25  $\mu$ M (hTop2 $\alpha$ D543C) or 25–100  $\mu$ M (hTop2 $\alpha$ D541C) Mn<sup>2+</sup> alone (gray bars), or a mixture of 5 mM Ca<sup>2+</sup> and 5–25  $\mu$ M (hTop2 $\alpha$ D543C) or 25–100  $\mu$ M (hTop2 $\alpha$ D541C) Mn<sup>2+</sup> (Ca<sup>2+</sup> + Mn<sup>2+</sup>, closed bars). The calculated arithmetic sum of DNA cleavage from reactions containing either Ca<sup>2+</sup> or Mn<sup>2+</sup> alone is shown (Sum, stacked open and gray bars, respectively). All data represent the average of at least two independent experiments. Error bars for reactions carried out in the presence of Ca<sup>2+</sup> + Mn<sup>2+</sup> are shown. Error bars for reactions carried out in the presence of Ca<sup>2+</sup> or Mn<sup>2+</sup> alone are not shown for simplicity.



**Figure 10.** Metal ion interactions in the DNA cleavage/ligation active site of human topoisomerase II $\alpha$ . Details are described in the text. The model postulates that metal ion 1 ( $M_1^{2+}$ , shown in red) interacts with E461, D543, and D545 and metal ion 2 ( $M_2^{2+}$ , shown in red) interacts with E461 and D541.  $M_1^{2+}$  makes a critical interaction with the 3'-bridging atom (shown in green) of the scissile phosphate (bond shown in blue), which most likely is needed to stabilize the leaving 3'-oxygen (left, shown in green).  $M_2^{2+}$  is required for DNA scission and likely interacts with the non-bridging oxygen (shown in green) during scission. This divalent cation may stabilize the DNA transition state and/or help deprotonate the active site tyrosine. The model was adapted from Ref. (14).