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## The Use of Divalent Metal Ions by Type II Topoisomerases

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### Abstract

Type II topoisomerases are essential enzymes that regulate DNA under- and overwinding and remove knots and tangles from the genetic material. In order to carry out their critical physiological functions, these enzymes utilize a double-stranded DNA passage mechanism that requires them to generate a transient double-stranded break. Consequently, while necessary for cell survival, type II topoisomerases also have the capacity to fragment the genome. This feature of the prokaryotic and eukaryotic enzymes, respectively, is exploited to treat a variety of bacterial infections and cancers in humans. All type II topoisomerases require divalent metal ions for catalytic function. These metal ions function in two separate active sites and are necessary for the ATPase and DNA cleavage/ligation activities of the enzymes. ATPase activity is required for the strand passage process and utilizes the metal-dependent binding and hydrolysis of ATP to drive structural rearrangements in the protein. Both the DNA cleavage and ligation activities of type II topoisomerases require divalent metal ions and appear to utilize a novel variant of the canonical two-metal-ion phosphotransferase/hydrolase mechanism to facilitate these reactions. This article will focus primarily on eukaryotic type II topoisomerases and the roles of metal ions in the catalytic functions of these enzymes.

### Keywords

Topoisomerase II $\alpha$ ; divalent metal ion; divalent cation; ATP hydrolysis; DNA cleavage; DNA ligation; topoisomerase II poisons

## 1. DNA Topology

DNA is essentially an extremely long double-stranded rope in which the two strands are plectonemically coiled about one another. As a result of these structural features, topological challenges profoundly affect every major nucleic acid process that requires opening or movement of the double helix.<sup>1–5</sup>

Topological properties of DNA, including under- and overwinding, knotting, and tangling, can only be changed when one or both strands of DNA are broken.<sup>2</sup> DNA underwinding (*i.e.*, negative supercoiling) is important because duplex DNA is the storage form for the genetic information. In order to replicate or express this information, the two strands of the double helix must be separated. Since negative supercoiling destabilizes the double-stranded

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nature of DNA, it greatly facilitates strand separation.<sup>2–7</sup> The importance of this attribute is underscored by the fact that genomes ranging from bacteria to humans are globally underwound by ~6%.<sup>2, 8–10</sup>

While negative supercoiling enhances many nucleic acid processes, DNA overwinding (*i.e.*, positive supercoiling) inhibits them. The linear movement of enzymes such as helicases and polymerases through the double helix induces DNA overwinding ahead of tracking systems.<sup>2, 8–10</sup> This positive supercoiling makes it increasingly difficult to open the two strands of the double helix and ultimately blocks critical cellular processes such as DNA replication and transcription.<sup>2–5, 11</sup>

Essential nucleic acid functions, such as DNA recombination and replication generate knots and tangles within the double helix.<sup>2, 4, 11–15</sup> DNA knots impair the ability to separate the two strands of the genetic material and reduce the tensile strength of the double helix. As a result, they prevent the completion of replication, adversely affect transcription, and induce genomic instability.<sup>16–20</sup> Intermolecular DNA tangles prevent segregation of chromosomes during mitosis.<sup>2, 4, 11–13</sup> Consequently, these two topological structures can be lethal to cells if they are not resolved.<sup>16–20</sup>

## 2. Topoisomerase II

The topological state of DNA is regulated in the cell by enzymes known as topoisomerases.<sup>1, 11–13, 21–23</sup> These enzymes are required for the survival of all organisms and alter DNA topology by generating transient breaks in the double helix.<sup>1, 11–13, 21–23</sup> There are two major classes of topoisomerases, type I and type II, that are distinguished by the number of DNA strands that they cleave.<sup>1, 11–13, 21–23</sup>

Bacterial and eukaryotic type I topoisomerases (topoisomerase I and III) are monomeric enzymes that require no high-energy cofactor.<sup>1, 21, 24</sup> These enzymes alter topology by creating a transient single-stranded break in the DNA, followed by passage of the opposite intact strand through the break or by controlled rotation of the helix around the break.<sup>1, 21, 24</sup> As a result of their reaction mechanism, type I topoisomerases can modulate DNA under- and overwinding (removing one supercoil per catalytic event), but cannot remove knots or tangles from duplex DNA. These enzymes play important roles in DNA replication and transcription and the maintenance of genomic stability.<sup>1, 11, 21, 24</sup> Type I topoisomerases will not be discussed in detail in this article. However, several excellent review articles on these enzymes have appeared recently if the reader is interested in obtaining additional information.<sup>21, 24–25</sup>

All type II topoisomerases require ATP for complete catalytic activity.<sup>12, 26–29</sup> These enzymes interconvert different topological forms of DNA by passing an intact double helix through a transient double-stranded break that they generate in a separate segment of DNA.<sup>12, 26–29</sup> As a result of this double-stranded DNA passage mechanism, type II topoisomerases can modulate DNA super-coiling (removing two supercoils per catalytic event) and also can remove DNA knots and tangles.

There are two bacterial type II enzymes, gyrase and topoisomerase IV.<sup>1, 26, 30–31</sup> These enzymes I, are encoded by separate genes, but are closely related and are heterotetrameric (A<sub>2</sub>B<sub>2</sub>) in structure.<sup>1, 22, 26, 30–31</sup> Gyrase is composed of two subunits: GyrA contains the active site for DNA cleavage and ligation and GyrB contains the site of ATP binding and hydrolysis. GyrB also contains conserved acidic amino acid residues that coordinate the divalent metal ions that are required for DNA cleavage and ligation. The homologous subunits in topoisomerase IV are ParC and ParE (named as genes required for partitioning in Gram-negative species) or GrlA and GrlB (named as gyrase-like genes in Gram-positive

species).<sup>1, 26, 30–32</sup> Gyrase is unique among type II topoisomerases, in that it can actively underwind (*i.e.*, negatively supercoil) the double helix.<sup>1, 22, 26, 30–31</sup> This enzyme is critical for maintaining the global balance of DNA supercoiling in bacteria and plays important roles in DNA replication and transcription.<sup>22, 26, 30–31</sup> Topoisomerase IV helps to alleviate positive DNA supercoiling that occurs during processes such as replication and removes the bulk of knots and tangles from the bacterial genome.<sup>22, 26, 30–31</sup>

Eukaryotic type II topoisomerases share homology with both gyrase and topoisomerase IV, but the two genes that encode the bacterial enzymes have fused into a single gene in eukaryotes.<sup>22, 26, 30, 33</sup> Consequently, the eukaryotic enzymes are homodimeric in nature. The N-terminal domain is homologous to GyrB and the central domain is homologous to GyrA.<sup>12, 22, 29, 33</sup> The C-terminal domain of eukaryotic topoisomerase II corresponds to the C-terminal domain of GyrA.<sup>22, 33</sup> This portion of the protein is highly variable across species and imparts unique functional and physiological properties to individual family members. For example, the C-terminal domain of gyrase contains elements that allow the enzyme to negatively supercoil DNA.<sup>31</sup> The corresponding portion of the eukaryotic type II enzyme contains nuclear localization signals<sup>34–36</sup> and allows the  $\alpha$  isoform of topoisomerase II (see below) to distinguish the handedness of DNA supercoils and relax (*i.e.*, remove) positive DNA supercoils ~10 times faster than it does negative.<sup>37–39</sup>

Lower eukaryotes and invertebrates encode only a single type II topoisomerase, called topoisomerase II.<sup>40–43</sup> In contrast, vertebrate species encode two closely related isoforms, topoisomerase II $\alpha$  and topoisomerase II $\beta$ .<sup>11–12, 21, 29, 44–51</sup> These isoforms differ in their protomer molecular masses (170 vs. 180 kDa, respectively) and are encoded by separate genes. Topoisomerase II $\alpha$  and topoisomerase II $\beta$  display a high degree (~70%) of amino acid sequence identity and similar enzymological characteristics.

Similarities notwithstanding, topoisomerase II $\alpha$  and topoisomerase II $\beta$  have distinct patterns of expression and separate cellular functions. Although topoisomerase II $\alpha$  is essential for the survival of proliferating cells, topoisomerase II $\beta$  is dispensable at the cellular level.<sup>52–54</sup> Furthermore, levels of topoisomerase II $\alpha$  protein rise dramatically during periods of cell growth,<sup>55–58</sup> while expression of the  $\beta$  isoform is independent of proliferative status.<sup>34, 59–60</sup> Topoisomerase II $\alpha$  is associated with replication forks and remains tightly bound to chromosomes during mitosis.<sup>58–59, 61–63</sup> Thus, it is believed to be the isoform that functions in growth-related processes, such as DNA replication and chromosome segregation.<sup>11, 58</sup> In contrast, topoisomerase II $\beta$  dissociates from chromosomes during mitosis and cannot compensate for the loss of topoisomerase II $\alpha$  in mammalian cells.<sup>58, 60, 64–65</sup> Although the physiological functions of the  $\beta$  isoform have yet to be fully defined, recent evidence indicates involvement in the transcription of hormonally- or developmentally-regulated genes.<sup>65–66</sup>

Many of the fundamental properties of type II topoisomerases were first established with bacterial systems. Several high quality review articles focus on these enzymes.<sup>22, 31, 67–68</sup> The present article will concentrate on eukaryotic type II topoisomerases, but will discuss the prokaryotic enzymes as appropriate.

Finally, much of what we understand regarding the mechanism of action of the eukaryotic type II enzymes comes from experiments with topoisomerase II from species that express only a single form of the protein. Furthermore, the double-stranded DNA passage mechanism utilized by all type II topoisomerases is fundamentally the same. Consequently, eukaryotic type II topoisomerases will be referred to collectively as topoisomerase II, unless the properties being discussed are specific to either the  $\alpha$  or  $\beta$  isoform.

### 3. Catalytic Cycle

As described above, type II topoisomerases alter nucleic acid topology using a double-stranded DNA passage reaction. This reaction can be separated into at least six discrete steps.<sup>26–28</sup> One round of catalysis is depicted in Fig. 1.

#### 1) DNA Binding

Topoisomerase II binds two segments of DNA to initiate a round of catalysis. Evidence suggests that the enzyme preferentially binds to regions of helix-helix juxtaposition formed by DNA supercoiling, knotting, or tangling.<sup>69–70</sup> The recognition of DNA crossovers and the subsequent binding of topoisomerase II requires no cofactor.<sup>71</sup>

#### 2) DNA Cleavage

All topoisomerases utilize active site tyrosine residues to mediate DNA cleavage (Tyr805 and Tyr821 in human topoisomerase II $\alpha$  and topoisomerase II $\beta$ , respectively).<sup>1, 12–13, 21–23, 26–28</sup> Each protomer subunit cleaves one strand of the double helix in a coordinated fashion.<sup>72–73</sup> DNA cleavage requires the presence of a divalent metal ion (detailed below),<sup>71, 74–75</sup> and takes place *via* a transesterification reaction that results in the formation of a covalent phosphotyrosyl bond between the protein and the newly generated 5'-terminus of the DNA chain.<sup>74–76</sup> Scission also generates a hydroxyl moiety on the 3'-terminus of the cleaved strand. The scissile bonds on the two strands of the double helix are staggered and located across the major groove from one another. Thus, topoisomerase II generates cleaved DNA molecules with 4-base 5'-single-stranded cohesive ends, each of which is covalently linked to a separate protomer subunit of the enzyme.<sup>74–76</sup>

Although topoisomerase II acts globally, it cleaves DNA at preferred sites.<sup>77</sup> The consensus sequence for cleavage is weak,<sup>77</sup> and the mechanism by which the enzyme selects its DNA sites is not apparent. Most likely, the specificity of topoisomerase II-mediated cleavage is determined by the local structure, flexibility, or malleability of the DNA that accompanies the sequence, as opposed to a direct recognition of the bases that comprise that sequence.<sup>78</sup>

It is notable that the scission reaction is reversible and that the enzyme establishes a DNA cleavage-ligation equilibrium. The transient covalent enzyme-DNA cleavage intermediate (known as the “cleavage complex”) that is formed during this process plays two important roles in the topoisomerase II reaction mechanism.<sup>1, 22, 26–28</sup> First, it conserves the bond energy of the sugar-phosphate DNA backbone. Second, because it does not allow the cleaved DNA chains to dissociate from the enzyme, the protein-DNA linkage maintains the integrity of the genetic material during the cleavage event.

The topoisomerase II-DNA cleavage complex is also important because it is the target for a number of clinically relevant drugs. Bacterial gyrase and topoisomerase IV are the targets of quinolone-based drugs such as ciprofloxacin and levofloxacin.<sup>30, 79–80</sup> These agents are the most active and broad-spectrum oral antibacterials currently available. Human topoisomerase II $\alpha$  and topoisomerase II $\beta$  are the targets of some of the most active anticancer agents used in the clinic.<sup>12–13, 28–29, 81–83</sup> Drugs such as etoposide, doxorubicin, and mitoxantrone are used routinely to treat a wide variety of hematological and solid tumors. All of these drugs kill cells by increasing the concentration of cleavage complexes, thereby generating high concentrations of DNA strand breaks.<sup>12, 28, 81–83</sup> Agents that act by this mechanism are known as topoisomerase II poisons.<sup>84</sup>

### 3) DNA Strand Passage

Topoisomerase II binds two ATP molecules, which induce critical conformation changes in the enzyme.<sup>85–86</sup> These structural rearrangements are transduced through an interaction between the  $\gamma$ -phosphate group of the bound ATP and a conserved lysine residue (Lys378 in human topoisomerase II $\alpha$ ) in the QTK loop of the protein.<sup>87–89</sup> Ultimately, the rearrangements close an N-terminal gate in the protein, open a gate in the cleaved double helix, and trigger the passage of an intact DNA segment through the nucleic acid gate.<sup>85–89</sup> DNA strand passage appears to be faster if one of the two ATP molecules is hydrolyzed.<sup>85–86</sup> The transported DNA is passed into a central cavity in topoisomerase II, such that the enzyme forms a protein clamp on the double helix.<sup>85, 90</sup> The functional interaction of topoisomerase II and ATP requires a divalent metal ion (discussed below).

### 4) DNA Ligation

Following DNA translocation, topoisomerase II reseals the original nucleic acid break, and the enzyme establishes a post-strand passage DNA cleavage-ligation equilibrium.<sup>91</sup> Ligation requires a divalent metal ion and is assumed to represent the reverse of the transesterification reaction used to cleave the DNA.<sup>92–93</sup> Once ligation has taken place, topoisomerase II and the DNA are no longer covalently linked. However, since the transported DNA segment is still engulfed between protein gates, the enzyme and DNA remain topologically complexed.<sup>85, 90</sup> It is notable that most clinically relevant topoisomerase II poisons increase the concentration of cleavage complexes primarily by inhibiting the ability of the enzyme to ligate cleaved DNA molecules.<sup>12–13</sup>

### 5) ATP Hydrolysis

Topoisomerase II hydrolyzes the second ATP molecule, which opens a C-terminal gate on the protein and allows the transported DNA segment to be released by the enzyme.<sup>85–86</sup> ATP hydrolysis requires the presence of a divalent metal ion.

### 6) Enzyme Turnover

The enzyme returns to its original conformation and regains the ability to begin a new round of catalysis. This process is not well understood.

## 4. Use of Divalent Metal Ions by Topoisomerase II

As discussed above, type II topoisomerases have two active sites: one that binds and hydrolyzes ATP and another that contains the catalytic tyrosine residue and mediates DNA cleavage and ligation. The use of metal ions in both active sites of topoisomerase II is discussed below. Additional information regarding the interactions of divalent metal ions with both type I and II topoisomerases can be found in an excellent recent review article by Sissi and Palumbo.<sup>68</sup>

### 4.1 ATPase Active Site

The ATPase domain of type II topoisomerases is a member of the GHKL superfamily (named for the founding members gyrase, Hsp90, histidine kinase, and MutL), which is characterized by an unconventional Bergerat ATP-binding fold.<sup>87–89</sup> It is located in the N-terminal, or GyrB homology domain of topoisomerase II (residues 29–264 in human topoisomerase II $\alpha$ ).<sup>87–89, 94</sup> The key catalytic residue, Glu87, activates a water molecule that hydrolyzes the  $\beta$ - $\gamma$  phosphodiester bond of the ATP moiety.<sup>87–89, 94</sup> The ATPase activity of topoisomerase II increases when DNA is bound.<sup>95</sup> Like other enzymes that hydrolyze ATP, the ATPase activity of topoisomerase II has an absolute requirement for a divalent metal ion.<sup>71</sup> In the absence of the metal ion, neither the conformational changes in

topoisomerase II that accompany ATP binding, nor hydrolysis of the ATP cofactor are observed.<sup>71, 87–89, 94</sup> The ATP-related functions of topoisomerase II appear to have a preference for  $Mg^{2+}$ .<sup>71</sup> Presumably, it is a  $Mg^{2+}$ -ATP complex that binds to the protein. Although  $Mn^{2+}$  and  $Ca^{2+}$  support the DNA cleavage/ligation activities of topoisomerase II,<sup>93, 96</sup> they do not allow the overall catalytic activity of the enzyme. In contrast,  $Co^{2+}$  supports DNA relaxation and decatenation catalyzed by human topoisomerase II $\alpha$ , suggesting that this divalent metal ion must also support the ATPase activity of the enzyme.<sup>96</sup>

The crystal structures of the ATPase domain of *Escherichia coli* gyrase and topoisomerase IV, *Saccharomyces cerevisiae* topoisomerase II, and human topoisomerase II $\alpha$  have been solved in the presence of a bound nucleotide triphosphate (Fig. 2).<sup>87–88, 94</sup> In all cases, the  $Mg^{2+}$  ion is octahedrally coordinated to all three phosphate groups of ATP, two water molecules, and the  $\delta^1$ -carbonyl oxygen of a conserved asparagine residue (Asn91 in human topoisomerase II $\alpha$ ). Following hydrolysis of the high-energy cofactor, a water molecule replaces the leaving  $\gamma$ -phosphate group that contacted the  $Mg^{2+}$  ion. It is notable that the catalytic residue, Glu87 in human topoisomerase II $\alpha$ , interacts with one of the water molecules that is coordinated by the metal ion.<sup>87–88, 94</sup>

## 4.2 DNA Cleavage/Ligation Active Site

The ability of topoisomerase II to cleave and ligate DNA is central to all of its catalytic functions.<sup>1, 12–13, 27–28</sup> While many of the amino acids involved in catalysis other than the active site tyrosine residues have not been elucidated, it is believed that the enzyme utilizes a general acid–base mechanism for DNA cleavage. Scission is initiated when a general base deprotonates the active site tyrosine hydroxyl moiety, allowing the oxyanion to attack the scissile phosphate. The base has not been identified but is believed to be a conserved histidine (potentially His758 in human topoisomerase II $\alpha$ ) residue or a metal-associated water molecule.<sup>97</sup>

The DNA cleavage reaction has an absolute requirement for a divalent metal ion.<sup>28, 33, 71, 98–99</sup> Although  $Mg^{2+}$  appears to fulfill this function *in vivo*,<sup>28</sup> other metal ions, such as  $Mn^{2+}$ ,  $Ca^{2+}$ , and  $Co^{2+}$  often support even higher levels of double-stranded DNA cleavage *in vitro*.<sup>93, 96, 99</sup> While divalent metal ions are necessary to promote the chemistry of DNA scission, they do not appear to be involved in site selection. To a large extent, cleavage maps generated in the presence of all of the above divalent cations are similar.<sup>93, 96</sup>

Topoisomerase II cuts the two strands of the double helix in a coordinated fashion.<sup>72, 76, 100</sup> It is believed that cleavage of the first strand introduces flexibility in the DNA that allows the substrate to attain an acutely bent transition state that is required for efficient cleavage.<sup>72–73, 101</sup> Thus, after the enzyme cleaves the first strand in the presence of  $Mg^{2+}$ ,  $Mn^{2+}$ , or  $Co^{2+}$  (average level of cleavage  $\approx 1\%$ ), it goes on to cut the second strand  $\geq 50\%$  of the time. In marked contrast, the two protomer subunits of topoisomerase II are considerably less coordinated when  $Ca^{2+}$  is utilized as the divalent metal ion.<sup>93</sup> Consequently, much higher levels of single-stranded DNA nicks are generated in the presence of this metal ion. The reason for the decreased coordination seen with  $Ca^{2+}$  is not known.

Commonly, enzymes that cleave nucleic acids utilize divalent metal ions in their active sites in order to stabilize both attacking (*i.e.*, amino acids and/or water) and leaving groups (*i.e.*, DNA backbone oxygen atoms).<sup>97, 102–120</sup> However, the number of metal ions per active site and the specific roles played by these cations in stabilizing leaving groups (bridging vs. non-bridging oxygen) varies. A series of enzymological, mutagenesis, and structural studies with prokaryotic and mammalian type II topoisomerases (discussed below) indicate that these enzymes cleave DNA using a two-metal-ion mechanism,<sup>97, 99, 121–125</sup> albeit a novel



variant<sup>125</sup> of the canonical two-metal-ion mechanism described for phosphotransferases and hydrolases such as DNA pol I and RNaseH.<sup>126–127</sup>

Many topoisomerases (type I and type II) and primases share a conserved region known as the *TOPRIM* domain.<sup>98</sup> This region has been implicated in coordinating divalent metal ions that are used during nucleic acid cleavage, ligation, and polymerization reactions.<sup>33, 98, 128</sup> The *TOPRIM* domain consists of ~125 amino acids (residues ~455–580 in human topoisomerase II $\alpha$ ) that is characterized by a compact  $\beta/\alpha$  fold. It includes a conserved glutamic acid and two (DXD) or three (DXDXD) conserved aspartic acid residues that are juxtaposed (Fig. 3). Structural studies indicate that the conserved glutamic acid and two of the aspartic acids interact with divalent metal ions during catalysis (E461, D541, and D543 in human topoisomerase II $\alpha$ ) (Figs. 4 and 5).<sup>99, 101, 123–124</sup> A recent crystallographic study suggests that the third aspartic acid residue (D545 in human topoisomerase II $\alpha$ ) does not play a role in metal ion binding.<sup>125</sup> Rather, it is involved in a salt bridge with a highly conserved arginine residue (R713 in human topoisomerase II $\alpha$ ) (Figs. 4 and 5).<sup>125</sup>

The first evidence for a two-metal-ion mechanism for DNA cleavage by type II enzymes came from work on *Escherichia coli* gyrase carried out by Noble and Maxwell.<sup>97</sup> These authors were able to distinguish the two metal ion sites in the DNA cleavage/ligation domain by mutating acidic amino acid residues that were proposed to bind divalent cations and found higher levels of DNA scission in the presence of Mg<sup>2+</sup> and Ca<sup>2+</sup> than with either metal ion alone. This study strongly suggested that type II topoisomerases utilized a two-metal-ion mechanism and provided a working model for the field. However it did not elucidate the specific roles played by divalent metal ions in the DNA cleavage reaction of type II enzymes.

Later studies by Deweese *et al.* extended the two-metal-ion mechanism to eukaryotic type II topoisomerases and began to define the roles of divalent cations in DNA cleavage.<sup>99, 122</sup> Two lines of evidence support the use of two metal ions by human topoisomerase II $\alpha$  and topoisomerase II $\beta$ . First, titration experiments with Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Ca<sup>2+</sup> all indicate a biphasic dependence on divalent cations for DNA cleavage. When Mg<sup>2+</sup> is utilized, the high affinity metal ion site (referred to alternatively as site A or 1) appears to have a  $K_D$  value of ~100  $\mu$ M, while the lower affinity site (referred to alternatively as site B or 2) appears to have a  $K_D$  that is approximately an order of magnitude higher (sites are shown in Figs. 4 and 5). There is an absolute requirement for both metal ion sites to be filled in order for DNA cleavage to take place.<sup>99, 122</sup>

Second, slightly higher rates and levels of scission are observed in the presence of two metal ions (Mn<sup>2+</sup> and Ca<sup>2+</sup>) than predicted by calculated levels derived from the sum of cleavage in the presence of the individual cations.<sup>99, 122</sup> More dramatic enhancements (in some cases, >20 – fold) are seen when modified DNA substrates are used to distinguish the two metal ions. These substrates contain a sulfur atom in place of the bridging or non-bridging oxygen atoms of the scissile phosphate (phosphorothiolate or phosphorothioate, respectively).<sup>99, 122</sup> Experiments take advantage of the fact that soft (or type B) metals (which tend to be larger and more polarisable), such as Mn<sup>2+</sup> often prefer sulfur over oxygen as an inner-sphere ligand, while hard (or type A) metals (which tend to have a smaller ionic radius and are non-polarisable), such as Ca<sup>2+</sup>, usually coordinate more readily with oxygen.<sup>105, 111, 130–135</sup>

Modified phosphorothiolate and phosphorothioate substrates also have been used to define interactions between metal ions and the scissile phosphate atoms.<sup>99–100, 122</sup> Relative levels and rates of cleavage determined for reactions mediated by human topoisomerase II $\alpha$  and topoisomerase II $\beta$  in Ca<sup>2+</sup>-supported reactions (relative to Mn<sup>2+</sup>-supported reactions)

decrease significantly when the bridging oxygen atom at the scissile bond is substituted by a sulfur atom.<sup>99–100, 122</sup>

Based on these findings, it was concluded that both type II enzymes utilize an interaction between the high affinity metal ion A and the 3'-bridging atom of the scissile phosphate to accelerate rates of enzyme-mediated DNA cleavage, most likely by stabilizing the leaving 3'-oxygen (Fig. 5).<sup>122, 125</sup> Based on similar experiments that examined cleavage on substrates that substituted a non-bridging sulfur atom for oxygen, there also appears to be an important contact between one of the metal ions and a non-bridging atom of the scissile phosphate in the active site of topoisomerase II $\beta$  (Fig. 5).<sup>122</sup> This latter interaction plays a significant role in DNA cleavage mediated by the  $\beta$  isoform and greatly stimulates scission.<sup>97, 99, 122, 124</sup> As proposed previously, this metal ion-DNA contact is believed to stabilize the DNA transition state. While a similar interaction between the metal ion and the non-bridging oxygen in the active site of topoisomerase II $\alpha$  cannot be ruled out, if it exists, its effects on rates of DNA cleavage appear to be equivocal.<sup>99, 124</sup>

Critical evidence for the roles of metal ions in topoisomerase II-mediated DNA cleavage comes from structural studies with the catalytic core of yeast topoisomerase II complexed with DNA substrates.<sup>101, 125</sup> The first study solved the structure of the DNA cleavage/ligation active site of the enzyme in the presence of non-covalently bound DNA.<sup>101</sup> While only one divalent metal ion was observed in the structure, its location was consistent with the assigned TOPRIM amino acid residues shown in Fig. 3. It should be noted, however, that the position of the active site tyrosine residue in this structure was not in a cleavage competent configuration.

A more recent study with the catalytic core of yeast topoisomerase II provided the first structure of a covalent enzyme-DNA complex for a eukaryotic type II topoisomerase.<sup>125</sup> This structure shows the coordination of two metal ions in the cleavage/ligation active site of the enzyme and reveals the constellation of amino acids required to form a cleavage-competent active site (Fig. 4). Metal ion A appears to interact with both the bridging oxygen and a non-bridging oxygen of the scissile bond in the enzyme-DNA cleavage complex.<sup>125</sup> Thus, it is well positioned to assist with transition state chemistry. In contrast, metal ion B does not contact either the active site tyrosine or any DNA atoms at the scissile bond.<sup>125</sup> Rather, it is located adjacent to the active site and appears to contact a non-bridging oxygen of the phosphate linking the (-1) and (-2) nucleotides upstream of the scissile bond.

Both metal ions in the covalent enzyme-DNA structure are coordinated by the highly-conserved acidic residues in the TOPRIM domain of yeast topoisomerase II. Metal ion A is anchored by E449 and D526, and metal ion B is anchored by D526 and D528 (Figs. 4 and 5). The functional importance of the conserved TOPRIM acidic residues in the catalytic activity of type II topoisomerases and their role in metal ion binding is strongly supported by mutagenesis studies that examined gyrase, topoisomerase II $\alpha$ , and topoisomerase II $\beta$ .<sup>97, 123–124, 136</sup> In all cases, substitution of the coordinating glutamic acid or aspartic acid residues with a non-acidic amino acid resulted in an enzyme with dramatically reduced DNA strand passage and cleavage activities. Furthermore, mutant enzymes required higher levels of divalent metal ions for activity than did the corresponding wild-type proteins. Finally, enzymes that substituted cysteine residues for the conserved aspartic acids displayed higher activity in the presence of Mn<sup>2+</sup>, a thiophilic metal ion, than did the corresponding alanine-containing mutants.<sup>136</sup>

In the canonical two-metal mechanism, one divalent cation activates a catalytic water or ribose hydroxyl for nucleophilic attack, while the other coordinates a leaving group.<sup>137</sup> Both metal ions stabilize the pentavalent transition state. Based on the collective data from the



enzymology, mutagenesis, and structural studies discussed above, there appear to be important interactions between divalent metal ions and the bridging atom of the scissile bond, the non-bridging atoms of the scissile bond and the bond one nucleotide upstream, and conserved acidic amino acid residues in type II topoisomerases.<sup>97, 123–125, 136</sup> Therefore, it is proposed that type II topoisomerases employ a novel variation of the canonical two-metal-ion mechanism for DNA cleavage (Fig. 5). In this model, metal ion A plays a direct role in the transition chemistry, most likely by promoting the leaving of the ribose 3'-OH. In contrast, metal ion B appears to play a structural role in anchoring the DNA during cleavage.

The fate of the metal ions when the cleaved DNA gate is opened is not known. Metal ion B could remain complexed with the phosphate of the upstream nucleotide during gate opening. However, since metal ion A is complexed to oxygen atoms that become the 3'-terminal hydroxyl and 5'-terminal phosphate moieties, it is clear that it cannot remain in contact with both atoms during this process. The resolution of this important issue likely will have to wait for future structural studies that capture the cleavage complex in a “gate-opened” form.

It is not obvious why type II topoisomerases have lost the need for a second metal in the transition chemistry required for DNA cleavage. However, it may be due to the relatively low  $pK_a$  (~10–11) of the active site tyrosine nucleophile, which makes it easier to deprotonate than either water or an attacking nucleic acid 3'-hydroxyl group ( $pK_a$  ~13–15). Alternatively, the presence of the positively-charged arginine immediately adjacent to the active site tyrosine residue may take the place of the second metal ion to assist with transition state stabilization or to further depress the  $pK_a$  of the tyrosine hydroxyl moiety.<sup>125</sup>

The DNA ligation reaction of type II topoisomerases is considerably less well understood than cleavage. Ligation has an absolute requirement for a divalent cation and is supported by a range of metal ions, including  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Ca^{2+}$ .<sup>92, 138–139</sup> The reaction is believed to represent the reverse of DNA cleavage/transesterification. However,  $Co^{2+}$ , which efficiently supports DNA scission mediated by human topoisomerase II $\alpha$ , does not support rapid rates of ligation.<sup>96</sup> Thus, there may be subtle, but important differences in the use of divalent metal ions during these two processes.

An important role of metal ions during ligation may be to properly align the DNA termini for resealing. To this point, the 3'-terminal ribose moiety in a metal-free structure adopts a C2'-endo (B-form DNA) sugar pucker that is not optimally oriented for ligation.<sup>125</sup> In contrast, the ribose adopts a C3'-endo state (A form) similar to its neighboring nucleotides in a structure that includes two divalent metal ions, and is positioned in line with the phosphotyrosyl linkage.<sup>125</sup>

## 5. Divalent Metal Ions as Probes of Topoisomerase II Function

The finding that type II topoisomerases are able to use a variety of divalent metal ions for DNA cleavage and ligation has played an important role in understanding these critical enzyme processes. For example, it has been known for more than 20 years that substituting  $Ca^{2+}$  for  $Mg^{2+}$  significantly raises levels of DNA cleavage mediated by bacterial gyrase and eukaryotic topoisomerase II (including yeast, *Drosophila*, bovine, and human).<sup>93, 96, 138, 140–141</sup> Prior to this finding, the study of gyrase-generated DNA scission was confined to reactions that contained quinolone antibacterials. The use of  $Ca^{2+}$  in DNA cleavage reactions greatly facilitated the ability to map sites of enzyme action and monitor DNA scission in a multitude of studies.<sup>93, 138, 140, 142</sup>

Topoisomerase II-mediated DNA cleavage and ligation normally exist in a tightly coupled equilibrium. Consequently, until the advent of suicide- or activated-substrates,<sup>143–148</sup> it was

impossible to carry out mechanistic studies of ligation. However, a unique property of  $\text{Ca}^{2+}$  allowed topoisomerase II-mediated ligation to be studied in isolation.<sup>93</sup> In contrast to  $\text{Mg}^{2+}$ , which appears to be inaccessible to chelating agents when involved in a cleavage complex,  $\text{Ca}^{2+}$  can be removed from the covalent topoisomerase II-cleaved DNA complex with EDTA or similar chelating agents. Thus, chelation of  $\text{Ca}^{2+}$  traps the cleavage complex in a kinetically competent form.<sup>93</sup> The subsequent addition of excess  $\text{Mg}^{2+}$  reactivates the trapped enzyme and allows a unidirectional ligation event. The use of this assay permitted the first analysis of ligation and demonstrated that topoisomerase II ligates double-stranded breaks one strand at a time.<sup>76, 93</sup> It also provided the first direct evidence that etoposide and several other anticancer drugs increase levels of topoisomerase II-generated DNA strand breaks primarily by inhibiting ligation.<sup>91, 149–150</sup>

Finally, the use of divalent metal ions with varying degrees of thiophilicity in conjunction with sulfur-containing DNA substrates provided important information regarding the role of divalent cations in the DNA cleavage reaction mediated by type II topoisomerases.<sup>99, 122, 136</sup> These studies demonstrated that human topoisomerase II $\alpha$  and topoisomerase II $\beta$  cleave DNA using a two-metal-ion mechanism and defined interactions between divalent metal ions and the scissile phosphate oxygen atoms.<sup>99, 122, 136</sup>

## 6. Summary

All type II topoisomerases require divalent metal ions to support their catalytic activities. Although we understand many aspects of the interactions between the metal ions, the enzymes, and their DNA substrates, a number of important issues have yet to be investigated. For example, what are the specific role(s) of  $\text{Mg}^{2+}$  in the chemistry of DNA cleavage and ligation? Do metal ions interact with anticancer drugs in the topoisomerase II-DNA cleavage complex? Can the finding that divalent metal ions affect levels of enzyme-mediated DNA scission be exploited in the design of novel topoisomerase II-targeted anticancer drugs? It is clear that future studies of metal ions and type II topoisomerases still hold considerable promise for important discoveries.

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## Biographies



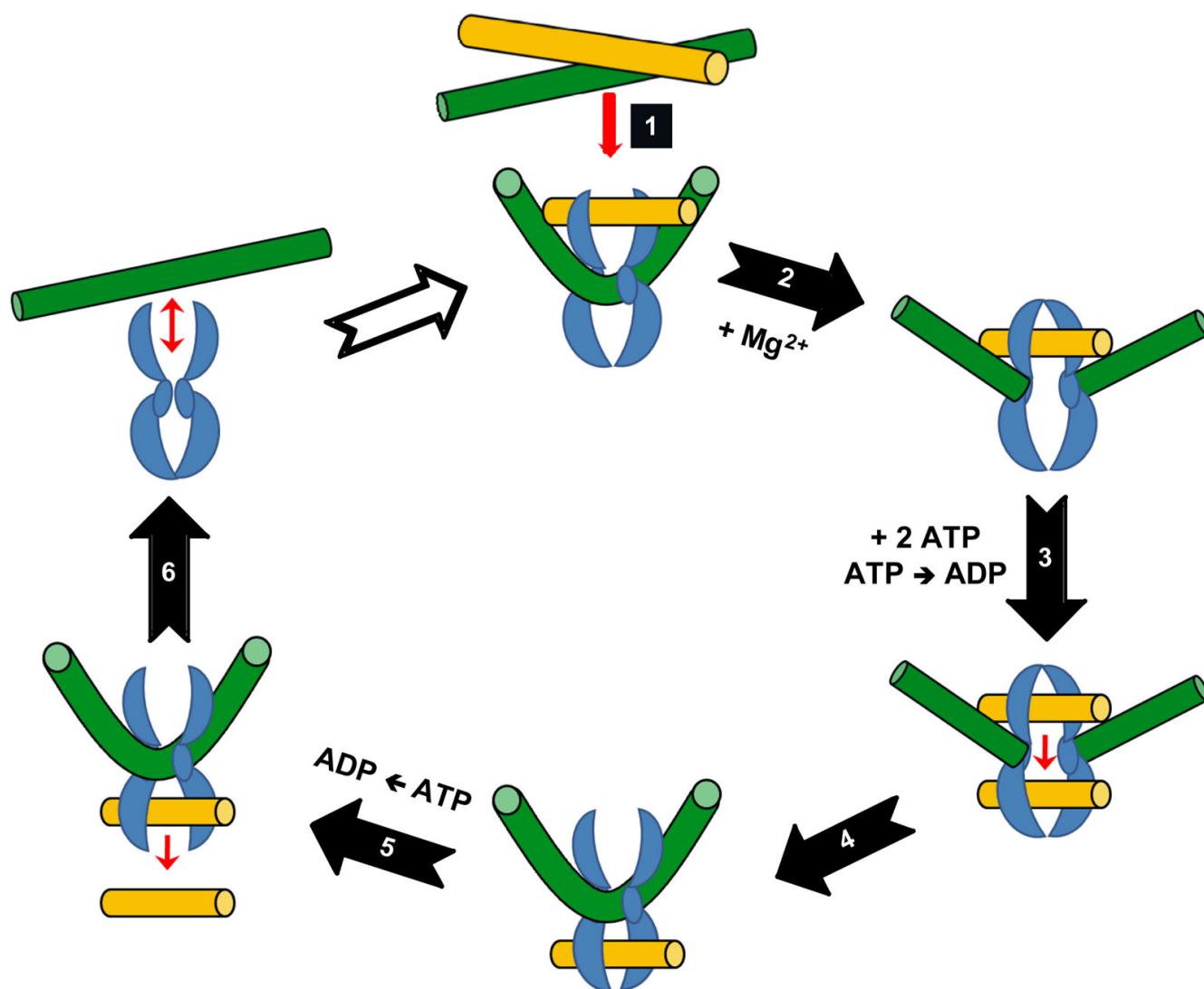
Neil Osheroff holds a B.A. in Chemistry from Hobart College (1974) and a Ph.D. in Biochemistry and Molecular Biology from Northwestern University (1979). He was a Helen Hay Whitney Foundation postdoctoral fellow in the Department of Biochemistry at the Stanford University School of Medicine from 1980–1983. In 1983, Dr. Osheroff moved to the Vanderbilt University School of Medicine where he currently holds Professorships in the Departments of Biochemistry and Medicine and was endowed with the John G. Conglio Chair in Biochemistry in 2003. Research in Dr. Osheroff's laboratory focuses on topoisomerases, enzymes that remove knots, tangles, and torsional stress from the genetic

material, and are the targets for several widely prescribed antibacterial and anticancer drugs. Among his work on these enzymes, he has investigated the role of metal ions in topoisomerase function for more than twenty years.



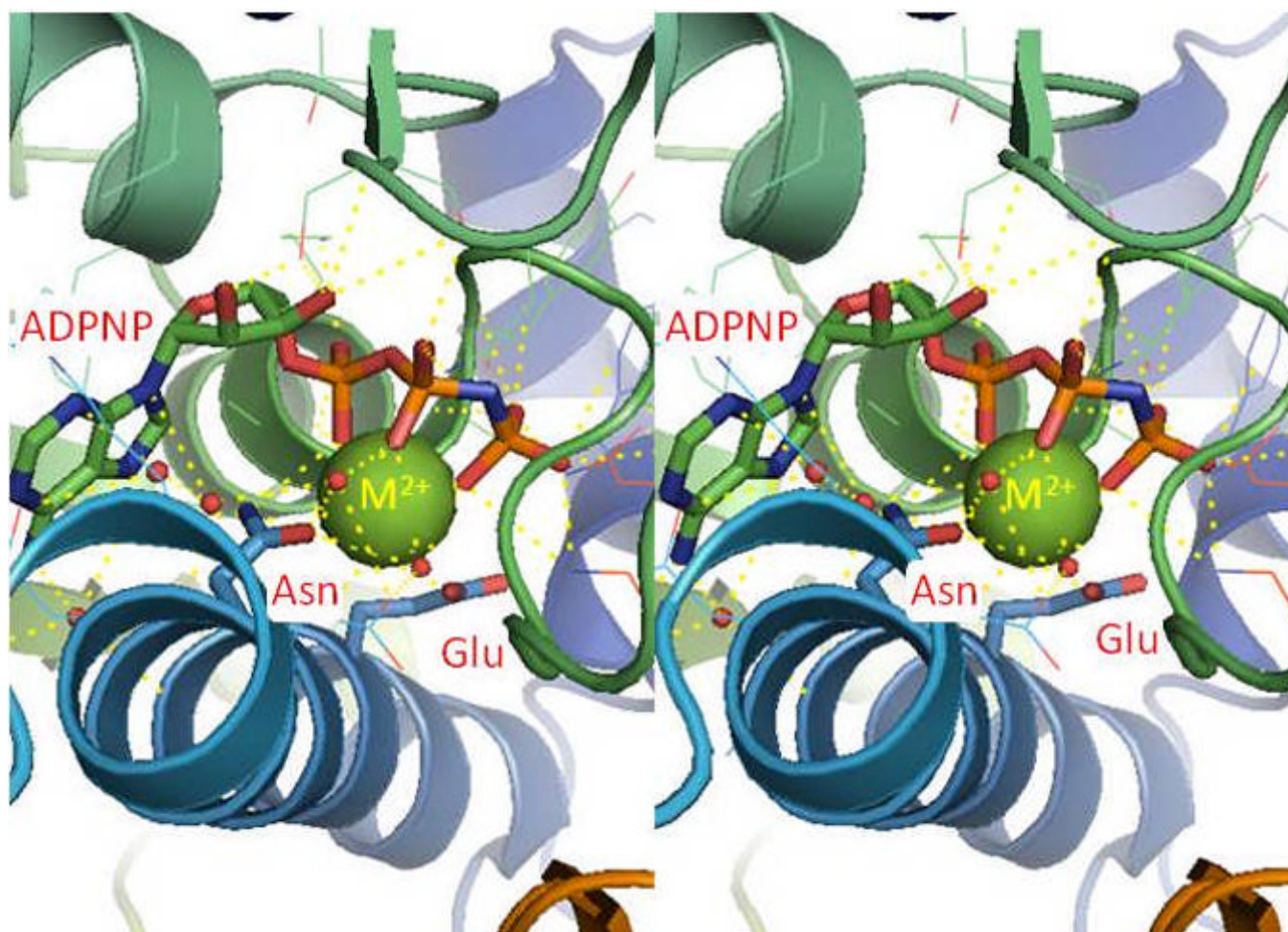
Joe Deweese holds a B.S. in Biochemistry from Freed-Hardeman University (2004) and a Ph.D. in Biochemistry from Vanderbilt University School of Medicine (2009). Dr. Deweese performed his graduate research in the laboratory of Dr. Neil Osheroff where he studied the involvement of metal ions in the cleavage reaction of mammalian type II topoisomerases. He currently is an Assistant Professor of Pharmaceutical Sciences at Lipscomb University

College of Pharmacy and an Adjunct Assistant Professor of Biochemistry at Vanderbilt University School of Medicine.



**Figure 1.** Catalytic cycle of topoisomerase II. The homodimeric enzyme is shown in blue, the DNA double helix that is cleaved is shown in green, and the double helix that is passed through the DNA gate is shown in yellow. The double-stranded DNA passage reaction of topoisomerase II can be separated into six discrete steps. 1) DNA binding to regions of helix-helix juxtaposition. 2) Double-stranded DNA cleavage, which requires the presence of  $Mg^{2+}$  (physiologically) or other divalent metal ions. 3) Binding of 2 ATP molecules, which promotes double-stranded DNA passage through the DNA gate generated by cleavage. Strand passage proceeds more rapidly if one of the two ATP molecules is hydrolyzed. 4) Ligation of the cleaved DNA. 5) Hydrolysis of the second ATP molecule, which allows release of the DNA through a C-terminal gate in the protein and promotes 6) enzyme turnover, which allows the enzyme to initiate a new round of catalysis.



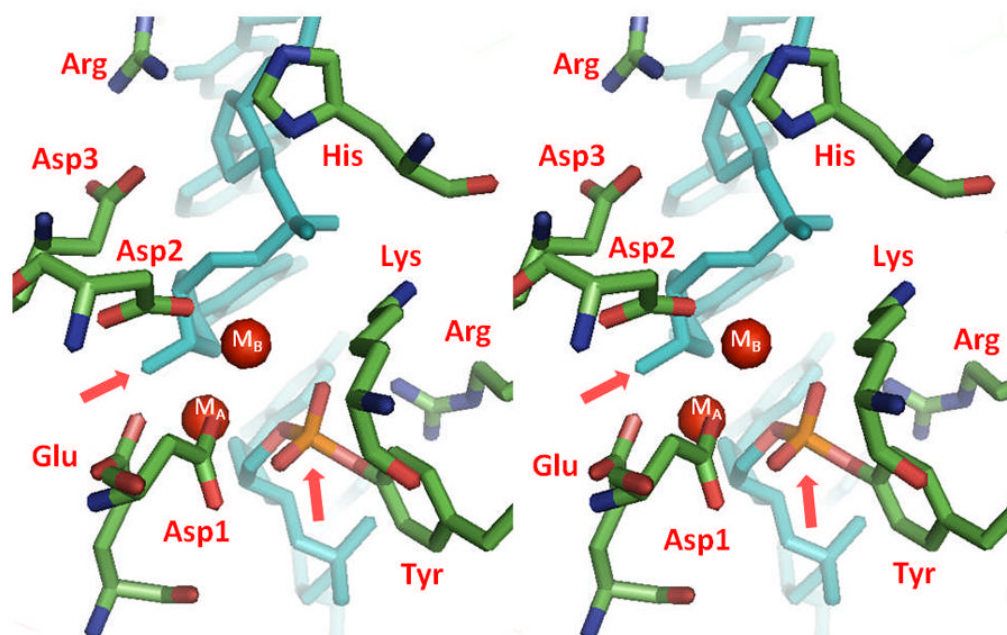


**Figure 2.** Crystal structure of the ATPase domain of yeast topoisomerase II (PDB ID 1QZR) with ADPNP bound.<sup>87</sup> The bound metal ion ( $Mg^{2+}$ , green) is coordinated by non-bridging oxygen atoms of the phosphate groups and by an asparagine residue (Asn91 in human topoisomerase II $\alpha$ ). The glutamic acid residue (Glu87 in human topoisomerase II $\alpha$ ) is thought to activate a water molecule (shown as red spheres) to initiate hydrolysis of the phosphate group. Yellow dashes represent ligand interactions. The structure is shown in stereo view.

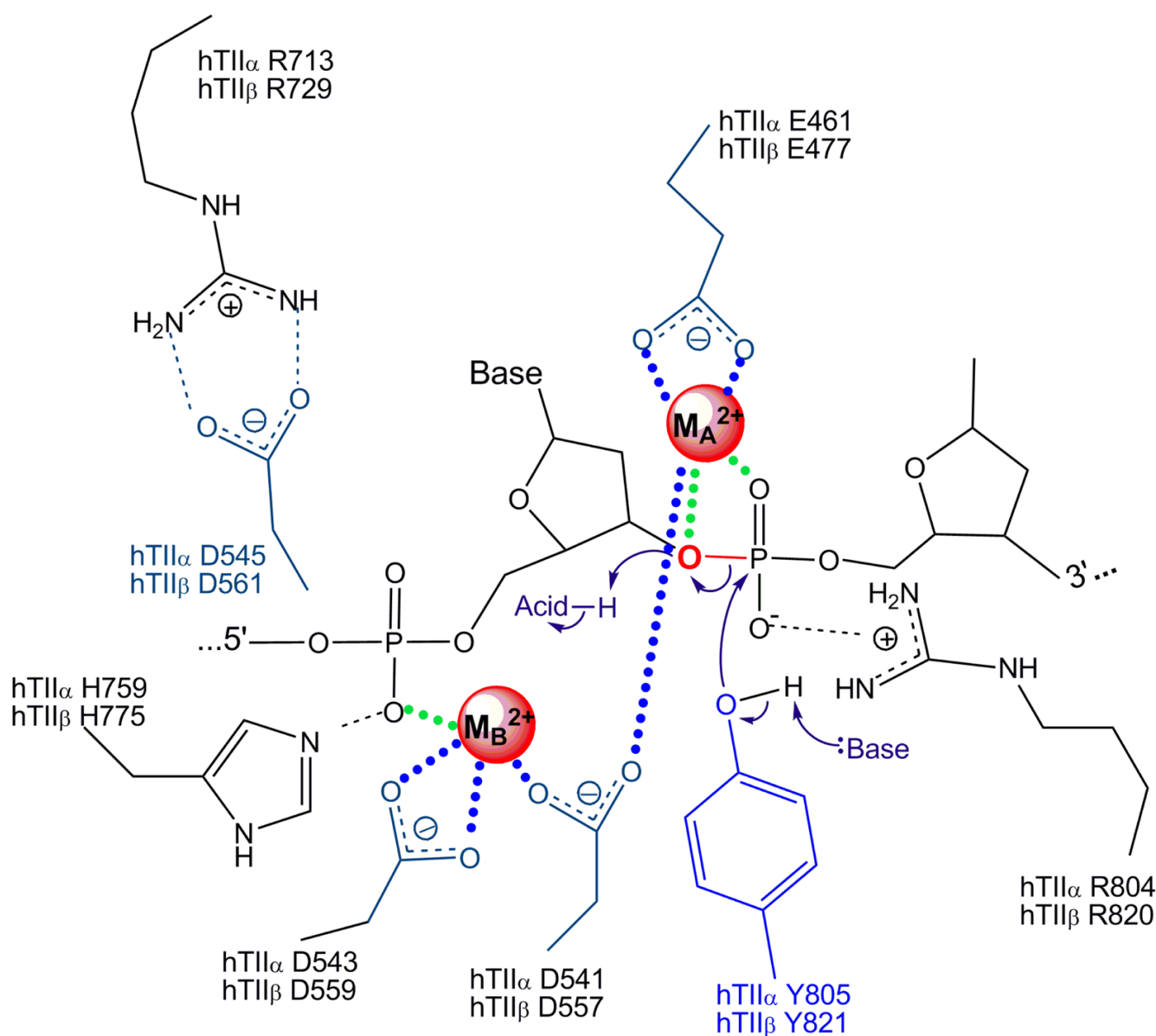
HsTop2a	459	LT	E	GD	538	IMT	D	Q	D	Q	D	G	S
HsTop2b	475	LT	E	GD	554	IMT	D	Q	D	Q	D	G	S
ScTop2	447	LT	E	GD	523	IMT	D	Q	D	H	D	G	S
EcGyrB	442	LV	E	GD	495	IMT	D	A	D	V	D	G	S

**Figure 3.**

Amino acids postulated to coordinate divalent metal ions in the active site of nucleic acid enzymes that contain a TOPRIM domain. Amino acid sequences are shown for human topoisomerase II $\alpha$  (HsTop2a), topoisomerase II $\beta$  (HsTop2b), *S. cerevisiae* topoisomerase II (ScTop2), and *E. coli* gyrase B subunit (EcGyrB). Conserved glutamic and aspartic acid residues that are proposed to bind divalent metal ions are highlighted in blue. Sequences are from Aravind *et al.* and Caron and Wang.<sup>98, 129</sup>



**Figure 4.** The DNA cleavage/ligation active site of topoisomerase II. Using the crystal structure of yeast topoisomerase II (PDB ID 3L4K) as a docked dimer, the key residues of the cleavage/ligation active site are shown and are labeled in red.<sup>125</sup> The residues include three aspartic acid residues (labeled 1, 2, and 3 corresponding to their sequence order), one glutamic acid, one histidine, the active site tyrosine, and two arginine residues. The DNA in the crystal structure (shown in blue) is covalently bound and has been cleaved by the enzyme. The 3'-terminal group and 5'-phosphate of the cleaved DNA are denoted by pink arrows. The red spheres represent the locations of divalent metal ions that are visible in the structure (labeled  $M_A$  and  $M_B$ ). The structure is shown in stereo view.



**Figure 5.**

Proposed mechanism of DNA cleavage and ligation by topoisomerase II. The type II enzyme utilizes a novel variant of the canonical two-metal-ion mechanism employed by primases and polymerases.<sup>98–99, 102, 105, 123–124</sup> Amino acids in the active site that are postulated to function in catalysis by topoisomerase II $\alpha$  and topoisomerase II $\beta$  are indicated. Metal ions ( $M_A^{2+}$  and  $M_B^{2+}$ ) are highlighted in red. Interactions between metal ions and nucleic acid or acidic amino acid residues are denoted in green or blue, respectively. In the proposed model,  $M_A^{2+}$  makes critical contacts with the 3'-bridging atom (red) and a non-bridging atom of the scissile phosphate. These interactions are likely to play a role in the transition chemistry and stabilizing the leaving 3'-terminal oxygen.  $M_B^{2+}$  also is required for DNA scission and contacts the non-bridging oxygen of the phosphate that connects the -1 and -2 bases upstream from the scissile bond. This metal ion appears to play a structural role in anchoring the DNA during scission. Cleavage is initiated when a general base deprotonates the active site tyrosine hydroxyl, allowing the oxyanion to attack the scissile

phosphate. The base has not been identified but is believed to be a conserved histidine residue or a metal-associated water molecule. Ligation is initiated when a general acid extracts the hydrogen from the 3'-terminal hydroxyl group. The acid may be a water molecule or an unidentified amino acid in the active site of topoisomerase II. Figure is adapted from Schmidt, *et al.*<sup>125</sup>