

## SURVEY AND SUMMARY

# Effects of magnesium and related divalent metal ions in topoisomerase structure and function

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Received September 25, 2008; Revised December 26, 2008; Accepted January 11, 2009

### ABSTRACT

The catalytic steps through which DNA topoisomerases produce their biological effects and the interference of drug molecules with the enzyme–DNA cleavage complex have been thoroughly investigated both from the biophysical and the biochemical point of view. This provides the basic structural insight on how this family of essential enzymes works in living systems and how their functions can be impaired by natural and synthetic compounds. Besides other factors, the physiological environment is known to affect substantially the biological properties of topoisomerases, a key role being played by metal ion cofactors, especially divalent ions ( $Mg^{2+}$ ), that are crucial to bestow and modulate catalytic activity by exploiting distinctive chemical features such as ionic size, hardness and characteristics of the coordination sphere including coordination number and geometry. Indeed, metal ions mediate fundamental aspects of the topoisomerase-driven transphosphorylation process by affecting the kinetics of the forward and the reverse steps and by modifying the enzyme conformation and flexibility. Of particular interest in type IA and type II enzymes are ionic interactions involving the Toprim fold, a protein domain conserved through evolution that contains a number of acidic residues essential for catalysis. A general two-metal ion mechanism is widely accepted to account for the biophysical and biochemical data thus far available.

Metal ions are important modulators of the biological response (1). Indeed, in nearly one-third of enzyme-mediated processes, metal ion cofactors are required

for activity. Metals are involved in redox (30%) and non-redox catalysis (70%). In the latter case, the most significant property is Lewis acidity, related to the power of a reaction centre to attract electrons. In fact, the metal removes electron density from the ligand and causes polarization of the substrate/cofactor reactive bonds, thereby increasing its electrophilic character.

### MAGNESIUM AS THE MAJOR METAL COFACTOR IN NUCLEIC ACID-DIRECTED ENZYMES

In cell systems, magnesium represents an abundant species as it is present in the millimolar range (2). In line with this, it is largely utilized for electrostatic stabilization and electrophilic activation of substrates by polarization of X–O (X = P,C) bonds. A special case is represented by water, which can be bound rather efficiently in the inner sphere coordination and is available for catalytic complex hydration as well as for hydrolytic processes. Magnesium is by far the most frequently found metal ion cofactor in enzymatic systems. This is most likely related to  $Mg^{2+}$  ability to form stable complexes with phosphate-containing species, including ATP, which is normally associated with the metal ion when acting in a physiological environment (1,3).

Moreover, essential biomacromolecules such as DNA and RNA efficiently bind to  $Mg^{2+}$  as it participates in neutralization of the polyanionic charge of the nucleic acid. In fact, this metal ion is characterized by a small atomic radius (0.72 Å) and an elevated hardness, which makes it especially suitable for coordination to oxygen atoms, which are hard ligands (3–6).

As a corollary of reduced ionic dimensions, large coordinating groups are inadequately accommodated by  $Mg^{2+}$  enzymes. Outer-sphere mechanisms, in which the substrate is connected to the enzymatic machinery through solvent bridge(s), are often operating to deal with coordination problems. In this connection, a hydrogen bonding

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network often serves for catalytic activation and proper presentation of the reacting species.

### ONE- VERSUS TWO ION MECHANISMS IN MAGNESIUM-DEPENDENT ENZYMATIC DNA MANIPULATION

An interesting issue arising from biochemical and biophysical studies has to do with the number of metal ions participating in hydrolytic processes involving the phosphodiester bond in nucleic acids (5,7).

There is not a unique metal ion:enzyme stoichiometry reported for DNA-processing enzymes such as nucleases (8). Indeed one, two or three metal ion-mediated mechanisms are currently proposed. Among these, a widely accepted mechanistic model envisages the concerted action of two  $Mg^{2+}$  ions located closely to each other and eventually sharing common coordination sites in the protein (9). Notable examples are T4 RNase H, T5 5'-exonuclease, Endonuclease V as well as other restriction endonucleases (10–14).

Two-metal ion mechanisms in non-redox processes bear a number of advantages over processes involving a single ion (15–17):

- Activation barriers arising from nuclear reorganization of the solvent and the enzyme can be reduced in binuclear versus mononuclear centres having the same net charge, due to greater charge delocalization.
- Polyatomic substrates can generally be accommodated better at binuclear centres.
- Electrostatic activation of substrate or ionization of a proton from an active-site water molecule will occur more readily at a charged binuclear centre, than at the corresponding mononuclear one. The binuclear electrostatic effect is estimated at least 3 pK units to the free energy of dissociation of a proton from a water ligand compared to the corresponding mononuclear aquo species.
- Formation of a low-energy transition state, for example the negatively charged trigonal-bipyramidal transition state formed during phosphodiester bond hydrolysis (–2 net charge) by nucleases and RNase H, is predicted to be energetically favoured by symmetrical coordination of the axial ligands to the metal ions of a binuclear centre. This geometry is achieved in exonucleases via formation of two bidentate bridges involving an equatorial phosphate O atom.

The second metal ion in binuclear complexes can attenuate the strength of the bridging-ligand field potential as shown by EPR studies on  $Mn^{2+}$ , an ion which can often replace magnesium in nucleic acid processing enzymes. A weakened bridging-ligand field potential may enhance the binding affinity to other ligands, above all water, thereby increasing the Lewis acidity (17).

The preference for  $Mg^{2+}$  should result from the chemical environment and the need for placing two metal ions at a distance of  $\approx 4 \text{ \AA}$ . The stringent requirement for two  $Mg^{2+}$  coordination is likely to be the basis for catalytic specificity by the two-metal ion mechanism. In fact, unlike

other divalent ions, two  $Mg^{2+}$  ions can be optimally coordinated at the appropriate distance (between 3 Å and 4 Å) to facilitate phosphoryl transfer. In contrast, catalytic specificity of one-metal ion-dependent nucleases, for which metal requirement and substrate specificity are less severe, seems to derive from substrate binding. Nevertheless, a link between one- and two-metal ion catalysis has been very recently proposed (18). In fact, structural and functional analysis of nucleotidyl transfer enzymes shows that the two apparently unrelated types of mechanism share a catalytically equivalent metal ion, the second metal ion being eventually replaced by a basic amino acid in the one-metal ion systems.

An additional interesting aspect of Mg-mediated biochemical reactions involving nucleic acid cleavage is the possibility of a 'moving metal mechanism' in which one of the  $Mg^{2+}$  ions moves from a more buried to a more exposed site as a result of the structural and electronic changes that occur during substrate cleavage. In fact, based on the different positions of the metal ions at the EcoRV active site a catalytic mechanism using two metal ions in three different positions has been proposed (19,20).

Further studies on the mechanism of T5 flap endonuclease are also consistent with a two-metal ion process for chemical catalysis. However, the overall biochemical process carried out by this enzyme is more complex and apparently requires three ions to be completely rationalized (21).

### MAGNESIUM IONS IN TOPOISOMERASE-MEDIATED DNA PROCESSING

Among DNA-backbone processing enzymes, topoisomerases play a central role. They participate in essential cellular processes, such as DNA replication, transcription and chromosome condensation, which require enzymes able to regulate the ensuing topological changes produced in the nucleic acid. Their biological, biophysical and structural properties have been carefully reviewed and their molecular mechanism(s) of action thoroughly described (22,23). Briefly, topoisomerases produce a topological change in a DNA chain by the unwinding or the supercoiling of the double helix thereby releasing the torsional strain imposed by DNA processing. This can occur only by transient formation of a break along the DNA phosphodiester backbone, followed by strand passage through the gate and finally by the resealing of the backbone break. The topoisomerase-mediated cleavage process consists of a nucleophilic attack of a tyrosine residue located in the catalytic pocket to a phosphodiester bond of the nucleic acid backbone. The transphosphorylation reaction produces a covalent protein–nucleic acid linkage and a free hydroxyl moiety at the split deoxyribose group. The topological change is generated by controlled rotation of the cleaved strand around the intact filament or by strand passing of a DNA filament through the gate produced by cleavage. This is followed by the resealing of the original bond through phosphoryl transfer from the tyrosine residue to the split deoxyribose hydroxyl. Release of the covalent enzyme–DNA linkage concludes the catalytic

**Table 1.** Principal biochemical features of the DNA topoisomerases

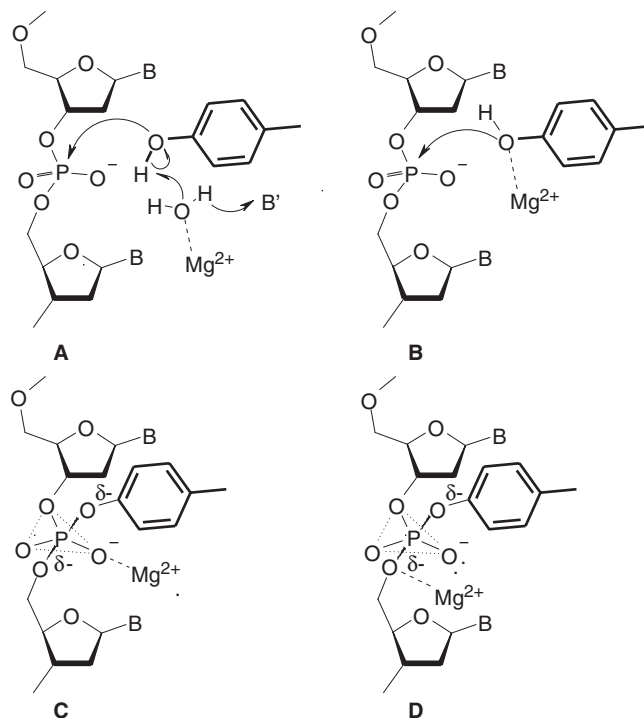
Type	Subfamily	Name	Organism(s)	ATP required	Mg required	Linkage to tyrosine	Mechanism	Toprim
I	IA	Topo I	Eubacteria, archaea, plants	/	+	5'	Strand passage	Yes
		Topo III	Eubacteria, archaea, eukaryotes	/	+	5'	Strand passage	Yes
		Reverse gyrase	Eubacteria, archaea	+	+	5'	Strand passage	Yes
	IB	Topo I	Eukaryotes, viruses, plants	/	/	3'	Controlled rotation	No
	IC	Topo V	Archaea	/	/	3'	Controlled rotation	No
II	IIA	Topo II	Eukaryotes	+	+	5'	Strand passage	Yes
		Gyrase	Eubacteria, archaea, plants	+	+	5'	Strand passage	Yes
		Topo IV	Eubacteria	+	+	5'	Strand passage	Yes
	IIB	Topo VI	Archaea, plants	+	+	5'	Strand passage	Yes

cycle (24,25). Depending upon the number of DNA strands involved in the cleavage reaction topoisomerases can be divided into type I (single-strand break) and type II (double-strand break). Each cellular organism contains at least one type I and one type II topoisomerase component. Further categorizations take into account the proteins' amino-acidic sequence and structure, the reaction mechanism and the sugar position (5' or 3') at which the protein becomes covalently linked to the nucleic acid through the phosphate group. Relevant topoisomerase properties are summarized in Table 1.

Interestingly, topoisomerase IA and topoisomerase II bind covalently to the 5'-phosphoryl group of the cleaved DNA, whereas topoisomerases IB and IC are linked to the 3'-phosphoryl group.

Topoisomerases often require cofactors for full catalytic activity. ATP (type II family and reverse gyrase) regulates the conformational changes required for enzyme action through binding and hydrolysis processes. Divalent metal ions, essentially  $Mg^{2+}$ , are also important as they perform both structural and catalytic functions, besides acting in complex with ATP. In fact, topoisomerase IA and topoisomerases II require  $Mg^{2+}$  to relax supercoiled DNA (22,26). In the case of the type IB enzymes a metal ion is not required to participate in the catalytic cycle and does not directly bind the protein. However,  $Mg^{2+}$  effectively stimulates catalytic activity, probably facilitating the rate-limiting DNA-release step at the end of the enzymatic cycle (27,28).

$Mg^{2+}$  can assist the transphosphorylation process in several ways, as summarized in Figure 1. In fact, it can operate according to a general base (outer sphere) mechanism, stabilize the incoming negative tyrosinate species, neutralize the pentacoordinate negatively charged transition state and/or assist in the stabilization of the negatively charged leaving group (3). Even if a number of investigations have been performed to simulate nucleic acid hydrolytic processes, no accurate quantum mechanical studies on this metal ion-mediated process are available as yet. In particular, it would be of interest to fully understand the factors that induce preferential attack of outer sphere tyrosine versus inner sphere water in topoisomerases, the reverse occurring in the related class of nucleases. Indeed, conserved tyrosine residues are often located in the active site of DNA nucleases and polymerases (Klenow fragment, T5 flap endonuclease,



**Figure 1.** Roles of  $Mg^{2+}$  in topoisomerase-mediated transphosphorylation catalysis: (A) activation of attacking nucleophile (outer sphere); (B) stabilization of tyrosinate; (C) stabilization of transition state; and (D) stabilization of leaving group.

staphylococcal nuclease), yet they do not appear to participate in catalysis but aid stabilizing ternary complex formation. This is an example of how nature can effectively produce different catalytic effects by enabling enzymes to perform distinct chemical reactions even in the presence of essentially the same functions at the active site.

### THE TOPRIM SEQUENCE AS THE CATALYTIC METAL ION(S) ANCHORING SITE IN TOPOISOMERASES IA AND II

Starting from the above mentioned mechanistic similarities in phosphodiester processing enzymes and from biochemical evidence, iterative profile searches were extended to a number of enzymes including DnaG-type primases,

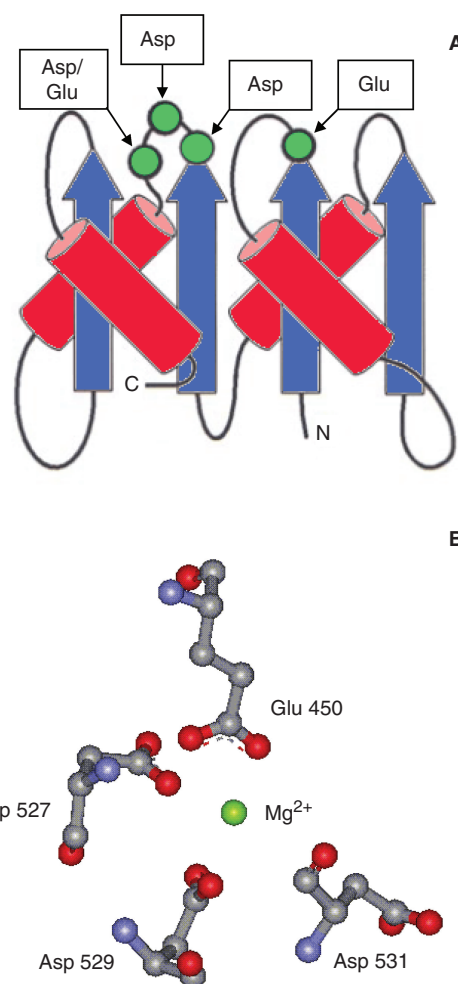
small primase-like proteins from bacteria and archaea, type IA and type II topoisomerases, bacterial and archaeal nucleases of the OLD family and bacterial DNA repair proteins of the RecR/M family (29). These studies revealed a conserved domain spanning about 100 residues, called Toprim, characterized by the invariant presence of a glutamate residue and an aspartate-x-aspartate (DxD) motif. The glutamate is located in a sharp turn connecting a  $\beta$ -strand to an  $\alpha$ -helix. A structurally similar arrangement is observed at the level of the DxD element. As a consequence of Toprim folding, the three acidic residues get close in space and become available for concerted interactions, as confirmed by the X-ray crystallographic studies of several Toprim-containing enzymes, including the *Saccharomyces cerevisiae* topoisomerase II (Figure 2) (30). Given its chemical nature and the requirement for  $Mg^{2+}$ , the triad motif has been proposed to represent the metal ion(s) binding element in the catalytic core.

The fact that both ATP-dependent and -independent topoisomerases, previously considered to be very diverse, share a common catalytic domain with a conserved glutamate plus DxD motif indicates that they basically share a unified reaction mechanism, with the single exception of topoisomerase IB (31), which was already shown to stand alone in the topoisomerase family (Table 1). Moreover, a distinct structural, functional and evolutionary relationship between topoisomerase IB and site-specific recombinases has been demonstrated (32), confirming that this family is indeed unrelated to the Toprim domain-containing topoisomerases.

The details of Toprim interactions with divalent ions and the mechanistic and catalytic consequences of metal ion binding have been the object of several investigations both on topoisomerase IA and II families. The positions of the key catalytic residues for several members of the above families are schematically presented in Table 2.

## MAGNESIUM IN TYPE IA TOPOISOMERASES

Early studies on the IA subfamily of type I DNA topoisomerases confirmed the importance of magnesium as a cofactor in enzyme activity. In fact, the classical Toprim



**Figure 2.** Structural organization of the Toprim domain in the topoisomerase family. (A) Schematic representation of the location of the conserved aspartate and glutamate residues with blue arrows representing tracts of  $\beta$ -barrel secondary structures and red cylinders helical arrangements. (B) Three-dimensional structures of the DxD and E catalytic Toprim residues coordinated to a  $Mg^{2+}$  ion in *S. cerevisiae* topoisomerase II bound to DNA (obtained from the PDB ID 2RGR).

**Table 2.** Sequence alignment of the catalytically relevant residues in Toprim-containing topoisomerases<sup>a</sup>

Type IA		Glu	Asp	Asp	Glu	Tyr	Chain length
Topo I ( <i>E. coli</i> )		9	111	113	115	319	865
Topo I ( <i>Y. pestis</i> )		9	117	119	121	325	871
Topo III ( <i>E. coli</i> )		7	103	105	107	328	653
Reverse gyrase ( <i>A. fulgidus</i> )		512	631	633	635	809	1054
Type IIA		Glu	Asp	Asp	Asp	Tyr	Chain length
TopoII (Human $\alpha$ )		454	541	543	545	805	1531
TopoII (Human $\beta$ )		477	557	559	561	826	1626
TopoII ( <i>S. cerevisiae</i> )		450	527	529	531	782	1428
Gyrase ( <i>E. coli</i> )	GyrB	424	498	500	502	122	804 + 875
TopoIV ( <i>E. coli</i> )	ParE	418	490	492	494	120	630 + 752
Type IIB		Tyr	Glu	Asp	Asp		Chain length
TopoVI ( <i>Sulfolobus shibatae</i> )	Subunit A	106	209	261	263		389

<sup>a</sup>Gyrase, topoisomerase IV and topoisomerase VI consist of two subunits.



sequence is reinforced by the generally found DxDxE motif (33,34), which contains a further acidic glutamate to grant more efficient metal ion binding, possibly allowing greater structural adaptability of the Toprim complex and more efficient transphosphorylation catalysis (35).

A positive relationship was found between the relaxation activity of the *Escherichia coli* enzyme and the concentration of  $Mg^{2+}$  from 0 mM to 10 mM, with near saturation of the metal requirement at 2.5 mM (36). Subsequently, the enzyme was found to be able to bind stoichiometric amounts of  $Mg^{2+}$ , producing a stable metal–enzyme complex. This promoted the relaxation of negatively supercoiled DNA and produced DNA cleavage without further addition of  $Mg^{2+}$ , inferring that  $Mg^{2+}$  ions could bind to specific site(s) on the enzyme even in the absence of the DNA substrate. This suggested that the contact of the metal ion to one or more DNA phosphates might occur through replacement of at least one of the original protein– $Mg^{2+}$  coordination bonds, yet still allowing catalysis without the need for extensive ion-mediated protein–DNA interactions. Among protein residues, Glu-9, Asp-111, Asp-113 and Glu-115 appeared to be the likely candidates involved in  $Mg^{2+}$  binding (34). Surprisingly, site-directed mutagenesis producing alanine substitution in several strictly conserved key residues showed that, in the Toprim domain, only substitution at Glu-9 was able to affect the transesterification process (37). The conclusion followed that  $Mg^{2+}$  is mainly required for conformational changes in the enzyme–DNA complexes rather than for DNA strand cleavage and rejoining and that the amount of  $Mg^{2+}$  required for removal of negative supercoils is much higher than that for DNA rejoining. Notably, Asp-113 was not selected for mutagenesis in this case, which might imply that metal ion binding is compatible with just one of the acidic groups in the DxD sequence.

This issue was considered by investigating Asp-111 and Asp-113 double mutants, which were found to lose over 90% of their activity in the relaxation assay in the presence of  $Mg^{2+}$ . In addition, metal ion concentrations well above those used for the wild-type enzyme (and therefore inhibitory) were needed by double mutants for maximal relaxation activity. Inductively coupled plasma analysis showed the loss of 1–2  $Mg^{2+}$  ions per enzyme molecule due to mutation, consistent with a 1:1 or 2:1 metal ion:topoisomerase stoichiometry in the wild-type enzyme. These results demonstrate a functional role for the acidic residues in the binding of  $Mg^{2+}$  to induce the conformational change required for the relaxation of supercoiled DNA (33).

Another interesting mutant in Toprim domain was characterized by *Yersinia pestis* topoisomerase I. Indeed, from sequence analysis and site-directed mutagenesis, it was shown that changing a single strictly conserved Gly residue to Ser (G116S) in the *Y. pestis* topoisomerase I (or in the corresponding position of the *E. coli* enzyme) can result in a mutant enzyme that entails SOS-inducing and cell-killing properties through a stabilization of the covalent complex with DNA (38).

Overexpression of this mutant topoisomerase I resulted in bacterial cell death. The purified enzyme lost relaxation

activity, yet it still formed a covalent complex and retained the ability to cleave DNA. These results suggest that a mutation close to the DxD diad could interfere with  $Mg^{2+}$  binding and so impair the enzyme re-ligation step. Perhaps, this might happen because the Ser OH displaces the deoxyribose hydroxyl from its correct location in the metal ion coordination sphere. Hence, perturbation of the active site region of bacterial topoisomerase I can result in stabilization of the cleavage complex, thereby producing bacterial cell death in a similar way to eukaryotic topoisomerase poisons.

Several other mutants at the Toprim glycine of *Y. pestis* topoisomerase I were examined (39). While the Gly to Ala substitution allowed both DNA cleavage and re-ligation, the 17 amino acid substitutions subsequently tested completely suppressed DNA cleavage activity. This is apparently a consequence of the steric hindrance generated when replacing the small and flexible, strictly conserved, Gly side chain with larger groups. On the other hand, activity retained by the Ser mutant could be remarkably increased by introducing a second mutation on the Met residue adjacent to the active site tyrosine. Induction of the double mutant topoisomerase resulted in up to 40-fold increase in cell killing rate when compared to the single Gly116Ser mutant. Fluorescence studies monitoring tryptophan emission upon addition of  $Mg^{2+}$  indicated very similar  $Mg^{2+}$ -binding affinity of the double mutant with reference to the single mutant. Accordingly, the relaxation activity of the double mutant did not increase by increasing  $Mg^{2+}$  concentration in the relaxation reaction buffer. Hence, reduced re-ligation has nothing to do with impaired metal ion binding, but with structural changes in the Toprim sequence.

Very recently, another significant Toprim substitution was investigated (40). Introduction of a new positive charge into the active site was achieved by producing a Met to Arg substitution next to the nucleophilic tyrosine. The mutant topoisomerase I showed a shift to basic pH values for optimal activity and a decreased  $Mg^{2+}$  binding affinity. As might be expected, the electrostatic potential map of the minimized mutant structure showed a significant increase in positive electrostatic potential with concomitant decrease in electron density at the level of the acidic triad. These changes in electrostatic potential in the active site region due to the Met to Arg substitution are clearly expected to affect the  $Mg^{2+}$  binding affinity of the mutant topoisomerase and inhibit DNA re-ligation.

## MAGNESIUM IN TYPE II TOPOISOMERASES

Given the close similarity in the catalytic metal ion binding domain and the  $Mg^{2+}$  requirement for activity, the results obtained with type II topoisomerases correlate well with those previously discussed for type IA enzymes.

The role of divalent metal ions in eukaryotic topoisomerase II-mediated reactions was recognized long ago (41–43).

Magnesium ions were found to promote enzyme–substrate interactions in at least two ways: (i) direct involvement in enzyme-mediated DNA cleavage reactions and

(ii) participation in 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 as shown by sedimentation velocity experiments in the presence/absence of  $Mg^{2+}$ . In addition, it did not affect the site-specificity of topoisomerase II binding to DNA, nor did it interfere with the enzyme's ability to discern the topological state of its nucleic acid substrate. It is useful to note that in the topoisomerase II family the DxD motif is generally extended to a DxDxD conserved pattern that offers an additional carboxylic site to generate a structural and electronic network for coordination and correct positioning of the catalytically relevant magnesium ion(s) once more in a similar way to the IA family characterized by the DxDxE motif (29,33).

Mutagenesis studies were performed on the human topoisomerase II  $\beta$ -isoform in the breakage-reunion domain containing the highly conserved acidic residues. In particular, a Glu 477 to Gln mutation was found to greatly impair both DNA decatenation and relaxation activity. In addition, it reduced the level of cleavage by more than two orders of magnitude and magnesium concentration had to be increased for DNA damage detection. Strand passage also required higher concentrations of the metal ion in the mutant with reference to the wild-type enzyme. This was considered as evidence for a reduced binding affinity of the topoisomerase to the magnesium ion(s) required for DNA cleavage. The above findings also highlight the participation of Glu477 in metal ion coordination during catalysis (44).

The prokaryotic topoisomerase, gyrase, was also examined in terms of  $Mg^{2+}$  effects on the enzymatic functions of DNA cleavage and re-ligation using site-directed mutagenesis. The Toprim acidic residues Glu424, Asp498, Asp500 and Asp502, located in the B-subunit, were mutated separately to alanine. Besides the above mutations, the glutamate 424 and the aspartate 498 residues were mutated to glutamine and asparagine, respectively (45). As expected, these residues were found to play an important role for the cleavage–re-ligation reaction. In fact, the activity of all mutants was reduced significantly to between 4% and 20% of the wild-type enzyme and, remarkably, it showed a dependence upon metal ion concentration which was modified in comparison to the wt activity profile. A notable decrease in DNA cleavage was also detected for the mutant enzymes when compared to their wt counterpart in the presence of gyrase poisons (quinolones) or calcium ions which are known to produce effects similar to the drugs. Also, a substantial increase in  $Mg^{2+}$  concentration was required to detect cleavage in the absence of poisons. To note, mutations were able to alter metal ion specificity and, using magnesium and calcium ions, the actual enzyme activity measured in the presence of both metals was greater than the sum of the individual metal ion concentration dependencies. The conclusion follows that at least two metal ions participate in the gyrase-mediated DNA cleavage–rejoining processes.

A model applicable to type IA and type II topoisomerases (and to other enzymes containing the Toprim fold) has been proposed for this process, according to which

polarization of the tyrosine residue and stabilization of the 3'-hydroxyl group are mediated by  $Mg^{2+}$ . In the two-metal ion model, one  $Mg^{2+}$  stabilizes the pentacovalent phosphate transition state as well as the 3'-oxyanion group to make it a better leaving group. The other metal ion stabilizes the negatively charged transition state, and polarizes the attacking tyrosine residue, so as to render it more susceptible to deprotonation. This corresponds to the modes of metal ion participation in the catalytic events depicted in Figure 1. Both ions are coordinated to the Toprim acidic residues, thus forming an organized structural network close to the active tyrosine and appropriate for processing the nucleic acid phosphodiester linkage. The DNA-rejoining reaction is considered to proceed similarly, with the first ion activating the 3'-hydroxyl group and the second facilitating the leaving of the tyrosine residue in a multi-centred relay mechanism.

For a deeper insight into the metal ion effects on DNA gyrase activity, the enzyme B subunit, its 43 kDa and 47 kDa domains, and two mutants in the Toprim domain (Asp498Ala and Asp500Cys) were examined in a biophysical study (circular dichroism and protein melting experiments) (46). It turned out that  $Mg^{2+}$  ions did not modify the conformational properties of the enzyme subunit at room temperature, but were able to produce selective and differential effects on protein stability. In particular, the protein's melting profiles were generally shifted towards lower temperature, showing that divalent cations produced destabilizing effects on gyrase B folding. These effects were essentially located in the 47 kDa C-terminal domain, containing the Toprim sequence. Melting studies on the gyrase B mutants showed that the Asp498 mutant had little effect on the binding of magnesium, whereas the Asp500 mutation impaired  $Mg^{2+}$  affinity to a large extent, suggesting effective contacts between this residue and the metal ion in the wild-type enzyme. Hence, the Toprim domain is confirmed as the principal selective site for metal ion complexation. These results point to a double role played by divalent metal ions in the catalytic steps in which the DNA gyrase B subunit participates: a direct involvement of cations bound to the Toprim domain in the DNA cutting–rejoining process and a striking increase in protein flexibility produced by ion binding. The latter event facilitates the huge conformational changes essential for the catalytic cycle to be performed and possibly allows the Toprim domain to be properly positioned relative to the Tyr122 residue in the active form of the enzyme. On the other hand, the GyrA subunit was much less prone to structural modifications upon addition of divalent ions. However, it is worth recalling that a Glu84Pro mutation in ParC, the GyrA equivalent in Topoisomerase IV, inhibits the formation of covalent topoisomerase IV–DNA complexes when  $Mg^{2+}$ , but not  $Ca^{2+}$ , is used as a cofactor (47). This points to the participation of residue 84 in interactions with magnesium, the mutation possibly affecting the coordination of the divalent cation in the topoisomerase IV–DNA complex. However, no  $Mg^{2+}$  was found in the crystal structures of GyrA or corresponding topoisomerase II sequences. On the other hand, the presence of the target DNA might be required to allow Glu84 (or its

equivalent in GyrA) to participate in metal ion-mediated interactions within the cleavable complex. This general issue was raised during an investigation on the effects of the presence of the nucleic acid in complex with reconstituted DNA gyrase (48).

The addition of divalent metal to the reconstituted enzyme required higher metal ion concentrations to produce structural changes comparable to those in GyrB, which confirms a supposed scaffold role of GyrA in regulating the overall enzyme structure. Chiroptical studies showed two distinct conformational changes in the presence of  $Mn^{2+}$  (a metal ion conferring full activity to the enzyme), which points to the presence of at least two discrete metal ion binding sites, each affecting protein folding to a different extent. Combining this and the previous information obtained using GyrB suggests that the two distinct conformationally relevant binding events occur in the Toprim region. The first structural transition is associated to a 1:1 stoichiometry, while the subsequent conformational change probably corresponds to the second coordination event. Most interestingly, however, the catalytically significant coordination arrangement can be observed only in the presence of the DNA chain, which points to an active role played by the nucleic acid within the gyrase catalytic complex. Apparently, the polynucleotide substrate represents a crucial component in properly recruiting the metal ion cofactors necessary to correctly perform the cleavage–rejoining cycle of the type II enzyme. Moreover, the data obtained after reconstitution of Gyrase with two GyrB mutants underline a differential role played by residues at position 498 and 500. It is proposed that residue Asp498 participates in the coordination sphere of the second metal ion, whereas residue Asp500 is mainly involved in the first complexation event.

The crystal structure of the DNA binding and cleavage core of *S. cerevisiae* type IIA topoisomerase bound to prospective gate-segment DNA has been recently reported and the structural organization of the catalytic site dissected at atomic resolution (30). Interestingly, a magnesium ion is modelled at the level of the Toprim Glu450, Asp527 and Asp 529 residues, essentially confirming the coordination scheme thus far discussed. However, only one metal ion is present. This would at first glance contradict the two-metal ion mechanism discussed above and supported by convincing evidence in both type IA and type II topoisomerases. Conceivably, a second metal ion could be accommodated in the catalytic site of the yeast topoisomerase, but is probably missing in the crystal structure because of the absence of a scissile phosphate in the cleavage position of the oligonucleotide substrate. Indeed, as suggested by recent work (48), the second metal ion needs the presence of the (scissile) DNA phosphate to be appropriately coordinated in the catalytic pocket. The proposed mechanism appears to hold also for the human type II $\alpha$  topoisomerase as reported in a very recent investigation using a series of divalent metal ions with varying thiophilicities in conjunction with DNA cleavage substrates in which the 3'-bridging oxygen of the scissile bond is replaced by a sulphur atom (49).

Critical interactions between the divalent cations and the latter bond, important for the DNA cleavage event mediated by the enzyme, were determined using the thiolated substrates. Kinetic measurements carried out on the above substrates using different cationic species alone or in combination strongly suggest that two divalent metal ions are required for catalysis. The emerging model closely resembles that discussed above for DNA gyrase. Amino acids likely to interact with the metal ions in the active site of topoisomerase II $\alpha$  are Asp541, Asp543, Asp545 (DxDxD motif) and Glu461. Conceivably, one of the divalent cations binds the 3'-phosphate oxygen to facilitate the cleavage event, while the other is postulated to stabilize the DNA transition state and/or help deprotonate the active site tyrosine (Figure 1).

## CONCLUDING REMARKS

The results thus far discussed show unambiguously that divalent metal ions, particularly magnesium, play a major, indispensable role in promoting the DNA cleavage–rejoining activity of topoisomerases through both participation in the catalytic events and assistance in the appropriate structural changes to assemble/disassemble the active site. Catalytic  $Mg^{2+}$  species are consistently bound to a specific region in the active site. Interestingly, in both IA and II enzymes the metal ions are tightly anchored to the protein backbone through four conserved, closely located, carboxyl side chain residues (Asp, Glu), more abundant than other Toprim-containing enzymes. Considering that topoisomerases perform two sequential phosphoryl transfer reactions with large conformational changes in-between, this might be related to the need to keep the 3'-OH end produced by the cleavage reaction firmly connected to the acidic sequence, hence in place to perform re-ligation (34). Losing contact with the enzyme would surely render the latter process less efficient. It is worth considering a key difference in the relaxation step between type IA and IB topoisomerases. In fact, while type IA enzymes perform a strand passage mechanism changing the linking number one by one, type IB enzymes conduct multiple linking number changes by a free rotation mechanism. Apparently, participation of the metal ion grants stricter control in the resealing of the cut DNA strand each time strand passage occurs. This is reasonable considering the multiple coordination ability of  $Mg^{2+}$  when compared to the proton relay catalytic mechanism characteristic of the IB topoisomerases (50).

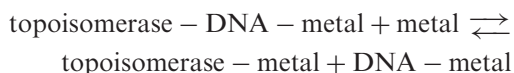
Hence, it appears that a very organized ionic network is formed close to the transphosphorylation centre, connecting the anionic centres in Toprim and the anionic centre in the nucleic acid through divalent (magnesium) ions. Remarkably, this is true also for DNA gyrase for which Toprim is located in one subunit and the active tyrosine in the other.

A two-ion model is generally suggested for Toprim containing type IA and Type II enzymes (33,45,48,49). This probably facilitates a relay-like mechanism, in which the two metals are possibly not fixed in space, but may undergo coordination rearrangements to cope with



the different spatial and electronic requirements for proper support of the cleavage and, subsequently, the rejoining process. Considering that the second  $Mg^{2+}$  is probably recruited by the nucleic acid binding to the enzyme, only one ion could be constitutively associated to the enzyme. This tightly bound magnesium would explain the discrepancies deriving from reports questioning the absolute requirement of the metal ion in the phosphodiester cleavage process. In addition to increasing relaxation activity in topoisomerase IA, increasing  $Mg^{2+}$  concentration also produces changes in the DNA cleavage patterns (51). This behaviour is not simply explicable in terms of the two-ion mechanism, but might be related to progressive changes in plasmid conformation (winding) caused by the metal ion (52), which is known to bind efficiently to the nucleic acid producing tightly bound ion pairs (53). Protein conformational changes at metal ion concentrations well above physiological could additionally contribute.

In connection with this, another invariant finding in ion-mediated topoisomerase function is the bell-shaped dependence of the cleavage process efficiency upon divalent ion concentration. This is probably the result of the competition between different binding events involving the three partners (topoisomerase, DNA and ion). As mentioned above, both the protein and the nucleic acid efficiently bind divalent metals. We can therefore envisage the formation of binary topoisomerase–metal and DNA–metal complexes, along with the productive ternary complex. Upon increasing metal ion concentration, ternary complex formation continues to increase up to the point at which the splitting of this complex into two binary systems occurs simply according to mass law. In fact, the process



is obviously shifted to the right by increasing the concentration of the metal ion above a given threshold. This is determined, besides the concentrations of the interacting species, by the relative affinities of the metal for ternary versus binary complexes and is further confirmed by the fact that different metals exhibit different concentration windows for optimal cleavage. It is, however, worth mentioning that active site-mutated type IA topoisomerases still exhibit single-stranded DNA (ssDNA) binding ability which is not affected by  $Mg^{2+}$ . Since the metal ion is likely to bind a double-stranded nucleic acid more tightly than a ssDNA due to the higher charge density exhibited by the former (54), the metal ion concentration required to split the ternary complex in topoisomerases IA could be significantly higher in comparison to that required in topoisomerases II. Moreover, type I and type II enzymes could be further conformationally affected to different extents by increasing ionic strength, thus exhibiting distinct profiles of inactivation as a function of added metal ion.

As pointed out above, a great deal of information on the molecular mechanisms of the action of topoisomerases has been gained not only by site-directed mutagenesis, but also by changing the nature of the catalytic metal ion. In particular, both  $Ca^{2+}$  and  $Mn^{2+}$  were able to modulate

enzyme activity and allowed the fine details of the mode of action to be understood (48,49). Like magnesium, calcium is redox-inert and prefers hard donors such as oxygen. It is characterized by a larger ionic radius (1 Å), hence a lower charge density and a higher flexibility to accommodate ligand groups in its coordination sphere (55). These properties render it less effective in transesterification catalysis, in particular in the re-ligation process.

The functional role of  $Mn^{2+}$  rests on its properties as a Lewis acid and an electrostatic stabilizer. It represents a good substitute for  $Mg^{2+}$  due to the similar length of its radius (0.83 Å) and its borderline hard–soft character (56). The latter feature can be helpful to detect specific contacts between the enzyme or the nucleic acid species since substitution of oxygen atoms with sulphur (or introduction of sulphur-containing groups) would substantially enhance manganese binding. These important details of the two-metal ion model would not be available without metal ion replacement studies.

Not only do divalent metal ions participate in topoisomerase catalysis, but they might also play a role in enzyme poisoning. In fact, a number of topoisomerase II-directed drugs (no compound aimed at topoisomerase IA has been thus far described), including quinolones (57), quinobenzoxazines (58), bisbenzoxazoles, (59) anthraquinones (60), anthracyclines (61) and epipodophyllotoxins (62) have been reported to participate in metal ion binding (in many instances  $Mg^{2+}$ ) when forming stable complexes with biological macromolecules. It is conceivable that metal ion binding ability can be exploited when the drug is sitting in the cleavage complex and inhibits the DNA resealing process. It would not be surprising that metal-chelating groups of the drug molecules participate in the  $Mg^{2+}$  coordination sphere, displacing the 3'-hydroxyl ligand from proper positioning and also reducing favourable electronic effects thus enhancing the activation energy barrier in the DNA resealing step. This would represent a further until now overlooked topoisomerase poisoning mechanism. Further investigations are warranted to dissect this point and to assess its potential use in rational drug design and development. In this connection, topoisomerase IA might be exploited as a novel target for antibacterial action by developing small molecules able to specifically interfere with the  $Mg^{2+}$ -dependent re-ligation process. This would be beneficial for successful treatment of bacterial infections resistant to current topoisomerase II inhibitors (63).

To confirm that such a strategy might be rewarding, a similar approach has proven fruitful in the inhibition of divalent ion-dependent strand transfer by HIV-1 integrase. In fact, raltegravir, which acts by chelating the ion cofactor(s) located at the active site of this enzyme, has recently been approved by the U.S. Food and Drug Administration for clinical treatment of AIDS (64). Therefore, a detailed knowledge of metal ion participation in topoisomerase-related processes might not only hold mechanistic relevance, but also open new avenues for targeted therapeutic intervention.

In conclusion, type IA and type II topoisomerases perform their catalytic job with a mechanism resembling several other types of DNA-processing and RNA-processing



enzymes including DNA- and RNA-polymerases, restriction endonucleases, transposases, primases and integrases (16). Interestingly, the apparently divergent functions of these enzymes exhibit a substantially conserved reaction pattern, which can yield a unified view of the making and cleaving phosphodiester bond reactions in nucleic acids. In fact, divalent ions, almost invariably magnesium in the two-ion mechanism, are required to allow and modulate polarization of the reacting phosphate group to facilitate some of the fundamental biochemical processes needed for cell life and proliferation. These ions are firmly held in the catalytic pocket by carboxylate groups belonging to invariant aspartic/glutamic acid residues and are ready to coordinate the DNA/RNA substrate to produce an activated complex. Interestingly, topoisomerases perform both the cleaving and rejoining step alternatively produced by the other enzymes, in this connection representing the bridge between the phosphodiester bond makers and disrupters and confirming that common active site chemical and structural features have evolved to govern apparently opposite biochemical processes for nucleic acids manipulation.

## FUNDING

Padova University (Grant number CPDA078422/07) and Associazione Italiana per la Ricerca sul Cancro. Funding for open access charge: Padova University (Grant number CPDA078422/07) and Associazione Italiana per la Ricerca sul Cancro.

*Conflict of interest statement.* None declared.

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