Florida International University

FIU Digital Commons

Biomolecular Sciences Institute: Faculty Publications

College of Arts, Sciences & Education

7-25-2013

Metal ion and inter-domain interactions as functional networks in E. coli topoisomerase I

Claudia Sissi

Dept. of Pharmaceutical and Pharmacological Sciences, v. Marzolo 5

Bokun Cheng

Valentina Lombardo

Yuk-Ching Tse-Dinh

Manlio Palumbo

Follow this and additional works at: https://digitalcommons.fiu.edu/biomolecular_fac

Recommended Citation

Claudia Sissi, Bokun Cheng, Valentina Lombardo, Yuk-Ching Tse-Dinh, Manlio Palumbo, Metal ion and inter-domain interactions as functional networks in E. coli topoisomerase I, Gene, Volume 524, Issue 2, 2013, Pages 253-260, ISSN 0378-1119, https://doi.org/10.1016/j.gene.2013.04.008.

This work is brought to you for free and open access by the College of Arts, Sciences & Education at FIU Digital Commons. It has been accepted for inclusion in Biomolecular Sciences Institute: Faculty Publications by an authorized administrator of FIU Digital Commons. For more information, please contact dcc@fiu.edu.

NIH Public Access
Author Manuscript
Gene. Author manuscript; available in PMC 2014 July 25.

Published in final edited form as:
Gene. 2013 July 25; 524(2): . doi:10.1016/j.gene.2013.04.008.

Metal ions and inter-domains interactions as functional networks in *E. coli* Topoisomerase I

Claudia Sissi[1,*], **Bokun Cheng**[2], **Valentina Lombardo**[1], **Yuk-Ching Tse-Dinh**[2,#], and **Manlio Palumbo**[1]

[1] Dept. of Pharmaceutical and Pharmacological Sciences, v. Marzolo 5, 35131 Padova, Italy

[2] Dept. of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

Abstract

Escherichia coli Topoisomerase I (EcTopoI) is a type IA bacterial topoisomerase which is receiving large attention due to its potential application as novel target for antibacterial therapeutics. Nevertheless, a detailed knowledge of its mechanism of action at molecular level is to some extent lacking. This is partly due to the requirement of several factors (metal ions, nucleic acid) to the proper progress of the enzyme catalytic cycle. Additionally, each of them can differently affect the protein structure.

Here we assess the role of the different components (DNA, metal ions, protein domains) in a dynamic environment as in solution by monitoring the catalytic as well as the structural properties of EcTopoI.

Our results clearly indicated the interaction among these components as functionally relevant and underlined their mutual involvement. Some similarities with other enzymes of the same family emerged (for example DNA prevents divalent metal ions coordination at non selective binding sites). Interestingly, same interactions (C- and N- terminal domains interaction) appear to be peculiar of this bacterial topoisomerase which suggest they could be favorable exploited to the design of selective inhibitors for this class of enzyme.

Keywords

Nucleic acid; metal ion; macromolecule interaction; circular dichroism; electrophoresis

INTRODUCTION

Topoisomerases constitute a family of enzymes fundamental for the proper processing of nucleic acids in all organisms[1; 2]. Indeed, they are required to finely control the supercoiling degree of DNA, a process which is fundamental to grant the stability of the nucleic acids as well as their proper replication, transcription and segregation. The topoisomerase mechanism of action is based on a controlled sequential cleavage-resealing reaction on the

* Claudia Sissi, Dept. of Pharmaceutical and Pharmacological Sciences, v. Marzolo 5, 35131 Padova, Italy. Tel +39 049 8275711; claudia.sissi@unipd.it.
#Current address: Dept. Chemistry and Biochemistry, Florida International University, Miami, FL33199, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

DNA chain. According to the number of DNA strands which are cleaved during the catalytic cycle, topoisomerases are divided into type I or II. Additionally, based on their amino acid sequence, three dimensional structure and mechanistic properties, they are further classified into subclasses (A, B and C for type I; A and B for type II). Common features of the type IA and type IIA topoisomerases are the formation of a 5' DNA-protein phospho-tyrosine bond and the presence of a conserved metal ion binding domain called TOPRIM[3; 4]. This domain is characterized by a combination of β-strand and α-helix which is required to bring close together a glutamate residue and an aspartate-x-aspartate (DxD) motif. This acidic triad is then located in the proximity of the protein catalytic core where it can efficiently coordinate divalent metal ions such as Mg^{2+}.

The conserved presence of this domain reflects the importance of divalent metal ions in modulating the DNA cleavage as well as the religation activity characterizing the mechanism of action of these enzymes[5]. Metal ions can assist these chemical reactions according to several mechanisms such as working as a general base, stabilizer of the negatively charged tyrosinate, stabilizer of the transition state, activator of attacking nucleophile or assistant of the leaving group[6]. Solution studies on mutant enzymes suggested that, in analogy to data acquired with several nucleases and polymerases, two metal ions actively participate in the topoisomerase cleavage reaction/rejoining reaction[7; 8; 9; 10; 11]. Nevertheless, structural studies indicate a peculiar two metal ions mechanism for type IA and type IIA topoisomerases. In this model only one metal ion is involved in stabilizing a pentavalent transition state thus in regulating the cleavage/religation chemistry, whereas the second ion is mainly responsible for proper anchoring of the DNA strand not covalently linked to the protein[12].

This picture clearly underlines how several functional groups work in a concerted way to modulate the protein properties along the catalytic pathway.

Escherichia coli Topoisomerase I (EcTopoI) is a type IA bacterial topoisomerase which is receiving large attention due to its potential application as novel target for antibacterial therapeutics[13]. This topoisomerase is a 97 kDa protein: the 67 kDa N-terminal domain (Top67) contains the active site as well as the Toprim domain where binding of two Mg^{2+} can occur; the remaining 30 kDa C-terminal portion (ZD) is formed by a Zn^{2+} binding domain comprising three repeated tetracysteine motifs and a 14 kDa C-terminal single stranded DNA binding domain[14; 15; 16; 17].

The biochemical properties of Topo I A have been extensively studied in the last years, providing several evidence to support relevant metal ion implications in its mechanism of action[18; 19; 20]. Additionally, a crystal structure of its covalent complex with a DNA fragment has been recently made available and support a concerted action of protein, nucleic acid and metal ions[21]. As a result, we considered it a good model to assess the role of the different components (DNA, metal ions, protein domain) in a dynamic environment as in solution. Previous studies have examined the effect of different divalent ions on cleavage of the small dT_8 oligonucleotide[19]. This DNA substrate may be too small to interact with all functional domains in EcTopoI and to provide a sufficient model on its own for steps involved in relaxation of supercoiled DNA. Thus we further examined the role of divalent ions in EcTopoI activity trying to correlate the effect on DNA cleavage and religation with the overall relaxation activity.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RESULTS

Addition of DNA prior to divalent ions results in higher catalytic efficiency for EcTopoI

It is well documented that divalent metal ions are crucial for the catalytic activity of topoisomerases[11]. This effect can result from their direct involvement in the catalytic process as well as from protein structural modifications upon binding. Additionally, it has been proven that, in order to properly coordinate these cofactors, topoisomerase-DNA binding can be required[10; 21]. To gain further insight into the role of metal ions on this prokaryotic enzyme, we monitored the effect of divalent metal ions on plasmid relaxation by EcTopoI under different conditions. In particular, to asses whether DNA binding can modulate the recruitment of divalent metal ions needed for the cleavage/rejoining processes, all activity assays were performed according to two different protocols: in a set (protocol A) we pre-equilibrated the protein with the divalent metal ion and then let it react with DNA, in another set (protocol B) we first mixed the protein with the nucleic acid and only subsequently introduced the required ions in the reaction mixture. Results are summarized in Fig. 1.

According to protocol A, upon addition of metal ions we observed a progressive increment in the amount of relaxed plasmid. In agreement with the behavior reported for other Topoisomerases[10], further increments of the metal ion concentrations resulted in an impairment of the enzyme relaxation activity. The concentration of metal ion required to grant optimal enzymatic activity is a function of the metal ion nature. Clearly, EcTopoI performed better when the second protocol was applied and showed optimal protein activity in a wide metal ion concentration range. This behavior was confirmed in the presence of Mg^{2+} as well as of Ca^{2+} and Mn^{2+} although with some interesting peculiarities (Fig. 1B). Indeed, only modest differences were observed when comparing relaxation promotion by Mg^{2+} and Ca^{2+}, whereas remarkable modulation of the enzyme efficiency occurred in the presence of Mn^{2+}. In particular, Mn^{2+} showed the highest activity at concentrations lower in comparison to the other tested metal ions. This behavior has been previously observed with DNA-gyrase and may represent a common feature for the TOPRIM containing topoisomerases of type A[22]. Increase of Mn^{2+} concentration to > 1mM resulted in more rapid decrease in relaxation activity when compared to Mg^{2+} and Ca^{2+}. This narrow range of effective concentration for Mn^{2+} probably accounted for relaxation activity not seen in the presence of Mn^{2+} in previous study with different buffer and incubation conditions[19]. Interestingly, no modulation of EcTopoI relaxation activity was observed by changing KCl concentration, thus supporting the relevance of divalent metal ion in the process.

EcTopoI can cleave DNA in the absence of divalent metal ions. Thus, according to protocol B, the equilibration step involving the protein and DNA could cause a partial plasmid relaxation. To avoid systematic errors due to the different time of contact between the protein and the nucleic acid, kinetic measurements were performed by monitoring the formation of relaxed plasmid as a function of incubation time (Fig. 2A). By subtracting the relaxation activity promoted by the protein in the absence of divalent ion added to the reaction mixture, we can directly compare the specific metalion dependent rates. These results are graphically reported in Fig. 2B and again confirm that the enzyme performs better according to protocol B.

Effect of different divalent metal ions on DNA cleavage intermediate

The efficiency of any topoisomerase in relaxing supercoiled plasmid results from the combination of at least two distinct catalytic steps: cleavage and resealing. To dissect the effect of divalent ions during these two events, we monitored the DNA cleavage pattern of

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

the full length protein as well as of its 67 kDa N-terminal domain (Top67) on different nucleic acid substrates.

First, supercoiled plasmid DNA was incubated with high enzyme concentrations and the cleavage products were trapped by SDS. In the absence of divalent ions, the nicked DNA cleavage intermediate formed by EcTopoI can be observed readily (Fig. 3), confirming that divalent ions are not required for the formation of the covalent cleavage intermediate. Ca^{2+} and Mg^{2+} ions were found to reverse accumulation of the nicked DNA formed in the absence of metal ions. However, the level of nicked DNA was not decreased by the addition of Mn^{2+}. This might account for the narrow range of Mn^{2+} concentration that can support the overall catalytic activity. Similar results were obtained for Top67 (Supplementary Material, Fig. S1) which would not remove negative supercoils from plasmid DNA due to the absence of the C-terminal domain.

DNA cleavage by EcTopoI and Top67 was examined further by using a 5'-end labelled 219 base single-stranded DNA substrate (Fig. 4). The cleavage products with faster electrophoretic mobility could be observed in the presence of 1 mM EDTA. The absence of the C-terminal Zn domain in Top67 resulted in more sites being cleaved. The cleavage products were not observed in the presence of 4 mM Mg^{2+}, likely due to DNA religation being favoured over DNA cleavage. For full length EcTopoI, Mn^{2+} and Ca^{2+} changed the DNA cleavage pattern, and did not shift the DNA cleavage-religation equilibrium strongly towards DNA religation as observed for Mg^{2+}. We can summarise the above results by attributing to Mn^{2+} a poor ability to reverse the cleavage reaction. Not the same for Ca^{2+}. Indeed, for Top67, its effect was similar to that of Mg^{2+}. This suggests that Ca^{2+} interacts with the enzyme-DNA complex and affects DNA cleavage differently when the C-terminal Zn domain is present.

In order to better monitor the effect of divalent ions on selectivity among potential cleavage sites, we used a shorter single stranded DNA oligonucleotide (32 residues long) that has three potential cleavage sites for E. coli topoisomerase I.

In the absence of metal ions Top67 was confirmed to cleave DNA more efficiently than the full length protein in a fast process that reaches saturation in about 10 min and that is not affected by the KCl concentration in the reaction mixture (Supplementary Material, Figs. S2 and S3). Three main cleavage sites were identified which confirmed the previously reported requirement for a cytosine at position -4[23].

Addition of divalent metal ions to the DNA-protein reaction mixture generally enhanced DNA cleavage by the two tested proteins (Fig. 5).

Not all sites were equally cleaved as it can be appreciated in Fig. 5. In particular, the preferential increments occurred at sites 1 - 2 and 2 - 3 with the full length and the truncated enzyme, respectively. This results further sustains that the C-terminal ZD domain (deleted in the Top67) can direct the enzyme activity to specific sites. It is important to underline how the metal ions cause differential levels of cleavage enhancement which is not always preserved on the two tested proteins (Supplementary Material, Fig. S4). As an example Ca^{2+} promotes DNA cleavage by Top67 but it does not in the presence of the full length enzyme.

The different behavior observed using the 219- vs. 32- residue ssDNA substrates suggests that proper anchoring of the protein requires a long DNA template. Indeed, only in this latter system reversal of cleavage can be monitored by metal ion addition. Additionally, removal of EcTopoI C-terminal domain further reduces the efficiency of DNA resealing on short DNA fragments at least in the presence of Ca^{2+}. This justifies why a clear cleavage pattern was observed only when this metal ion was added to the Top67-32-mer mixture. This result

combined with the more pronounced DNA cleavage reversal observed on the longer DNA fragment when the metal ion was added to truncated enzyme, apparently indicated that Ca^{2+} performs better on Top67.

Finally, we followed both proteins cleavage activity according to the A and B protocols (different order of component addition) previously used for monitoring metal ions effect on DNA relaxation activity. A kinetic analysis of the cleavage products formation fails to show any effect of incubation order (data not shown). Possibly, the reaction is too fast to allow monitoring clear-cut differences within the applied time scale. Alternatively, the interaction with metals is likely to be more significant for other steps of the catalytic cycle.

Spectroscopic characterization of EcTopoI in solution

The data above indicated an altered DNA processing according to the presence of metal ions and protein domain compositions. This can be the result of protein modification in terms of overall protein structure or flexibility: to asses this point we applied CD spectroscopy which can allow monitoring both parameters.

To avoid systematic errors related to protein structural rearrangements upon mixing, all our data were acquired after one single addition of concentrated protein solution to the required buffer. According to these experimental protocols, we performed CD scans to monitor the overall protein conformation whereas the protein thermal stability was monitored by recording the proteins CD signal at 220 nm while incrementing the temperature at a constant rate (Fig. 6).

In Tris buffer at low ionic strength (20 mM KCl) the CD spectrum showed a main band at 222 nm with a shoulder at 208 nm, in line with the presence of relevant content in α-helix secondary structure. In these experimental conditions, upon increasing the temperature, one single transition at 50.8°C is detected. By incrementing KCl concentration up to 150 mM no changes in the overall CD spectrum were observed (data not shown) at 25°C but a progressive change in melting profile occurred. This ultimately leads to identify two thermal transitions at 48.3 and 56.7°C. Interestingly, their resolution was even more pronounced in other buffers (for ex. in phosphate buffer they occur at 45.5 and 59.6°C, in acetate buffer at 42.4 and 54.4°C) in which the protein retains catalytic activity.

Thus, this behavior does not reflect a loss of protein active form driven by the ionic strength. It can be easily attributed to a two steps unfolding process which occurs at the level of two distinct structural domains. To asses if these correspond to the two C- and N- terminal functional domains contained in the full length protein, we took advantage of the availability of the truncated form of EcTopoI, Top67 as well as of its ZD domain.

A direct comparison of the CD spectra (Fig. 7A) in 50 mM Tris, 150 mM KCl, pH 7.7 relative to the tested proteins confirmed that in our experimental conditions the ZD domain is poorly folded (random coil features of the dichroic spectrum). In turn, the N-terminal 67 kDa domain showed a slightly higher α-helix content in comparison to the full-length protein. The algebraic sum of the ZD and Top67 components (math in Fig. 7) indicates a lower content in ordered structure when compared to the full-length Topo I, whereas an equimolar mixture of the ZD and Top67 protein domains provides an experimental dichroic spectrum that is comparable to the one corresponding to the full-length protein. This indicates that the two domains likely interact with each other, leading to a more ordered global structure.

The melting profiles of the tested proteins (Fig. 7B) evidenced that the ZD domain, poorly folded, does not present a clear structural transition. An increment in signal intensity

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

occurred at high temperature (Tm ≈68.8 °C) which reflects a modest increment in β-sheet content. A comparison of the melting profile of the two folded proteins showed always two distinct thermal transitions, thus supporting that Top67 is already organized into two structural domains. Interestingly, the structural transition occurring at higher temperature matches for the two proteins whereas, in Top67, the first transition (39.7 °C) is clearly shifted to a lower temperature in comparison to the full length enzyme (48.3 °C). We might attribute this change to the interaction between the C- and N-terminal domains. However, in our conditions, combination of equimolar amounts of the two separated domains did not significantly affect Top67 thermal transition (see Table I). Conceivably, when the subunits are not covalently linked, only easily reversible interactions occur which can make the complex to fall apart even at low temperature, thus preventing us from monitoring Tm shift of the T67 domain.

A comparable analysis was performed in the presence of a constant concentration of metal ions (Fig. 8). By using protein concentration and buffer composition (absence of BSA, glycerol) suitable for spectroscopic analysis, upon stirring, Mn^{2+} induced protein precipitation, hence, we did not include data relative to this ion in our spectroscopic evaluation.

The presence of tested metal ions did not alter significantly the CD spectra of the proteins suggesting modest rearrangements of the secondary structure content as a function of ion nature/concentration (data not shown).

Addition of 4 mM Mg^{2+} did not modify EcTopoI melting profile. However, in the presence of 4 mM Ca^{2+} a remarkable variation was observed as the two structural rearrangement events recorded in the absence of the metal ion are turned into a single melting transition occurring at 52.4 °C (Fig. 8A). This behavior was not shared by the truncated protein Top67: indeed, irrespectively of the presence/absence of divalent metal ion, its melting profile did not change (Fig. 8B).

It is worth to remind that Ca^{2+} allowed preserving the relaxation enzymatic activity; however, only the truncated form was not affected by this metal ion in terms of DNA cleavage/cleavage reversal. This point is in agreement with the above reported apparent better linear DNA processing by Top67 in the presence of Ca^{2+} in comparison to the full length enzyme.

To fully correlate the activity profile with the solution structural information, we finally investigated the role of DNA on protein folding. As the nucleic acid we choose the 32-base oligonucleotide previously used for cleavage experiments, which is efficiently processed by both the full length and the Top67 proteins. Divalent metal ions were not included to avoid significant DNA processing during the analysis.

On all tested proteins, addition of equimolar amounts of DNA did not significantly alter protein folding as shown by CD at room temperature (data not shown). However, the melting profile of the full length protein is remarkably affected by the polynucleotide, as it produces a ≈10 C increase in Tm (Fig. 9A). This supports an effective interaction of the protein with DNA which can actually work as a template to preserve the protein folding. On the other hand, the melting profiles of Top67 and ZD domains are not significantly affected by the nucleic acid (Fig. 9B and 9C). Indeed, only a slight increment of the higher Tm relative to Top67 was detected. These results point to the need for the full length protein to grant effective DNA recognition and highlight a role for the ZD domain in stabilizing the protein-DNA complex when bound to the Top67 fragment.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

DISCUSSION

The catalytic cycle of topoisomerases is quite complex. It is made up by several steps where distinct DNA processing reactions (DNA binding, cleavage and resealing) actually occur in conjunction with several DNA and protein structural rearrangements. To accomplish the change in DNA topology, DNA, protein domains and metal ions must properly interact to work in a finely concerted fashion. The experimental data herein presented were collected to better define the role played by each single component in EcTopoI to better understand this complex mechanism.

The already assessed requirement of divalent metal ions to promote EcTopoI activity has been clearly evidenced in all tested activity assays (relaxation and cleavage)[7; 18; 19; 24]. In addition, the relaxation reaction, which offers the possibility of monitoring the reaction products at the end of several catalytic cycles, allowed us to experimentally confirm a precise role of DNA as coordination site for metal ions likely holding them in the proper position to grant enzymatic activity. Indeed, in order to obtain a catalytically active enzyme, the DNA-protein complex turned out to be a better metal ion coordination system than the protein alone. Additionally, the wider range of metal ion concentrations that is accepted by the DNA-enzyme complex in comparison to the protein alone likely indicates that DNA prevents divalent metal ions coordination at non selective binding sites which could impair protein activity.

In line with the chemical similarities between the two metal ions, the effect of Ca^{2+} closely resembles the one of Mg^{2+} during plasmid relaxation. In the presence of Mn^{2+} we showed good activity but in a narrower concentration range in comparison to Mg^{2+}. This behavior was previously reported for example for DNA-gyrase and can reflect the composition of the conserved Toprim domain[10]. Indeed, these two divalent metal ions have different coordination properties (Mn^{2+} well accepts nitrogen to fill its coordination sphere and it does not share the same coordination geometry with Mg^{2+}) which can result in a differential binding affinity in proximity of the catalytic site.

It is relevant that protein interaction with DNA or divalent metal ions does not lead to significant changes in the protein CD signal although literature data reported variations in the EcTopoI fluorescence response upon Mg^{2+} or DNA binding[7; 25; 26]. However, a comparison of the currently available X-ray data indicated relative domain interactions as the main difference between the DNA-free and DNA-bound topoisomerase[21]. Thus, since the chiroptical signal of the protein is related to the overall secondary structure content, it is not surprising that it is not significantly affected by tested protein ligands (DNA and ions).

According to the nature of the metal ion we should expect different results in the cleavage assay if the cleavage and resealing reactions were differently assisted by the metal ions. Generally this was not the case but in the presence of Ca^{2+} that, unexpectedly, does not favor the formation of DNA cleavage sites by the full length enzyme. This is not related to its inability to provide a catalytically active enzyme (Ca^{2+} promotes relaxation) thus it should be the results of an altered balance between the rate of cleavage (kc) and the one of resealing (kr) in comparison to Mg^{2+} and Mn^{2+}. To justify this behavior, we must assume that Ca^{2+} comparably produce a situation corresponding to $kr > kc$ by increasing kr or reducing kc. Our results can help dissecting this point.

First, we must consider that the truncated enzyme Top67 cleaves efficiently DNA in the presence of Ca^{2+}, thus lack of cleavage must be somehow related to the ZD domain. Second, it must be underlined that Ca^{2+} reduces the thermal stability only of the full length enzyme thus sustaining its involvement within the ZD domain. Since ZD is required to increase the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

DNA affinity, it is feasible that the lack of cleavage is due to a reduction in *kc* which should be the only rate constant sensitive to the amount of reversible protein-DNA complex. Similar conclusions were drawn directly monitoring the cleavage rate of short poly(dT) oligonucleotides[19].

The above discussion is related mainly to the role of metal ion in promoting at least one enzymatic activity. However, cleavage data can be discussed also in terms of location of cleavage sites.

Conversely from cut promotion, trapping of the protein at different DNA sites is not significantly linked to the metal ion nature but is clearly affected by the presence/absence of the protein ZD domain. It is already known that Top67 has a reduced affinity for DNA when compared to EcTopoI[27] and, indeed, by CD melting we showed that only the full length protein is able to form a DNA complex which is conserved at temperatures well above the physiological one, thus sustaining the relevance of C-and N-terminal domains interactions to fully account for DNA recognition. Indeed, we confirmed that effective interactions actually occur between the two domains, a no trivial result taking into account the poor folding of the ZD domain. The biological relevance of such a DNA binding requirement emerged clearly from the cleavage site distribution pattern. Indeed, an impaired DNA holding after the cleavage step can cause subsequent resealing to take place at reduced rate. This can occur on DNA sites more or less preferential for EcTopoI giving rise to an altered cleavage pattern by Top67 in comparison to the full length enzyme.

In conclusion, our results bring new insight into the multiple functional interactions occurring in EcTopoI which appear to be only partly shared with other enzymes of the topoisomerase family. Due to the significant impact they play on the enzyme activity they can be favorably exploited to design Top IA selective inhibitors/poisons as conceptually new antimicrobial agents.

MATERIALS AND METHODS

Materials

E. coli topoisomerase I (EcTopoI) was produced and purified as previously reported[28]. Its activity was checked by DNA relaxation assay in the required reaction buffer. Stock solutions of $Mn(ClO_4)_2$, $CaCl_2$ and $MgCl_2$ were prepared freshly in milliQ water.

Relaxation Activity Assay

Supercoiled plasmid pBR322 (Fermentas, 0.125 μg) was incubated with EcTopoI (0.7 nM) in 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9 for 1 h at 37°C. Reaction products were resolved on 1% agarose gels in TBE 0.5X (45 mM Tris, 45 mM boric acid, 1 mM Na_2EDTA) and the DNA bands were visualized by ethidium bromide staining and photographed. The relative amounts of different DNA topoisomers were quantified using a Geliance 2000 apparatus.

DNA Cleavage Assay

Plasmid DNA—Supercoiled plasmid DNA (300ng) was incubated with 200 ng of enzyme in buffer of 10 mM Tris-HCl, pH 7.5, 0.4 mM EDTA at 37°C for 10 min. Metal ions were added at the indicated concentrations and incubation was continued for 5 min. SDS at 1% was added to denature the enzyme and proteinase K (0.1 mg/ml) was added to digest the protein for 30 min before analysis by agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide in TAE buffer.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

5'-end labeled single-stranded DNA—Single-stranded DNA substrate, labeled at the 5'-end with ^{32}P, was generated by PCR and denaturation as previously described (219 base long) [29] or by [γ-^{32}P]ATP and T4 polynucleotide kinase labeling of the synthetic oligonucleotides used in previous studies. [27]

Cleavage reactions using the 219 base long single-stranded DNA substrate were carried out as described.[29] Labeled 32 base long oligonucleotide (30000 cpm) was incubated with protein (1 μg) in 20 mM Tris, 0.5 mM EDTA, 100 μg/ml BSA, 12 % glycerol, pH 8.0 in the presence of variable concentration of KCl or divalent metal ions for 20 min at 37°C. The reaction was stopped by incubation with SDS and proteinase K.

After ethanol precipitation the DNA fragments were analyzed by electrophoresis in a 7-20% sequencing gel.

Circular Dichroism Measurements—Circular dichroism measurements were performed using 1-10 mm path length cells on a Jasco J810 spectropolarimeter in 50 mM TRIS, pH 7.7, added of 20, 100 or 150 mM KCl. Selected control experiment were performed also in phosphate buffer (20 mM K_2PO4, 100 mM KCl, pH 7.7) and acetate buffer (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9). When required, 4 mM divalent metal ions were included in the buffer. CD spectra were recorded using proteins concentrations ranging from 0.2 up to 0.4 μM. For each measurement 3 scans were run and recorded with 1-nm step resolution. Observed ellipticity was converted to mean residue ellipticity $[\theta] = \text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}$. For thermal denaturation experiments, protein solutions were equilibrated at 25°C. Then the signal at 220 nm was recorded while increasing the temperature at 0.8°C/min. T_m was determined by locating the maxima/minima of the first derivative of the curve describing the melting profile (CD vs. T). Melting experiments were performed in triplicate and the corresponding curves were practically superimposable.

Acknowledgments

This research is supported by University of Padova (Prat funds grant # CPDA114388) and MIUR (Ministero dell'Istruzione, dell'Universita' e della Ricerca) (PRIN # 2009MFRKZ8_004); YT by National Institute Of General Medical Sciences (Grant Number R01GM054226). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

REFERENCES

1. Schoeffler AJ, Berger JM. DNA topoisomerases: harnessing and constraining energy to govern chromosome topology. Q Rev Biophys. 2008; 41:41–101. [PubMed: 18755053]
2. Vos SM, Tretter EM, Schmidt BH, Berger JM. All tangled up: how cells direct, manage and exploit topoisomerase function. Nat Rev Mol Cell Biol. 2011; 12:827–41. [PubMed: 22108601]
3. Podobnik M, McInerney P, O'Donnell M, Kuriyan J. A TOPRIM domain in the crystal structure of the catalytic core of Escherichia coli primase confirms a structural link to DNA topoisomerases. J Mol Biol. 2000; 300:353–62. [PubMed: 10873470]
4. Aravind L, Leipe DD, Koonin EV. Toprim--a conserved catalytic domain in type IA and II topoisomerases, DnaG-type primases, OLD family nucleases and RecR proteins. Nucleic Acids Res. 1998; 26:4205–13. [PubMed: 9722641]
5. Sissi C, Palumbo M. Effects of magnesium and related divalent metal ions in topoisomerase structure and function. Nucleic Acids Res. 2009; 37:702–11. [PubMed: 19188255]
6. Cowan J. Chem. Rev. 1998; 98:1067–1087. [PubMed: 11848925]

NIH-PA Author Manuscript

7. Zhu CX, Tse-Dinh YC. The acidic triad conserved in type IA DNA topoisomerases is required for binding of Mg(II) and subsequent conformational change. J Biol Chem. 2000; 275:5318–22. [PubMed: 10681504]
8. Sorokin EP, Cheng B, Rathi S, Aedo SJ, Abrenica MV, Tse-Dinh YC. Inhibition of Mg2+ binding and DNA religation by bacterial topoisomerase I via introduction of an additional positive charge into the active site region. Nucleic Acids Res. 2008; 36:4788–96. [PubMed: 18653534]
9. Noble CG, Maxwell A. The role of GyrB in the DNA cleavage-religation reaction of DNA gyrase: a proposed two metal-ion mechanism. J Mol Biol. 2002; 318:361–71. [PubMed: 12051843]
10. Sissi C, Chemello A, Vazquez E, Mitchenall LA, Maxwell A, Palumbo M. DNA gyrase requires DNA for effective two-site coordination of divalent metal ions: further insight into the mechanism of enzyme action. Biochemistry. 2008; 47:8538–45. [PubMed: 18642932]
11. Deweese JE, Osheroff N. The use of divalent metal ions by type II topoisomerases. Metallomics. 2010; 2:450–9. [PubMed: 20703329]
12. Schmidt BH, Burgin AB, Deweese JE, Osheroff N, Berger JM. A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases. Nature. 2010; 465:641–4. [PubMed: 20485342]
13. Tse-Dinh YC. Bacterial topoisomerase I as a target for discovery of antibacterial compounds. Nucleic Acids Res. 2009; 37:731–7. [PubMed: 19042977]
14. Viard T, de la Tour CB. Type IA topoisomerases: a simple puzzle? Biochimie. 2007; 89:456–67. [PubMed: 17141394]
15. Beran-Steed RK, Tse-Dinh YC. The carboxyl terminal domain of Escherichia coli DNA topoisomerase I confers higher affinity to DNA. Proteins. 1989; 6:249–58. [PubMed: 2560191]
16. Ahumada A, Tse-Dinh YC. The Zn(II) binding motifs of E. coli DNA topoisomerase I is part of a high-affinity DNA binding domain. Biochem Biophys Res Commun. 1998; 251:509–14. [PubMed: 9792804]
17. Baker NM, Rajan R, Mondragon A. Structural studies of type I topoisomerases. Nucleic Acids Res. 2009; 37:693–701. [PubMed: 19106140]
18. Domanico PL, Tse-Dinh YC. Cleavage of dT8 and dT8 phosphorothioyl analogues by Escherichia coli DNA topoisomerase I: product and rate analysis. Biochemistry. 1988; 27:6365–71. [PubMed: 2851323]
19. Domanico PL, Tse-Dinh YC. Mechanistic studies on E. coli DNA topoisomerase I: divalent ion effects. J Inorg Biochem. 1991; 42:87–96. [PubMed: 1649911]
20. Zhu CX, Tse-Dinh YC. Binding of Zn(II) to Escherichia coli DNA topoisomerase I. Biochem Mol Biol Int. 1994; 33:195–204. [PubMed: 8081208]
21. Zhang Z, Cheng B, Tse-Dinh YC. Crystal structure of a covalent intermediate in DNA cleavage and rejoining by Escherichia coli DNA topoisomerase I. Proc Natl Acad Sci U S A. 2011; 108:6939–44. [PubMed: 21482796]
22. Sissi C, Marangon E, Chemello A, Noble CG, Maxwell A, Palumbo M. The effects of metal ions on the structure and stability of the DNA gyrase B protein. J Mol Biol. 2005; 353:1152–60. [PubMed: 16223508]
23. Tse YC, Kirkegaard K, Wang JC. Covalent bonds between protein and DNA. Formation of phosphotyrosine linkage between certain DNA topoisomerases and DNA. J Biol Chem. 1980; 255:5560–5. [PubMed: 6155377]
24. Zhu CX, Roche CJ, Tse-Dinh YC. Effect of Mg(II) binding on the structure and activity of Escherichia coli DNA topoisomerase I. J Biol Chem. 1997; 272:16206–10. [PubMed: 9195920]
25. Cheng B, Annamalai T, Sorokin E, Abrenica M, Aedo S, Tse-Dinh YC. Asp-to-Asn substitution at the first position of the DxD TOPRIM motif of recombinant bacterial topoisomerase I is extremely lethal to E. coli. J Mol Biol. 2009; 385:558–67. [PubMed: 19013470]
26. Cheng B, Feng J, Gadgil S, Tse-Dinh YC. Flexibility at Gly-194 is required for DNA cleavage and relaxation activity of Escherichia coli DNA topoisomerase I. J Biol Chem. 2004; 279:8648–54. [PubMed: 14711811]
27. Ahumada A, Tse-Dinh YC. The role of the Zn(II) binding domain in the mechanism of E. coli DNA topoisomerase I. BMC Biochem. 2002; 3:13. [PubMed: 12052259]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

28. Zhu CX, Tse-Dinh YC. Overexpression and purification of bacterial DNA topoisomerase I. Methods Mol Biol. 1999; 94:145–51. [PubMed: 12844870]
29. Annamalai T, Dani N, Cheng B, Tse-Dinh YC. Analysis of DNA relaxation and cleavage activities of recombinant Mycobacterium tuberculosis DNA topoisomerase I from a new expression and purification protocol. BMC Biochem. 2009; 10:18. [PubMed: 19519900]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Highlights

- A network of interactions among DNA, metal ions, protein domains assessed in EcTopoI
- DNA prevents divalent metal ions coordination at non selective binding sites
- Protein C- and N- terminal domains functionally interact
- Localization of DNA-protein trapping sites is affected by the protein ZD domain.
- The EcTopoI functional interactions network is not fully shared among topoisomerases

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

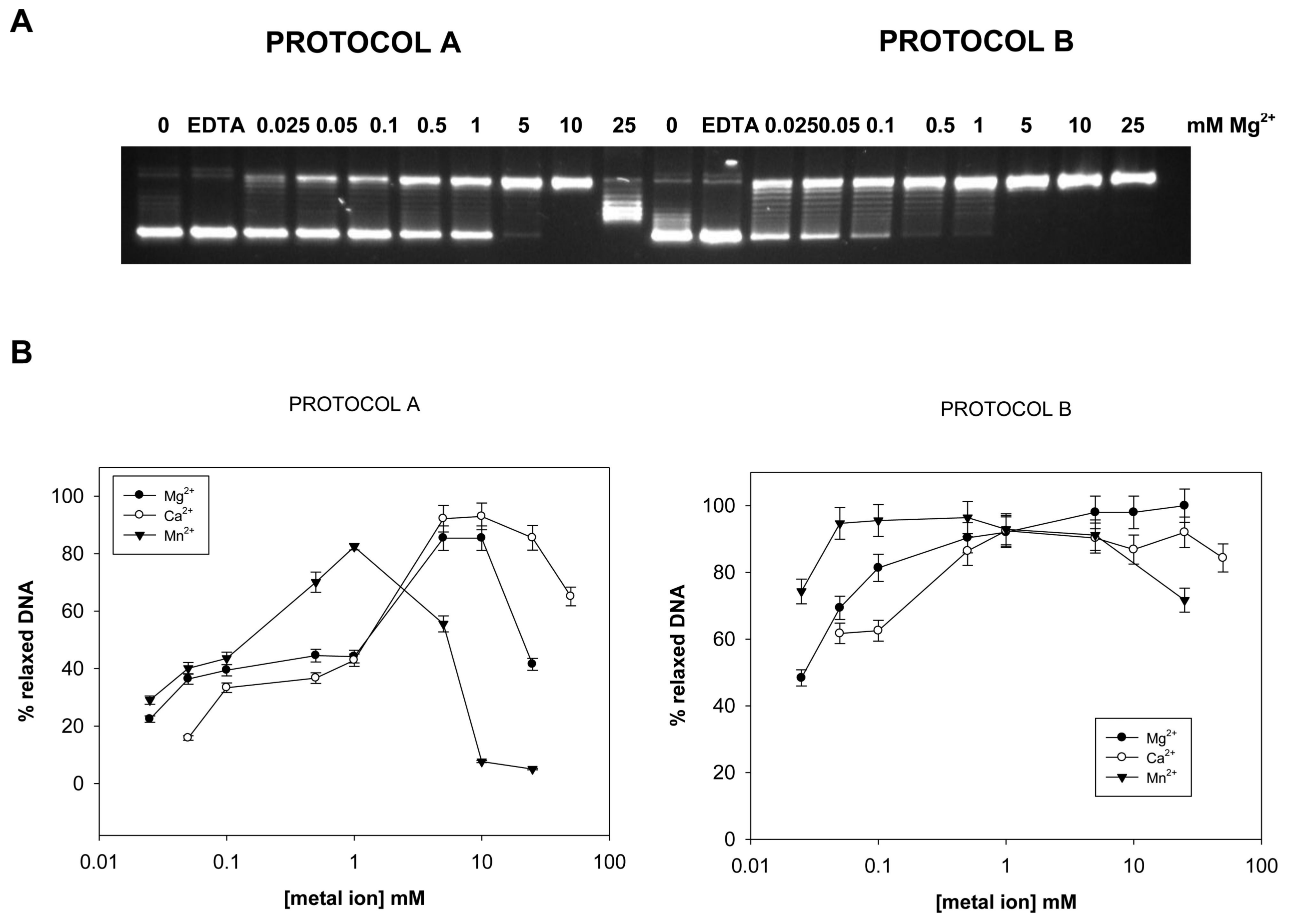

Fig. 1.

pBR 322 (0.15 μg) relaxation promoted by EcTopoI (0.7 nM) in the presence of variable metal ion concentration. In PANEL A, effect of increasing Mg^{2+} concentrations, according to two different protocols (see text). In PANEL B the percentage of relaxed DNA obtained after incubation of the nucleic acid with EcTopoI in the presence of increasing concentrations of metal ions according to two different protocols.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

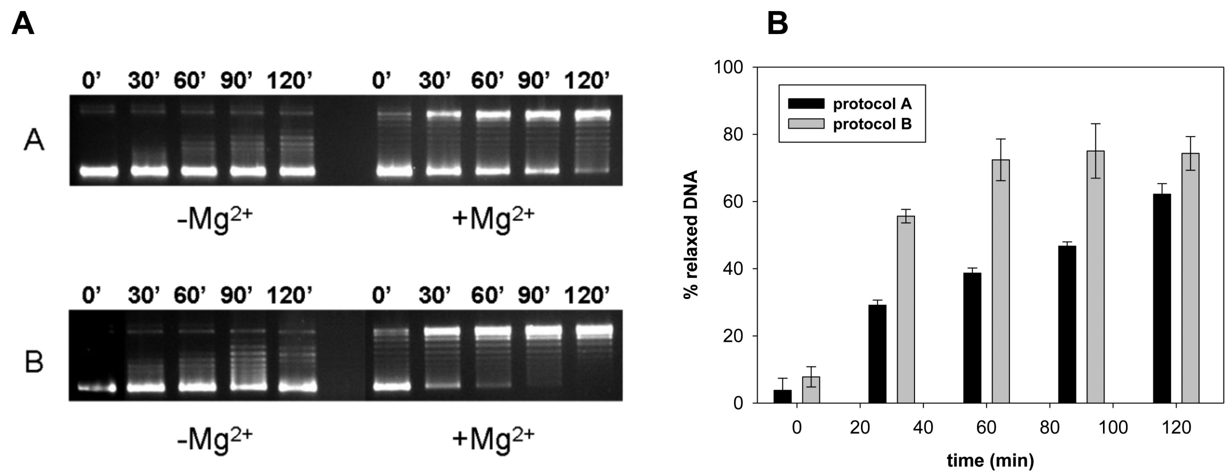

Fig. 2.

pBR 322 (0.15 μg) relaxation promoted by EcTopoI (0.7 nM) in the presence/absence of 10 mM Mg^{2+} after increasing incubation time, according to two different protocols (see text). In Panel A, resolution of reaction products by agarose gel is reported, in Panel B the percentage of relaxed DNA obtained in the presence of Mg^{2+} subtracted for the relaxation induced in the absence of metal ion are plotted as a function of incubation time.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

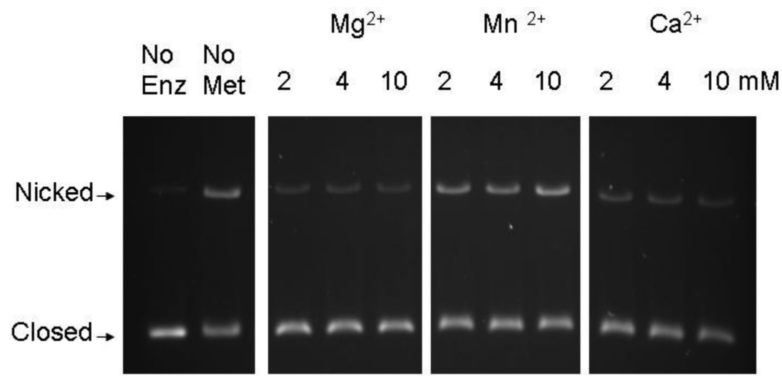

Fig. 3.

Effect of divalent ions on the level of nicked plasmid DNA resulting from cleavage of supercoiled plasmid DNA (0.3 μg) by EcTopoI (200 ng). The nicked DNA from the covalent cleavage intermediate was separated from the closed DNA by agarose gel electrophoresis in the presence of ethidium bromide.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

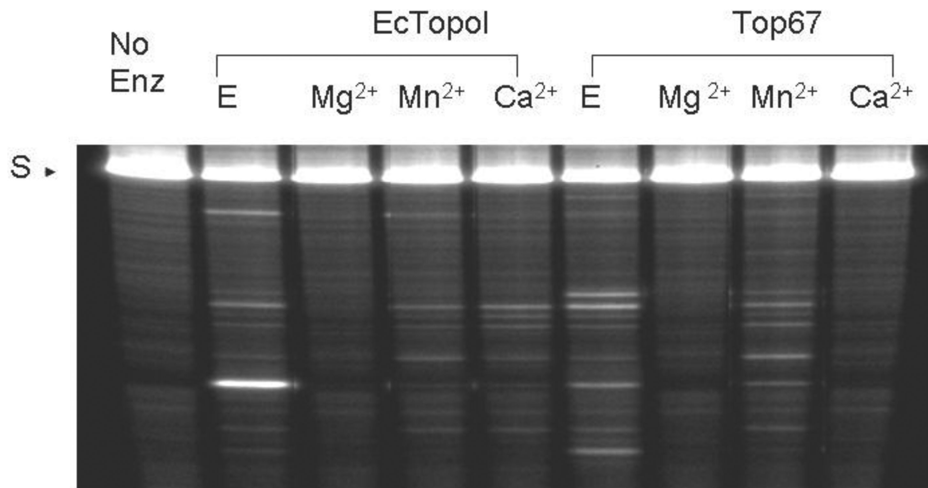

Fig. 4.

Effect of divalent ions on cleavage of single-stranded DNA by topoisomerase I. 5’-^{32}P labelled single-stranded DNA 216 base in length (S) was incubated with 200 ng of EcTopoI or Top67 in the presence of 1 mM EDTA (E) or different divalent ions at 4 mM concentration.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

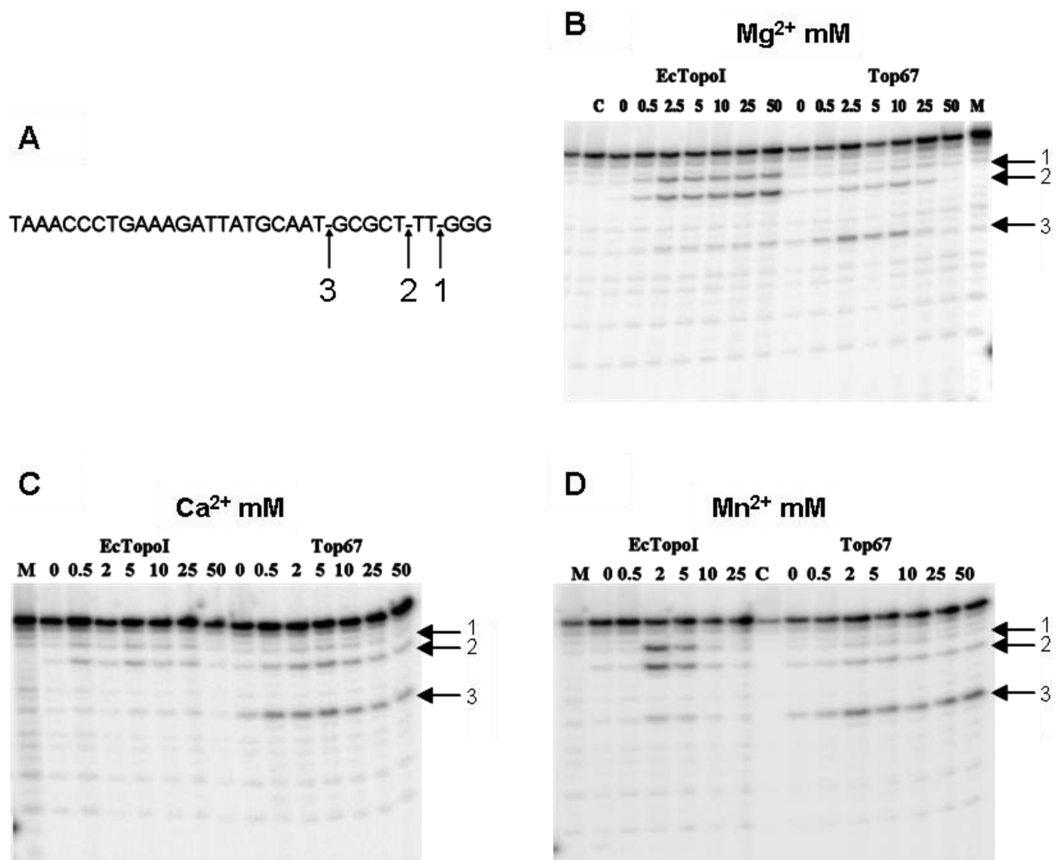

Fig. 5.

Main cleavage sites identified along a 32-residue sequence. The cleavage products produced on the same sequence after 30 min incubation at room temperature with EcTopoI and Top67 (1 μM), in the presence of increasing divalent metal ions concentrations (0-50 mM) in 20 mM Tris, pH 8.0, 100 μg/ml BSA, 12 % glycerol were resolved by DNA sequencing PAGE. Panel B, C and D refer to addition of Mg^{2+}, Ca^{2+} and Mn^{2+}, respectively. Lanes marked M and C refer to purine marker and DNA treated in the absence of the protein, respectively.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

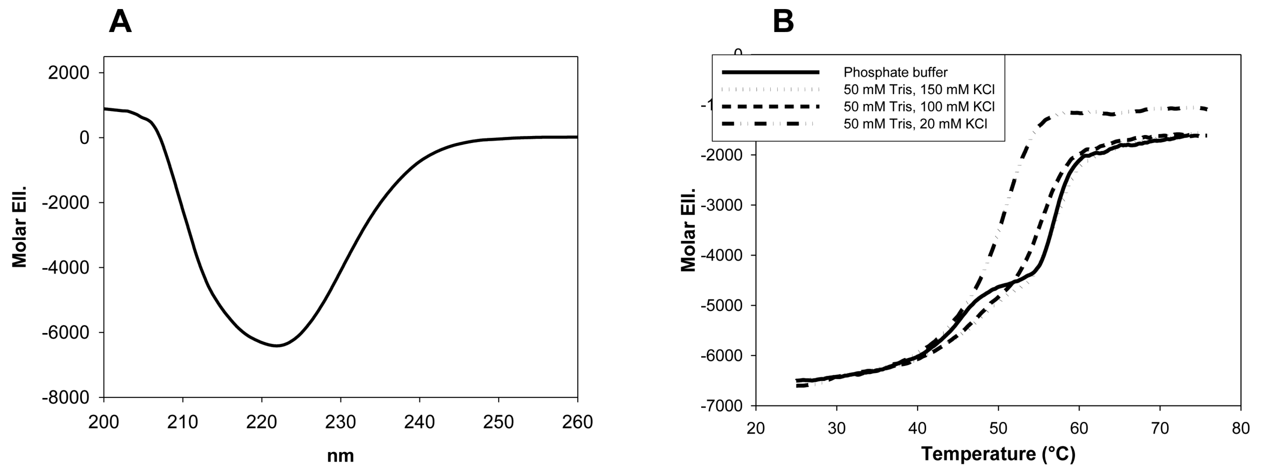

Fig. 6.
CD spectrum of EcTopoI (0.35 μM) recorded in 50 mM Tris, 20 mM KCl, pH 7.7 (PANEL A) and melting profiles acquired by reading the protein CD signal at 220 nm (PANEL B) in the reported buffers.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

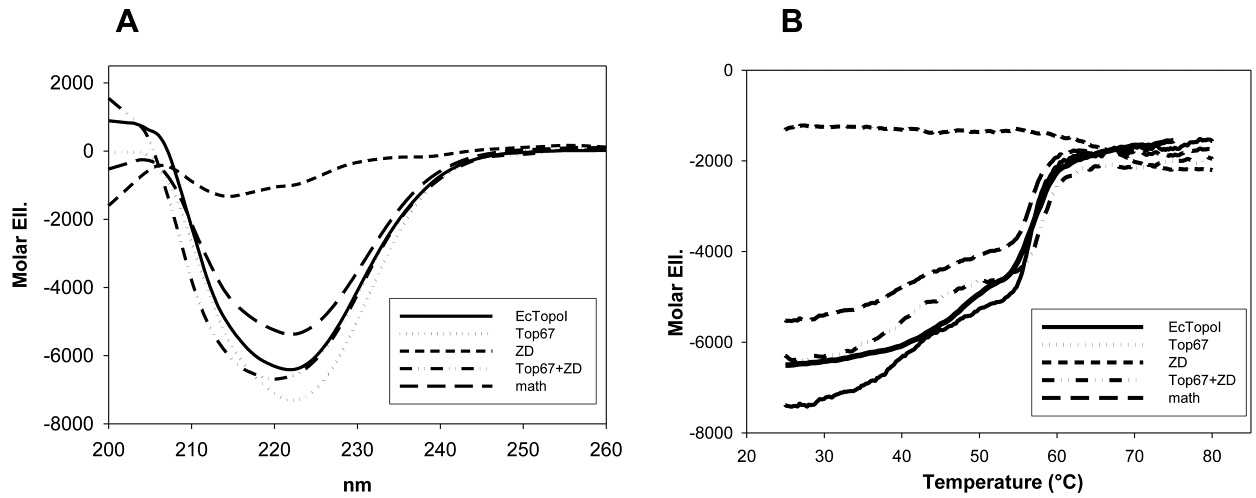

Fig. 7.

Dichroic spectra (PANEL A) and melting profiles (PANEL B) of EcTopoI and its N- and C-terminal domains (Top67 and ZD, respectively) recorded in 50 mM Tris, 150 mM KCl, pH 7.7. Protein concentration: 0.3 μM. Math refers to the algebraic combination of the Top67 and ZD domain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

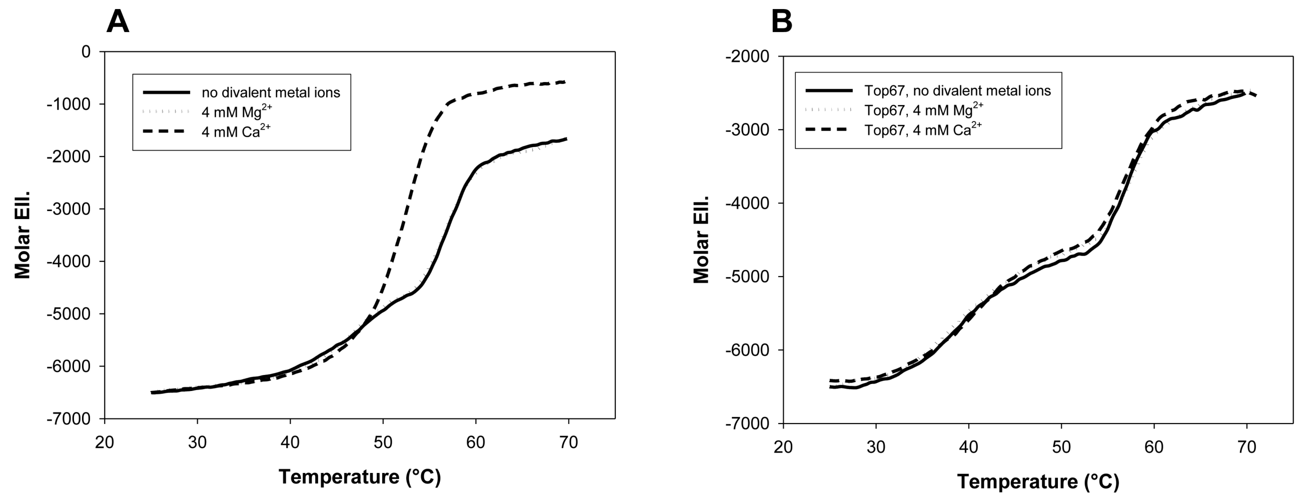

Fig. 8.

Melting profiles of 0.3 μM EcTopoI or Top67 (PANEL A and B, respectively) acquired by reading the protein CD signal at 220 nm in 50 mM Tris-HCl, 150 mM KCl, pH 7.7, in the presence/absence of 4 mM divalent metal ions. Protein concentration was 0.3 μM.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

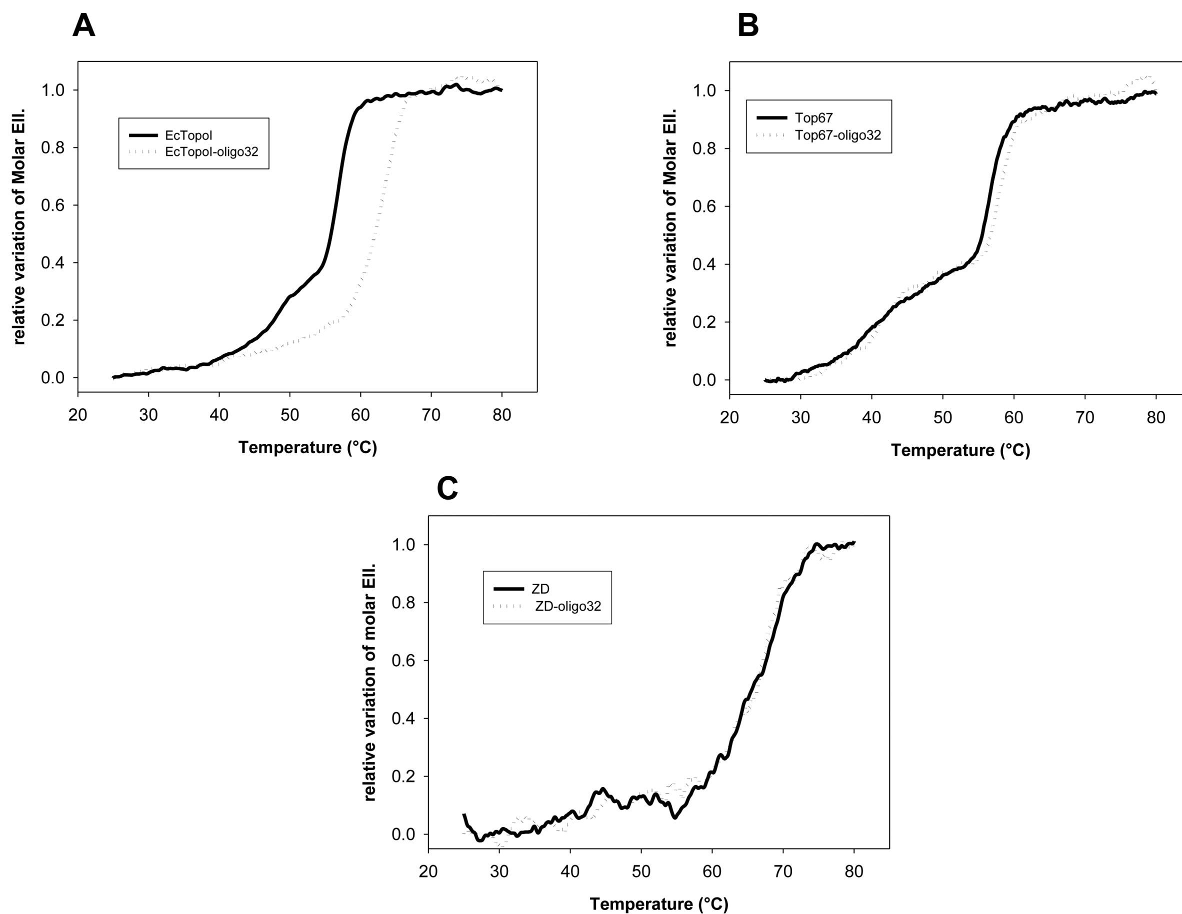

Fig. 9.

Melting profiles of EcTopoI (Panel A) and its N- and C-terminal domains (Panel B and C, respectively) acquired by reading the protein CD signal at 220 nm in the presence (dotted line) or absence (solid line) of oligo32 in 50 mM Tris, 150 mM KCl, pH 7.7. The relative Molar Ellipticity variation (Δ Molar Ellipticity /(Δ Molar Ellipticity)$_{max}$) is reported. Protein concentration: 0.3 μM, DNA concentration: 0.3 μM.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table I

Melting temperatures (Tm °C) for EcTopoI and its C- and N-terminal domains determined by recording the variation of the protein CD signal at 220 nm in 50 mM Tris, 150 mM KCl, pH 7.7. Data obtained in the presence or absence of stoichiometric amounts of oligo32 are included. Errors were ± 0.1 °C

	EcTopoI	Top67	ZD	Top67+ZD
50 mM Tris, 150 mM KCl, pH 7.7	48.3 and 56.7	39.7 and 56.7	68.8	40.6 and 58.1
+ 0.3 μM oligo32	63.7	42.0 and 58.0	68.7	nd
+ 4 mM Mg^{2+}	48.2 and 56.7	39.6 and 56.5	nd	nd
+ 4 mM Ca^{2+}	52.4	39.9 and 56.7	nd	nd