ATP independent type IB topoisomerase of *Leishmania donovani* is stimulated by ATP: an insight into the functional mechanism

Souvik Sengupta[1], Agneyo Ganguly[2], Amit Roy[1], Somdeb BoseDasgupta[3], Ilda D'Annessa[4], Alessandro Desideri[4] and Hemanta K. Majumder[1,*]

[1]Molecular Parasitology Laboratory, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India, [2]Biophysical Chemistry, [3]Infection Biology, Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056 Basel, Switzerland and [4]Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome 00133, Italy

ABSTRACT

Most type IB topoisomerases do not require ATP and Mg^{2+} for activity. However, as shown previously for vaccinia topoisomerase I, we demonstrate that ATP stimulates the relaxation activity of the unusual heterodimeric type IB topoisomerase from *Leishmania donovani* (LdTOP1L/S) in the absence of Mg^{2+}. The stimulation is independent of ATP hydrolysis but requires salt as a co-activator. ATP binds to LdTOP1L/S and increases its rate of strand rotation. Docking studies indicate that the amino acid residues His93, Tyr95, Arg188 and Arg190 of the large subunit may be involved in ATP binding. Site directed mutagenesis of these four residues individually to alanine and subsequent relaxation assays reveal that the R190A mutant topoisomerase is unable to exhibit ATP-mediated stimulation in the absence of Mg^{2+}. However, the ATP-independent relaxation activities of all the four mutant enzymes remain unaffected. Additionally, we provide evidence that ATP binds LdTOP1L/S and modulates the activity of the otherwise ATP-independent enzyme. This study establishes ATP as an activator of LdTOP1L/S in the absence of Mg^{2+}.

INTRODUCTION

DNA topoisomerases are ubiquitious enzymes that solve the topological problems associated with DNA replication, transcription, recombination and chromatin remodeling by introducing temporary single- or double-strand breaks in the DNA (1). They are broadly classified into two types, type I and type II. Type I topoisomerases act by making transient single-stranded breaks in the DNA and allow strand passage (type IA) or controlled rotation (type IB) across the nick. In contrast, type II topoisomerases act by making transient double-stranded breaks in DNA and pass a separate double-stranded molecule through the break (2). The two types are further divided into four subfamilies: IA, IB, IIA and IIB (3). Type IB DNA topoisomerases relax DNA supercoils via a reaction pathway entailing non-covalent binding of the enzyme to the duplex DNA, cleavage of one DNA strand with the formation of a covalent DNA-(3′-phosphotyrosyl)-protein intermediate thereby creating a single stranded nick, strand rotation or swiveling of another strand across the nick by a 'controlled rotation' mechanism and strand religation (1,4,5). In all the instances, strand cleavage is accompanied by the formation of a covalent intermediate (6).

Eukaryotic type I enzymes display no requirement for a divalent cation or ATP for their catalytic activity. However, their activity, measured by relaxation of supercoiled DNA, can be stimulated by Mg^{2+} (1). Although none of the eukaryotic type I enzymes require a nucleotide cofactor to relax supercoiled DNA, there has been varying reports in the literature regarding the effect of ATP on the activity of topoisomerase I from different sources. Sekiguchi and Shuman (7) have reported stimulatory effect of ATP on vaccinia topoisomerase I; whereas Chen and Hwang (8) have reported an inhibitory effect of ATP on human topoisomerase I relaxation activity.

The possibility of ATP being able to regulate the activity of eukaryotic type I enzymes, either positively or

---

negatively, has led us to revisit this issue using the unusual topoisomerase IB from *Leishmania donovani* (LdTOP1L/S) as the model enzyme. Topoisomerase I in these parasites is a heterodimeric protein consisting of a large subunit and a small subunit. The two subunits are encoded by two distinct genes. Our group has previously reported the *in vitro* reconstitution of the bi-subunit topoisomerase I from *L. donovani* (9). The large subunit (LdTOP1L) consists of 635 amino acids (73 kDa) and contains the DNA binding motif, whereas the small subunit (LdTOP1S) consists of 262 amino acids (29 kDa) and harbors the catalytic tyrosine residue. Each subunit, by itself is catalytically inactive (9,10). However, a catalytically active heterodimeric protein in which the two subunits are present at a molar ratio of 1:1 was reconstituted (9,11). However, despite unusual subunit structure, the *L. donovani* enzyme is functionally similar to other eukaryotic type IB topoisomerases (9,12).

In this article, we have investigated the effect of ATP on the relaxation activity of LdTOP1L/S *in vitro*. In agreement with the results previously published for Vaccinia topoisomerase I (7), we show that ATP stimulates the topoisomerase IB activity of the parasite. This stimulatory effect is seen only in the absence of Mg^{2+}. We provide evidence that ATP affects the strand rotation rate of LdTOP1L/S and also decreases its DNA binding capacity. Using a fluorescent analog of ATP (TNP-ATP), we confirm the binding of ATP to LdTOP1L/S. Docking studies and subsequent experimental validation of the results have identified one of the amino acid residue crucial for ATP binding to LdTOP1L/S. Based on these observations, the possibility of subtle changes occurring in the enzyme activity of the *Leishmania* topoisomerase IB is discussed.

MATERIALS AND METHODS

Chemicals

ATP, Adenosine 5'-(β,γ–imido) triphosphate tetralithium salt hydrate (ADPNP) and Agarose were purchased from Sigma Chemicals (St Louis, MO, USA). 2'-(or-3')-O-(trinitrophenyl)-adenosine 5'-triphosphate (TNP-ATP) was purchased from Molecular Probes (Eugene, OR, USA). Unless otherwise mentioned, all other routinely used chemicals were purchased from Sigma Chemicals.

Cloning and site-directed mutagenesis

The mutants H93A, Y95A, R188A and R190A were generated from pET16bLdTOP1L as the template DNA using Stratagene Quick change XL kit and the appropriate primers according to the manufacturer's protocol. Bacterial colonies were selected for mutants, DNA samples prepared from the mutant bacterial colonies were screened by sequencing and then transformed into

Escherichia coli BL21 (DE3) pLysS cells for expression and purification of proteins.

Overexpression, purification and reconstitution of recombinant proteins

All the procedures were performed as described previously (9). Briefly, *E. coli* BL21 (DE3) pLysS cells harboring pET16bLdTOP1L, pET16bLdTOP1L-H93A, pET16bLd TOP1L-Y95A, pET16bLdTOP1L-R188A, pET16bLd TOP1L-R190A, pET16bLdTOP1S, pET16bLdTOP1 Δ33S and pET16bLdTOP1Δ140S were separately induced at $A_{600} = 0.6$ with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) for 12 h at 22°C (9). Cells harvested from 1 l culture were lysed separately by lysozyme/sonication and each mutant protein was purified through Ni–NTA (Ni^{2+}–nitrilotriacetate)–agarose columns (Qiagen) followed by a phosphocellulose (P11 cellulose; Whatman) chromatography as described previously (9). The purified proteins LdTOP1L, LdTOP1L-H93A, LdTOP1L-Y95A, LdTOP1L-R188A, LdTOP1L-R190A, LdTOP1S, LdTOP1Δ33S and LdTOP1Δ140S were stored at −70°C. Purified wild-type and the mutant LdTOP1L subunits were each mixed with purified small subunit (LdTOP1S) and deletion constructs of LdTOP1S separately at a molar ratio of 1:1 at a total protein concentration of 0.5 mg/ml in a reconstitution buffer (50 mM potassium phosphate, pH 7.5, 0.5 mM DTT (dithiothreitol), 1 mM EDTA, 0.1 mM PMSF and 10% (v/v) glycerol). The mixtures were dialyzed overnight at 4°C and the dialyzed fractions were used for the plasmid relaxation activity (9,13).

Plasmid relaxation assay

The type I DNA topoisomerase was assayed by measuring the decreased electrophoretic mobility of the relaxed isomers of supercoiled pBS (SK+) [pBluescript (SK+)] DNA in an agarose gel as described (9,14) with the exception that 10 mM $MgCl_2$ was not included in the relaxation assay buffer, unless stated otherwise. Standard reaction mixtures (25 μl) containing 25 mM Tris–HCl, pH 7.5, 50 mM KCl, 5% glycerol, 0.5 mM DTT, 2.5 mM EDTA and 150 μg/ml bovine serum albumin (BSA), purified wild-type (LdTOP1L/S) or the mutant enzymes, supercoiled pBS (SK+) DNA (85–95% of which were negatively supercoiled, while the remaining fractions being nicked circles) and other components as indicated were incubated at 37°C for 30 min. The kinetics of relaxation assay were performed under different experimental conditions, as indicated and the reactions stopped at the indicated time by adding a solution containing glycerol, bromophenol blue, xylene cyanol and 0.5% SDS. For the non-turnover relaxation experiment, 30-fold molar excess of LdTOP1L/S was used over supercoiled pBS (SK+) DNA and the reactions were stopped at the indicated time.

For quantitative estimation of the effect of different concentrations of KCl on plasmid relaxation assay, the

percentage of relaxation was calculated using the following equation:

$$\% \text{ of Relaxation} = \frac{(\text{Int}_{\text{initial}} - \text{Int}_{\text{final}})}{\text{Int}_{\text{initial}}} \times 100 \qquad (1)$$

where $\text{Int}_{\text{initial}}$ is the area under the supercoiled DNA band in the absence of enzyme and $\text{Int}_{\text{final}}$ is the area under the supercoiled DNA in the presence of enzyme.

For estimation of the velocity of reaction, the amount of supercoiled monomer DNA band fluorescence after EtBr (ethidium bromide; 0.5 µg/ml) staining was quantified by integration using Gel Doc 2000 under UV illumination (Bio-Rad Quality One software), as described previously (9). Initial velocities (nM of DNA base pairs relaxed/min) were calculated using the equation:

$$V_{\text{i}} = \frac{\{([\text{Supercoiled DNA}]_0) - (\text{Int}_{\text{t}}/\text{Int}_0 \times [\text{Supercoiled DNA}]_0)\}}{t} \qquad (2)$$

where V_{i} is the initial velocity, $[\text{Supercoiled DNA}]_0$ is the initial concentration of the supercoiled DNA, Int_0 is the area under the supercoiled band at time zero and Int_{t} is the area at reaction time t (15). The effect of DNA concentration on the kinetics of relaxation was examined over the range of 4–40 nM supercoiled pBS (SK+) DNA at a constant concentration of 0.3 nM enzyme (LdTOP1L/S) at 37°C for 1 min. The respective initial velocities were fitted to the Michaelis–Menten Equation by non-linear regression using GraphPad Prism version 5 for Windows and the respective values of V_{max} and turnover number were obtained.

Analysis of LdTOP1L/S-DNA binding by gel mobility shift assay

The end labeling of the 25-mer duplex oligonucleotides and subsequent annealing were carried out as described (13). A DNA binding assay was performed by incubating the labeled oligonucleotide 1/oligonucleotide 2 in 25 µl reaction mixtures containing 25 mM Tris–HCl pH 7.5, 50 mM KCl, 2 nM labeled duplex 25-mer, 1 nM LdTOP1L/S and increasing concentrations of ATP at 15°C for 15 min. The samples were then electrophoresed in a 6% non-denaturing polyacrylamide gel in 0.167×TBE buffer (45 mM Tris–borate, 1 mM EDTA) at 4°C. Due to the high pI values for the reconstituted topoisomerase I proteins (>9.0), free protein and protein–DNA complexes migrated to the cathode and therefore only the free oligonucleotides entered the gel (13). The unbound oligonucleotides in the gel were quantified following autoradiography by film densitometry and the percentage of bound DNA was calculated using the following equation:

$$\text{DNA Bound } (\%) = \frac{(\text{Int}_{\text{ctrl}} - \text{Int}_{\text{expt}})}{\text{Int}_{\text{ctrl}}} \times 100 \qquad (3)$$

where Int_{ctrl} is the intensity of control unbound DNA and Int_{expt} is the intensity of experimental unbound DNA.

Fluorescence binding assay

Fluorescence emission scan of each protein was performed in a PerkinElmer LS55 luminescence spectrometer. Wild-type or the mutant protein (1 µM concentration each) was allowed to react with 10 µM of TNP-ATP in 50 mM Tris–HCl pH 7.5. The samples were excited at λ_{ex} of 403 nm (16) and λ_{em} was scanned in the range of 500–600 nm. Excitation and emission slit widths were 5 and 10 nm, respectively. An excess of ATP (10 mM) was then added to each set to displace the bound TNP-ATP from the enzyme and the emission spectra were then recorded. The spectra of the protein (1 µM) and TNP-ATP (10 µM) alone in 50 mM Tris–HCl pH 7.5 were also recorded.

Molecular docking procedure

Two different sets of docking experiments were carried out, one between the large subunit of LdTOP1L/S and ATP and the other one between the LdTOP1L/S dimer and ATP, with the Autodock 4 program, using the AutodockTools suite v. 4 to prepare the structures of the ligand and the receptor (17). The three-dimensional coordinates of the LdTop1L/S receptor have been taken from the PDB structure 2B9S (18), where residues 27–456 and 221–262 of the large and small subunits, respectively, are present. Residues 427–430, missing in the X-ray structures, were reconstructed with the program Swiss-PdbViewer v. 4.0.1 (19) and regularized to avoid clashes using the GROMOS force field implemented in the program. This program was also used to eliminate the DNA molecule present in the crystal structure in order to perform the docking experiments. In both docking experiments, the protein has been immersed in a simulative cubic box (48×48×48 Å) that contains the whole protein structure and 250 docking runs for each system have been performed using the Lamarckian Genetic Algorithm (20). A modified version of the program g_mindist from the Gromacs 3.3.3 package (21) has been used to calculate the protein–ligand contacts in all the 250 + 250 resulting complexes, using a threshold value of 3.5 Å. Images have been produced with the VMD visualization package (22).

RESULTS

Effect of Mg^{2+} and ATP on the supercoiled DNA relaxation activity catalyzed by *Leishmania* topoisomerase IB (LdTOP1L/S)

Although type IB topoisomerases are not ATP requiring enzymes, Sekiguchi and Shuman (7) have reported that ATP can stimulate the DNA relaxation activity of the vaccinia virus topoisomerase I. In contrast, the human enzyme has been shown to be inhibited by ATP (8). Since topoisomerase IB from *L. donovani* (LdTOP1L/S) is a novel heterodimeric protein, we examined the effect of ATP on the DNA relaxation activity of this novel enzyme by measuring the conversion of supercoiled plasmid DNA to relaxed closed circular forms under standard *in vitro* assay conditions using the bacterially expressed recombinant protein (Figure 1). In agreement
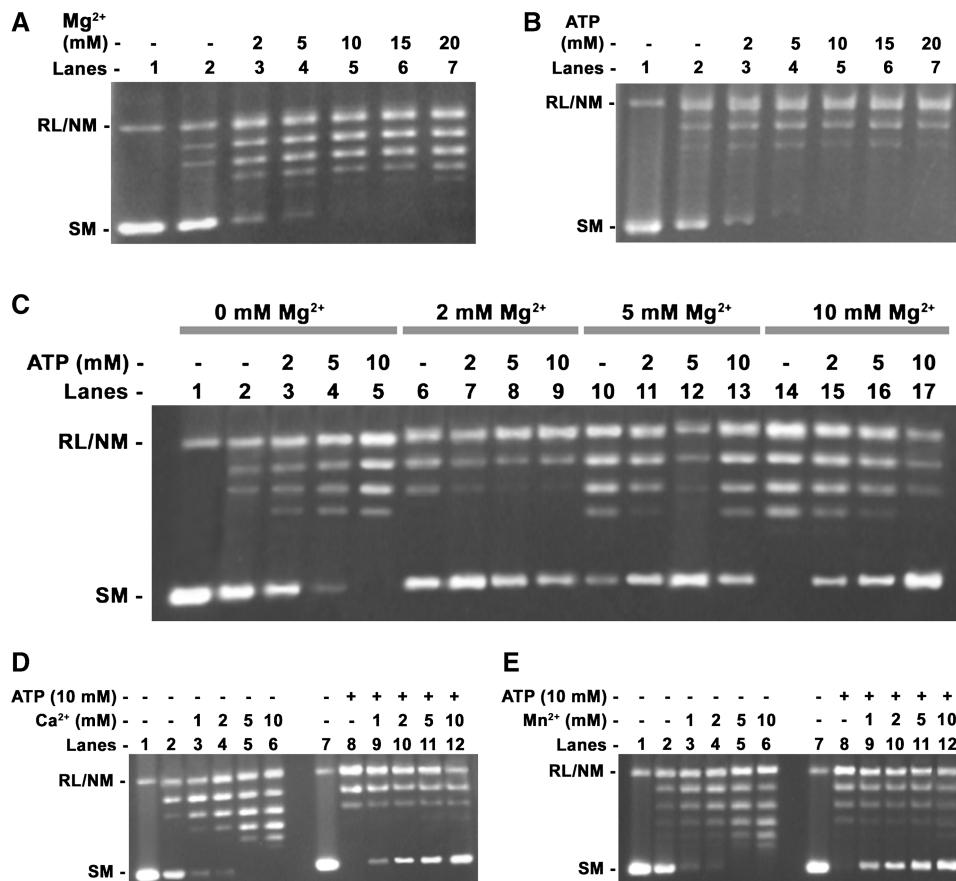
Figure 1. Plasmid relaxation assay. Relaxation of supercoiled pBS (SK+) DNA with enzyme LdTOP1L/S at a molar ratio of 3:1 in the presence of increasing concentrations of Mg^{2+} (**A**) and increasing concentrations of ATP (**B**). Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lane 2, same as lane 1 but incubated with 40 fmol of enzyme at 37°C for 30 min; lanes 3–7, same as lane 2 but in the presence of 2, 5, 10, 15 and 20 mM Mg^{2+} or ATP (A and B), respectively. (**C**) Differential effect of Mg^{2+} and ATP. Relaxation of supercoiled pBS (SK+) DNA with enzyme LdTOP1L/S at a molar ratio of 3:1 in the presence or absence of indicated concentrations of Mg^{2+} and ATP. Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lane 2, same as lane 1 but incubated with 40 fmol of enzyme at 37°C for 30 min; lanes 3–17, same as lane 2 but in the presence of indicated concentrations of ATP and Mg^{2+}; lanes 6, 10 and 14 did not contain any ATP but contained indicated concentrations of Mg^{2+}. (**D**) and (**E**) Relaxation of supercoiled pBS (SK+) DNA with enzyme LdTOP1L/S at a molar ratio of 3:1 in the presence or absence of indicated concentrations of Ca^{2+} and ATP (D) and Mn^{2+} and ATP (E). Reactions were stopped by the addition of 0.5% SDS and samples were electrophoresed on 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RL/NM) are indicated.

with the results previously reported from this laboratory (9,23), in the absence of Mg^{2+}, LdTOP1L/S-mediated relaxation of supercoiled DNA occurred with reduced efficiency (Figure 1A, lane 2). Addition of increasing concentrations of Mg^{2+} stimulated the reaction considerably (Figure 1A, lanes 3–7). However, in the absence of Mg^{2+}, addition of ATP to the reaction mixtures also caused marked stimulation of DNA relaxation activity in a concentration-dependent manner (Figure 1B, lanes 3–7). In contrast, when the reaction mixtures contained both Mg^{2+} and ATP, the stimulation of relaxation activity observed when ATP or Mg^{2+} was added individually to reactions was abolished (Figure 1C). At equimolar concentration of Mg^{2+} and ATP in the reaction mixture, the extent of relaxation of supercoiled plasmid DNA was the same as that observed when both Mg^{2+} and ATP were absent (Figure 1C, compare lane 2 with lanes 7, 12 and 17). It has been suggested (7,24) that simultaneous presence of both Mg^{2+} and ATP in reaction mixtures may lead to Mg^{2+}-ATP complex formation in solution and thus rendering free Mg^{2+} and free ATP unavailable

to participate in the enzymatic reactions. In support of this postulate, we observed that addition of EDTA to the relaxation assay containing both Mg^{2+} and ATP restored ATP-mediated stimulation of the supercoiled DNA relaxation activity (data not shown).

Effect of other divalent cations

Other divalent cations, e.g. Ca^{2+} or Mn^{2+} can substitute for Mg^{2+} in the plasmid relaxation assay catalyzed by LdTOP1L/S (Figure 1D and E). Complete relaxation occurred at 5 mM concentration of each. Addition of 10 mM ATP to the reactions caused inhibition of the LdTOP1L/S activity (Figure 1D and E, lanes 9–12) similar to that observed with Mg^{2+} and ATP in the reactions.

Dependence on KCl

The effect of KCl on Mg^{2+} and ATP-induced stimulation of topoisomerase activity was also investigated (Figure 2A). In the absence of Mg^{2+} or ATP, the
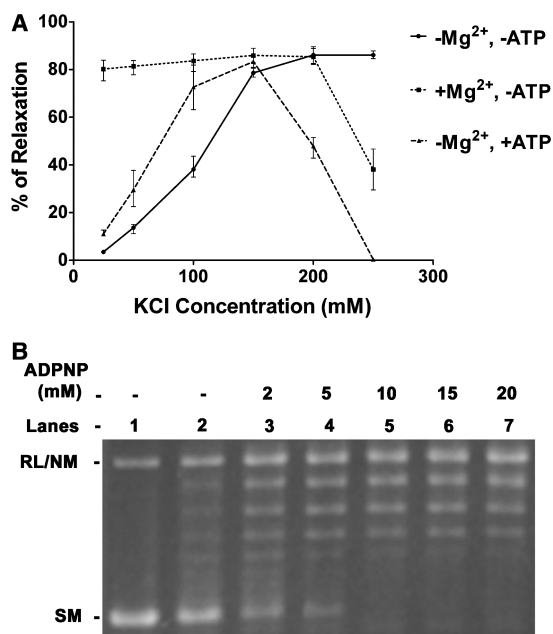
Figure 2. (A) Quantitative representation of the effect of varying concentration of KCl on relaxation activity under different conditions. Plasmid relaxation assay was carried out as described previously with 25, 50, 100, 150, 200 and 250 mM of KCl in the assay buffer, respectively, under different conditions [absence of Mg^{2+} and ATP (closed circles), presence of 10 mM Mg^{2+} only (closed squares) and presence of 10 mM ATP only (closed triangles)]. The percentage of relaxation under each condition was calculated by quantifying the left over supercoiled band as described in 'Materials and Methods' section. The percentage of relaxation is plotted as a function of KCl concentration as indicated. Data presented are mean ± S.E. ($n = 3$). (B) Relaxation of supercoiled pBS (SK+) DNA with enzyme LdTOP1L/S at a molar ratio of 3:1 in the presence of increasing concentrations of ADPNP in the absence of Mg^{2+}. Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lane 2, same as lane 1 but incubated with 40 fmol of enzyme at 37°C for 30 min; lanes 3–7, same as lane 2 but in the presence of 2, 5, 10, 15 and 20 mM of ADPNP, respectively. Reactions were stopped and electrophoresed as described above. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RL/NM) are indicated.

optimal KCl concentration for DNA relaxation activity mediated by *Leishmania* topoisomerase IB was between 200 and 250 mM KCl. Similar observation was also reported by Stewart *et al.* (24). When the relaxation assays were carried out in the presence of 10 mM ATP over a range of KCl concentrations (0–250 mM), ATP showed its maximal stimulatory activity at about 100 mM KCl (the extent of relaxation was ~73% in presence of 10 mM ATP while it was only 38% in its absence). Higher concentrations of KCl were inhibitory (Figure 2A). In contrast, when the topoisomerase reaction contained 10 mM Mg^{2+}, the maximum stimulatory effect was achieved at only 25 mM KCl (80% relaxation in presence of 10 mM Mg^{2+} and 25 mM KCl *vis a vis* 4% relaxation in presence of 25 mM KCl alone). The relaxation activity remained unaltered between 25 and 200 mM KCl concentration (Figure 2A). At 250 mM KCl, however, the extent of relaxation dropped to 38%. These observations are in agreement with those reported by Stewart *et al.* (24).

Stimulation of LdTOP1L/S activity is independent of ATP hydrolysis

The stimulation of DNA relaxation activity by ATP does not involve hydrolysis of ATP since a non-hydrolyzable ATP analog ADPNP [Adenosine 5'-(β, γ-imido) triphosphate tetralithium salt hydrate] was able to substitute for ATP in stimulating the relaxation activity in the absence of Mg^{2+} (Figure 2B).

Kinetics of relaxation under different conditions

To obtain a clearer view of the stimulation or inhibition of relaxation activity by ATP and the role of KCl therein, a time course relaxation assay was performed under different experimental conditions. The rate of DNA relaxation by LdTOP1L/S was stimulated in the presence of 50 mM KCl (Figure 3A) or 10 mM Mg^{2+} (Figure 3B). Mg^{2+} alone was more potent than KCl in stimulating relaxation activity of LdTOP1L/S (compare Figure 3A and B). Maximal activation was achieved by a combination of KCl and Mg^{2+} (Figure 3C). However, unlike KCl and Mg^{2+}, 10 mM ATP alone was unable to stimulate LdTOP1L/S activity (Figure 3D). ATP-mediated stimulation of relaxation activity was completely dependent on the presence of KCl in the reaction mixture (Figure 3E). In the presence of 10 mM Mg^{2+}, 10 mM ATP was unable to show its stimulatory effect (Figure 3F) probably due to Mg^{2+}-ATP complex formation. Addition of 50 mM KCl to reactions containing 10 mM Mg^{2+} and 10 mM ATP restored LdTOP1L/S activity (Figure 3G) to the extent similar to the activity at 50 mM KCl alone (Figure 3A). For comparison, a time course assay was performed without added cofactors (Figure 3H).

These observations suggest that ATP-mediated stimulation of LdTOP1L/S activity is KCl dependent. Mg^{2+} alone is capable of stimulating LdTOP1L/S activity but ATP alone cannot do so. KCl alone can also stimulate LdTOP1L/S activity to a lesser extent, but KCl and Mg^{2+} together cause maximal stimulation. Taken together, these observations suggest that the mechanism of ATP activation is distinct from that exhibited by KCl and Mg^{2+}.

To understand the extent of stimulation separately by ATP and Mg^{2+}, we examined their effect on the velocity of LdTOP1L/S mediated relaxation reaction. The kinetics of relaxation by LdTOP1L/S under different conditions was examined over a range of supercoiled pBS (SK+) DNA (4–40 nM) and the respective initial velocities were fitted to the Michelis–Menten Equation by non-linear regression using GraphPad Prism version 5 for Windows (Figure 3I). Actual velocity data used for fitting of the curves have been given in Supplementary Table 1. The enzyme: DNA ratio was kept within the steady-state assumption. The maximal velocity (V_{max}) for LdTOP1L/S in the absence of both Mg^{2+} and ATP was 0.7167×10^{-9} M base pairs of supercoiled DNA relaxed/min/0.3 nM of enzyme that corresponds to a turnover number of about two plasmid molecules relaxed/min/molecule of enzyme. However, V_{max} for LdTOP1L/S in presence of Mg^{2+} only was 19.37×10^{-9} M base pairs of supercoiled DNA relaxed/min/0.3 nM of enzyme that corresponds to a
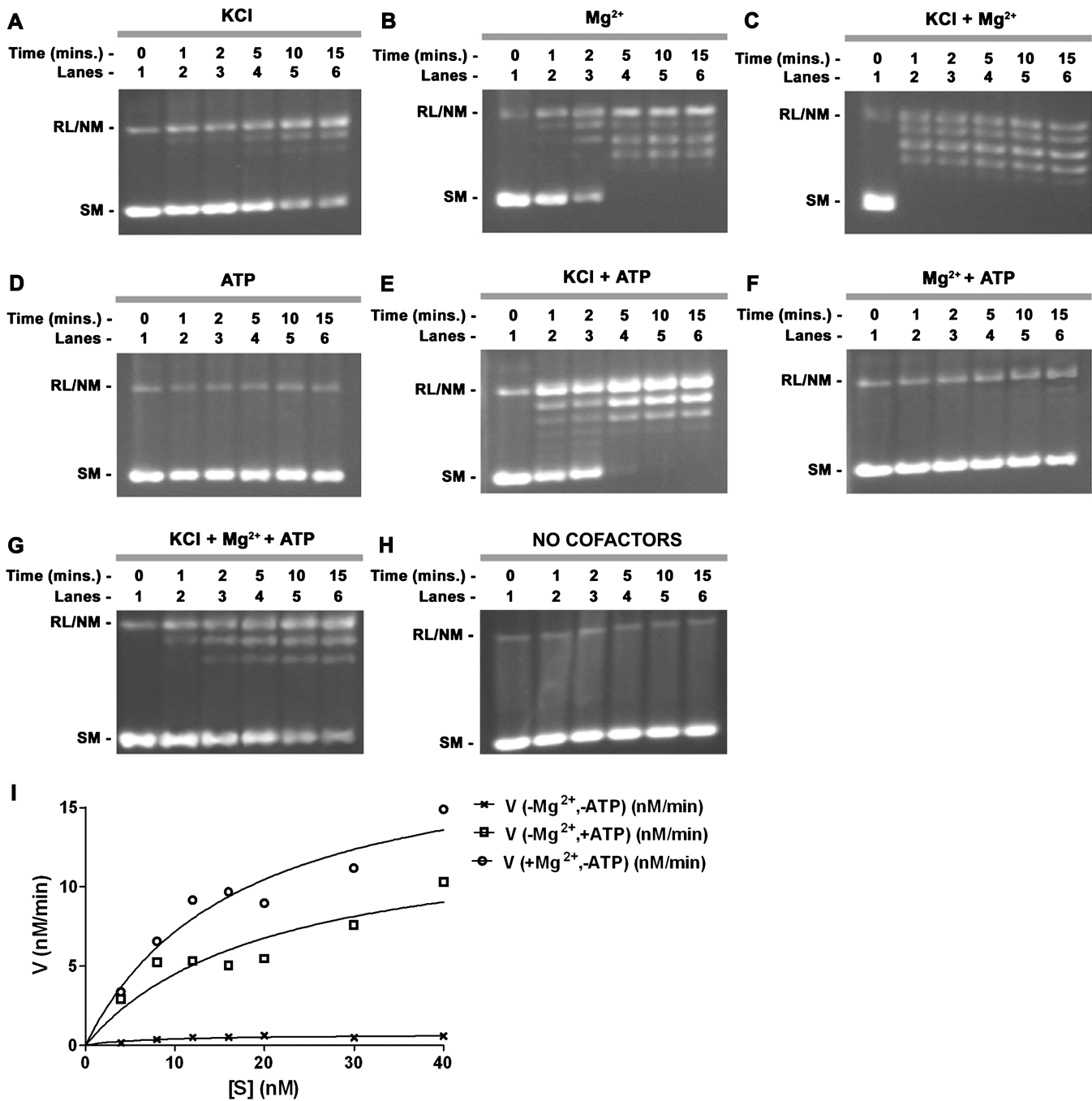
Figure 3. Kinetics of relaxation under different conditions. Relaxation of supercoiled pBS (SK+) DNA with enzyme LdTOP1L/S at a molar ratio of 3:1 in the presence of 50 mM KCl (**A**), 10 mM Mg^{2+} (**B**), 50 mM KCl and 10 mM Mg^{2+} (**C**), 10 mM ATP (**D**), 50 mM KCl and 10 mM ATP (**E**), 10 mM Mg^{2+} and 10 mM ATP (**F**), 50 mM KCl, 10 mM Mg^{2+} and 10 mM ATP (**G**) and in the absence of any cofactor (**H**). Lane 1, 120 fmol of supercoiled pBS (SK+) DNA. Lanes 2–6, same as lane 1 but incubated with 40 fmol of enzyme at 37°C for different time periods under different conditions as indicated in the figure. All reactions were stopped by the addition of 0.5% SDS at the indicated time points, and samples were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. Analysis of velocity of reaction (**I**). Relaxation of supercoiled pBS (SK+) DNA with LdTOP1L/S. DNA concentrations ranged from 4 to 40 nM, Mg^{2+} or ATP when included were at 10 mM each and enzyme was at 0.3 nM. The respective initial velocities were fitted to the Michelis–Menten Equation by non-linear regression using GraphPad Prism version 5 for Windows and the respective values of V_{max} and turnover number were obtained.

turnover number of about 65 plasmid molecules relaxed/min/molecule of enzyme. In contrast, the V_{max} for LdTOP1L/S in the presence of ATP alone was 13.61×10^{-9} M base pairs of supercoiled DNA relaxed/min/0.3 nM of enzyme that corresponds to a turnover

number of about 45 plasmid molecules relaxed/min/molecule of enzyme.

These results confirm that ATP does have a role in stimulating the relaxation activity of LdTOP1L/S in the absence of Mg^{2+}. Also, ATP does not affect the

interaction between the large and the small subunit of the enzyme (Supplementary Figure S1).

Assessment of the strand rotation event

The turnover number of LdTOP1L/S in the presence of ATP is high compared with that in the absence of Mg^{2+} and ATP. The difference in the catalytic activity can be at the initial cleavage step just after binding to the substrate or during strand rotation. ATP has no effect on the initial cleavage step (Supplementary Figure S2). Therefore, we have studied the effect of ATP on the strand rotation rate of LdTOP1L/S. To address this issue, a relaxation assay was performed as described (12,25). The reaction mixtures contained 30-fold molar excess of LdTOP1L/S over supercoiled plasmid molecules in the absence or in the presence of 10 mM ATP. The excess enzyme eliminated the need for enzyme turnover and dissociation during the reaction. Moreover, the DNA substrate pBS (SK+) used in the assay has a size of 2.9 kb, which corresponds to roughly 14 negative supercoils per DNA molecule. Thus, 30-fold molar excess of the enzyme is used to achieve conditions in which the reaction rates are independent of the association or dissociation rates. It was found that in the absence of ATP, relaxed intermediates start appearing from 10 s of incubation and complete relaxation under this condition is achieved after 300 s of incubation (Figure 4A). On the other hand, in the presence of 10 mM ATP, relaxed intermediates start appearing from 5 s of incubation and complete relaxation is achieved after 30 s of incubation (Figure 4B). The result indicates faster completion of catalytic cycle of

Figure 4. Relaxation assay under condition of enzyme excess. The relaxation of supercoiled pBS (SK+) DNA was carried out with LdTOP1L/S in the absence (**A**) and in the presence (**B**) of 10 mM ATP. Relaxation reactions were carried out from 0 to 600 s (lanes 1–10) as indicated in the presence or absence of ATP using 150 nM enzyme and 5 nM DNA. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RL/NM) are indicated.

LdTOP1L/S in the presence of ATP compared with LdTOP1L/S alone. The same assay was also run in a gel containing 3 mg/l chloroquine (Supplementary Figure S4). We observed a more rapid appearance of the relaxed band in the presence of ATP (Supplementary Figure S4, compare panel A with B). Moreover, assuming the possibility of multiple strand rotations for each cleavage event, as suggested by the controlled strand rotation model, the rate of strand rotation is likely to be rate-limiting for catalysis under these conditions. Taken together, the faster relaxation rate of LdTOP1L/S in the presence of ATP compared with LdTOP1L/S alone can best be explained by a faster rate of strand rotation.

Effect of ATP on DNA binding by LdTOP1L/S

To test whether the observed changes in the relaxation activity in the presence or absence of ATP affect the DNA binding capacity of LdTOP1L/S, native gel mobility shift assays were performed with reconstituted LdTOP1L/S complexed with the $5'-^{32}P$-labeled duplex oligomer containing the high-affinity topoisomerase IB binding site (26). LdTOP1L/S is a positively charged protein and because the bound oligonucleotide only partially neutralizes the positive charge of the protein, the protein–DNA complexes formed is still positively charged and thus failed to enter the native gel (13). As evident from Figure 5A, the amount of unbound oligonucleotide was quite small as compared with the oligonucleotide control when the enzyme was allowed to react with the oligonucleotide in the absence of ATP (Figure 5A, lanes 1 and 2). The percentage of bound DNA was estimated indirectly by quantifying the amount of unbound DNA by film densitometry. About 79% of the input DNA was bound under these conditions (Figure 5B). The amount of unbound oligonucleotide increased gradually with increasing concentrations of ATP (Figure 5A, lanes 3–7). The effect of ATP was to cause a concentration dependent decrease in the extent of LdTOP1L/S-DNA complex formation between 2 and 20 mM of ATP (Figure 5B).

Binding of TNP-ATP to LdTOP1L/S

We used a fluorescent ATP analog 3′ (2′)-o-(2, 4, 6-trinitrophenyl)-adenosine triphosphate (TNP-ATP) that was previously described for other proteins (16,27,28) to study the interaction of ATP with LdTOP1L/S. This fluorescent analog exhibits changes both in its visible spectrum as well as in its fluorescence when bound to a protein. It exhibits higher affinity than ATP for its interacting proteins (16,27). Free TNP-ATP is weakly fluorescent. However, upon binding to proteins, its fluorescence is enhanced by several fold.

Figure 6A shows the fluorescence emission spectrum of TNP-ATP in the presence of LdTOP1L/S. The fluorescence emission of TNP-ATP in buffer alone was maximal at ~553 nm, while LdTOP1L/S alone displayed little fluorescence at this wavelength. In the presence of LdTOP1L/S, the fluorescence of TNP-ATP was enhanced nearly 3-fold at the emission wavelength of 553 nm indicating that TNP-ATP was bound to
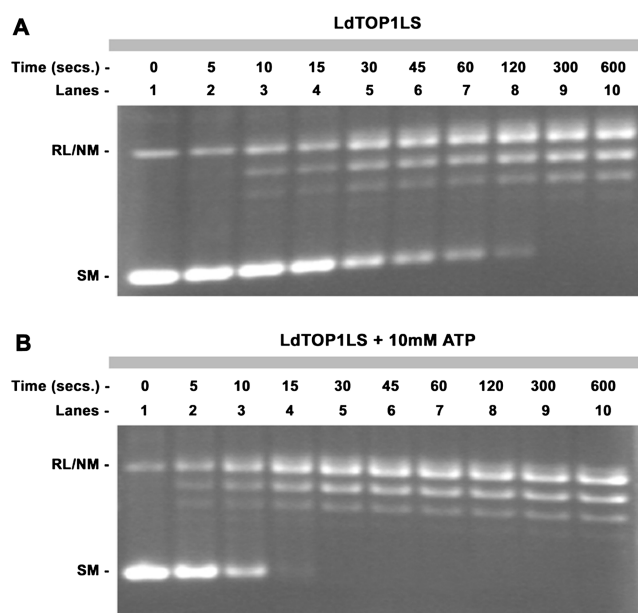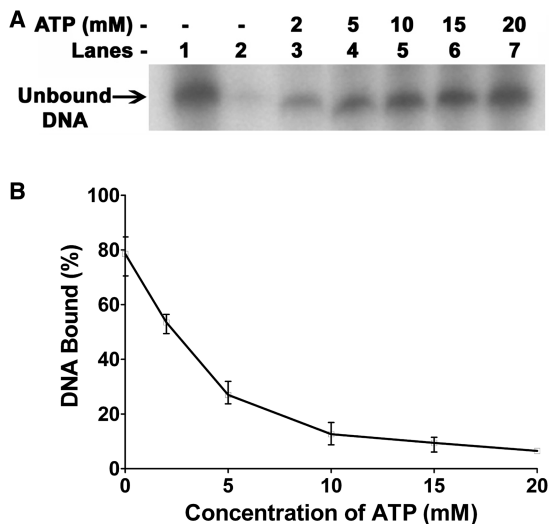
Figure 5. Effect of ATP on DNA binding. (**A**) Binding of LdTOP1L/S to a radiolabeled 25-base pair DNA was assayed as described in 'Materials and Methods' section. Lane 1, 2 nM of labeled input DNA; lane 2, same as lane 1 but incubated with 1 nM of LdTOP1L/S; lanes 3–7, same as lane 2 but in the presence of 2, 5, 10, 15 and 20 mM ATP, respectively. Position of the unbound DNA is indicated.Quantitative representation of the percentage of DNA bound in the presence of increasing concentrations of ATP (**B**). The percentage of bound DNA (open squares) is plotted as a function of ATP concentration. Data presented are mean ± S.E. (*n* = 3).

LdTOP1L/S. TNP-ATP stimulates the DNA relaxation activity of LdTOP1L/S in the absence of Mg^{2+} in a concentration dependent manner (Supplementary Figure S3). TNP-ATP inhibits the DNA relaxation activity of the enzyme in the presence of Mg^{2+} (data not shown). Furthermore, addition of a 1000-fold excess of ATP to the LdTOP1L/S-TNP-ATP complex resulted in the rapid decrease in the fluorescence. These results show that the binding of TNP-ATP to LdTOP1L/S was specific and is successfully competed out by the addition of ATP.

The TNP-ATP binding assay was also performed with each of the two subunits (LdTOP1L and LdTOP1S) comprising LdTOP1L/S. The fluorescence of TNP-ATP was enhanced in the presence of the large subunit (LdTOP1L) and upon addition of a 1000-fold excess of ATP to LdTOP1L-TNP-ATP complex, the fluorescence decreased (Figure 6B). In contrast, addition of the small subunit (LdTOP1S) to TNP-ATP did not change the fluorescence of TNP-ATP (Figure 6C). It was nearly same as that of TNP-ATP in buffer alone. Addition of a 1000-fold excess of ATP to LdTOP1S-TNP-ATP complex did not cause any change in the fluorescence. Taken together, these observations suggest that TNP-ATP binds to LdTOP1L specifically but not to LdTOP1S.

Additional confirmation for this observation was obtained when we examined the effect of increasing concentrations of ATP on the relaxation assay using two different deletion constructs of LdTOP1S, each reconstituted with LdTOP1L to make the holoenzyme. When LΔ33S (LdTOP1L reconstituted with N-terminal 33 amino acid-deletion construct of LdTOP1S) and LΔ140S

(LdTOP1L reconstituted with N-terminal 140 amino acid-deletion construct of LdTOP1S) were assayed in the absence of Mg^{2+}, increasing concentrations of ATP was able to gradually stimulate the relaxation activity of LΔ33S (Figure 6D, compare lanes 3–7 with lane 2) and also of LΔ140S (Figure 6E, compare lanes 3–7 with lane 2). It should be noted that ATP stimulates the activity of both the deletion mutants, however, the activity of the larger mutant (LΔ140S) is enhanced to a lesser extent. These observations suggest that ATP does not bind to the N-terminal 140 amino acid region of LdTOP1S. The possibility of ATP binding to the C-terminal region of LdTOP1S is unlikely because the catalytic tyrosine is located at the amino acid 222 position.

Computational model of the ATP binding pocket

In order to identify the preferential binding region of the ATP molecule on the LdTOP1 large subunit, 250 Molecular Docking runs were carried out. The resulting 250 docked ATP molecules were found in the cavity that accommodated the DNA (Figure 7A). The free energy of the complexes ranges from −6.3 to −9.5 Kcal/mol. The percentage of contacts between the protein and the ATP molecule found in the 250 complexes and reported in Table 1, indicates that the ligand interacts with only four residues of the protein. Both His93 and Arg188 mainly interact with the phosphate group, Tyr95 interacts with the base moiety and Arg190 with the sugar moiety (Figure 7C).

A second experiment using the heterodimeric form of the protein has been carried out, to detect if the presence of the small subunit could contribute to the binding. The 250 docked molecules were located in a position quite close to the one observed in the previous experiment (Figure 7A and B). The free energy of the complexes in this case ranged between −7 and −12.5 Kcal/mol, indicating that the presence of the small subunit enhances the affinity of the ATP molecule for the protein. Moreover, ATP is still in contact with residues 93, 95, 188 and 190 of the large subunit, with Arg190 being having contacts in >80% of the total complexes. New interactions with both subunits appear (Table 1). In detail, in 50% of the complexes ATP is in contact with Lys352 of the large subunit, one of the residues of the catalytic pentad and Ser218 of the small subunit, a residue in close contact with the catalytic Tyr222 (Figure 7D). The proximity of ATP to the active site could explain its role in enhancing the activity of the protein.

Effect of ATP on the mutant enzymes

Docking studies of LdTOP1L/S with ATP suggest the possibility that four amino acid residues, His93, Tyr95, Arg188 and Arg190 of the large subunit may interact with ATP. To characterize the properties of these mutant enzymes further, each of the four amino acid residues was mutated to alanine individually by site directed mutagenesis and assayed for its effect on *in vitro* DNA relaxation activity. We observed that each
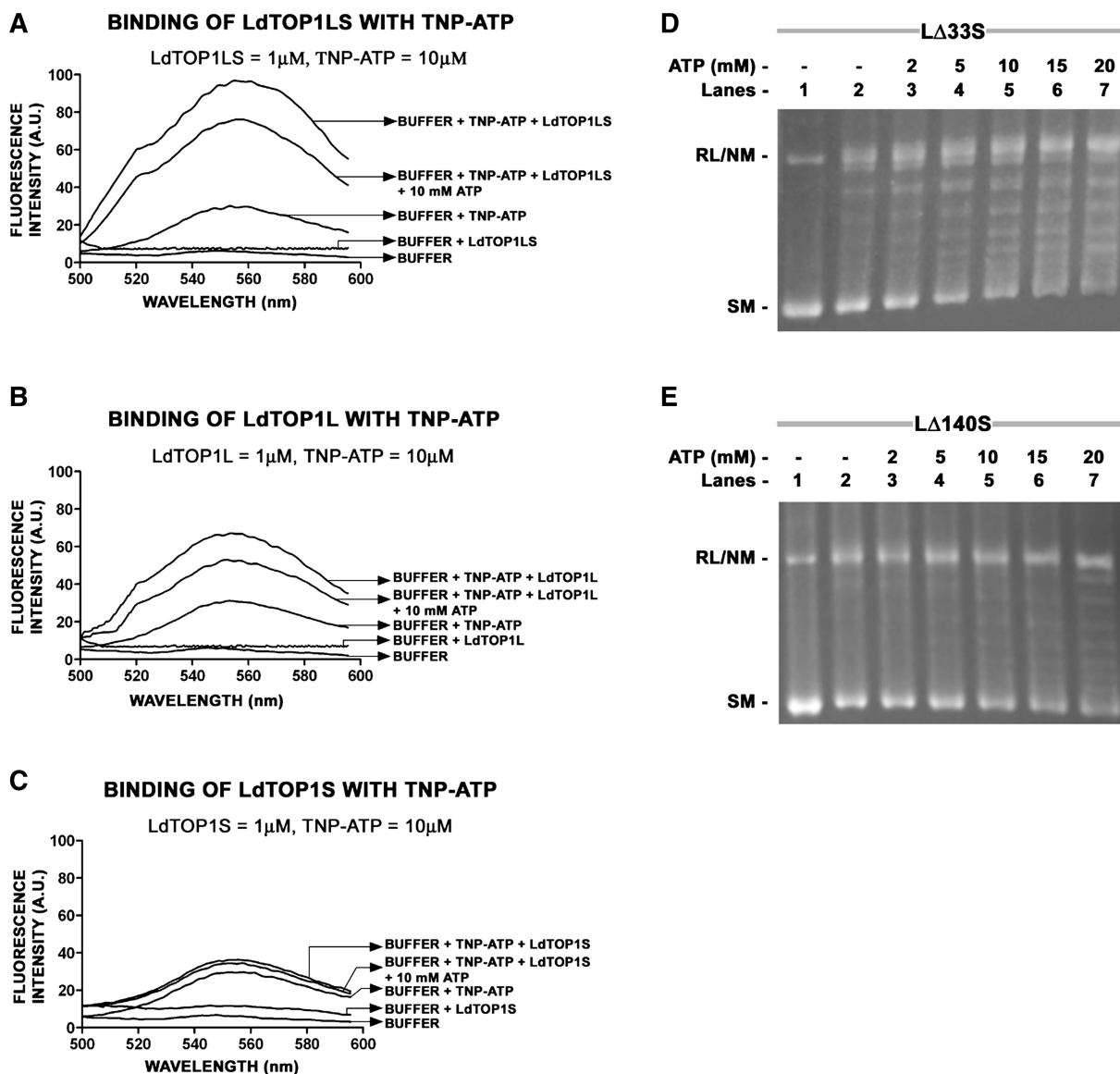
Figure 6. Binding of TNP-ATP to the protein. Fluorescence emission spectra of 10 μM TNP-ATP bound to LdTOP1L/S (**A**), LdTOP1L (**B**) and LdTOP1S (**C**). Emission spectra of buffer, 10 μM TNP-ATP in buffer, protein (1 μM) alone in buffer, 10 μM TNP-ATP and 1 μM protein in buffer and competition of 10 μM TNP-ATP and 1 μM protein in buffer with 10 mM ATP are shown individually in (A), (B) and (C). Fluorescence excitation was at 403 nm and emission scan was performed between 500 and 600 nm. Plasmid relaxation assay of LΔ33S and LΔ140S in the absence of Mg²⁺. Relaxation of supercoiled pBS (SK+) DNA with LΔ33S (**D**) and LΔ140S (**E**) at a molar ratio of 3:1 in the presence of increasing concentrations of ATP. Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lane 2, same as lane 1 but incubated with 40 fmol of enzyme at 37°C for 30 min; lanes 3–7, same as lane 2 but in the presence of 2, 5, 10, 15 and 20 mM ATP, respectively, as indicated in the figure. Reactions were stopped by the addition of 0.5% SDS and electrophoresed on 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RL/NM) are indicated.

mutant enzyme resembles the wild-type enzyme in its ability to relax supercoiled plasmid DNA under standard *in vitro* assay conditions containing Mg²⁺ (Figure 8A, compare lanes 10, 15, 20 and 25 for the mutant enzymes with lane 5 for the wild-type protein). Furthermore, like the wild-type enzyme, inclusion of both ATP and Mg²⁺ in the topoisomerase reaction caused inhibition of DNA relaxation activity in all cases (Figure 8A, lanes 6, 11, 16, 21 and 26). Finally, in the absence of KCl, ATP was unable to exert its stimulatory effect (Figure 8A, lanes 2, 7, 12, 17 and 22). It should be

noted that in the absence of Mg²⁺, the relaxation activity of the mutant LH93A/S decreased considerably (Figure 8A, lane 8) while the activity was not even detectable for the other three mutant enzymes (Figure 8A, lanes 13, 18 and 23). Importantly, ATP was able to exert its stimulatory effect with LH93A/S, LY95A/S and LR188A/S mutant enzymes (Figure 8A, lanes 9, 14 and 19) although to a lesser extent than the wild-type enzyme (Figure 8A, lane 4). However, ATP was inactive in stimulating the activity of the LR190A/S mutant enzyme (Figure 8A, lane 24). Similar conclusions were derived
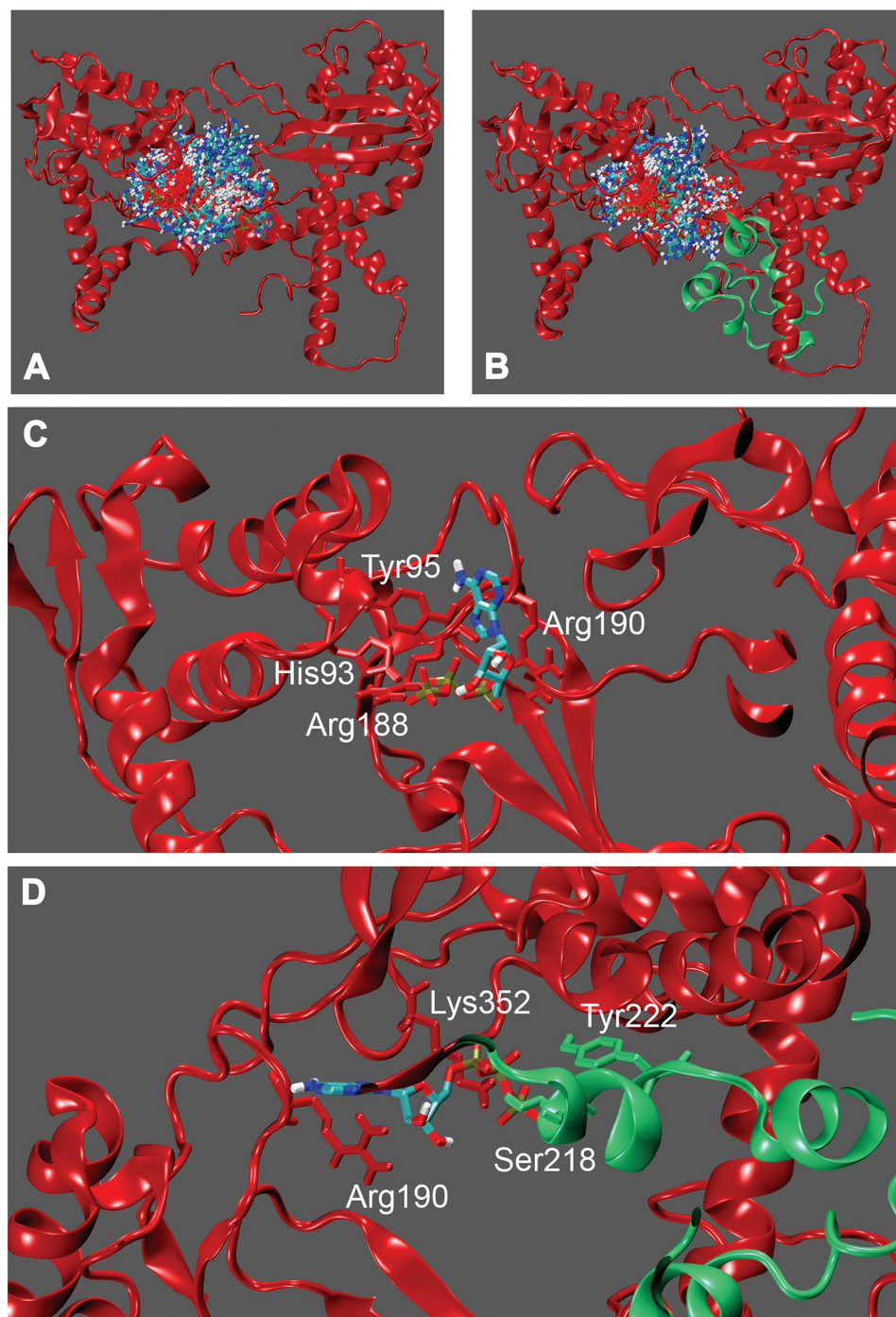
Figure 7. Spread of the 250 ATP docked molecules in the docking performed on the large subunit (**A**) and in the heterodimeric complex (**B**). The protein is represented in ribbons, with the large subunit colored in red and the small subunit colored in green. The best complex for the docking with the large subunit (**C**) and with the heterodimeric protein (**D**) is reported evidencing the residues contacting the ATP molecule for >50%. ATP molecules are represented in licorice with the C, N, O, P, H atoms colored cyan, blue, red, gold and white, respectively. Residues contacting ATP are reported in licorice and colored following the subunit code. In (**D**) the catalytic Tyr222 residue is also evidenced, although not directly contacted by ATP.

with all four mutant enzymes when the DNA relaxation assays were carried out with increasing concentrations of ATP in the absence of Mg^{2+} (Figure 8B–E). These results suggest that the Arg190 residue of the large subunit of the *Leishmania* topoisomerase IB plays an important role in eliciting the stimulatory effect of ATP in the DNA relaxation reaction.

Binding of TNP-ATP to the mutant enzymes

The ability of all the four mutant LdTOP1L/S enzymes to bind TNP-ATP was also investigated. The fluorescence of TNP-ATP was enhanced in the presence of LdTOP1LH93A/S mutant enzyme and addition of ATP caused decrease in the fluorescence (Figure 9A).

Table 1. Percentage of contacts among the 250 complexes between ATP and protein in the two docking experiments

Contacting residues	Percentage of contacts	
	Large subunit	Complex
His93 (large)	93	46
Tyr95 (large)	71	33
Arg188 (large)	95	51
Arg190 (large)	91	81
Arg314 (large)	–	48
Lys352 (large)	–	49
Asp353 (large)	–	35
Ser218 (small)	–	53

Fluorescence of TNP-ATP was also enhanced in the presence of LdTOP1LY95A/S (Figure 9B) and LdTOP1LR188A/S (Figure 9C). In each case, the fluorescence of the mutant enzymes was successfully competed by ATP. However, the enhancement in fluorescence exhibited by these three mutant enzymes was much less as compared with that observed with the wild-type enzyme (Figure 6A). This observation suggests that although TNP-ATP binds to these three mutants, the individual mutations do have some effect on the binding of ATP to LdTOP1L/S. Interestingly, neither the mutant enzyme LdTOP1LR190A/S caused any significant enhancement in TNP-ATP fluorescence, nor the addition of ATP caused any significant decrease in the fluorescence (Figure 9D). Furthermore, docking studies indicate that only the Arg190 residue is contacted by ATP in >90% of the total complexes formed with the large subunit and >80% of the total complexes formed with the heterodimeric form of the protein (Table 1). Additionally, the mutant enzyme LdTOP1LR190A/S is insensitive to ATP-mediated stimulation. Taken together, these observations suggest that the Arg190 residue of the large subunit is important for ATP binding and subsequent stimulation of LdTOP1L/S activity by ATP. Although the other mutant enzymes are able to elicit ATP-mediated stimulation (albeit to a lesser extent) and do exhibit binding with TNP-ATP, we surmise that they may also have a role in ATP binding and consequent stimulation of LdTOP1L/S activity.

DISCUSSION

Type I DNA topoisomerases do not require an energy cofactor such as ATP. The energy of the broken phosphodiester bond is conserved in a covalent linkage with the enzyme and is thus available to restore that bond (29,30). In contrast, type II DNA topoisomerases are catalytically inactive in the absence of ATP (29). There are, however, varying reports in the literature regarding the effect of ATP on the DNA relaxation activity of type IB DNA topoisomerases. Human topoisomerase I activity has been reported to be inhibited *in vitro* by ATP (8,31) while ATP has been reported to have a stimulatory effect on the activity of Vaccinia viral topoisomerase IB

(7,29). In this work, we have examined the role of ATP on the DNA relaxation activity catalyzed by the novel heterodimeric topoisomerase IB from *L. donovani*.

Using purified bacterially expressed recombinant *Leishmania* protein; we show that ATP stimulates the DNA relaxation activity of LdTOP1L/S *in vitro* only when Mg^{2+} is omitted from the reaction. ATP-induced stimulation requires salt, e.g. KCl as a co-activator and does not involve hydrolysis of ATP. While in the absence of ATP, Mg^{2+} alone is also able to stimulate the DNA relaxation activity of LdTOP1L/S, the presence of both Mg^{2+} and ATP in the topoisomerase reaction abolished the stimulatory effect observed when Mg^{2+} or ATP was added individually to the topoisomerase reactions. Our results also suggest that ATP and Mg^{2+} probably form complex in solution that in turn might decrease the amount of free Mg^{2+} or free ATP in solution rendering both of them ineffective to individually stimulate the activity of LdTOP1L/S at that concentration.

Analyses of kinetics of relaxation reveal that KCl is essential for ATP-mediated stimulation. This supports the observation of Sekiguchi and Shuman (7) who have reported that stimulation of vaccinia topoisomerase I by ATP was completely dependent on inclusion of NaCl in the reaction mixtures. However, the salt requirement for ATP mediated stimulation of LdTOP1L/S activity is very stringent compared with Mg^{2+}, which is able to stimulate even in the absence of KCl. ATP increases the velocity of reaction nearly 20-fold and also causes an increase in the enzyme turnover.

The unusual heterodimeric nature of LdTOP1L/S prompted us to test whether ATP plays any role in the interaction between the large and the small subunit. However, we were unable to detect any significant change in the K_D values in the presence of ATP. The next question is whether ATP affects any step in the catalytic cycle of LdTOP1L/S.

The catalytic cycle of topoisomerase I comprises of four steps: (i) non-covalent binding of enzyme to duplex DNA; (ii) cleavage of one strand with simultaneous formation of a covalent protein-DNA adduct; (iii) release of superhelical tension through one or more cycles of controlled strand rotation; and (iv) religation across the bond originally broken (1,4,7,32,33). These cascade of events release DNA with reduced superhelicity, which allows the enzyme to undergo another cycle of DNA binding and relaxation (33). ATP stimulates LdTOP1L/S-mediated relaxation of supercoiled DNA, i.e. it enhances the LdTOP1L/S reaction rate in the absence of Mg^{2+} and this enhancement might occur ideally at any one or more than one step of the catalytic cycle. Chen and Castora (34) have reported that ATP does not affect the kinetics of the topoisomerase I-mediated cleavage process. Our assessment of the equilibrium cleavage experiment also shows that kinetics of LdTOP1L/S-mediated cleavage is not affected by ATP. Under conditions of an excess of enzyme, LdTOP1L/S was found to relax supercoiled plasmids faster in presence of ATP than in its absence. The slower rate of relaxation under these conditions indicates a slower strand rotation. Thus, it is conceivable that ATP causes faster completion of the catalytic
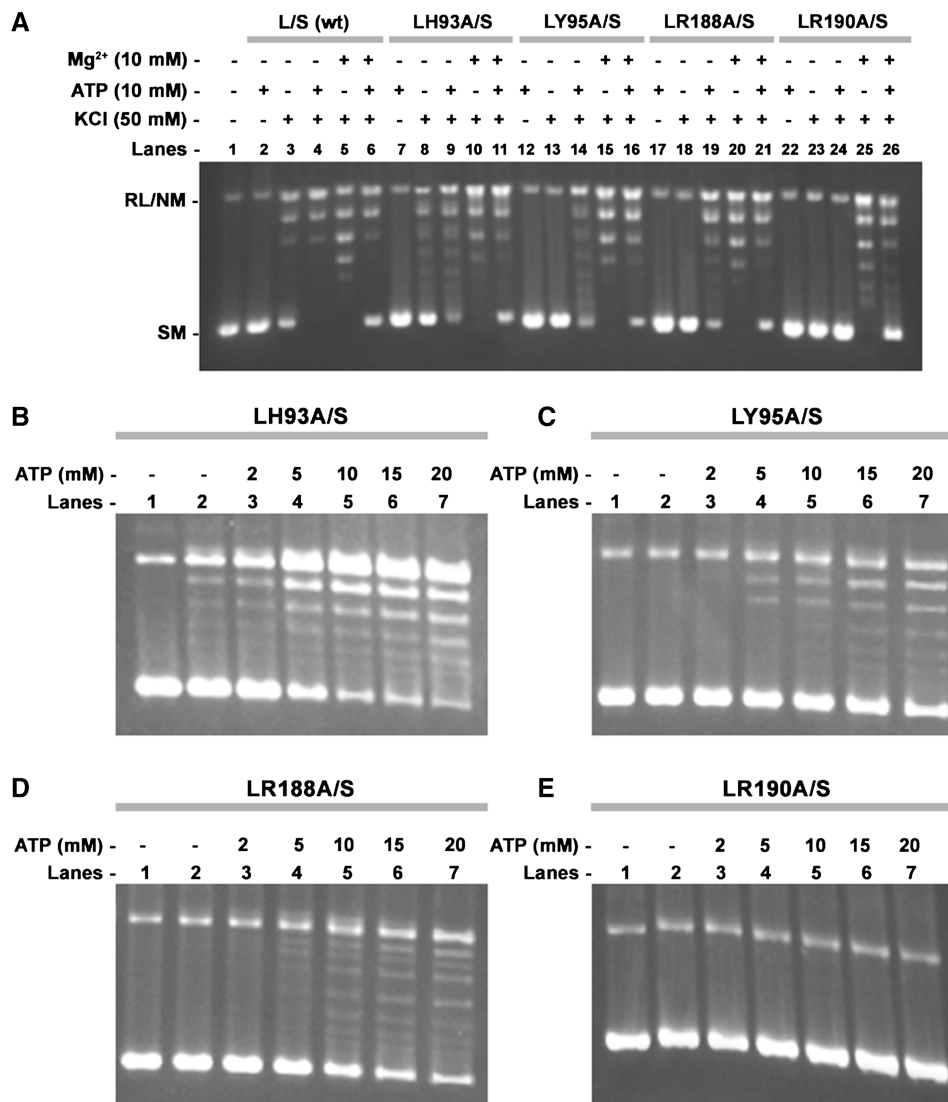
Figure 8. Differential effect of KCl, Mg^{2+} and ATP on the mutant enzymes (**A**). Relaxation of supercoiled pBS (SK+) DNA with the enzyme LdTOP1L/S (lanes 2–6), LdTOP1LH93A/S (lanes 7–11), LdTOP1LY95A/S (lanes 12–16), LdTOP1LR188A/S (lanes 17–21) and LdTOP1LR190A/S (lanes 22–26) at a molar ratio of 3:1 in the presence or absence of indicated concentrations of KCl, Mg^{2+} and ATP. Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lanes 2–26, same as lane 1 but incubated with 40 fmol of the indicated enzymes at 37°C for 30 min but in the presence of indicated concentrations of KCl, Mg^{2+} and ATP. Plasmid relaxation assay of the mutant enzymes in the absence of Mg^{2+} (**B–E**). Relaxation of supercoiled pBS (SK+) DNA with the enzyme LdTOP1LH93A/S (B), LdTOP1LY95A/S (C), LdTOP1LR188A/S (D) and LdTOP1LR190A/S (E) at a molar ratio of 3:1 in the presence of increasing concentrations of ATP. Lane 1, 120 fmol of supercoiled pBS (SK+) DNA; lane 2, same as lane 1 but incubated with 40 fmol of indicated enzymes at 37°C for 30 min; lanes 3–7, same as lane 2 but in the presence of 2, 5, 10, 15 and 20 mM ATP, respectively. Reactions were stopped and electrophoresed as described above. Positions of supercoiled monomer (SM) and relaxed and nicked monomers (RL/NM) are indicated.

cycle of LdTOP1L/S by increasing the strand rotation rate. This observation is unique in the context of the steric effect of ATP on topoisomerase I.

The increase of any reaction rate fundamentally implies the acceleration of the rate limiting step. Stivers *et al*. (35) have shown that during multiple turnover reactions, the release of the product (i.e. dissociation of topoisomerase from the DNA) is rate-limiting in the steady state. Divalent cations are known to accelerate the rate of DNA relaxation by 10-fold (36) and have been shown to increase the rate constant for dissociation of the product (35). This correlation suggests that dissociation of enzyme

from DNA is likely to be rate-limiting during relaxation of supercoiled DNA by LdTOP1L/S. It has been reported previously that salt and Mg^{2+} both cause modest concentration-dependent decrease in the binding of vaccinia virus topoisomerase I to duplex DNA at equilibrium (37), which is consistent with their effect of stimulating the DNA relaxation. LdTOP1L/S relaxation activity is stimulated by Mg^{2+} (9) although Mg^{2+} is not required for its activity. Our results show that ATP stimulates the DNA relaxation activity of *Leishmania* topoisomerase IB strictly in the presence of salt by increasing the rate of strand rotation. We postulate that the faster strand
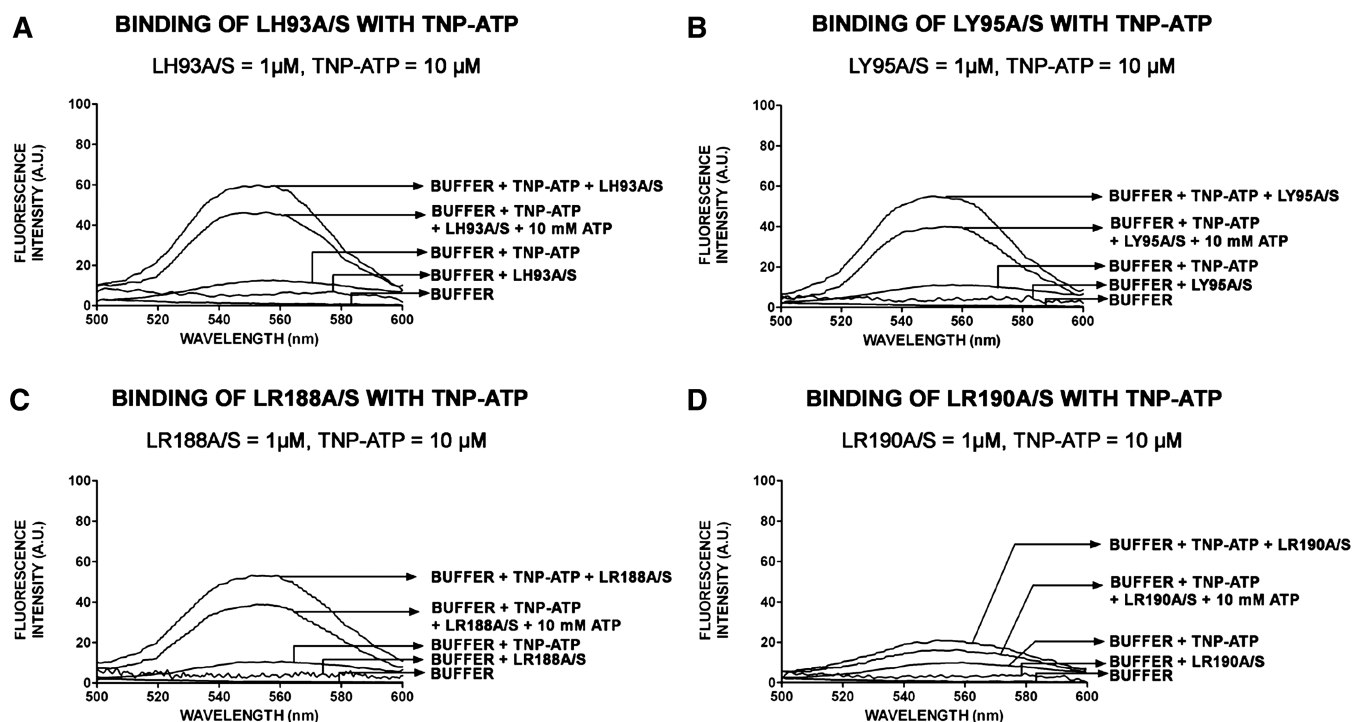
Figure 9. Binding of TNP-ATP to the mutant proteins. Fluorescence emission spectra of 10 μM TNP-ATP bound to LdTOP1LH93A/S (**A**), LdTOP1LY95A/S (**B**), LdTOP1LR188A/S (**C**) and LdTOP1LR190A/S (**D**). Emission spectra of buffer, 10 μM TNP-ATP in buffer, protein (1 μM) alone in buffer, 10 μM TNP-ATP and 1 μM protein in buffer and competition of 10 μM TNP-ATP and 1 μM protein in buffer with 10 mM ATP are shown individually in (A), (B), (C) and (D), respectively. Fluorescence excitation was at 403 nm and emission scan was performed between 500 and 600 nm.

rotation in the presence of ATP causes rapid completion of the catalytic cycle of LdTOP1L/S and ultimately causes increase in the product off-rate. The results of Figure 5 showing the ATP-dependent decrease in the binding of LdTOP1L/S to DNA at equilibrium, strengthens this view.

Divalent cations can affect the stimulation of DNA relaxation by several potential mechanisms. For example Mg^{2+} can make it more favorable for two duplexes to lie on the top of each other to form a node. Mg^{2+}-facilitated nodes recruit topoisomerase I to supercoiled DNA, thereby effectively increasing its activity. Alternatively, topoisomerase I may simply prefer to relax DNA with a Mg^{2+}-shielded phosphate backbone (24). Divalent cations do not bind directly to type IB topoisomerase (34,38). Hence, it is likely that the effect of divalent cations on the relaxation by topoisomerase I is mediated by metal cation binding to DNA, presumably to the phosphate backbone (7,24). Nevertheless, when ATP and Mg^{2+} are both present at equal concentrations, as in Figure 3F, it is conceivable that the triphosphate group of ATP binds Mg^{2+} and prevents its binding to the DNA. Apparently, ATP cannot exert its effect via the same mechanism as Mg^{2+} does, because there is no electrostatic basis for ATP binding to nucleic acid. It has been reported previously that ATP binds to the C-terminal domain of human DNA topoisomerase I (39). Consequently, we anticipated that ATP binds to LdTOP1L/S. Our TNP-ATP binding experiment confirms this view. The fact that ATP binds to

the C-terminal domain of human DNA topoisomerase I (39) suggested the possibilty that ATP might interact with the small subunit of LdTOP1L/S since this subunit, like the C-terminal domain of human topoisomerase IB, harbors the catalytic tyrosine (9) while the large subunit is known to contain the DNA binding motif. However, we observed that TNP-ATP binds to the large subunit and does not bind to the small subunit. These results directly demonstrate the binding of ATP to the unusual heterodimeric topoisomerase IB of *L. donovani* and also suggest that the ATP binding property is conferred by the large subunit.

In silico docking experiments reveal that His93, Tyr95, Arg188 and Arg190 residues on the large subunit are probably responsible for ATP binding. Mutation of these four residues individually to alanine and subsequent determination of the effect of ATP on each mutant enzyme validated the result of the docking experiments and led to some interesting observations. The mutant enzymes LdTOP1LH93A/S, LdTOP1LY95A/S and LdTOP1LR188A/S are able to elicit the ATP-mediated stimulation of relaxation, although to a lesser extent as compared with the wild-type enzyme. Interestingly, the mutant LdTOP1LR190A/S, which fails to bind TNP-ATP, is insensitive to ATP-mediated stimulation of *in vitro* DNA relaxation activity. It is noteworthy that the other three mutant enzymes are able to bind with TNP-ATP and also elicit the stimulatory effect of ATP. Importantly, the ATP-independent DNA relaxation

activity is unaffected in case of all the four mutant enzymes. Altogether, we confirm that Arg190 residue of the large subunit is essential for ATP binding and subsequent stimulation of the activity of LdTOP1L/S. The sugar moiety of ATP probably interacts with the arginine residue and thus elicits the stimulatory effect.

In the DNA-cocrystal structure of *Leishmania* topoisomerase I as a vanadate transition state mimetic, there is no comment on the residues involved in DNA binding (18). However, careful examination of the crystal structure reveals that Arg190 of the large subunit penetrates into the minor grove of the DNA duplex and makes a hydrogen bond with guanine N3 and the O4 atom of the deoxyribose sugar of the same nucleoside on the nicked strand. This could account for why docking a purine nucleotide to apoenzyme would place ATP near Arg190 via putative contacts to the adenosine sugar moiety and not the phosphates. We hypothesize that binding of ATP to the Arg190 residue of the large subunit via the adenosine sugar moiety probably disrupts the contacts between Arg190 and guanine downstream of the nick. Under these circumstances, the phosphate group of ATP is free and probably causes steric repulsion of the DNA phosphate backbone. This, in turn, causes faster movement of the DNA strand, which experimentally causes an increase in the strand rotation.

It is worth noting that Mg^{2+} is a cofactor in many enzymatic reactions and is especially important for those enzymes that use nucleotides as cofactors or substrates. This is because, as a rule, it is not the free nucleotide but its Mg^{2+}-complex that is the actual cofactor or the substrate of the enzymatic reaction (40). Our observation that Mg^{2+} and ATP individually are able to stimulate the DNA relaxation reaction but together they cause inhibition is an unique finding of its kind. Although physiological relevance awaits accomplishment, we surmise that since most of the cellular magnesium remains in the bound form (~90%) (41), the salt dependent ATP pathway of stimulating topoisomerase I may serve as a bypass pathway to stimulate the activity of topoisomerase I function to counteract the topological impediments during various nuclear processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

FUNDING

REFERENCES

1. Champoux,J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**, 369–413.
2. Osheroff,N. (1998) DNA topoisomerases. *Biochim. Biophys. Acta*, **1400**, 1–2.
3. Wang,J.C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat. Rev. Mol. Cell Biol.*, **3**, 430–440.
4. Shuman,S. (1998) Vaccinia virus DNA topoisomerase: a model eukaryotic type IB enzyme. *Biochim. Biophys. Acta*, **1400**, 321–337.
5. Bosedasgupta,S., Das,B.B., Sengupta,S., Ganguly,A., Roy,A., Tripathi,G. and Majumder,H.K. (2008) Amino acids 39-456 of the large subunit and 210-262 of the small subunit constitute the minimal functionally interacting fragments of the unusual heterodimeric topoisomerase IB of Leishmania. *Biochem. J.*, **409**, 481–489.
6. Wang,J.C. (1985) DNA topoisomerases. *Annu Rev Biochem.*, **54**, 665–697.
7. Sekiguchi,J. and Shuman,S. (1994) Stimulation of vaccinia topoisomerase I by nucleoside triphosphates. *J. Biol. Chem.*, **269**, 29760–29764.
8. Chen,H.J. and Hwang,J. (1999) Binding of ATP to human DNA topoisomerase I resulting in an alteration of the conformation of the enzyme. *Eur. J. Biochem.*, **265**, 367–375.
9. Das,B.B., Sen,N., Ganguly,A. and Majumder,H.K. (2004) Reconstitution and functional characterization of the unusual bi-subunit type I DNA topoisomerase from Leishmania donovani. *FEBS Lett.*, **565**, 81–88.
10. Broccoli,S., Marquis,J.F., Papadopoulou,B., Olivier,M. and Drolet,M. (1999) Characterization of a Leishmania donovani gene encoding a protein that closely resembles a type IB topoisomerase. *Nucleic Acids Res.*, **27**, 2745–2752.
11. BoseDasgupta,S., Ganguly,A., Das,B.B., Roy,A., Khalkho,N.V. and Majumder,H.K. (2008) The large subunit of Leishmania topoisomerase I functions as the 'molecular steer' in type IB topoisomerase. *Mol. Microbiol.*, **67**, 31–46.
12. Ganguly,A., Das,B.B., Sen,N., Roy,A., Dasgupta,S.B. and Majumder,H.K. (2006) 'LeishMan' topoisomerase I: an ideal chimera for unraveling the role of the small subunit of unusual bi-subunit topoisomerase I from Leishmania donovani. *Nucleic Acids Res.*, **34**, 6286–6297.
13. Das,B.B., Sen,N., Dasgupta,S.B., Ganguly,A. and Majumder,H.K. (2005) N-terminal region of the large subunit of Leishmania donovani bisubunit topoisomerase I is involved in DNA relaxation and interaction with the smaller subunit. *J. Biol. Chem.*, **280**, 16335–16344.
14. Roy,A., Das,B.B., Ganguly,A., BoseDasgupta,S., Khalkho,N.V., Pal,C., Dey,S., Giri,V.S., Jaisankar,P., Dey,S. *et al.* (2008) An insight into the mechanism of inhibition of unusual bi-subunit topoisomerase I from Leishmania donovani by 3,3′-di-indolylmethane, a novel DNA topoisomerase I poison with a strong binding affinity to the enzyme. *Biochem. J.*, **409**, 611–622.
15. Osheroff,N., Shelton,E.R. and Brutlag,D.L. (1983) DNA topoisomerase II from Drosophila melanogaster. Relaxation of supercoiled DNA. *J. Biol. Chem.*, **258**, 9536–9543.
16. Yao,H. and Hersh,L.B. (2006) Characterization of the binding of the fluorescent ATP analog TNP-ATP to insulysin. *Arch. Biochem. Biophys.*, **451**, 175–181.
17. Morris,G.M., Huey,R., Lindstrom,W., Sanner,M.F., Belew,R.K., Goodsell,D.S. and Olson,A.J. (2009) *J. Comput. Chem.*, **30**, 2785–2791.
18. Davies,D.R., Mushtaq,A., Interthal,H., Champoux,J.J. and Hol,W.G. (2006) The structure of the transition state of the heterodimeric topoisomerase I of Leishmania donovani as a

vanadate complex with nicked DNA. *J. Mol. Biol.*, **357**, 1202–1210.

19. Guex,N., Diemand,A. and Peitsch,M.C. (1999) Protein modelling for all. *TiBS*, **24**, 364–367.

20. Morris,G.M., Goodsell,D.S., Halliday,R.S., Huey,R., Hart,W.E., Belew,R.K. and Olson,A.J. (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.*, **19**, 1639–1662.

21. Lindahl,E., Hess,B. and van der Spoel,D. (2001) GROMACS 3.0: A package for molecular simulation and trajectory analysis. *J. Mol. Mod.*, **7**, 306–317.

22. Humphrey,W., Dalke,A. and Schulten,K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.*, **33**, 27–28.

23. Das,B.B., BoseDasgupta,S., Ganguly,A., Mazumder,S., Roy,A. and Majumder,H.K. (2007) Leishmania donovani bisubunit topoisomerase I gene fusion leads to an active enzyme with conserved type IB enzyme function. *FEBS J.*, **274**, 150–163.

24. Stewart,L., Ireton,G.C., Parker,L.H., Madden,K.R. and Champoux,J.J. (1996) Biochemical and biophysical analyses of recombinant forms of human topoisomerase I. *J. Biol. Chem.*, **271**, 7593–7601.

25. Dickey,J.S. and Osheroff,N. (2005) Impact of the C-terminal domain of topoisomerase IIalpha on the DNA cleavage activity of the human enzyme. *Biochemistry*, **44**, 11546–11554.

26. Yang,Z. and Champoux,J.J. (2001) The role of histidine 632 in catalysis by human topoisomerase I. *J. Biol. Chem.*, **276**, 677–685.

27. Hiratsuka,T. (2003) Fluorescent and colored trinitrophenylated analogs of ATP and GTP. *Eur. J. Biochem.*, **270**, 3479–3485.

28. Huang,S.G., Weisshart,K. and Fanning,E. (1998) Characterization of the nucleotide binding properties of SV40 T antigen using fluorescent 3′(2′)-O-(2,4,6-trinitrophenyl)adenine nucleotide analogues. *Biochemistry*, **37**, 15336–15344.

29. Foglesong,P.D. and Bauer,W.R. (1984) Effects of ATP and inhibitory factors on the activity of vaccinia virus type I topoisomerase. *J. Virol.*, **49**, 1–8.

30. Depew,R.E., Liu,L.F. and Wang,J.C. (1978) Interaction between DNA and Escherichia coli protein omega. Formation of a complex between single-stranded DNA and omega protein. *J. Biol. Chem.*, **253**, 511–518.

31. Low,R.L. and Holden,J.A. (1985) Inhibition of HeLa cell DNA topoisomerase I by ATP and phosphate. *Nucleic Acids Res.*, **13**, 6999–7014.

32. Pommier,Y., Pourquier,P., Fan,Y. and Strumberg,D. (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim. Biophys. Acta*, **1400**, 83–105.

33. Stewart,L., Redinbo,M.R., Qiu,X., Hol,W.G. and Champoux,J.J. (1998) A model for the mechanism of human topoisomerase I. *Science*, **279**, 1534–1541.

34. Chen,B.F. and Castora,F.J. (1988) Mechanism of ATP inhibition of mammalian type I DNA topoisomerase: DNA binding, cleavage, and rejoining are insensitive to ATP. *Biochemistry*, **27**, 4386–4391.

35. Stivers,J.T., Shuman,S. and Mildvan,A.S. (1994) Vaccinia DNA topoisomerase I: single-turnover and steady-state kinetic analysis of the DNA strand cleavage and ligation reactions. *Biochemistry*, **33**, 327–339.

36. Shuman,S., Golder,M. and Moss,B. (1988) Characterization of vaccinia virus DNA topoisomerase I expressed in Escherichia coli. *J. Biol. Chem.*, **263**, 16401–16407.

37. Shuman,S. and Prescott,J. (1990) Specific DNA cleavage and binding by vaccinia virus DNA topoisomerase I. *J. Biol. Chem.*, **265**, 17826–17836.

38. Sissi,C. and Palumbo,M. (2009) Effects of magnesium and related divalent metal ions in topoisomerase structure and function. *Nucleic Acids Res.*, **37**, 702–711.

39. Rossi,F., Labourier,E., Gallouzi,I.E., Derancourt,J., Allemand,E., Divita,G. and Tazi,J. (1998) The C-terminal domain but not the tyrosine 723 of human DNA topoisomerase I active site contributes to kinase activity. *Nucleic Acids Res.*, **26**, 2963–2970.

40. Saris,N.E., Mervaala,E., Karppanen,H., Khawaja,J.A. and Lewenstam,A. (2000) Magnesium. An update on physiological, clinical and analytical aspects. *Clin. Chim. Acta*, **294**, 1–26.

41. Wolf,F.I. and Cittadini,A. (1999) Magnesium in cell proliferation and differentiation. *Front. Biosci.*, **4**, D607–D617.