N-terminal Region of the Large Subunit of Leishmania donovani Bisubunit Topoisomerase I Is Involved in DNA Relaxation and Interaction with the Smaller Subunit*

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Topoisomerases are enzymes that can modify the tertiary structure of DNA without altering the primary structure (1).

DNA topoisomerases accomplish their function by passing one strand of DNA duplex through a transient break in the other strand (type I topoisomerase), resulting in changes in linking number in steps of one (2) or by passing of a duplex DNA from the same or another molecule through a double-stranded break generated in the DNA in an ATP-dependent manner (type II topoisomerase), resulting in changes in the linking number in steps of two (3) and are involved in vital cellular processes (4).

Trypanosoma and Leishmania are ancient eukaryotes. The distinctive features include structurally and metabolically unusual kinetoplast DNA within the single mitochondrion of the organism. DNA topoisomerases from the kinetoplastid parasites play a key role in many aspects of nucleic acid metabolism (5).

Recently, the emergence of the bisubunit topoisomerase I of Trypanosoma (6) and Leishmania (7) in the kinetoplastid family have brought a new twist in topoisomerase research related to evolution and functional conservation of the type IB family. The core DNA binding domain and the catalytic domain harboring the consensus SKXXY motif lies in separate subunits. The two subunits are synthesized by two different genes, which associate with each other through protein-protein interaction to form an active heterodimeric topoisomerase I within the parasite. This unusual structure of DNA topoisomerase I may provide a missing link in the evolution of type IB enzyme.

Camptothecin (CPT), an important class of antitumor agent (8), is an uncompetitive inhibitor that traps the enzyme-DNA covalent complex and slows the religation step of the nicking closing cycle (9, 10). In Trypanosoma brucei, Trypanosoma cruzi, and Leishmania donovani, CPT promotes protein-DNA complex formation with nuclear as well as kinetoplast DNA (11).

We have previously demonstrated the in vitro reconstitution of bisubunit topoisomerase I of L. donovani. The reconstituted enzyme (LdTOP1LS) is characterized by direct 1:1 molar interaction. Under standard relaxation assay conditions, reconstituted enzyme showed reduced processivity as well as 2-fold reduced affinity for DNA compared with eukaryotic monomeric rat liver topoisomerase I. The enzyme is conventional in its Mg²⁺ independence and binds with the eukaryotic type IB-specific recognition site. CPT enhanced the formation of “cleavable complex” at low salt (12). CPT induces cellular dysfunction in L. donovani promastigotes and amastigotes with features that are well characterized by several cytoplasmic and nuclear events of apoptosis (13, 14).

Eukaryotic type IB topoisomerases are monomeric and consist of highly conserved structure (15). The enzymes contain a central DNA binding core domain and a C-terminal catalytic domain harboring SKINYL motif. The linker domain, which is poorly conserved and variable in length, links the two domains.
Cleavage occurs by a trans-esterification reaction involving nucleophilic attack by an active tyrosine (Tyr^{223} in human topoisomerase I) on a DNA phosphodiester bond, resulting in the formation of a covalent DNA 3'-phosphothieryl linkage. In the religation phase, a similar trans-esterification reaction involves attack by the free DNA 5'-hydroxyl that releases the enzyme from DNA (16, 17).

The crystal structure of human topoisomerase I demonstrates that the core and C-terminal domains form a clamp-like structure embedding the DNA helix in a central pore, with two lobes of the protein binding each their site of the helix (18). The conserved subdomains I and II contribute the upper part "CAP," which is connected by a flexible hinge to the bottom part of the clamp of subdomain III. This architecture facilitates the opening and closing of protein clamp during binding and release of DNA. The N-terminal domain of human topoisomerase I is the only part of the enzyme still not crystallized. Knudsen and co-workers demonstrated that the amino acid residues located between positions 191 and 206 coordinate "controlled and co-operative rotation" during the topoisomerization step and sensitivity to interstrand cleavage (19). This finding is consistent with the most recent crystal structure of human topoisomerase I, which reveals a close interaction of Trp^{205} with Gly^{357}, which controls protein dynamics including spatial rearrangements required for strand opening (20, 21).

In the present study, we have addressed the possible role of the large subunit of the bisubunit topoisomerase I of L. donovani. Two N-terminal deletion mutants of the large subunit (LdTOP139L and LdTOP1399L) were generated and mixed with intact small subunit (LdTOP1S). The mutant enzymes were analyzed for topoisomerase I activity. Our findings reveal that amino acid residues 1–39 of the large subunit have a modulating role in noncovalent interaction with DNA and CPT sensitivity, whereas the residues within the amino acid 40–99 region of LdTOP1L appear to be important in relation to interaction with LdTOP1S. Taken together, our study provides the first insight into the mechanistic detail for understanding the unusual structure of bisubunit topoisomerase I of L. donovani.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Plasmid—**The full-length large subunit gene (LdTOP1L) was cloned in the NdeI/BamHI site of bacterial expression vector pET16b (12). For construction of the N-terminal truncation mutants, the region corresponding to amino acids 40–635 and 100–635 were amplified by PCR using pET16bLdTOP1L as a template. The sense primers were 5'-GGGCTTCAATGATTGGGCGGA GCCGGTCGTTGG-3' and 5'-GGGAATTCATGGTGTTCCGGCGCAC CTCC-3', respectively, containing a NdeI site created at the initiation codon, whereas the antisense primer was the same 5'- CGGATCCCTACACCCTGAGCCAAAGGA-3' with a BamHI site immediately downstream from the termination codon. The amplified products were cloned in the NdeI/BamHI site of pET16b. The resultant constructs pET16bLdTOP1399L and pET16bLdTOP1399L were transformed in Escherichia coli BL21 (DE3) pLysS as described (12).

The full-length small subunit gene (LdTOP1S) was previously cloned in the NdeI/BamHI site of bacterial expression vector pET28c (22). The open reading frame was PCR-amplified using sense primer 5'-GGGATCCCTACACCCTGAGCCAAAGGA-3' with a BamHI site created at the initiation codon of the open reading frame, and an antisense primer 5'-CGGATCCCTACACCCTGAGCCAAAGGA-3' with a HindIII site immediately downstream from the termination codon. The PCR-amplified fragment was cloned in the BamHI/HindIII site of pET28c expression vector pET28c, resulting in the construct pET28cLdTOP1S.

For co-expression, we used two compatible T7-based expression vectors (pET16b and pET28c) with two different antibiotic resistances as selectable markers (22). E. coli BL21(DE3)pLysS cells harboring pET16bLdTOP1L and pET16bLdTOP1399L expressing ampicillin resistance were separately co-transformed with pET28cLdTOP1S with kanamycin resistance. These transformants were selected using ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (34 μg/ml) for the E. coli BL21 (DE3) pLysS strain in an LB agar plate.

**Overexpression and Purification Procedures—**E. coli BL21(DE3)pLysS cells harboring pET16bLdTOP1L, pET16bLdTOP1399L, and pET16bLdTOP1S were separately induced at 30 °C with 0.6 with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 22 °C for 12 h. Cells harvested from 1 liter of culture were separately lysed by lysozyme/sonication, and the proteins were purified through a Ni²⁺-NTA-agarose column (Qiagen) followed by a phosphocellulose column (P11 cellulose; Whatman) as described previously (12). Finally, the purified protein LdTOP1L, LdTOP1399L, LdTOP1399L, and LdTOP1S were stored at –70 °C.

**Reconstitution of Mutant Large Subunit with Small Subunit in Vitro—**Purified LdTOP1399L and LdTOP1399L were mixed with purified LdTOP1S separately at a molar ratio of 1:1. A total protein concentration of 0.5 mg/ml in reconstitution buffer (50 mM potassium phosphate, pH 7.5, 0.5 mM dithioreitol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol). The mix was dialyzed overnight at 4 °C, and the dialyzed fractions were used for the plasmid relaxation activity.

**Plasmid Relaxation Assay—**The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled plBlueScript (SK⁺) DNA in an agarose gel. Relaxation assay was carried out as described (12) with LdTOP1L99S, LdTOP1399L, and LdTOP1399LS serially diluted in the relaxation buffer (25 mM Tris-HCl, pH 7.5, 5% glycerol, 0.5 mM dithioreitol, 10 mM MgCl₂, 2.5 mM EDTA, 150 μg/ml bovine serum albumin), supercoiled plBlueScript (SK⁺) DNA (85–95%) were negatively supercoiled, with the remainder being nicked circles), and 50 mM KCl. The amount of supercooled monomer DNA band migration was quantitated by integration using Gel Doc 2000 (Bio-Rad Quality One software). Initial velocities (Vₐ₀ DNA base pairs relaxed/min -1) were calculated by the following equation:

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V_{\text{max}} = \frac{\text{[supercoiled DNA]} - \text{Int}_{t_0} \text{[supercoiled DNA]}_0}{t} (\text{Eq. 1})
\]

where [supercoiled DNA]₀ is the initial concentration of supercoiled DNA, Int₀ is the area under the supercoiled DNA band at time zero, and Intₜ is the area at the reaction time t (23). The effect of DNA concentration on the kinetics of relaxation was examined over a range of 2.5–40 nM supercoiled plBlueScript (SK⁺) DNA (0.1–2 μg/μl of reaction mix) at a constant concentration of 10 mM MgCl₂ and 0.9 mM enzyme. Recombinant LdTOP1L99LS, LdTOP1399LS, and LdTOP1399LS were negatively supercoiled, with the remainder being nicked circles), and 50 mM KCl. The amount of supercooled monomer DNA band migration was quantitated by integration using Gel Doc 2000 (Bio-Rad Quality One software). Initial velocities (Vₐ₀ DNA base pairs relaxed/min – molecule of enzyme).

**Suicidal Cleavage Assay—**A 14-mer (5'-GAAAAAAGACTT-3') oligonucleotide containing the topoisomerase IB-specific cleavage site was 5'-P-end-labeled and annealed to 25-mer (5'-TTTTTTTCTTTTTTTTTCTTT-3'), where "p" is an unlabeled phosphate) oligonucleotide as described (24). The suicidal cleavage reaction was carried out with 5 nM DNA substrate with 0.1 μM enzymes (LdTOP1L99S, LdTOP1399LS, and LdTOP1399LS) in 20 μl of reaction mix under standard assay conditions (10 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA, and 50 mM KCl) at 23 °C for the indicated time periods. All reactions were stopped by the addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with 0.4 mM NaCl and three volumes of ethanol and subsequently digested with 5 μl of 1 mg/ml trypsin in 10 mM Tris-Cl (pH 7.5) and 5 mM EDTA for 30 min at 37 °C. For analysis, samples were mixed with 5 μl of formamide dye (80% deionized formamide, 10 mM Tris, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromphenol blue), boiled, and electrophoresed in 12% polyacrylamide gel and autoradiographed (19). Quanti-
analyzed by 10% SDS-PAGE. The gel was stained with Coomassie Blue to visualize the protein bands and dried before exposure to the film to detect the radiolabeled proteins.

**Substrate: pET16b-LdTOP1** Covalent complexes were generated by incubating 5 nm suicide DNA substrate with 0.15 µM enzymes (LdTOP1LS, LdTOP1AS9LS) for 4 h at 23 °C as above. Under these conditions, suicidal cleavage of 70–80% of the input DNA was observed. Excess salt was not added in the reaction due to the unusual salt sensitivity of the wild type enzyme. The reactions were transferred to 37 °C and preincubated for 2 min. Religation was initiated by the addition of a 300-fold molar excess of the 11-mer recombination acceptor oligonucleotides (5′-OH-AGAAAAATTTC-3′) as described in Ref. 24. The reaction was carried out under standard conditions (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA, and 50 mM KCl) in a 30-µl reaction volume at 37 °C for the indicated time periods. All of the reactions were stopped by adding SDS, and DNAs were subsequently precipitated by ethanol. The precipitated samples were treated with trypsin and analyzed as described for the suicidal cleavage reaction.

**Analysis of Duplex Oligonucleotide Cleavage Assay—** The 25-mer duplex of oligonucleotide 1 (5′-GAAAAGACTTGAGAAAAATTTC-3′) and oligonucleotide 2 (5′-TTAAACATTTTTAGCTTCTTTC-3′) containing a topoisomerase I binding motif was labeled and annealed as described in Ref. 24. Cleavage was carried out using a 20-fold molar excess of wild type and mutant enzymes over duplex containing labeled oligonucleotides (enzymes, 0.2 µM; DNA, 10 nM). The reactions were carried out in standard assay mix containing 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA, 50 mM KCl in the presence or absence of CPT (60 µM) at 37 °C for 30 min. All of the reactions were stopped by the addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with ethanol, digested with 5 µl of 1 mg/ml trypsin, and analyzed by 12% denaturing polyacrylamide gel followed by autoradiography as described above. The amounts of strand cleavage in the presence or absence of CPT for the wild type and mutant enzymes were determined by film densitometry.

**Analysis of Topoisomerase I DNA Interaction by Electrophoretic Mobility Shift Assay—** The γ-32P end labeling and annealing of the 25-mer duplex of oligonucleotide have been carried out as described above. A DNA duplex was carried out by incubating the labeled oligonucleotide 1/oligonucleotide 2 (1 nM) in a 25-µl reaction as described previously (12). For LdTOP1AS9LS and LdTOP1AS99LS, the protein concentrations used in the assay ranged from 2.5 to 0.06 µM. For lysozyme used as a negative control, the concentration extended from 1 to 12 µM. The reaction mixtures were incubated at 15 °C for 15 min and electrophoresed in 6% nondenaturing polyacrylamide gel at 4 °C in 0.18% TBE buffer (45 mM Tris borate, 1 mM EDTA). 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. The unbound oligonucleotides in the gel were quantified by using a phosphor imager (Bio-Rad Molecular Imager system). The K₅₀ value was estimated from the protein concentration at which one-half of the duplex oligonucleotide was bound to the protein (25).

Ni²⁺-NTA-Agarose Co-immobilization Binding Assay—Protein complexes of hexahistidine-tagged LdTOP1AS9LS or LdTOP1AS99LS with GST-LdTOP1S (10 µg) were mixed with pre-equilibrated Ni²⁺-NTA agarose beads (Qiagen) in 100 µl of reconstitution buffer and incubated at 4 °C for 2.5 h with gentle shaking as described previously (12). The beads were pelleted by centrifugation, flow-through was collected, and the beads were washed twice with 500 µl of reconstitution buffer containing 20 mM imidazole so that all of the unbound proteins were removed. The protein samples were eluted from the beads with 50 µl of reconstitution buffer containing 250 mM imidazole and analyzed by 10% SDS-PAGE.

**Coexpression, Immunoprecipitation, and Immunoblot Analyses—** E. coli BL21(DE3)pLysS cells harboring plasmid pET16b-LdTOP1 and pET28c-LdTOP1S or pET16b-LdTOP1AS99L and pET28c-LdTOP1S were grown in 150 ml of Luria-Bertani medium containing the appropriate antibiotics at 37 °C until reaching an A₅₇₀ value of 0.6. The cultures were then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 22 °C for 12 h. Cells were harvested by centrifugation, washed twice with ice-cold 50 mM HEPES (pH 7.5), lysed by sonication, and subjected to immunoprecipitation analyses. The antibodies used in the study were raised against LdTOP1 and LdTOP1S separately as described previously (12).

For immunoprecipitation, 2–5 mg/ml of cleared bacterial total lysate were incubated overnight at 4 °C either with anti-LdTOP1L or anti-LdTOP1S antibody (5 µl/ml) with 150 µl of protein A-Sepharose beads (Sigma) in buffer containing 50 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 0.25 mM dithiothreitol, and a mixture of protease inhibitors. Isolated immunocomplex was recovered by centrifugation washed in accordance with the manufacturer's instructions and subjected to 10% SDS-PAGE and Western blot analysis as described (26).

**RESULTS**

**Purification of Recombinant Proteins—** A schematic representation of all recombinant constructs used in the present study is shown in Fig. 1A. The overexpressed proteins from E. coli BL21(DE3)pLysS cells harboring plasmid pET16b-LdTOP1AS99L (amino acid 1–39 deletion mutant from the N-terminal region of the large subunit) and pET16b-LdTOP1AS99L (amino acid 1–99 deletion mutant from the N-terminal region of the large subunit) were purified separately through an Ni²⁺-NTA-agarose column. Mutant proteins were further purified through a phosphocellulose column as described under “Experimental Procedures.” The other recombinant proteins (i.e. LdTOP1L (large subunit), LdTOP1S (small subunit), and GST-LdTOP1S) were purified as described previously (12). Analysis of the purified proteins by SDS-PAGE (Fig. 1B) showed that all of the recombinant proteins are essentially homogeneous.

**Reconstitution of Relaxation Activity with Wild Type and Mutant Subunits—** Reconstitution of enzyme activity was examined by a plasmid relaxation assay as described previously (12). The purified wild type and mutant large subunits (LdTOP1L, LdTOP1AS9LS, and LdTOP1AS99L) were mixed separately with intact LdTOP1S at a molar ratio of 1:1 in reconstitution buffer and dialyzed overnight at 4 °C as described under “Experimental Procedures.”

The reconstituted enzymes LdTOP1LS and LdTOP1AS9LS show the topoisomerase I relaxation activity (Fig. 2, A and B). We have shown earlier that the parasite reconstituted enzyme is active at low salt, and the optimum activity is at 50 mM KCl and 10 mM Mg²⁺ concentration (12). Therefore, the subsequent relaxation experiments were performed at the above mentioned conditions.

To investigate the relaxation activity of mutant reconstituted enzymes, we compared it with that of LdTOP1LS. Time course relaxation experiments were performed in a standard assay mix, where the plasmid DNA and the enzymes (LdTOP1LS, LdTOP1AS9LS, and LdTOP1AS99LS) were mixed at a molar ratio of 3:1. The velocities for LdTOP1LS and LdTOP1AS9LS enzymes were linear for the first 5 min of the reaction. All of the subsequent initial velocities during kinetic studies were calculated for the time points up to 1 min, well within the linear range for the velocity examined. It was observed that LdTOP1AS9LS relaxes supercoiled DNA at a slower rate than that of LdTOP1LS (Fig. 2; compare lanes 2–10 of A with lanes 2–10 of B), whereas LdTOP1AS99LS failed to show the generation of topological isomers in a plasmid DNA relaxation assay (Fig. 2C). When the molar ratio between LdTOP1AS99L and LdTOP1S was changed to 2:1, relaxation activity was still not achieved. The loss of activity of the mutant enzyme was further analyzed in subsequent experiments.

The kinetics of relaxation by LdTOP1AS99LS was examined over a range of supercoiled pBS (SK) DNA (2.5–40 nm), and the initial velocity was plotted in a Lineweaver-Burk plot (Fig. 2D). The maximal velocity (V_max) for LdTOP1AS9LS was 4.5 × 10⁻⁸ base pairs of supercoiled DNA relaxed/min/0.9 µM of enzyme, which corresponds to a turnover number of about 50 plasmid molecules relaxed/min/molecules of enzyme. This turnover number appears to be about 1.5-fold slower than that of LdTOP1LS (73 plasmid molecules relaxed/min/molecule of enzyme), as previously demonstrated (12).

Hence, the slower relaxation rate of mutant enzyme (LdTOP1AS99LS) compared with wild type reconstituted enzyme (LdTOP1LS) suggests that deletion of amino acid resi-
dues 1–39 from the N terminus of the large subunit affects the initial steps of catalysis (i.e., noncovalent binding of DNA with the enzyme followed by cleavage of DNA and formation of a transient covalent complex between the enzyme and DNA).

**Effect of CPT on the Relaxation Activity of Wild Type and Mutant Enzymes**—We examined the effect of CPT on the relaxation activity of the wild type (LdTOP1LS) and mutant (LdTOP1Δ39LS) reconstituted enzymes. LdTOP1Δ99LS was not included in this experiment, since it was enzymatically inactive in the relaxation assay. The plasmid DNA and the enzymes (LdTOP1LS and LdTOP1Δ39LS) were mixed at a molar ratio of 1:2 to circumvent a possible effect due to a slow dissociation rate and enzyme turnover number in the presence of CPT. Under these conditions in the absence of CPT, the rate of relaxation of LdTOP1LS was faster than that of LdTOP1Δ39LS (Fig. 3; compare lanes 2 and 3 of A with lanes 2 and 3 of B). However, in the presence of CPT, it can be seen that the time required to complete relaxation for LdTOP1LS is increased ~15-fold (from 1 to 15 min; Fig. 3A, compare lane 3 with lane 14), whereas the drug has a reduced effect on the rate of relaxation by LdTOP1Δ39LS (Fig. 3B, compare lane 4 with lane 12). At low salt concentration, reconstituted human topoisomerase I is significantly inhibited by CPT (24). Hence, our observation regarding the CPT sensitivity at low salt is not surprising, and it is consistent with our recent in vivo studies where we have shown that CPT induces programmed cell death in L. donovani promastigotes mediated through the endogenous topoisomerase I (13, 14).

**Suicidal Cleavage Activity of the Wild Type and Mutant Enzymes**—To determine which step of the topoisomerase reaction was affected by the deletion of amino acids 1–39, we examined the trans-esterification reaction under suicidal conditions by reacting LdTOP1Δ39LS, LdTOP1LS with a synthetic suicide DNA substrate. The substrate consisted of a 5'-32P-labeled 14-bp duplex with an 11-base 5'-tail (24). Upon cleavage and formation of a covalent protein-DNA complex, the AG dinucleotide at the 3'-end of the scissile strand is released. Cleavage was performed at 23 °C for the indicated time periods as described under “Experimental Procedures.” The position and molecular masses of protein standards are indicated on the left.

**Fig. 1.** Protein constructs. A, structure of recombinant L. donovani topoisomerase I proteins. The first line shows the full-length larger subunit (dark) as the core DNA binding subunit. The second line shows the N-terminal truncated large subunit LdTOP1Δ39L that spans amino acids 40–635. The third line shows another N-terminal truncation mutant of the large subunit LdTOP1Δ99L that spans amino acids 100–635, corresponding to the most conserved DNA binding region of eukaryotic type I DNA topoisomerase (dark). The fourth line shows the smaller catalytic subunit. The fifth line is the same as the fourth line but with a GST tag (lightly shaded). B, Coomassie-stained 10% SDS-PAGE analysis of the purified recombinant proteins with 5 μg/lane. Lanes 1–3 and 5, LdTOP1L, LdTOP1Δ39L, LdTOP1Δ99L, and LdTOP1S proteins purified through the Ni2+-NTA column, respectively, followed by phosphocellulose column. Lane 4, GST-LdTOP1S purified through a GST-Sepharose column as described under “Experimental Procedures.” The position and molecular masses of protein standards are indicated on the left.
of incubation, whereas LdTOP1Δ39LS completed the reaction after 180 min of incubation (Fig. 4A). This observation indicates an ~6-fold reduction in the apparent suicidal cleavage rate of LdTOP1Δ39LS over LdTOP1LS. Hence, we surmise that deletion of amino acids 1–39 from the N-terminal end of the large subunit affects either the trans-esterification reaction or a step in the reaction pathway that occurs after initial binding prior to strand rotation. This difference leads to an explanation for the relative slow plasmid relaxation rate by LdTOP1Δ39LS.

To gain further insights into the fate of the covalent complexes produced by LdTOP1LS, LdTOP1Δ39LS, and LdTOP1Δ99LS with labeled oligonucleotide substrate, the reaction mixtures were analyzed by SDS-PAGE. A Coomassie Blue-stained SDS-polyacrylamide gel shows the mobility of free LdTOP1S (Fig. 4B, lanes 1–3). An autoradiograph of the same dried gel shows that the label appears associated with LdTOP1S (Fig. 4C, lanes 1 and 2) that causes a slightly slower migration of LdTOP1S-DNA complex compared with free LdTOP1S. No LdTOP1S-DNA bands are visible with Coomassie Blue staining (Fig. 4B), since only a small amount of protein became covalently attached with DNA, and that became visible after autoradiography (Fig. 4C, lanes 1 and 2). Suicide cleavage by LdTOP1Δ99LS was not achieved under the same conditions (Fig. 4C, lanes 3) or even after 24 h of incubation (data not shown). These results confirmed that combination of LdTOP1Δ99L and LdTOP1S cannot reconstitute the topoisomerase I cleavage activity.

**Single Turnover Religation Activity**—Religation was studied under single turnover conditions by assaying the ability of the covalent intermediate to attach a 5'-hydroxyl-terminated 11-mer to the covalently cleaved 12-mer to form a 23-mer product (24). The ligation reactions of LdTOP1LS and LdTOP1Δ39LS were performed as described under “Experimental Procedures.” The results indicated that religation kinetics for both the wild type (LdTOP1LS) and mutant enzyme (LdTOP1Δ39LS) were similar and were completed within 60 s of incubation (Fig. 5). Hence, the religation kinetics for both enzymes are relatively faster than that of the cleavage rate, indicating that 6-fold reduction in the suicidal cleavage kinetics accounts for relatively slow plasmid relaxation by LdTOP1Δ39LS compared with LdTOP1LS.

**Cleavage Assay and CPT Sensitivity**—Trans-esterification was also examined under equilibrium conditions by reacting LdTOP1LS, LdTOP1Δ39LS, and LdTOP1Δ99LS with 5'-32P-end-labeled 25-mer duplex oligonucleotide as described under “Experimental Procedures” in the absence or presence of 60 μM CPT.

For LdTOP1LS, CPT enhanced cleavage approximately 30–35% with respect to the extent of cleavable complex observed without CPT (Fig. 6). These results indicate that CPT does bind to the covalent complex formed between 25-mer duplex DNA and LdTOP1LS and correlate with the reduction of relaxation activity in the presence of CPT. However, only a low level of cleavage was observed in the absence or presence of CPT for LdTOP1Δ39LS (Fig. 6, lanes 4 and 5). Interestingly, LdTOP1Δ99LS was deficient in the cleavage of 25-mer duplex oligonucleotides both in the absence and the presence of CPT (Fig. 6A, lanes 6 and 7). The lower cleavage activity obtained for LdTOP1Δ39LS was consistent with the modest reduction in suicidal cleavage activity.

**DNA Binding Affinity**—We compared the DNA binding affinity of mutant LdTOP1Δ39LS and LdTOP1Δ99LS reconstituted enzymes for the 5'-32P-labeled duplex oligomer containing the high affinity topoisomerase IB binding site by native gel mobility shift assay (12). Similar to LdTOP1LS, LdTOP1Δ39LS and

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**Fig. 2.** Reconstitution of relaxation activity with wild type and mutant subunits. Relaxation of supercoiled pBS (SK−) DNA with reconstituted enzyme LdTOP1LS (A), LdTOP1Δ39LS (B), and LdTOP1Δ99LS (C) at a molar ratio of 3:1. Lane 1, 90 fmol of pBS (SK−) DNA. Lanes 2–10, same as lane 1 but incubated with 30 fmol of topoisomerase I variants at 37 °C for different time periods as described in the figure. All reactions were stopped by the addition of 0.5% SDS, and samples were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated. D, Lineweaver-Burk representation of the kinetics of relaxation of supercoiled pBS (SK−) DNA by LdTOP1Δ39LS. DNA concentrations ranged from 2.5 to 40 nM, MgCl2 was at 10 mM, and enzyme was at 0.9 nM.

percentage of substrate converted to products, were plotted as a function of time (19). In a suicidal cleavage assay for LdTOP1LS, about 80% of the input DNA become covalently bound to protein and reached its cleavage plateau after 30 min.
LdTOP1Δ99LS are positively charged, and because the bound oligonucleotide only partially neutralizes the positive charge, the protein-DNA complexes failed to enter the native gel. Under these conditions, $K_d$ is equal to the protein concentration at which the amount of unbound oligonucleotides observed in the gel has been reduced by a factor of 2 (25). The binding assays yielded a $K_d$ value of $6 \times 10^{-7}$ M for the interaction of LdTOP1Δ39LS with the DNA substrate, whereas LdTOP1Δ99LS shows a $K_d$ value of $1.2 \times 10^{-6}$ M (Fig. 7). Lysozyme, a positively charged protein, was therefore used as a negative control. The binding of lysozyme with DNA is relatively nonspecific ($K_d$ value of $6.9 \times 10^{-6}$ M). Our previous finding demonstrates that LdTOP1LS binds with the DNA substrate with a $K_d$ value of $3.1 \times 10^{-7}$ M (12). This result indicates that LdTOP1Δ99LS has a lower affinity for DNA compared with LdTOP1LS, whereas LdTOP1Δ39LS shows about 3.8-fold reduced affinity for DNA.

**FIG. 3.** Effect of CPT on the relaxation activity with LdTOP1LS and LdTOP1Δ39LS. Relaxation of supercoiled pBS (SK+) DNA with reconstituted LdTOP1LS (A) or LdTOP1Δ39LS (B) at a molar ratio of 1:2 assayed in the presence or absence of CPT. Lanes 1 and 9, 50 fmol of pBS (SK+) DNA. Lanes 2–8, same as lane 1 but incubated with 100 fmol of LdTOP1LS or LdTOP1Δ39LS in the absence of CPT. Lanes 10–16, same as lanes 2–8 but in the presence of 60 μM CPT incubated at 37 °C for the indicated time periods as described in the figure. All reactions were stopped by the addition of SDS to a final concentration of 0.5% (w/v) and were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM) and relaxed and nicked monomer (RL/NM) are indicated.

**FIG. 4.** Suicidal cleavage activity of LdTOP1LS, LdTOP1Δ39LS, and LdTOP1Δ99LS. A, DNA cleavage rate for LdTOP1LS and LdTOP1Δ39LS with 5'-32P-end-labeled suicide DNA substrate (14-mer/25-mer). The reaction mixtures were incubated with the topoisomerase I variants for 5, 10, 15, 20, 30, 60, 120, 180, and 240 min at 23 °C as described under “Experimental Procedures.” Cleavage products were analyzed by denaturing polyacrylamide sequencing gel electrophoresis, and the percentage of cleaved DNA substrate was plotted as a function of time. The results depicted are the average of three independent experiments normalized relative to the highest amount of substrate converted to cleaved product by LdTOP1LS and indicated by open circles. The filled squares represent LdTOP1Δ39LS. B, Coomassie Blue-stained SDS-polyacrylamide gel; C, autoradiograph of the same gel. Lanes 1–3, 3 μg of reconstituted LdTOP1LS, LdTOP1Δ39LS, and LdTOP1Δ99LS incubated with 5'-32P-end-labeled suicide DNA substrate (as indicated above), respectively, in the reaction buffer for 2 h at 23 °C. The reaction was stopped with SDS-PAGE sample buffer, boiled, and loaded onto a 10% SDS-polyacrylamide gel. The covalent attachments of the labeled DNA to the small subunits are shown in C. The positions and molecular masses of protein standards are indicated on the left, and the migrations of the nucleoprotein complex are shown on the right.
and mutant subunits in interaction between the two subunits. LdTOP1L (amino acids 40–99) may be responsible for the in-

8 lane E2

A

GST-LdTOP1S in the Ni2+/H9004 active enzyme, whereas LdTOP1, A LdTOP1S (Fig. 8 co-immobilization assay.

Our earlier studies demonstrated that purified subunits LdTOP1L and LdTOP1S failed to show generation of topological isomers when assayed separately in a plasmid DNA relaxation assay. Therefore, a protein-protein interaction should occur to reconstitute active DNA topoisomerase I in L. donovani (12).

The in vitro protein-protein interaction between His6-tagged LdTOP1L and GST-LdTOP1S in a molar ratio of 1:1 was shown through a Ni2+-NTA co-immobilization assay (12). We extend our study with the His6-tagged mutant large subunits with the GST-tagged LdTOP1S in the Ni2+-NTA co-immobilization assay.

Hexahistidine-tagged LdTOP1Δ39L can pull down GST-LdTOP1S (Fig. 8A, lane E1) in a Ni2+-NTA co-immobilization assay, indicating that the two subunits can interact to form an active enzyme, whereas LdTOP1Δ59L was unable to pull down GST-LdTOP1S in the Ni2+-NTA co-immobilization assay (Fig. 8A, lane E2), suggesting that the N-terminal region of LdTOP1L (amino acids 40–99) may be responsible for the interaction between the two subunits.

For further analysis, we directly co-expressed the wild type and mutant subunits in E. coli and analyzed the interaction between the two subunits through co-immunoprecipitation and Western blot experiments. We have previously described that antibodies raised against the two recombinant subunits pGEX-GST-LdTOP1L (from amino acid 125–528 of LdTOP1L) and LdTOP1S recognize LdTOP1L and LdTOP1S in induced bacterial crude extracts and L. donovani promastigote extract in immunoblot analysis (12). Deletion of amino acid residues 1–39 from the N-terminal end of the large subunit (LdTOP1Δ39L) does not hamper the interaction between the two subunits (LdTOP1Δ39L and LdTOP1S), as evidenced by a Ni2+-NTA co-immobilization assay (Fig. 8A), and the reconstituted en-

zyme LdTOP1Δ39LS shows topoisomerase I activity (Fig. 2B). Therefore, co-expression of LdTOP1Δ39L and LdTOP1S genes was not performed. The mutant LdTOP1Δ99LS was compared with wild type enzyme (LdTOP1LS) in the above experiments.

For co-expression, we used two compatible T7-based expression vectors with two different antibiotic resistances as selectable markers (22). Induced E. coli BL21DE3pLysS cells harboring recombinant plasmids pET16bLdTOP1L and pET28cLdTOP1S express both proteins and were in the soluble fraction, as determined by Western blotting using specific antibodies (Fig. 8B). The lysate after 200 times dilution in relaxation buffer shows topoisomerase I activity under standard assay conditions (data not shown). In the control experiments, bacterial crude extracts harboring pET16b and pET28c (uninduced and induced) were diluted 200 times as above in the final assay mix. Under these conditions, no bacterial topoisomerase
I activity was observed. The interaction between LdTOP1L and LdTOP1S was further studied using in vivo co-precipitation experiments. The soluble complex was immunoprecipitated with anti-LdTOP1L antibody or anti-LdTOP1S antibody separately as described under “Experimental Procedures.” The immune complex was Western blotted with anti-LdTOP1L antibody or anti-LdTOP1S antibody. Both immunoprecipitations resulted in the appearance of LdTOP1L (Fig. 8B, left, lane 1) or LdTOP1S (Fig. 8B, right, lane 1), respectively, whereas crude extracts of induced bacterial cells harboring pET16b and pET28c were negative to co-precipitation experiments (Figs. 8, B and C, lane 2). These results provide evidence in favor of in vivo protein-protein interaction between LdTOP1L and LdTOP1S in bacterial cell.

Induced E. coli BL21(DE3)pLysS cells harboring pET16b-LdTOP1Δ39LS and pET28c-LdTOP1S express both proteins and were in the soluble fraction as determined by Western blotting using specific antibodies (Fig. 8C). The crude lysate was unable to relax the supercoil DNA (data not shown), which is consistent with our observation in Fig. 2C. This crude lysates was further tested for co-precipitation experiments. The soluble proteins were immunoprecipitated with anti-LdTOP1L antibody or anti-LdTOP1S antibody separately as described above. The result indicates that LdTOP1L lacking amino acids 1–99 from the N-terminal portion of the protein was significantly deficient in interaction with LdTOP1S (Fig. 8C), since the LdTOP1Δ39LS could not be precipitated with anti-LdTOP1S and LdTOP1S by anti-LdTOP1L antibody. However, the antibody against LdTOP1L and LdTOP1S recognized the co-expressed protein LdTOP1Δ399L and LdTOP1S, respectively, in the bacterial lysates when probed separately (Fig. 8C) in Western blotting analysis.

**DISCUSSION**

The multidomain structure for monomeric eukaryotic topoisomerase I is revealed by many lines of evidence, including sequence comparison, mutagenesis, and crystal structure (2, 15–18). Recently, emergence of the bisubunit topoisomerase I in the kinetoplastid family has brought a new twist to topoisomerase research related to evolution and functional conservation of the type IB family. Very little is known about the characteristics of the bisubunit enzyme. Although the bisubunit nature of topoisomerase I from kinetoplastid parasites has been reported by two groups (6, 7), we have demonstrated for the first time the in vitro reconstitution of the two recombinant proteins with functional activity (12).

Champoux and co-workers (27) have previously demonstrated their unique findings on the domain association, CPT sensitivity, and salt sensitivity of the human topoisomerase I that has been artificially divided into two proteins (topoisomerase 58/12 or topoisomerase 58/6.3). Most interestingly, the kinetoplastid topoisomerase I is endogenously bisubunit, and some of our findings are in keeping with that of reconstituted human topoisomerase I. However, a closer look reveals that differences do exist in the sequences, some biochemical properties and preferential sensitivities to inhibitors. In this study, we have tried to assess the importance of the large subunit that resembles the core DNA binding domain with an identity score of 37% and a similarity score of 49% with human topoisomerase I. This homology allowed us to generate two deletion mutants of the large subunit of the bisubunit topoisomerase I of *L. donovani* in order to gain an insight into the mechanistic details of the bisubunit enzymes.

**Effect of Deletion of Amino Acids 1–39 from the N Terminus of LdTOP1L—**We describe here for the first time the significant modulation of in vitro DNA relaxation mediated by the residues within the first 39 amino acids of the N-terminal region (LdTOP1L) of bisubunit topoisomerase I of *L. donovani*. The salient findings from our present work may be briefly summarized as follows.

Under standard assay conditions, LdTOP1Δ39LS distinguishes from the wild type enzyme LdTOP1LS by a decreased relaxation rate regardless of enzyme/DNA ratio. LdTOP1Δ39LS shows an ~1.5-fold decrease in turnover number compared with LdTOP1LS. Moreover, the mutant enzyme shows decreased sensitivity toward CPT in plasmid DNA relaxation experiments, whereas LdTOP1LS is 15 times more sensitive to the drug. The reduced relaxation activities of LdTOP1Δ39LS correlated well with decreased cleavage rates under suicidal conditions (i.e. LdTOP1Δ39LS shows a 6-fold reduction in cleavage rate over LdTOP1LS). In the cleavage assay with 25-mer duplex oligonucleotides, a low level of cleavage was observed for LdTOP1Δ39LS in the presence or absence of CPT compared with cleavage by LdTOP1LS (Fig. 6). Interestingly, the reconstituted enzymes have similar religation rates, which are relatively faster than the cleavage rates. This perhaps accounts for the reduced processivity of LdTOP1LS in the relaxation assay compared with the monomeric enzyme (12). Hence, we surmise that amino acid residues 1–39 from the N-terminal end of the large subunit have a prominent role in the cleavage step or in the steps preceding cleavage (i.e. DNA binding), since LdTOP1Δ39LS has a ~1.9-fold decreased binding affinity (Kd of 6 × 10^{-7} M) compared with that of LdTOP1LS (Kd = 3.1 × 10^{-7} M).

These observations are in keeping with that of the human topoisomerase I, where it was demonstrated that deletion of amino acids 1–206 from the N-terminal region (28) or substitution of Trp^{206} by glycine has a major role in strand rotation and DNA binding (21). The analogous residue Trp^{30} is also conserved in the large subunit of the *Leishmania* enzyme, which probably interacts with the bases in the DNA. Loss of residues 1–39 from the N-terminal end results in decreased affinity for the target site, which is manifested in the slow cleavage and relaxation rate, compared with LdTOP1LS. This result also corresponds with that of vaccinia topoisomerase I, where it was shown that deletion of 81 amino acids from the
N-terminal domain reduces DNA binding and the region is required for the precleavage conformation step (29). Taken together, our data argue in favor of the interpretation that amino acids 1–39 of LdTOP1L of the unusual bisubunit enzyme regulates DNA dynamics during relaxation by controlling non-covalent DNA binding or by coordinating DNA contacts by other parts of the enzyme.

Effect of Deletion of Amino Acids 1–99 from the N Terminus of LdTOP1L—
The four catalytic residues conserved in the core DNA binding domain of human topoisomerase I (i.e. Arg488, Lys532, Arg590, and His632) are highly conserved in the type IB family (15, 18, 30). The analogous residues, also conserved in the large subunit of the Leishmania enzyme, are Arg314, Lys352, Arg410, and His453 (7). Although the deletion mutant LdTOP1Δ99L contains all of these amino acid residues, the mutant cannot reconstitute topoisomerase I activity with the small subunit (LdTOP1S) containing the SKXY motif. We have shown that LdTOP1Δ99L was deficient at interacting with LdTOP1S both in the Ni2+-NTA-agarose co-immobilization assay and in vivo co-precipitation experiments (Fig. 8). In light of our results, two possibilities can be considered. First, the residues within the amino acid 40–99 region of LdTOP1L contain few polar patches, determined by ProtScale analysis (available on the World Wide Web at www.expasy.org/tools/protscale.html). Polar interaction between the subunits of heterocomplex proteins is very common (31). Hence, the residues in this region may be directly involved in ionic interaction for the subunit association of the bisubunit enzyme. Second, comparing the crystal structure of human and vaccinia top I, it is well evidenced that a precleavage conformational change in the core and catalytic domain is necessary to establish the correct position of the active site tyrosine for nucleophilic attack on DNA.

FIG. 8. In vitro and in vivo analysis for subunit interaction. A, Ni2+-NTA agarose co-immobilization binding between (His6-tagged) LdTOP1Δ39L and LdTOP1Δ99L with GST-LdTOP1S. Lane L1, His6-LdTOP1Δ39L and GST-LdTOP1S reconstituted complexes before loading onto Ni2+-NTA-agarose beads. Lane F1, flow-through. Lane W1, excess unbound proteins after washing with 20 mM imidazole. Lane E1, bound proteins eluted with 250 mM imidazole. Lane L2, His6-LdTOP1Δ99L and GST-LdTOP1S reconstituted complexes before loading onto Ni2+-NTA-agarose beads. Lane F2, flow-through contains the uninteracted subunit. Lane W2, excess unbound proteins after washing with 20 mM imidazole. Lane E2, bound protein eluted with 250 mM imidazole. Proteins were electrophoresed in 10% SDS-PAGE, B, crude extract prepared from induced E. coli BL21(DE3)pLysS cells co-expressing LdTOP1L and LdTOP1S was immunoprecipitated (IP) with antibodies against LdTOP1L and submitted to Western blot analysis (WB) using antibodies against LdTOP1S (left, lane 1) or immunoprecipitated with antibodies against LdTOP1S and submitted to Western blot analysis using antibodies against LdTOP1L (right, lane 1). B, lane 2, same as lane 1, but the above experiment was performed with induced bacterial crude extracts harboring pET16b and pET28a. The presence of a soluble fraction of LdTOP1L and LdTOP1S protein in the total lysates was detected by Western blotting with specific antibodies as indicated. Crude extracts from induced E. coli cells co-expressing LdTOP1Δ99L and LdTOP1S were immunoprecipitated with antibodies against LdTOP1L and were submitted to Western blot analysis using antibodies against LdTOP1S (C, left, lane 1) or immunoprecipitated with antibodies against LdTOP1S and submitted to Western blot analysis using antibodies against LdTOP1L (C, right, lane 1). C, lane 2, used as vector control. The presence of a soluble fraction of LdTOP1Δ99L and LdTOP1S protein in the total lysates was detected by Western blotting with specific antibodies as indicated. The arrowheads mark the positions and molecular masses of blotted bands, indicated on the right.
(29, 30, 32, 33). This may imply that loss of amino acids 1–99 of LdTOP1L may lead to a conformational change in the large subunit and that thereby LdTOP1Δ99L cannot form a complex with LdTOP1S to attain the precleavage conformation. Thus, it is justifiable to say that association between the large and small subunits of the bisubunit enzyme appears to be an absolute requirement for cleavage step of topoisomerase activity.

In conclusion, our studies demonstrate the role of the N-terminal region of the large subunit in enzymatic action of the bisubunit topoisomerase I of *L. donovani*. In this way, structural insights gained from this study can be translated into better understanding of the molecular mechanisms of enzyme action in *vivo*. The interaction of the enzyme with specific inhibitors and poisons screened from natural or synthetic sources will help in the quest to selectively target the topoisomerase-based replication apparatus as a means to therapeutically control the parasitic menace in the foreseeable future.

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