Molecular & Biochemical Parasitology 165 (2009) 57–66

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Mutational studies reveal lysine 352 on the large subunit is indispensable for catalytic activity of bi-subunit topoisomerase I from *Leishmania donovani*

Agneyo Ganguly, Souvik Sengupta, Somdeb BoseDasgupta, Amit Roy, Hemanta K. Majumder*

Molecular Parasitology Laboratory, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata, 700032, India

ARTICLE INFO

Article history:
Received 20 October 2008
Received in revised form 31 December 2008
Accepted 8 January 2009
Available online 20 January 2009

Keywords:
Leishmania donovani
Topoisomerase I
General acid
Cleavable complex
Mutagenesis
Transesterification

ABSTRACT

From the vanadate complex crystal structure of *Leishmania donovani* topoisomerase I, several amino acid residues have been implicated to be involved in the catalytic reaction. Although several predictions and propositions have been made, the exact role of these amino acids has not yet been clearly demonstrated *in vitro*. Among these residues, lysine 352 and arginine 314 stand as potential candidates for playing the role of a general acid during the cleavage step. In this study, we have characterized the role of lysine 352 on the large subunit, by site-directed mutagenesis and have tried to identify the general acid that can protonate the 5′-O atom of the leaving strand. Studies with the mutant enzymes reveal that, relaxation activity was severely affected when Lys352 was mutated to arginine or alanine (K352R or K352A). Mutation of Arg314 to Lys (R314K) has very little effect on the relaxation activity. Detailed study reveals that, both cleavage and religation steps are severely affected in case of K352R and K352A and the cleavage religation equilibrium is shifted towards the cleavage. On the contrary, the R314K mutant exhibits only a slightly slower rate of cleavage compared to wild-type enzyme. Cleavage assays with an oligonucleotide containing 5′-bridging phosphorothiolate indicate that Lys352 acts as a general acid in the cleavage step. Altogether, this study establishes the indispensable role of lysine 352 in the catalytic reaction of *L. donovani* topoisomerase I.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The structure and catalytic mechanism of type IB topoisomerases have been the subject of intensive study because topoisomerase I is critical for cellular processes such as DNA replication and transcription. The mechanism by which this enzyme alters the DNA topology involves three major steps: (i) nucleophilic attack by the hydroxyl group of the active site tyrosine on the scissile phosphate resulting in covalent attachment of enzyme to the 3′ end of the broken strand. (ii) a topoisomerization step involving strand passage or free rotation and (iii) religation of the DNA strand and release of the enzyme [1–4].

The catalytic activity of type IB topoisomerases is derived chiefly from five strictly conserved amino acid residues. In human topoisomerase I (hTopo I) these residues are Arg488, Lys532, Arg590, His632, and Tyr723 [5,6]. Studies with vaccinia topoisomerase I have revealed similar role for the amino acids Arg130, Lys167, Arg223, His265 and Tyr274 [7]. The transesterification reaction by topoisomerase has been shown to involve a general acid and general base catalysis mechanism. A general base on the enzyme accepts a proton from the attacking tyrosine nucleophile during covalent adduct formation and a separate general acid donates a proton to expel the 5′-OH leaving group during the cleavage reaction [8]. Evidence has been presented that, Lys532 of human topoisomerase I acts as general acid to protonate the leaving 5′-oxygen atom [9]. In vaccinia topoisomerase I Arg130 and Lys167 are implicated in a proton relay mechanism, which contributes to the acid–base reaction involved in transesterification [10].

L. donovani possesses an unusual heterodimeric topoisomerase I which is expressed from two open reading frames to produce a heterodimer consisting of a 635 residue large subunit (LdTOP1L) and a 262 residue small subunit (LdTOP1S) [11,12]. The individually expressed proteins LdTOP1L and LdTOP1S are inactive *in vitro* [13]. The enzyme is a type IB enzyme with conserved tyrosine as the catalytic residue residing on the small subunit. It has been shown that, the enzyme is active *in vitro* when the two proteins are mixed in equimolar ratio [14]. Recent studies with the enzyme has revealed that, the large subunit is essential for DNA binding and driving the enzyme to its specific substrate DNA [15] while the small subunit supplies the catalytic tyrosine for transesterification reaction [16].

The mechanism of the transesterification reaction by the bi-subunit enzyme and the amino acids involved in each step needs to be deciphered. Although, a general type IB mechanism derived from that of the human and vaccinia topoisomerase I has been proposed for the enzyme, involvement of specific amino acids and their roles are not yet clear. From sequence alignment and crystal structure data of the *L. donovani* topoisomerase I five amino acids have been identified as active site residues which also exhibit conservation

* Corresponding author. Tel.: +91 33 2412 3207; fax: +91 33 2473 5197.
E-mail address: hkmajumder@iicb.res.in (H.K. Majumder).

0166-6851/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2009.01.002

with human and vaccinia topoisomerase I. The vanadate complex crystal structure has helped to predict amino acids constituting the active site pentad [17]. These are Arg314, Lys352, Arg410, His453 and Tyr222. Among these, Tyr222 resides in the SKXXY motif in the small subunit and is responsible for the nucleophilic attack on the DNA backbone and subsequent formation of phosphotyrosine linkage. Mutation of the histidine at position 453 has been shown to alter DNA binding of the enzyme. Of the remaining three, Arg314 and Lys352 stand as potential candidates for acting as the general acid and thereby play a vital role in transesterification reaction.

The exact nature of the general acid responsible for protonation of the 5′-oxygen atom of leaving group during cleavage needs to be investigated in case of *L. donovani* topoisomerase. In all structures of eukaryotic topoisomerases I, the active site lysine makes contact with the −1 base, and may be responsible for stabilizing the enzyme–DNA interaction. Experiments with hTopo I and vaccinia virus topoisomerase I have demonstrated that the DNA cleavage activity of lysine to alanine mutants (K532A in hTopo I and K167A in vaccinia topoisomerase I) can be rescued through the use of 5′-phosphorothiolate substrates [7,9], suggesting strongly that the conserved lysine residue is the general acid. The vanadate complex structure of *L. donovani* topoisomerase I [17] provides evidence that, Lys352 or Arg314 can act as a general acid in the cleavage reaction since both the amino acids are within hydrogen bond distance of the 5′-O atom of the leaving group. Among these, Lys352 is the more probable candidate because of the low pK_a value of lysine. A proton relay mechanism between K352 and R314 is unlikely since these residues are on the opposite sides of the leaving group with a distance of over 5 Å.

In this work we have investigated the effects of site-directed mutations of the amino acid residues Lys352 and Arg314 so as to elucidate their role in catalysis. We have generated K352R, K352A and R314K mutants of the large subunit of *L. donovani* topoisomerase I and reconstituted them with the small subunit. The mutants were tested for their *in vitro* relaxation activity, cleavage efficiency, and religation kinetics. A drastic fall of cleavage and religation rates of both K352 mutants indicates that, the amino acid has significant role in catalysis. Restoration of the cleavage by using oligonucleotide substrate containing 5′-bridging phosphorothiolate clearly establishes Lys352 as the general acid which protonates the leaving oxygen during cleavage step. Thus our study provides insight into the role of the active site lysine and its functional conservation as a general acid in *L. donovani* topoisomerase I.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis

The mutants K352R, K352A and R314K were generated using *pet28cLdTOP1L* as the template DNA using Stratagene Quick change XL kit and the primers listed in Table 1. Reactions were performed according to the manufacturer's protocol. Bacterial colonies were selected for the mutants; DNA samples were prepared and screened by sequencing of the corresponding DNA samples. The DNA for the selected mutants was then transformed into *E. coli* BL21 (DE3) pLysS cells for expression and purification of proteins.

2.2. Overexpression and purification of proteins from bacterial cells

E. coli BL21 (DE3) pLysS cells harboring the mutants pet28cLdTOP1L-K352R, pet28cLdTOP1L-K352A, pet28cLdTOP1L-R314K and wild-type pet16bLdTOP1L and pet16bLdTOP1S were separately induced at 0.6 O.D. (600 nm) with 0.5 mM IPTG at 22 °C for 12 h. Cells harvested from 1 L of culture were separately suspended in lysis buffer containing 1 mg/mL lysosyme, sonicated

Table 1
Forward and reverse primers used to generate the site-directed mutagenesis constructs: R314K, K352R and K352A.

SDM	Primer type	Sequence
R314K	Forward	5′-GAC CGC CTC GCC CTC AAG GTT GGT AAT GAG AAG GGC -3′
	Reverse	5′- GCC CTT CTC ATT ACC AAC CTT GAG GGC GAG GCG GTC -3′
K352R	Forward	5′- CGG TTC GAC TTC CTG GGC AGG GAC TCG ATC CGC TAC C -3′
	Reverse	5′ G GTA GCG GAT CGA GTC CCT GCC CAG GAA GTC GAA CCG -3′
K352A	Forward	5′- CGG TTC GAC TTC CTG GGC GCG GAC TCG ATC CGC TAC C -3′
	Reverse	5′- G GTA GCG GAT CGA GTC CGC GCC CAG GAA GTC GAA CCG -3′

and proteins were purified using Ni^{2+}–NTA–agarose column (Qiagen). Proteins were dialyzed and purified using a phosphocellulose column (P11, Whatman) as described previously [18]. Finally, the purified proteins were stored at −70 °C.

2.3. In vitro reconstitution

Purified LdTOP1L mutants were mixed with purified LdTOP1S separately in 1:1 molar ratio (as determined by Ni^{2+}–NTA co-immobilization assay) following standard protocol [14]. The total protein concentration was 0.5 mg/mL in the reconstitution buffer containing 50 mM potassium phosphate, pH7.5, 0.5 mM dithiothreitol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol. The mix was dialyzed at 4 °C for 12 h and the dialyzed proteins were used for all subsequent assays.

2.4. Plasmid relaxation assay

DNA topoisomerase I was assayed by decreased mobility of the relaxed isomers of supercoiled plasmid pBluescript (SK^+) DNA in agarose gel. Relaxation assays were carried out separately with the mutant enzymes serially diluted in the relaxation buffer (25 mM Tris–HCl, pH 7.5, 5% glycerol, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, 25 mM EDTA and 150 μg/mL bovine serum albumin) and supercoiled plasmid pBluescript (SK^+) DNA (85–95% were negatively supercoiled, with remainder being nicked circles). For all kinetic studies, the reaction mixtures containing the buffer and DNA were heated to 37 °C prior to addition of the enzymes. The reactions were rapidly quenched using stop solution and kept on ice. The gels were stained with ethidium bromide (EtBr) and were visualized on UV transilluminator.

2.5. Preparation of radiolabeled oligonucleotide substrates

The 25-mer oligonucleotide ML25 (5′-GAAAAAAGACTTAGAAAAATTTTTA-3′) was radiolabeled at its 5′ end with [γ^{32}P]-ATP and T4 polynucleotide kinase (Roche) in a suitable buffer. The 5′ end labeled oligonucleotide was annealed with the complementary sequence MC25 (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) following standard annealing conditions [19], to generate the 25-mer duplex DNA substrate. The 14-mer oligonucleotide ML14 (5′-GAAAAAAGACTTAG-3′) was radio labeled at its 5′ end following similar procedure as mentioned above. The radiolabeled ML14 was annealed to MC25 as described [19] to generate the suicidal substrate ML14/MC25. The oligonucleotide containing phosphorothiolate modification MPf22 (5′-AAAAAGACTT-O-P-S-AGAAAAATTTTT-3′) was also labeled and annealed to MPr22 (5′-AAAAATTTTTCTAAGTCTTTTT-3′) similarly as ML25 and MC25 as described above. The 61-oligonucleotide sequence present in the pHOT1 plasmid DNA was synthesized from IDT, USA. The oligo was

Download English Version:

https://daneshyari.com/en/article/2830033

Download Persian Version:

https://daneshyari.com/article/2830033

Daneshyari.com