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AN ATP-INDEPENDENT CATENATING ENZYME FROM THE KINETOPLAST HEMOFLAGELLATE Leishmania donovani

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SUMMARY : An enzyme from Leishmania donovani that catenates monomeric pBR322 into huge catenanes has been isolated and characterized. The enzyme also decatenates kinetoplast DNA networks into covalently closed monomeric circles and relaxes supercoiled pBR322. The catenation, decatenation and relaxation reactions do not require ATP. The formation of topological isomers of unique linking numbers suggest that the enzyme is a type II DNA topoisomerase.

The kinetoplast DNA of Leishmania and the related organisms is an unusual assembly of DNA composed of several thousands of minicircles (1-3 kb) and a few maxicircles (20-25 kb), all held together by extensive catenated network (1). A topological problem arises when the replication of the network is taken into consideration (2). The topology of DNA inside cells is governed by the enzymes DNA topoisomerases (3). The DNA topoisomerases are classified into two types according to their mechanism of actions (3,4). Type I DNA topoisomerases work by making reversible single strand breaks and Type II enzymes work by passing a segment of DNA through a transient double strand break. Although the precise function of many of these enzymes is not clear, their participation in a variety of genetic processes has been suggested (5).

In Leishmania donovani we have reported earlier the presence of an ATP-dependent DNA topoisomerase that catalyzes the relaxation of supercoiled plasmid DNA and decatenation of kDNA network into monomeric circles. The enzyme resembles type II DNA topoisomerase (6). However, this enzyme fails to catenate monomeric circles into huge catenanes at a variety of conditions tested in which catenating activity can be detected

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in a large variety of organisms (7,8). Because of the presence of intercatenated network structure of DNA in the kinetoplast, a catenating activity is expected to be present which would form the progeny networks by catenating replicated free minicircles. In the present communication we report an ATP-independent catenating enzyme from L. donovani which also carries out reactions specific for type II DNA topoisomerases. The enzyme has been partially purified and initial characterization shows that the enzyme resembles type II DNA topoisomerase isolated from another kinetoplastid hemoflagellated parasite Trypanosoma cruzi (9,10).

MATERIALS AND METHODS

Materials. L. donovani strain UR6 (MHOM/IN/1978/UR6) promastigotes were grown in Ray's modified media (11) and subcultured at 72 h intervals.
Reagents and Buffers. Reagents used for agarose gel electrophoresis and hydroxyl apatite (HTP) were obtained from Bio-Rad and phosphocellulose (p11) from Whatman. All other chemicals were of reagent grade or equivalent. Buffers were made as follows : buffer A was 10 mM TrisHCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine hydrochloride, 5 mM DTT. Buffer B was 50 mM TrisHCl, pH 7.5, 2 M NaCl, 10 mM β-mercaptoethanol, 1 mM PMSF. Buffer C was 50 mM TrisHCl, pH 7.5, 1 M NaCl, 10 mM β-mercaptoethanol, 1 mM PMSF. Buffer D was 10% glycerol, 10 mM β-mercaptoethanol, 1 mM PMSF. Buffer E was 50 mM TrisHCl, pH 7.5, 25% glycerol, 0.5 mM DTT, 5 mM β-mercaptoethanol, 0.1 mM PMSF. Storage buffer was 50 mM TrisHCl, pH 7.5, 50% glycerol, 50 mM KCl, 0.5 mM DTT, 5 mM β-mercaptoethanol, 0.1 mM EDTA.
Enzyme assays. The principle of the assay is the decreased mobility in an agarose gel of supercoiled DNA after treatment with topoisomerase. The standard assay mixture (25 μl) contained : 25 mM TrisHCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, 10% glycerol, 0.5 μg of pBR322 DNA and 5 μl of enzyme. The reaction was carried out at 30°C for 15 min. The catenation assay was the same except that NaCl was 20 mM and spermidine 10 mM or histone H1 8 μg/ml. The decatenation assay was the same as topoisomerase assay except that spermidine was omitted and the substrate was catenated kDNA network from L. donovani (12). Reactions were stopped by adding 1% SDS, 10 mM EDTA, 0.25 mg/ml bromophenol blue and 15% glycerol. Samples were applied to a horizontal 1% agarose slab gel (BRL Model H4) and subjected to electrophoresis in TAE buffer (0.04M Tris acetate, 0.002 M EDTA, pH 8.0) at 1.5 v/cm for 14-16 h at room temperature. The gels were stained with ethidium bromide (5 μg/ml) destained in water and photographed under UV illumination. One unit of enzyme activity is defined as the amount of enzyme needed to convert half of the 0.1 μg of monomeric pBR322 DNA into catenated forms.
Preparation of crude nuclear extract of L. donovani. All procedures were carried out at 4°C. 4 g (wet weight) of cells were suspended in 20 ml of buffer A and homogenized in Sorvall Omnimixer at 4000 rpm for 2 min with an interval of 1 min after 1 min homogenization. To the homogenate equal volume of 0.5 M sucrose in buffer A was added, mixed well, kept for 20 min and then centrifuged at 5000xg for 10 min at 4°C. The pellet containing nuclei was suspended in 15 ml of buffer B and kept for 30 min in ice. The nuclei were lysed by slow addition with stirring of 15 ml of buffer C. After another 30 min at 0°C, 15 ml of buffer C containing 18% PEG was slowly added with constant stirring. The solution was incubated for 30 min and centrifuged at 12000g for 60 min. The

supernatant (fraction I) containing topoisomerase activity was either directly subjected to hydroxyl apatite column chromatography or stored at -70°C until further use.

Hydroxylapatite column chromatography. Fraction I (42 ml), containing 5.2 mg protein was loaded onto a hydroxyl apatite column (1.6 cm x 4.5 cm), previously equillibrated with buffer C containing 6% PEG. The column was washed with 25 ml of buffer D containing 200 mM potassium phosphate pH 7.0 and DNA topoisomerase activities were eluted using a linear gradient of 0.2 M to 0.8 M of potassium phosphate in buffer D at a flow rate of 20 ml/h for 3 h. The topoisomerase activities were pooled and dialysed against buffer E (fraction II). Fraction II has been found to efficiently catenate the covalently closed circular pBR322 DNA into huge network.

Phosphocellulose chromatography. Fraction II (14 ml) containing 0.12 mg protein was loaded on to a phosphocellulose column (0.9 cm x 3.2 cm), previously equillibrated with buffer E containing 100 mM KCl. The column was washed with the same buffer and eluted in steps with two column volumes each of 0.2, 0.3, 0.4, 0.6 and 0.8 M KCl in buffer E respectively at a flow rate of 10 ml/h. Active fractions obtained in different steps were pooled separately and dialysed against buffer E. The pooled fraction containing both topoisomerase and catenating activities was dialysed against storage buffer and stored at -20°C (fraction III).

RESULTS AND DISCUSSION

The topoisomerase activities eluted from the hydroxylapatite column relaxes supercoiled pBR322 in absence of ATP (Fig.1A). Relaxation assays in presence of ATP do not show any other topoisomerase activities (data not shown). This ATP-independent relaxing activity eluting between 0.25 and 0.45 M potassium phosphate (Fig.1B) after dialysis against buffer E (fraction II) efficiently catenates covalently closed circular pBR322 DNA in presence of 10 mM spermidine or 8 μg/ml histone H1 (Fig.2). Fraction II also decatenates kDNA networks into free minicircles in absence of ATP (Fig.2, lane 6).

Fig.3 shows the fractionation of DNA topoisomerases (fraction II) on phosphocellulose column. ATP independent relaxing activities were eluted at 0.3-0.4 M and 0.6-0.8 M KCl respectively. The pooled fraction eluting at 0.3-0.4 M KCl after dialysis against buffer E possess both catenating and decatenating activities (Fig.4). Decatenation of kDNA is ATP independent as evidenced by the release of free minicircles from the network after treatment with phosphocellulose enzyme (fraction III) in absence of ATP (Fig.4, lanes 1 and 2). In a standard decatenation reaction, 10 μl of the enzyme completely decatenates 0.5 μg of the kDNA networks into free minicircles. When pBR322 (Fig.4, lane 3) was treated with fraction III enzyme (5 μl and 10 μl) in presence of 8 μg/ml histone H1 or 10 mM spermidine as condensing agents the DNA forms catenanes which do not enter the gel (Fig.4, lanes 4-7). These catenanes involve topological interlocking of DNA rings as the network that is formed is

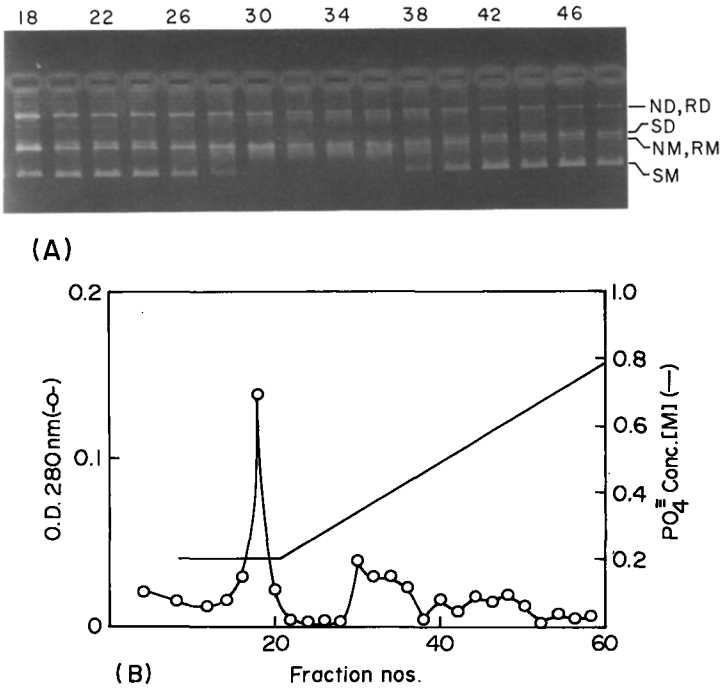

Fig.1. Fractionation of PEG-supernatant on a hydroxylapatite column. (A) Relaxation assays of the column fractions. A sample of each alternative fraction (1 µl) was incubated in the standard ATP-independent relaxation mixture as described in "Materials and Methods". Position of supercoiled monomer (SM), nicked or relaxed monomer (NM, RM), supercoiled dimer (SD) and nicked dimer (ND) are indicated. (B) Potassium phosphate gradient (-) and protein profile (-o-) of the column fractions.

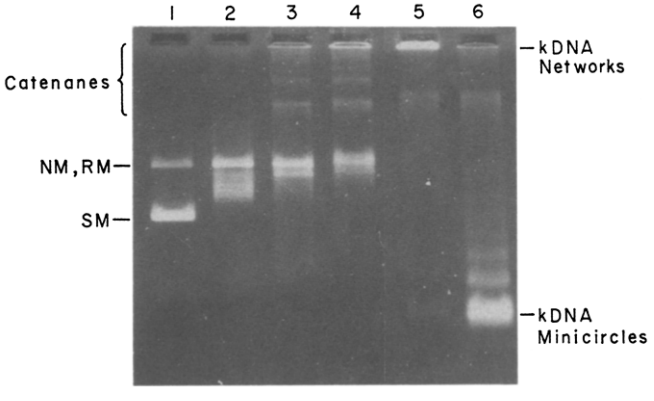

Fig.2. Reactions catalyzed by fraction II enzyme to demonstrate catenation of pBR322 DNA and decatenation of kDNA. pBR322 DNA (lane 1) was incubated with 10 µl of fraction II enzyme (lane 2), 10 µl enzyme plus 10 mM spermidine (lane 3) and 10 µl enzyme plus 8 µg/ml histone H1 (lane 4) in standard catenation reaction mixture (see Materials and Methods). Control kDNA (lane 5) was incubated with 10 µl enzyme (lane 6) in a standard decatenation reaction (see Materials and Methods).

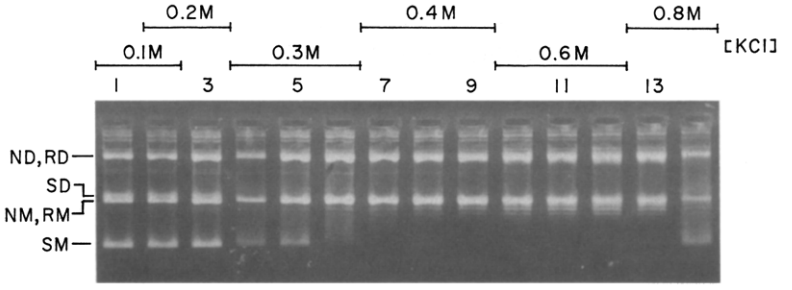

Fig.3. Fractionation of DNA topoisomerases (fraction II) on a phosphocellulose column. Fraction II (14 ml containing 0.12 mg protein) was loaded onto a 2 ml phosphocellulose column and eluted stepwise with increasing concentrations of KCl. 2.5 µl of each fraction was assayed as described in "Materials and Methods".

resistant to SDS, pronase and phenol, but is readily converted to monomeric linear DNA when it is digested with restriction enzyme PstI that cleaves each pBR322 molecules once (data not shown).

Fig.5 shows the effect of different high energy cofactors on catenating activity of fraction III enzyme. It was found that the reaction catalyzed by the enzyme is ATP-independent (Fig.5, lane 2). Moreover, 2 mM each of ATP, GTP, CTP or dATP has no significant effects on the catenating activity in presence of 10 mM spermidine (Fig.5, lanes 3-6).

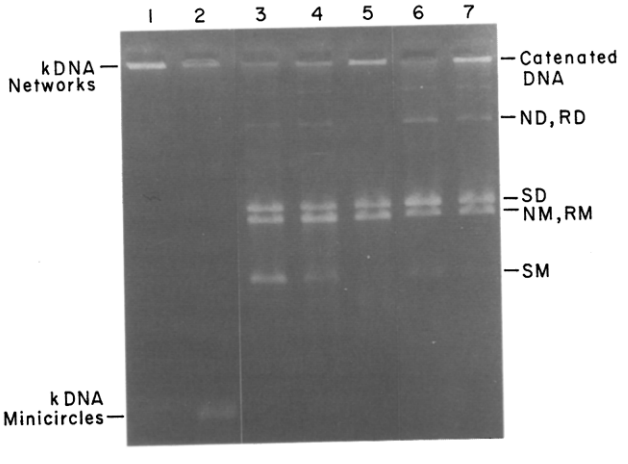

Fig.4. Reactions catalyzed by fraction III enzyme. Lane 2 shows the decatenation of kDNA (lane 1) with 5 µl enzyme (see Materials and Methods). pBR322 DNA (lane 3) was treated with 5 µl of fraction III enzyme in presence of 8 µg/ml histone H1 and 10 mM spermidine (lanes 4 and 6) and with 10 µl of enzyme in presence of 8 µg/ml histone H1 and 10 mM spermidine (lanes 5 and 7) in standard catenation reactions (see Materials and Methods).

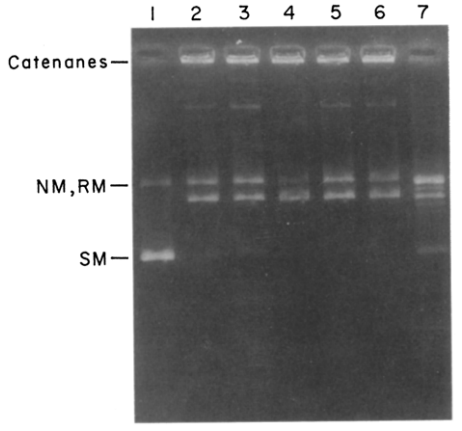

Fig.5. Effect of high energy cofactors on catenating activity of fraction III enzyme. Standard catenation reactions (see Materials and Methods) were carried out with 10 µl enzyme and 10 mM spermidine. Lane 2 shows catenation of pBR322 (lane 1) in absence of ATP. Lanes 3-6 contain 2 mM each of ATP, CTP, GTP and dATP. Lane 7, no condensing agent.

The catenating activity was not detected in PEG supernatant (data not shown). However, using similar procedure a catenating activity can be detected in the crude extract from rat liver nuclei which can effectively catenate monomeric pBR322 into huge catenane (6).

This enzyme resembles ATP-independent type II topoisomerase from T. cruzi (9) in many of its properties viz., catenation of pBR322, decatenation of kDNA and relaxation of supercoiled pBR322. None of these reactions require ATP. However, whether this enzyme is a proteolytic cleavage product of ATP-dependent topoisomerase II of L. donovani (6) or a separate enzyme is yet to be determined. Wang (5) suggested that T. cruzi ATP-independent enzyme is a proteolytic cleavage product of ATP-dependent topoisomerase II. Preliminary results are in agreement with this idea because using this procedure we were unable to isolate an ATP-dependent enzyme from L. donovani nuclei.

We have also detected an endonuclease in post-nuclear supernatant which decatenates kDNA into linear molecules as judged by Bal 31 digestion (data not shown). But we have been unable to isolate any ATP-dependent decatenating activity from the post nuclear supernatant. This suggests that during purification of catenating enzyme involving high salt extraction of nuclei, PEG fractionation, hydroxylapatite and phosphocellulose column chromatography the ATP-dependent enzyme either gets inactivated or can form some cofactor association or dissociation to show these properties.

Table 1. Partial purification of ATP-independent catenating enzyme from L. donovani

Fractions	Volume (ml)	Protein (mg)	Catenating units	Specific activity (units/mg protein)
PEG supernatant (fraction I)	42	5.2	-	-
Hydroxylapatite (fraction II)	14	0.12	1400	1.2×10^4
Phosphocellulose (fraction III)	4	0.03	400	1.3×10^4

Purification was carried out with 4 g (wet weight) of L. donovani promastigotes as described in Materials and Methods.

Another difficulty regarding purification of catenating enzyme is that, the enzyme is very unstable as the activity loses markedly during purification (Table 1). There is very little increase in specific activity after phosphocellulose purification step. The stabilization, purification and characterization of the enzyme are in progress.

The properties of both ATP-dependent decatenating activity (6) and ATP-independent catenating activity suggest that DNA topoisomerases in L. donovani differ from other eukaryotic DNA topoisomerases. Therefore elaborate studies on the nature of these enzymes will be necessary to understand their mode of actions and to improve our knowledge on the mechanism of kDNA replication.

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