DNA topoisomerase from *Agrobacterium tumefaciens*: purification and catalytic properties

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SUMMARY

The DNA topoisomerase from *Agrobacterium tumefaciens* has been purified to apparent homogeneity. The enzyme is a single polypeptide of about 100,000 in molecular weight. No apparent separation of the nicking and sealing activities could be obtained in attempts to separate the two activities by a variety of methods, including limited protease digestion, thermal denaturation, and differential inhibition. Monoclonal antibodies obtained from hybridomas likewise did not preferentially inhibit one of the two activities. These results suggest that the two catalytic functions are carried by the same essential residues of the active enzyme site.

INTRODUCTION

Type I DNA topoisomerases relax superhelical DNA by introducing a nick in one strand of the DNA, which they later reseal. The *E. coli* enzyme, the prokaryotic enzyme most studied, forms knotted rings on single-stranded DNA and relaxed circles on negatively supercoiled double-stranded DNA (1-3). In contrast, the eukaryotic enzyme can untwist both positive and negative superhelical DNA (4-5). Because no exogenous energy cofactor is required, the energy for resealing the phosphodiester bond is thought to be stored in a covalent protein-DNA bond (2). The basis of the assignment is the isolation of a complex of single-stranded DNA and the prokaryotic topoisomerase (6). The linkage was determined to be at the 5' terminus of the DNA in a phosphotyrosyl linkage that was isolated under alkaline or denaturing conditions (7). Under similar conditions, the eukaryotic enzyme was found covalently bound to the 3'-phosphoryl end of the DNA on denaturation (8). Differences were found in the requirements for nicking and resealing. Nicking was observed only in the

optimal range of salt concentrations, but resealing occurred at low and high salt concentrations (8-9).

In this paper, we report the purification to apparent homogeneity of the DNA topoisomerase from A. tumefaciens and describe its basic physicochemical characteristics. The two steps of catalysis were examined to determine approaches to the separation of the two activities. A partial purification of the enzyme has been previously reported (10).

EXPERIMENTAL PROCEDURES

A. tumefaciens strain 1D135 was obtained from Dr. C.I. Kado. Single-stranded calf thymus DNA agarose, SV40 and ϕ X174 R.F. DNA were obtained from Bethesda Research Laboratories.

Protein content was determined by the method of Lowry, et al., with BSA as a standard (11) and SDS polyacrylamide gels were prepared according to the method of Laemmli (12).

DNA topoisomerase reactions were carried out in a 50 μ l volume in a mixture containing 20 mM Tris-HCl (pH, 7.4) and 2 mM $MgCl_2$ at 37°C for 15 min. A unit of enzyme is defined as the amount of activity that relaxes 1 μ g of superhelical SV40 DNA in 30 min. Reactions were stopped and electrophoresis in agarose took place in a vertical slab gel as described previously (10).

To determine the molecular weight of the topoisomerase we used several protein markers to calibrate a Sephadex G-150 column (46 x 0.5 cm) equilibrated with buffer C - 50 mM Tris-HCl (pH, 8.0) and 0.3 M NaCl. All column chromatography was performed at 4°C. We applied the DNA topoisomerase and the protein markers to the column separately in a 1.0 ml volume and assayed fractions (0.40 ml) for activity or determined their protein content spectrophotometrically at 280 nm. The peak of DNA topoisomerase activity was determined by assaying under limiting enzyme conditions.

Linear sucrose gradients (4.8 ml) were prepared with 5% and 20% sucrose in a buffer containing 20 mM Tris-HCl (pH, 7.5), 500 mM NaCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 10% glycerol. The gradients were centrifuged at 100,000g at 4°C for 17 h. We estimated the sedimentation value and molecular weight of the DNA topoisomerase by comparison with several standards.

Monoclonal antibodies against purified DNA topoisomerase were obtained by the procedure developed by Kohler and Milstein for producing hybrid cell lines that synthesize antibody against selected antigens (13,14). Antibodies were assayed on polyvinyl chloride (PVC) microtiter plates which were coated with topoisomerase at 50 ng/well in the presence of 1 mM carbodiimide in sodium bicarbonate buffer (pH, 9.6) and held overnight at 4°C. The plates were washed with PBS (0.05 M NaCl and 0.1 M sodium phosphate at a pH of 7.2) containing 0.01% Tween 20 and 0.02% NaN₃; and reaction with 0.1 M NH₄Cl proceeded (pH 7.2) for 30 min at 37°C. The plates with bound antigen were then washed with PBS and stored at 4°C.

The culture supernatants obtained from hybrid cell cultures reacted with the antigen-coated PVC plates for 2 h at room temperature, and the plates were then washed four times with PBS. ¹²⁵I-Labeled sheep anti mouse IgG (heavy and light-chain) F (ab')₂ (50,000 counts/min) in a total of 100 µl PBS was added to each well, and plates were incubated for another 2 h at 37°C and washed five times with PBS. Each well was then removed from the PVC plate and counted. As little as 0.5 ng of anti-DNA topoisomerase antibody could be detected in the supernatants by this procedure. A total of 80 positive antitopoisomerase antibody producing cell lines were identified by solid phase radioimmunoassay system. Cells in the wells showing presence of antibody were cloned in single cell cultures by limiting dilutions in HY medium containing 10⁷ thymocytes per ml (13). Supernatants from clones that appeared 10-14 days after cloning were again tested for the presence of antibody. The clones producing antibodies were further expanded in HY medium. Supernatants of these clones were concentrated, purified on ion exchange columns and labelled with ¹²⁵I. Each antibody was then competed with unlabelled antibodies derived from other clones. By making the assumption that a non-competing antibody would bind an antigenic site different than the site on the topoisomerase molecule bound by the labelled antibody, 27 different clones were identified. These antibodies were reacted with the enzyme in functional assays.

RESULTS

Purification:

Frozen *A. tumefaciens* cells (75 g) were broken in 1.5 volumes of buffer A - 20 mM phosphate (K⁺) (pH 7.0), 7 mM 2-mercaptoethanol, and 1 mM EDTA - by sonication on 30-s pulses. Unbroken cells and cell debris were then removed by ultracentrifugation at 100,000 g for 90 min. The supernatant (140 ml) was applied to a phosphocellulose column (25x3.5 cm) equilibrated with buffer A. The column was washed until absorbance at 280 nm dropped to less than 0.1. A 2-L gradient was applied to the column (0-1 M KCl in buffer A). Fractions (250 drops each) were assayed for topoisomerase with SV40 I DNA as the substrate. The topoisomerase came off the column at 0.2-0.6 M KCl (Figure 1).

Fig. 1. Fractionation of *A. tumefaciens* extract by phosphocellulose chromatography. Aliquots (5 μ l) were assayed for topoisomerase activity with SV40 DNA as substrate. Reactions were carried out as described in the text. Fraction numbers represent relative elution from the column. Also shown is *Atu* I restriction endonuclease activity, which appears in fractions that elute later than those containing the DNA topoisomerase activity.

The peak of activity was pooled, diluted to a conductivity less than 20 mS, applied to a single-stranded DNA agarose column (5 x 2.2 cm) equilibrated with buffer B - 10 mM Tris-HCl (pH 7.8), 0.5 mM EDTA, and containing 0.25 M NaCl. The column was washed until the A_{280} absorbance was less than 0.1, and a 500 ml step (1 M NaCl and buffer B) was applied. The DNA topoisomerase remained on the column. A step with a mixture of 4.2 M NaCl, 0.1 M $MgCl_2$, and buffer B was required to elute the topoisomerase. The yield of topoisomerase was 0.5×10^6 units (Table 1). The enzyme was dialyzed for several hours against buffer B and against 50% glycerol and buffer B overnight. It was then stored at -20° .

Structural Characterization and Reaction Requirements:

Analysis by denaturing SDS-polyacrylamide gels showed the purified DNA topoisomerase to be a single polypeptide (Figure 2). When relative mobilities of the markers were plotted against the log of the molecular weight, a molecular weight of $100,000 \pm 5,000$ was obtained for the topoisomerase (Figure 2).

For molecular-weight determination, we used a Sephadex G-150 column equilibrated in buffer C and standardized with known protein standards. When K_{av} was plotted against the log of the molecular weight, a value of 90,000-115,000 was obtained for the DNA topoisomerase (Figure 2).

TABLE I

Purification of DNA topoisomerase from 75 g of *A. tumefaciens*

Fraction	Volume ml	Protein mg/ml	Total Protein mg	Total Activity units 10^{-6}	Specific Activity units/mg 10^{-3}	Purification fold
1. Crude	140	60.0	8400	a	a	--
2. Phosphocellulose column	210	3.4	714	0.67	0.94	1
3. Single-stranded DNA agarose column	77	0.023	1.77	0.49	276.8	295

^avalues could not be obtained by agarose-gel electrophoresis because of interfering nucleases.

Fig. 2. Molecular weight estimation of DNA topoisomerase.
 A. Denaturing polyacrylamide gel electrophoresis. A sample (24,000 units) of DNA topoisomerase from the ss DNA agarose fraction was subjected to 10% polyacrylamide gel electrophoresis under denaturing conditions. Arrow indicates mobility of the topoisomerase. The protein standards used were α -phosphorylase, bovine serum albumin and ovalbumin. The molecular weight obtained was $100,000 \pm 5,000$.
 B. Determination of molecular weight on Sephadex G-150 column. A sample of DNA topoisomerase (100,000 units) from the ss DNA agarose fraction was fractionated on a Sephadex G-150 column. The protein standards used were alkaline phosphatase, bovine serum albumin, and ovalbumin. Arrow indicates the elution of the DNA topoisomerase from *A. tumefaciens*. The molecular weight was 90,000-115,000.
 C. Tracing at A₅₉₅ of denaturing SDS polyacrylamide gel of DNA topoisomerase.

The topoisomerase has a pH optimum in Tris-HCl of 7.5-8.5 and requires 2 mM $MgCl_2$ for activity. NaCl above 100 mM inhibits the topoisomerase. The enzyme uses negatively supercoiled DNA, and positively supercoiled DNA was found not to be a substrate. N-Ethylmaleimide is not an effective inhibitor of the enzyme at concentrations as high as 35 mM.

Studies on the Active Site and Mechanism of Catalysis:

Inhibition with increasing amounts of monoclonal antibody and with combinations of different monoclonal antibodies was analyzed on regular and magnesium-containing agarose gels. In all cases, there was either no inhibition or total inhibition; in no case was there a preferential inhibition of one activity or the other (Figure 3).

We subjected the enzyme to thermal inactivation, to determine whether nicking or resealing activities could be preferentially inactivated. The enzyme was incubated at 55°C and aliquots were withdrawn at various times and placed on ice. With increasing duration of heat inactivation, inhibition of enzyme activity increased (Figure 4A). Analysis by polyacrylamide gels in the presence of Mg^{+2} also showed no increase in the nicked DNA form (Figure 4B).

Purified enzyme was treated with trypsin and subtilisin under limiting conditions in an attempt to separate the nicking from the ligation activity. Limited exposure of the enzyme to either trypsin or subtilisin resulted in a decrease in both activities which was directly related to exposure to proteases (Figure 5).

In all of the above experiments incubation mixtures were fractionated on two parallel gels. We added 5 mM magnesium acetate in the agarose electrophoresis system, to allow the separation of nicked circular from covalently closed circular (or unwound) DNA. Under such conditions, the closed circular DNA moves in an intermediate position between the nicked circular and supercoiled. To monitor the inhibition of the nicking activity, we determined the overall enzyme inhibition that is detectable on 1% agarose gels and the inhibition of ligation detectable on 1% agarose gels containing 5 mM magnesium acetate.

Fig. 3. Effect of monoclonal antibody on DNA topoisomerase activity. Monoclonal antibodies were incubated singly or in combination with ϕ X174 RF under appropriate conditions with the topoisomerase. Selected typical examples of DNA patterns obtained upon adding of ab to reaction mixtures are shown. Reactions were stopped and reaction products were displayed on agarose (Panel A) and magnesium-containing agarose gels (Panel B). Lane 1, ϕ X174 RF DNA. Lane 2, DNA after incubation with ab. Lane 3, DNA incubated with DNA topoisomerase. Lanes 4-6, DNA and increasing amounts (5-15 μ l) of ab 17. Lanes 7-9, DNA and increasing concentrations of pooled ab 9 and 14 in equal amounts (5,10,15 μ l) respectively. As demonstrated, various amounts of inhibition were noted for the different antibodies. Panel B demonstrates the absence of accumulation of nicked circular forms of DNA.

Fig. 4. Thermal inactivation of DNA topoisomerase. DNA topoisomerase was incubated at 55° without substrate. Aliquots (2 μ l) were withdrawn, assayed, and run on regular (A) and magnesium-containing (B) gels. Lane 1, ϕ X174 RF DNA (0.5 μ l). Lanes 2-8, times of 1, 2, 4, 8, 16, 32, and 64 min at 55°. Lane 9, 32 min at 40°.

DISCUSSION

The DNA topoisomerase from *A. tumefaciens* has been purified and has been shown to be a single polypeptide of 100,000 $\pm$ 5,000. It requires negatively supercoiled DNA and MgCl_2 for activity, as do other prokaryotic topoisomerases. In contrast, the eukaryotic topoisomerases have been found to have molecular weights of 70,000-100,000 (4,15-17). Unlike the prokaryotic enzyme, which is inhibited by NaCl at the same concentrations, the eukaryotic topoisomerases require monovalent cations at 0.15-0.2 M

Fig. 5. Protease treatment of DNA topoisomerase. The DNA topoisomerase (100 μ l) was treated with various amounts of trypsin and subtilisin for 10 min at 25 $^{\circ}$. Aliquots (10 μ l) were removed, incubated with DNA and reaction products separated on regular (A) and magnesium-containing (B) gels. Lane 1, ϕ X174 RF DNA (0.5 μ g). Lane 2, enzyme under the same conditions. Lanes 3-6, DNA topoisomerase preincubated with 0.65, 1.3, 2.5 and 3.9 units of trypsin respectively. Lanes 7-10 DNA topoisomerase preincubated with 0.05, 0.1, 0.2 and 0.4 units of subtilisin respectively. Protease units are those defined in the Worthington manual.

for activity. Both positively and negatively supercoiled DNA were found to be substrates for the eukaryotic enzymes (4,5).

Enzymatic cleavage of DNA does not occur at high salt concentrations. Resealing, however, can occur in both low and high concentrations. In these studies, we explored whether nicking and resealing steps of the *A. tumefaciens* DNA topoisomerase could be separated from each other or differentially inhibited.

We used several approaches in an attempt to separate the

two activities. Such approaches included limited thermal denaturation, protease digestion and examination of inhibition of the two steps of catalysis. In a different approach, the effect of a battery of monoclonal antibodies was tested against the DNA topoisomerase activity. In all cases the monoclonal antibodies either inhibited both steps of the enzyme activity or had no effect on catalysis. Previous reports have shown DNA to be covalently bound to the topoisomerases through specific amino acid residues and within the active site of the enzyme. Our results based on the multiple experimental approaches suggest that both catalytic steps of A. tumefaciens DNA topoisomerase are undertaken by amino acid residues that are localized either in a single active site or in very closely associated sites with common elements.

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