A Catalytic Domain of Eukaryotic DNA Topoisomerase I*

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Eukaryotic type IB topoisomerases catalyze the cleavage and rejoining of DNA strands through a DNA-(3'→5' phosphotyrosyl)-enzyme intermediate. The 314-amino acid vaccinia topoisomerase is the smallest member of this family and is distinguished from its cellular counterparts by its specificity for cleavage at the target sequence 5'-CCCTT. Here we show that Topo-(81–314), a truncated derivative that lacks the N-terminal domain, performs the same repertoire of reactions as the full-sized topoisomerase: relaxation of supercoiled DNA, site-specific DNA transesterification, and DNA strand transfer. Elimination of the N-terminal domain slows the rate of single-turnover DNA cleavage by 10–3.6 but has little effect on the rate of single-turnover DNA religation. DNA relaxation and strand cleavage by Topo-(81–314) are inhibited by salt and magnesium; these effects are indicative of reduced affinity in noncovalent DNA binding. We report that identical properties are displayed by a full-length mutant protein, Topo(Y70A/Y72A), which lacks two tyrosine side chains within the N-terminal domain that contact the DNA target site in the major groove. We speculate that Topo-(81–314) is fully competent for transesterification chemistry, but is compromised with respect to a rate-limiting precleavage conformational step that is contingent on DNA contacts made by Tyr-70 and Tyr-72.

The eukaryotic type IB DNA topoisomerase family includes topoisomerase I, a ubiquitous nucleo enzyme, and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (1). These proteins relax supercoiled DNA via a common reaction pathway, which involves noncovalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with concomitant formation of a covalent DNA-(3'→5' phosphotyrosyl)-protein intermediate, strand passage, and strand religation. Our aim is to understand the structural requirements for DNA recognition and transesterification chemistry. Toward that end, we have undertaken a structure-function analysis of the vaccinia topoisomerase.

The 314-amino acid vaccinia enzyme is the smallest topoisomerase known and thus affords a more tractable target for structure-function studies than the cellular type IB enzymes, which range from 765 to 1019 amino acids (2, 3). Another attractive feature of the vaccinia topoisomerase is its sequence specificity in transesterification; it forms a covalent adduct at sites containing the sequence 5'-CCCTT. Here we show that Topo-(81–314) is fully competent for transesterification chemistry, but is compromised with respect to a rate-limiting precleavage conformational step that is contingent on DNA contacts made by Tyr-70 and Tyr-72.

and DNA-bound states (8), mapped specific DNA contact points on the enzyme by UV photo-cross-linking (9), and performed targeted mutagenesis of 140 individual amino acid residues (10–18).

The vaccinia virus topoisomerase consists of three protease-resistant polypeptide fragments separated by two protease-sensitive interdomain segments, which we have referred to as the bridge and hinge (Fig. 1) (8). Specific functional groups identified through mutagenesis as being required for transesterification chemistry are situated near the hinge and within the C-terminal domain. These include the active site nucleophile (Tyr-274) and four other residues (Arg-130, Lys-167, Arg-223, and His-265) that are essential for the DNA cleavage and religation steps (11–15, 17, 19). Two other residues (Gly-132 and Tyr-136) are critical for the cleavage reaction, but not for religation (17). None of the essential residues appears to play a role in target site affinity, insofar as alanine substitutions that elicit from 10^−3 to 10^−7 decrements in transesterification rate have no significant effect on the noncovalent binding of topoisomerase to CCCTT-containing duplex DNA (17).

The interdomain bridge is defined by trypsin-accessible sites at Arg-80, Lys-83, and Arg-84 (8, 20). Residues implicated in noncovalent DNA binding are situated within the bridge and in the N-terminal domain just proximal to the bridge. Tyr-70 and Tyr-72 were identified as the sites of UV cross-linking between topoisomerase and the +4 and +3 bromocytosine-substituted bases, respectively, of the CCCTT element (9). Alanine-scanning mutagenesis of the N-terminal domain suggests that Arg-67, Tyr-70, Tyr-72, and Arg-80 contribute to target site affinity (18). Mutational effects on DNA binding are evinced by inhibition of topoisomerase activity in the presence of magnesium and salt (16, 18).

These results have prompted the suggestion (9) that low affinity DNA binding and reaction chemistry are performed by the carboxyl two-thirds of the vaccinia enzyme, the sequence of which is similar to that of the cellular topoisomerases, whereas discrimination of the DNA sequence at the cleavage site is facilitated by the N-terminal domain, which is divergent in sequence and three-dimensional structure between the viral and cellular enzymes (20, 21). Here, we demonstrate that a deleted version of vaccinia topoisomerase, Topo-(81–314), that lacks the N-terminal 80-amino acid domain (Fig. 1), is active in relaxing supercoiled DNA, but is exquisitely sensitive to inhibition by salt or magnesium. The capacity of Topo-(81–314) to cleave a CCCTT-containing substrate under nonstringent conditions suggests that the catalytic domain per se can discriminate the target site. We propose a revised model for catalysis whereby the N-terminal domain enhances DNA binding and is required for a pre-cleavage conformational step.

EXPERIMENTAL PROCEDURES

The segment of the vaccinia virus topoisomerase gene encoding amino acids 81–314 was polymerase chain reaction-amplified using a sense-strand oligonucleotide primer that introduced an internal NdeI restriction site (CATATG) with an in-frame methionine codon in lieu of the codon for Arg-80. An NdeI-BglII restriction fragment containing the...
truncated topoisomerase gene was cloned into the T7-based expression vector pET3c to yield pET-Topo-(81–314). The entire insert of this plasmid was sequenced to confirm that no unwanted mutations had been introduced during amplification or cloning. The pET-Topo-(81–314) plasmid was transformed into Escherichia coli BL21. Topo-(81–314) expression was induced by infection with bacteriophage λC5E6 (22). Topo-(81–314) was purified from soluble bacterial lysates by phosphocellulose column chromatography and glycerol gradient sedimentation. The elution and sedimentation profiles of the Topo-(81–314) polypeptide were monitored by SDS-PAGE of the column and gradient fractions. Topo-(81–314) adsorbed to phosphocellulose in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 0.1% Triton X-100) containing 0.5 mM NaCl, and was recovered by step elution with buffer A containing 1.0 mM NaCl. An aliquot of the phosphocellulose preparation (250 μg) was applied to a 4.8-ml 15–30% glycerol gradient (Fig. 2). The DNA relaxation assays were also performed in the presence of 100 mM NaCl or 5 mM MgCl2. Either salt or magnesium blocked activity almost completely (Fig. 3). The combination of salt and magnesium was similarly deleterious.

Prior studies indicated that product dissociation is rate-limiting during relaxation by WT enzyme in the absence of salt or magnesium. Salt and magnesium stimulate relaxation by enhancing product off-rate, without affecting the rate of DNA cleavage by the WT topoisomerase (23, 24). In the presence of salt plus magnesium, the DNA cleavage step is apparently

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; WT, wild type; DTT, dithiothreitol; bp, base pair(s).
rate-limiting. The paradoxical response of Topo-(81–314) to salt and magnesium is strongly suggestive of reduced affinity of the truncated protein for the DNA substrate. Strand cleavage is likely to be rate-limiting in the absence of salt or magnesium. However, in the presence of salt or magnesium, the DNA binding step becomes limiting for Topo-(81–314). The relative rates of DNA cleavage can be gauged crudely by comparing the WT relaxation rate in the presence of salt and magnesium to the relaxation rate of Topo-(81–314) in the absence of added solutes. By this criteria, we estimate that the rate of DNA cleavage by Topo-(1–314) was less than 1% of the WT rate.

**Topo-(81–314) Forms a Covalent Adduct on CCCTT-containing DNA**—A DNA substrate containing a single CCCTT site was used to examine DNA cleavage under single-turnover conditions. The substrate consisted of a 5’-32P-labeled 18-mer scissile strand 5’-pCGTGTCGCCCTTATTCCC annealed to a 30-mer strand 3’-GCACAGCGGGAATAAGGCTATCACTGA-TGTT to produce an 18-mer duplex with a 12-mer 5’-ATTCCC is released and the enzyme re-

The site specificity of cleavage of the 18-mer scissile strand by Topo-(81–314) was initially compared with that of the WT enzyme by treating the respective covalent adducts with proteinase K in the presence of SDS, then resolving the digestion products by electrophoresis through a 17% polyacrylamide gel containing 7 M urea. Digestion of the wild type covalent adduct yielded a cluster of labeled species migrating faster than the 18-mer input strand, but slower than a free 12-mer strand (data not shown). These digestion products consisted of the 12-mer oligonucleotide 5’-pCGTGTCGCCCTTp linked to short peptides of heterogeneous size. Digestion of the Topo-(81–314) covalent adduct yielded an identical cluster of 32P-labeled DNA-peptide adducts (data not shown). Because any alteration in the site of covalent adduct formation on the 32P-labeled scissile strand would result in an easily detectable shift in the mobility of the proteinase K digestion products (25), we surmise that Topo-(81–314) transesterified at the same phosphodiester bond as the WT enzyme.

**DNA Religation by the Covalent Topo-(81–314)-DNA Intermediate**—The religation reaction was studied under single-turnover conditions by assaying the ability of the covalently intermediate to transfer the covalently held 5’-32P-labeled 12-mer strand to a 5’-hydroxyl-terminated 18-mer strand to form a 30-mer product (26). WT topoisomerase and Topo-(81–314) were first preincubated with the 18-mer/30-mer substrate. DNA analysis by denaturing gel electrophoresis established that virtually all of the input 32P-labeled 18-mer strand had reacted with the WT topoisomerase (as gauged by disappearance of the 18-mer scissile strand; note that the covalent protein-DNA adduct does not enter the gel), whereas Topo-(81–314) reacted with ~70% of the substrate (Fig. 5, right panel,
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Effects of Salt and Magnesium on DNA Cleavage by Topo-(81–314)—Single turnover cleavage reactions are routinely performed at low ionic strength in the absence of a divalent cation. The findings that DNA relaxation by Topo-(81–314) was inhibited by 0.1 M NaCl and 5 mM MgCl$_2$ suggested that pre-cleavage binding might be rate-limiting under those more stringent conditions. To address this issue, we examined the effects of salt and magnesium on cleavage of the 18-mer/30-mer substrate. The amounts of covalent adduct formed in the presence of 50, 100, 150, and 200 mM NaCl or 1, 2, 4, 6, and 8 mM MgCl$_2$ were measured and normalized to the extent of cleavage in unsupplemented control reactions. WT topoisomerase cleavage reactions were quenched after 10 s, whereas Topo-(81–314) reactions were terminated after 6 h. The reaction times were chosen to attain comparable sensitivity for the effects of solution parameters on the cleavage reaction. The salt effects are shown in Fig. 6A; magnesium effects are shown in Fig. 6B. We observed that the WT topoisomerase was unaffected by up to 150 mM NaCl, but was inhibited by 26% at 200 mM NaCl. In contrast, covalent adduct formation by Topo-(81–314) was salt-sensitive; Topo-(81–314) was inhibited 29% and 85%, respectively, by 50 and 100 mM NaCl. Topo-(81–314) cleavage activity was abolished at 150 mM and 200 mM NaCl (Fig. 5A). WT topoisomerase was unaffected by magnesium up to 8 mM, whereas Topo-(81–314) was inhibited progressively by 1–8 mM MgCl$_2$. 52% inhibition occurred at 1 mM MgCl$_2$ and 88% inhibition was observed at 6 mM MgCl$_2$ (Fig. 5B). Susceptibility to salt and magnesium inhibition indicates that deletion of the N-terminal domain results in decreased affinity for the CCCTT-containing DNA substrate.

Noncovalent DNA Binding by Topo-(81–314)—The noncovalent binding of Topo-(81–314) to a 32P-labeled 60-bp DNA containing a single centrally placed CCCTT site (17) was assessed by native gel electrophoresis (27). The full-sized active site mutant protein Topo(Phe-274) was analyzed in parallel (Fig. 7). The binding reaction mixtures contained no added salt or magnesium. The Phe-274 protein bound to the 60-mer ligand to form a single discrete complex of retarded electrophoretic mobility (indicated by the asterisk in Fig. 7). The extent of complex formation was proportional to input Phe-274 topoisomerase and was near quantitative at a 2:1 molar ratio of protein to DNA. Increasing the concentration of Phe-274 to attain 5:1 and 10:1 molar ratios of protein to DNA resulted in the appearance of at least two more slowly migrating complexes (Fig. 7). We presume that this reflects the binding of one or two more topoisomerase monomers to the 60-bp DNA. We showed previously that Phe-274 topoisomerase binds to nontoposites on duplex DNA with 7–10-fold lower affinity than to CCCTT sites (27). The same is true of noncovalent binding by WT topoisomerase (27). Incubation of Topo-(81–314) with the 60-mer ligand resulted in the formation of a somewhat diffuse smear of slowly migrating material (Fig. 7). We estimate from the protein concentrations required to attain a comparable decrease in the residual unbound DNA that Topo-(81–314) bound the 60-mer with one-fifth the affinity of the full-sized enzyme.

Limited Proteolysis of Topo-(81–314) with Chymotrypsin—To address the possibility that the altered biochemical properties of recombinant Topo-(81–314) were caused by aberrant protein folding, we probed the structure of Topo-(81–314) by digestion with increasing concentrations of chymotrypsin. At limiting protease concentrations, Topo-(81–314) was cleaved to yield a predominant polypeptide fragment of 20 kDa and a minor fragment of 18 kDa (Fig. 8). The 20-kDa carboxyl species was resistant to digestion by a level of chymotrypsin sufficient to cleave all the input Topo-(81–314). The 18-kDa polypeptide increased in abundance at the higher levels of protease. Sequencing of these polypeptides by automated Edman chemistry after transfer to a polyvinylidene difluoride membrane revealed that the 20-kDa species arose via cleavage between amino acids Tyr-136 and Leu-137 and the 18-kDa species arose via cleavage between residues Leu-146 and Thr-147. These two
FIG. 6. Inhibition of covalent adduct formation by salt and magnesium. Reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 0.5 pmol of 18-mer/30-mer DNA substrate, and 2.5 pmol of WT topoisomerase, Topo-(81–314), or Y70A/Y72A were supplemented with NaCl (A) or MgCl₂ (B) as indicated. Reactions were initiated by adding protein and quenched after incubation at 37 °C for either 10 s (WT) or 6 h (Topo-(81–314) and Y70A-Y72A). The reaction products were analyzed by SDS-PAGE. The extents of covalent complex formation were normalized to that of the unsupplemented control reaction (defined as 100%) and then plotted as a function of salt or magnesium concentration.

FIG. 7. Assay of DNA binding by native gel electrophoresis. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.5 pmol of 60-bp DNA 5'-32P-labeled on the CCCTT-containing strand (17), and increasing amounts of either Phe-274 topoisomerase or Topo-(81–314) as indicated (0.25, 0.5, 1, 2.5, or 5 pmol, proceeding from left to right within each titration series) were incubated at 37 °C for 5 min. A control reaction contained no topoisomerase (lane –). Glycerol was added to 5% and the samples were electrophoresed through a 6% native polyacrylamide gel in 0.25× TBE (22.5 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h. Free DNA and a topoisomerase-DNA complexes of retarded mobility were visualized by autoradiographic exposure of the dried gel.

The results presented in this study of vaccinia topoisomerase Y70A/Y72A were comparable to that of Topo-(81–314) with respect to rate and the accumulation of partially relaxed topoisomers (Fig. 9). Y70A/Y72A also displayed the same profound inhibition of relaxation by 100 mM NaCl or 5 mM MgCl₂ that was observed for Topo-(81–314) (Fig. 9). Y70A/Y72A formed a covalent intermediate with the 18-mer/30-mer CCCTT-containing DNA under nonstringent conditions; however, the rate of single turnover cleavage was extremely slow ($k_{cl} = 3.6 \times 10^{-5}$ s⁻¹) (data not shown). The cleavage rate decrement elicited by the double-alanine replacement was nearly identical to that caused by removal of the entire N-terminal domain. The sensitivity of covalent adduct formation by Y70A/Y72A to inhibition by salt and magnesium paralleled that of Topo-(81–314) (Fig. 6).

DISCUSSION

The results presented in this study of vaccinia topoisomerase enhance our understanding of the eukaryotic type IB enzyme family as follows. (i) They define a catalytically active domain that is conserved between the cellular and poxvirus enzymes; (ii) they illuminate a clear distinction between structural elements required for transesterification chemistry in general and those required specifically for the cleavage reaction; (iii) they...
were performed as described in the legend to Fig. 3.

contain (per 20 μm)
tacts made by the two tyrosines induce a conformational
same effects on relaxation and cleavage as does removal of the
face. Elimination of the two tyrosine side chains responsible for
suggests a post-binding alteration of the protein-DNA inter-
make major groove contacts with the CCCTT target site (9).
available evidence that residues within the N-terminal domain
age reaction, which is not pertinent during religation. Although
that a post-binding, pre-chemical step applies during the cleav-
results indicate that the N-terminal domain is nonessential for
synthesis, in the cleavage reaction specifically,
esterification chemistry, in the cleavage reaction specifically,
alteration in the protease sensitivity of the hinge occurs prior to
transesterification. Moreover, single alanine substitutions at
residues Gly-132 and Tyr-136 within the hinge cause a reduc-
tion by more than 2 orders of magnitude in the rate of DNA
 cleavage, but only a modest effect on religation (17). These
effects are strikingly similar to those reported here for deletion of the N
term. We speculate that N-terminal domain and hinge dynamics may activate cleavage by properly orienting the catalytically essential residues with respect to the scissile phosphate.

The scissile phosphate and six other phosphates that contact the topoisomerase are located on the minor groove of the CCCTT target site (7). Our observation that the catalytic domain retains specificity for cleavage at CCCTT implies that vaccinia topoisomerase (i) interacts with the bases in the minor groove, (ii) senses the target site through indirect readout of backbone conformation, or (iii) recognizes the major groove through contacts intrinsic to the catalytic domain per se. Loss of contacts made by the N-terminal domain resulted in decreased affinity for the target site, which was manifest by salt and magnesium inhibition of single-turnover cleavage and DNA relaxation by Topo-(81–314). The salt and magnesium inhibition curves were essentially identical for Topo-(81–314) and Y70A/Y72A. We surmise that the two tyrosine side chains are largely responsible for the contribution made by the N-terminal domain to target site affinity. Studies of the effects of conservative replacement for Tyr-70 or Tyr-72 point to the aromatic character of these side chains as being important for enhancing DNA binding (18).

The present findings suggest that the distinctive target site specificities of the poxvirus and cellular type IB topoisomerases are not simply a function of structural differences between the domains N-terminal to the conserved catalytic core (20, 21). For the vaccinia enzyme, the specificity of cleavage is an intrinsic property of the core domain. This may also be the case for the cellular enzyme, to the extent that a core domain has been defined. Stewart et al. (28) have shown that active human topoisomerase can be reconstituted from a 58-kDa fragment derived from the central portion of the protein (this fragment contains all the residues of the vaccinia enzyme essential for transesterification except the active site tyrosine) plus a C-terminal 6.3-kDa fragment that includes the active site tyrosine. The fragment-reconstituted human enzyme cleaved DNA with the same sequence specificity as the full-sized protein. However, the cleavage-religation equilibrium of the reconstituted enzyme was skewed toward religation, DNA binding affinity was reduced, and the enzyme relaxed distributively (28). These properties of fragment-reconstituted human topoisomerase I vis à vis the intact enzyme are broadly similar to what we observe for the vaccinia catalytic domain. Nonetheless, the reconstituted human fragments together are still more than twice the size of the catalytic domain of the vaccinia enzyme.

Although mutational analysis of the vaccinia topoisomerase has implicated individual amino acid residues in general transesterification chemistry, in the cleavage reaction specifically, and in noncovalent DNA binding, a complete interpretation of these results will ultimately hinge on the availability of a crystal structure of the enzyme in both the free and DNA-

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2 C. Cheng, unpublished data.
bound states. Efforts in this and other laboratories to crystal-
lize the full-sized vaccinia topoisomerase have been unsuccess-
ful. We suspect this is due to flexibility of the protein at the
interdomain bridge and hinge segments. We recently achieved
success in crystallizing the catalytically active domain charac-
terized in this paper. The structure of the domain is under
refinement and will be reported separately.

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