Mutational analysis of vaccinia virus topoisomerase identifies residues involved in DNA binding

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ABSTRACT

Vaccinia DNA topoisomerase catalyzes the cleavage and re-joining of DNA strands through a DNA–(3′-phosphothioyl)–enzyme intermediate formed at a specific target sequence, 5′-(C/T)CCTT. The 314 aa protein consists of three protease-resistant structural domains demarcated by protease-sensitive interdomain segments referred to as the bridge and the hinge. The bridge is defined by trypsin-accessible sites at Arg80, Lys83 and Arg84. Photocrosslinking and proteolytic footprinting experiments suggest that residues near the interdomain bridge interact with DNA. To assess the contributions of specific amino acids to DNA binding and transesterification chemistry, we introduced alanine substitutions at 16 positions within a 24 aa segment from residues 63 to 86 (DSKRQRQFYGKMHVNQRNAKRD). Assays of the rates of DNA relaxation under conditions optimal for the wild-type topoisomerase revealed significant mutational effects at six positions; Arg67, Tyr70, Tyr72, Arg80, Arg84 and Asp85. The mutated proteins displayed normal or near-normal rates of single-turnover transesterification to DNA. The effects of amino acid substitutions on DNA binding were evinced by inhibition of covalent adduct formation in the presence of salt and magnesium. The mutant enzymes also displayed diminished affinity for a subset of cleavage sites in pUC19 DNA. Tyr70 and Tyr72 were subjected to further analysis by replacement with Phe, His, Gln and Arg. At both positions, the aromatic moity was important for DNA binding.

INTRODUCTION

The type IB DNA topoisomerase family includes eukaryotic topoisomerase I, a ubiquitous nuclear enzyme, and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (1). These proteins relax supercoiled DNA via a common reaction pathway, which involves non-covalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with concomitant formation of a covalent DNA–(3′-phosphothioyl)–protein intermediate, strand passage and strand religation. A shared structural basis for transesterification is inferred from the cellular and virus-encoded enzymes (2). However, the cellular and viral proteins display different site-specificities for covalent adduct formation on DNA. Vaccinia topoisomerase cleaves at sites containing the pentamer sequence 5′-(C/T)CCTT, immediately 5′ of the scissile bond (3,4). The cellular topoisomerases exhibit a loose preference for a four base motif, 5′-(A/T)(G/C)(A/T)T  (5).

Our aim is to understand how the vaccinia topoisomerase accomplishes target site recognition and transesterification chemistry. Four complementary experimental strategies have been employed, including: (i) identification of key contacts on the DNA target site by enzymatic and chemical footprinting; (ii) mapping of the protein side of the protein–DNA interface by proteolysis of the enzyme in the free and DNA-bound states; (iii) identification of specific DNA contact points on the enzyme by UV photocrosslinking; and (iv) comprehensive mutational analysis of the enzyme.

The 314 aa vaccinia virus topoisomerase consists of three protease-resistant structural domains demarcated by two protease-sensitive interdomain segments, which we refer to as the bridge and hinge (Fig. 1). Upon non-covalent binding of topoisomerase to duplex DNA, the bridge and hinge are protected from proteolysis (6), suggesting that the interdomain segments comprise part of the DNA binding surface. DNA footprinting and modification interference studies show that the topoisomerase makes contact with the base pairs and with the sugar–phosphate backbone of DNA within the CCCTT recognition site (7–9). Base-specific contacts are made in the major groove of the DNA, whereas contacts with specific phosphates, including the scissile phosphate, are made on both strands along the minor groove. The phosphate contacts are situated on the opposite face of the DNA helix from the base-specific contacts, which implies that vaccinia topoisomerase binds DNA circumferentially (8).

Available evidence, derived from mutational analysis of >100 aa of the vaccinia topoisomerase, indicates that transesterification reaction chemistry is carried out by the hinge and the adjacent C-terminal domain. The 20 kDa C-terminal domain includes the active site nucleophile (Tyr274) and three other residues (Lys167, Arg223 and His265) that are essential for DNA cleavage (10–15). Three more essential residues (Arg130, Gly132 and Tyr136) are located within or near the hinge (12,16). Kinetic analysis of the mutant enzymes prompted the hypothesis that the side chains of Arg130, Lys167, Arg223 and His263 interact directly with the scissile phosphate during the strand cleavage and religation steps (15,17). A priori, the active site Tyr274 must also be positioned at the scissile phosphate. None of these essential residues appears

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to play a role in site affinity, insofar as alanine substitutions that elicit from $10^{-2}$ to $10^{-7}$ decrements in transesterification rate have no significant effect on the non-covalent binding of topoisomerase to CCCTT-containing duplex DNA (14,15,17).

To date, we have identified only three residues of the topoisomerase at which alanine substitution decreases non-covalent DNA binding affinity. One of these is Ser204, which is located in the C-terminal domain. Replacement of Ser204 by alanine reduced the affinity of topoisomerase for CCCTT-containing DNA by a factor of 6 and slowed the rate of DNA cleavage by a factor of 70, but did not alter the site-specificity of the vaccinia topoisomerase for CCCTT and related pentamer sequences (18). A specific DNA contact point for Ser204 has not been delineated.

The two other residues implicated in site affinity are Tyr70 and Tyr72. These neighboring tyrosines were identified as the sites of UV crosslinking between topoisomerase and the +4 and +3 bromocytosine-substituted bases, respectively, of the CCCTT element (9). Tyr70 and Tyr72 are situated in the N-terminal domain just upstream of the interdomain bridge (Fig. 1). Sharma et al. (19) have solved the crystal structure of an N-terminal 77 aa fragment of vaccinia topoisomerase. We have used their structure to build a molecular model of the domain bound to CCCTT-containing B-form DNA (9). The protein fits into the major groove such that the side chains of Tyr70 and Tyr72 are in proximity to the C-5 atoms of the +4 and +3 cytosine bases (Fig. 2). The DNA docks into a concave surface of the protein from Arg67 to Val77. Our initial mutational studies suggested that Tyr70 and Tyr72 contribute to site affinity (9).

Here, we examine in greater detail the roles of Tyr70 and Tyr72 in DNA binding and covalent catalysis. In addition, we have conducted a mutational analysis of 14 other residues within a 24 aa segment that flanks these tyrosines and includes the interdomain bridge. The results suggest a role in DNA binding for residues Arg67, Arg80, Arg84 and Asp85.

**MATERIALS AND METHODS**

**Topoisomerase expression and purification**

Mutations were introduced into the vaccinia virus topoisomerase gene by using the two-stage PCR-based overlap extension method (20). NdeI–BglII restriction fragments containing the mutated topoisomerase genes were cloned into the T7-based expression vector pET3c. All mutations were confirmed by dideoxy sequencing. pET-based plasmids were transformed into Escherichia coli BL21. Topoisomerase expression was induced by infection with bacteriophage λCE6. Wild-type and mutant topoisomerases were purified from soluble bacterial lysates by phosphocellulose column chromatography (21). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (BioRad) with bovine serum albumin as the standard.

**Suicide cleavage assays**

An 18mer CCCTT-containing DNA oligonucleotide was 5’-end-labeled by enzymatic phosphorylation in the presence of [γ-32P]ATP and T4 polynucleotide kinase, then gel-purified and hybridized to a complementary 30mer strand. Cleavage reaction mixtures (20 µl) containing 50 mM Tris–HCl (pH 8.0), 0.5 pmol 18mer/30mer DNA and topoisomerase were incubated at 37°C. The reactions were quenched by adding SDS to 1%. The samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide. The extent of covalent adduct formation (expressed as the percent of the input 5’-32P-labeled oligonucleotide that was transferred to protein) was quantitated by scanning the dried gel using a FUJIX BAS1000 Bio-Imaging Analyzer.

Kinetic analysis of single-turnover cleavage was performed as described (14,15). Reaction mixtures containing (per 20 µl) 50 nM Tris–HCl (pH 8.0), 0.5 pmol 18mer/30mer DNA and 65 ng topoisomerase were incubated at 37°C. The reactions were initiated by adding the enzyme to prewarmed reaction mixtures. Aliquots (20 µl) were withdrawn at 5, 10, 20, 30 and 45 s, 1, 2 and 5 min, and
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We performed a mutational analysis of the segment of vaccinia topoisomerase from aa 63 to 86 (Fig. 1). This region is conserved in the topoisomerases encoded by six different genera of poxviruses: vaccinia virus, Shope fibroma virus, Orf virus, fowlpox virus, molluscum contagiosum virus and Ansmacta mooreie entomopoxvirus (22–27). Arg80 of the vaccinia enzyme is the principal site of proteolysis by trypsin (denoted by the arrow in Fig. 1). Tryptic cleavages also occur at nearby residues Lys83 and Arg84. These tryptic sites, which define the interdomain bridge, are protected when topoisomerase binds to duplex DNA (6).

We assessed the role of individual amino acid side chains by alanine-scanning mutagenesis. Sixteen positions were substituted singly by alanine to yield the following mutants: D63A, S64A, K65A, R67A, R68A, Y70A, F71A, Y72A, Q78A, N79A, R80A, N81A, K83A, R84A, D85A and R86A. In addition, we replaced mutating residues G66, G73, M75 and V77, because we felt that the side chains in these side chains would be unlikely to interact with DNA. H76 was mutated in a previous study and found to be non-essential for topoisomerase function in vitro (14). The mutant alleles of vaccinia topoisomerase were expressed in E.coli. The wild-type and mutant proteins were purified from soluble bacterial extracts. The topoisomerase polypeptide constituted the major species in the protein preparations, as determined by SDS–PAGE, and the extents of purification were essentially equivalent (Fig. 3).

Relaxation of supercoiled DNA

To assess the impact of these mutations, all proteins were tested for their ability to relax supercoiled plasmid DNA. Screening assays were performed in 0.1 M NaCl in the absence of MgCl2. The rate limiting step under these conditions is the dissociation of topoisomerase from the relaxed plasmid product (28,29). The rates of relaxation were determined at a fixed level of input protein. Wild-type topoisomerase (2.7 ng) relaxed 0.3 µg supercoiled pUC19 DNA to completion within 5 min (Fig. 4, –MgCl2). Relatively few reaction products of intermediate superhelicity were observed with the wild-type topoisomerase, suggesting that the enzyme relaxed individual DNA molecules to completion before dissociating and engaging a new DNA. The DNA relaxation assays were also performed in the presence of 5 mM MgCl2. Magnesium enhances product off-rate, without affecting the rate of DNA cleavage by the wild-type topoisomerase (28). Magnesium stimulated the activity of the wild-type enzyme such that 2.7 ng enzyme relaxed all supercoils in 30 s (Fig. 4, +MgCl2).

We observed that the relaxation rates of 10 of the 16 alanine-substituted proteins were equivalent to that of the wild-type enzyme in the absence of presence of magnesium: these were D63A, S64A, K65A, R68A, F71A, Q78A, N79A, N81A, K83A and R86A (data not shown). These 10 mutants also displayed wild-type activity in covalent adduct formation on a CCCTT-containing suicide cleavage substrate (not shown). Similar results had been reported for another mutant in this region, H76A (14). On the basis of these findings, we concluded that the side chains of Asp63, Ser64, Lys65, Arg80, Phe71, His76, Gln78, Asn79, Asn81, Lys83 and Arg86 are unimportant for topoisomerase function in vitro. These proteins were not analyzed further.
Effect of Tyr70 mutations on DNA relaxation

Substitution of Tyr70 by alanine had no effect on the kinetics of DNA relaxation in the absence of magnesium. However, the Y70A mutant displayed an aberrant response to magnesium. Y70A was not stimulated by magnesium; indeed, its rate of relaxation was actually lower by a factor of 5 in the presence of 5 mM MgCl2 than in its absence (Fig. 4). Paradoxical inhibition of Y70A by magnesium (in contrast to the stimulatory effect on wild-type enzyme) suggest that this mutant has altered affinity for magnesium and was inhibited further in its presence (Fig. 4).

In order to gauge which structural features of the amino acid side chain were relevant to this effect, we introduced alternative functional groups. Replacement of Tyr70 by Phe, His, Gln or Arg had no detrimental effect on the rates of relaxation in the absence of magnesium; in fact, the Y70H and Y70F mutants relaxed slightly faster than did the wild-type enzyme (Fig. 4). Yet, each of the Y70 mutants responded abnormally to divalent cation. The conservative changes in Y70F and Y70H caused a pronounced inhibition of relaxation by magnesium. Under conditions optimal for the wild-type topoisomerase (100 mM NaCl, 5 mM MgCl2), Y70F and Y70H relaxed DNA at one-fourth to one-half the wild-type rate, whereas Y70A, Y70Q and Y70R reacted at 1–5% of the wild-type rate.

Effect of Tyr72 mutations on DNA relaxation

Replacement of Tyr72 by alanine slowed the rate of DNA relaxation by about a factor of 8 in the absence of magnesium (Fig. 5). Intermediate topoisomers were prominent during relaxation by Y72A. This suggests that the Y72A mutant is more distributive than wild-type in its action; i.e. it is more prone to dissociate to a new substrate molecule before relaxing to completion. Inclusion of magnesium nearly abolished DNA relaxation by Y72A (Fig. 5).

Conservative substitutions of Tyr72 with Phe and His had no deleterious effect on relaxation in the absence of magnesium; indeed, Y72F and Y72H relaxed slightly faster than wild-type topoisomerase (Fig. 5). Y72F and Y72H were slowed by factors of 2 and 8, respectively in the presence of magnesium. Non-conservative replacements by Arg and Gln were considerably more deleterious than simple side-chain removal (i.e. by alanine substitution). Y72R was inactive in the presence or absence of divalent cation, whereas Y72Q relaxed freely in the absence of magnesium and was inhibited further in its presence (Fig. 5).

Conditions optimal for the wild-type topoisomerase (NaCl plus MgCl2), Y72F and Y72H relaxed DNA at one-eighth to one-twentieth the wild-type rate, whereas Y70A, Y70Q and Y70R reacted at <1% of the wild-type rate.

To determine which component step(s) of the topoisomerase reaction were affected by the Y70 and Y72 mutations, we subjected the mutant proteins to a detailed biochemical characterization as described below. The wild-type topoisomerase was analyzed in parallel.

Effects of Y70 and Y72 mutations on DNA cleavage

A suicide substrate containing a single CCCTT cleavage site for vaccinia topoisomerase was used to examine the transesterification reaction under single-turnover conditions in the absence of added salt and magnesium. The substrate consisted of an 18mer scissile strand annealed to a 30mer strand (Fig. 6). Upon formation of the covalent protein–DNA adduct, the distal cleavage product 5′-ATTCCC is released and the topoisomerase becomes covalently trapped on the DNA. The extent of cleavage by the wild-type topoisomerase during a 5 min reaction was proportional to added enzyme; >90% of the input DNA became covalently bound at saturation (Fig. 6A). The concentration-dependence of the cleavage activity profiles of each of the Y70 mutants (Y70A, Y70F, Y70H, Y70Q and Y70R) was similar to that of the wild-type, with 85–95% of the input substrate becoming covalently bound in 5 min (Fig. 6A). Among the Y72 mutants, the titration profile of Y72F was identical to the wild-type (Fig. 7A). Y72H, Y72A and Y72Q attained similar extents of cleavage at saturation, although the titration curves were shifted slightly to the right. The yield of covalent adduct by Y72R at saturation was less than half the wild-type value (Fig. 7A).

Measurements of covalent adduct formation in our screening cleavage assay (5 min reaction reflecting the yield of covalent protein–DNA complex) could obscure or underestimate mutational effects on reaction rate. We therefore measured the rates of DNA cleavage by wild-type topoisomerase and selected Y70 and Y72 mutants under conditions of enzyme excess. The apparent rate constant (kcl) for wild-type enzyme was 0.36 s⁻¹ (data not shown). The observed rate constants for Y70A (0.12 s⁻¹), Y70F (0.22 s⁻¹), Y70H (0.28 s⁻¹), Y70R (0.17 s⁻¹) and Y70Q (0.13 s⁻¹) indicated that these mutations had, at most, a 2–3-fold effect on cleavage. The Y72A (kcl = 0.16 s⁻¹) and Y72F (kcl = 0.06 s⁻¹) mutations caused 9-fold and 6-fold decrements, respectively, whereas Y72H (kcl = 0.16 s⁻¹) and Y72F (kcl = 0.18 s⁻¹) caused only a 2-fold rate effect (data not shown). Control experiments employing a native gel mobility shift assay of topoisomerase–DNA complex formation on a 32P-labeled CCCTT-containing 24 bp DNA duplex ligand (15) indicated that the levels of input wild-type and mutant proteins used in the kinetic analyses were sufficient to bind the input DNA nearly quantitatively under the solution conditions of the cleavage assay, i.e., in the absence of salt or magnesium (data not shown).

In previous studies (14–18), we have classified mutational effects on cleavage rate as follows. Residues at which side chain removal by alanine substitution results in a ≥10⁻² effect on cleavage rate are defined as essential. Residues at which side-chain removal elicits a ≥10⁻¹ but ≤10⁻² effect on reaction rate are deemed important. Residues at which alanine substitution causes less than an order of magnitude rate effect are regarded as...
Figure 6. Effect of Y70 mutations on suicide DNA cleavage. Suicide cleavage was assayed as described in Materials and Methods. The structure of the 5′-32P-labeled suicide substrate is depicted at the top of the figure. (A) Enzyme titration. Cleavage reaction mixtures containing 0.5 pmol of the suicide DNA substrate and wild-type or mutant topoisomerase as indicated were incubated for 5 min at 37°C. Covalent complex formation is plotted as a function of input protein. (B) Effect of NaCl. (C) Effect of MgCl2. Reaction mixtures containing 0.5 pmol DNA substrate and 65 ng topoisomerase were supplemented with NaCl (B) or MgCl2 (C) as indicated. Reactions were initiated by adding protein and terminated after incubation at 37°C for 30 s. The extents of covalent complex formation were normalized to that of the unsupplemented control reaction (defined as 100%). The normalized cleavage data are plotted as a function of the concentration of added salt (B) or magnesium (C).

Figure 7. Effect of Y72 mutations on suicide DNA cleavage. (A) Enzyme titration. Cleavage reaction mixtures containing 0.5 pmol suicide DNA substrate and wild-type or mutant topoisomerase as indicated, were incubated for 5 min. (B) Effect of NaCl. (C) Effect of MgCl2. Reaction mixtures containing 0.5 pmol DNA substrate and 65 ng topoisomerase were supplemented with NaCl or MgCl2 as indicated. Reactions were initiated by adding protein and terminated after incubation at 37°C for either 30 s (WT, Y72F, Y72H, Y72A and Y72Q) or 5 min (Y72R). The extents of covalent complex formation were normalized to that of the unsupplemented control reaction (defined as 100%).

Effects of NaCl and MgCl2 on DNA cleavage by Y70 and Y72 mutants

Suicide cleavage reactions are routinely performed at low ionic strength in the absence of a divalent cation. Prior studies showed that the rate of single-turnover cleavage by the wild-type vaccinia topoisomerase was unaffected by the levels of salt and magnesium that strongly stimulate DNA relaxation under steady-state conditions (28,29). It has been argued that salt and magnesium stimulate relaxation by enhancing product dissociation, rather than by affecting the chemical steps of transesterification. The finding that DNA relaxation by Y70 and Y72 mutant enzymes was either unstimulated or actually inhibited by magnesium suggested that pre-cleavage binding might be limiting under these conditions.

To address this issue, we examined the effects of salt and magnesium on suicide cleavage. The amounts of covalent adduct formed in 30 s in the presence of 50, 100, 150 and 200 mM NaCl, or 1, 2, 5 and 7.5 mM MgCl2 were measured and normalized to the extent of cleavage in unsupplemented control reactions. The salt effects on Y70 mutants are shown in Figure 6B and magnesium effects are shown in Figure 6C. We observed that the wild-type topoisomerase was unaffected by up to 150 mM NaCl, but was inhibited by 24% at 200 mM NaCl. In contrast, covalent adduct formation by the Y70 mutants was salt-sensitive. Y70F and Y70H were least affected, whereas Y70R, Y70A and Y70Q were inhibited significantly at 150 mM NaCl and were virtually inactivated at 200 mM (Fig. 4B). Wild-type topoisomerase, Y70F and Y70H were unaffected by magnesium up to 7.5 mM, whereas Y70R, Y70A and Y70Q were inhibited progressively by 1–7.5 mM MgCl2. Susceptibility to salt and magnesium inhibition suggested that Y70R, Y70A and Y70Q bound less avidly to the non-essential. These definitions are reasonable when one considers that the wild-type topoisomerase accelerates the rate of transesterification by an estimated factor of 10⁹ (28). According to these criteria, Tyr70 and Tyr72 do not make a significant contribution to transesterification chemistry. The 10⁻² effect on kₐ seen with the Y72R mutant (data not shown) is presumably caused by the introduction of a positive charge and is, therefore, not directly instructive regarding the contribution of Tyr72 to enzyme activity.
Figure 8. DNA relaxation by R67A, R80A, R84A and D85A. Assays were performed as described in the legend to Figure 4.

CCCTT-containing DNA substrate under the solution conditions used to assay DNA relaxation.

The Y72 mutants were more sensitive to salt and magnesium than were the Y70 mutants (Fig. 7B and C). An instructive hierarchy of mutational effects was evident: Y72F was affected least by salt and magnesium; Y72H displayed intermediate sensitivity; and mutants Y72A, Y72Q and Y72R were inhibited significantly. These data explain the profound defects in DNA relaxation by Y72Q, Y72R and Y72A in the presence of 0.1 M NaCl ± 5 mM MgCl₂.

Effects of R67A, R80A, R84A and D85A mutations on topoisomerase activity

Mutant enzymes R67A, R80A and R84A each relaxed supercoiled DNA about four times faster than wild-type topoisomerase in 0.1 M NaCl, in the absence of magnesium (Fig. 8). This finding was noted consistently in multiple experiments. Inclusion of 5 mM MgCl₂ reduced the rates of relaxation by R67A, R80A and R84A by about a factor of 4 (Fig. 8). R80A and R84A also appeared to generate slightly higher levels of intermediate topoisomers than wild-type enzyme. Mutant D85A relaxed supercoils at ~25% the wild-type rate in the absence of magnesium and was neither stimulated nor inhibited when magnesium was included.

Mutational effects on suicide DNA cleavage were assessed. The dependence of covalent adduct formation on R67A, R80A, R84A and D85A protein concentration was similar to that of the wild-type topoisomerase (Fig. 9A). Kinetic analysis showed that the cleavage rate constants were either unaffected (R67A $k_1 = 0.35$ s⁻¹) or minimally affected (R80A $k_1 = 0.22$ s⁻¹, R84A $k_1 = 0.23$ s⁻¹, D85A $k_1 = 0.23$ s⁻¹) (data not shown). Hence, Arg67, Arg80, Arg84 and Asp85 are not involved in transesterification chemistry.

Suicide cleavage by R67A, R80A and R84A was salt sensitive (Fig. 9B). Covalent adduct formation was reduced by 39–56% at 150 mM NaCl and 91–97% at 200 mM NaCl. D85A was unaffected by 150 mM NaCl and only slightly more sensitive than wild-type to 200 mM NaCl. The extents of cleavage by R67A, R80A, R84A and D85A were not affected by magnesium in the range 1–7.5 mM (data not shown). However, magnesium did inhibit cleavage by R67A, R80A and R84A when added in the presence of 0.1 M NaCl (Fig. 9C). Note that 0.1 M NaCl by itself had no effect on these proteins. D85A was not affected to the same extent by the combination of 0.1 M NaCl and MgCl₂.

Cleavage of pUC19 DNA

Topoisomerase action on a complex DNA molecule like pUC19 is not restricted to a single site as it is in the suicide cleavage substrate. Rather, the wild-type enzyme can bind and cleave at multiple sites on the plasmid DNA; such sites, when mapped at nucleotide resolution, contain the pentameric motif 5′-(C/T)CCTT immediately preceding the site of strand scission (3). We examined the effects of alanine-substitution mutations on cleavage site choice by incubating wild-type and mutant topoisomerases with linear pUC19 DNA that had been cut with XbaI and 3′-end-labeled with [α-32P]dCMP on both DNA strands (Fig. 10). These assays were performed at low ionic strength in the absence of a divalent cation. Addition of SDS to the mixture traps the covalently bound protein on the unlabeled portion of the DNA strand, permitting localization of the sites of strand cleavage by size analysis of the cleavage products under denaturing conditions. The sizes of the radiolabeled cleavage products reflect the distance of the cleavage sites from the 3′-ends of the cleaved strand. The wild-type enzyme cleaved at two ‘high-affinity’ sites (indicated by the arrows in Fig. 10) and multiple other lower

![Figure 9](https://academic.oup.com/nar/article-abstract/25/18/3649/1046341/Mutational-analysis-of-vaccinia-virus/16563260)
affinity sites (3,9). The high affinity sites are occupied at lower enzyme concentrations and cleavage at these sites is relatively insensitive to salt, divalent cations and temperature as reaction variables (3). Note that the signal intensity of the cleavage product at saturating enzyme concentration is dictated by the internal cleavage–religation equilibrium, which can differ among the various cleavage sites. (Also, when multiple topoisomerase molecules cleave the DNA, the signal intensity at sites located furthest away from the labeled 3'-end may diminish.)

The mutant proteins that had normal topoisomerase activity generally displayed a wild-type or near-wild-type cleavage pattern on pUC19 DNA (Fig. 10). In contrast, cleavage of pUC19 by the Y70A, Y72A, R80A, R84A and D85A proteins was limited to a subset of the sites that were cleaved by the wild-type topoisomerase (Fig. 10). The sites that were cleaved best by these mutants were the two high affinity sites. Cleavage at representative low affinity sites (denoted by circles in Fig. 10) was most sensitive to mutagenesis, as would be expected if the mutations in question affect site affinity, but not reaction chemistry. None of the mutations caused the topoisomerase to cleave at sites not normally cleaved by the wild-type enzyme.

**DISCUSSION**

Multiple points of contact between vaccinia topoisomerase and DNA are likely to contribute to site specificity and site affinity. Few of these are documented. The capacity of the 20 kDa C-terminal segment for non-specific low-affinity DNA binding (6) suggests that this domain may interact with the phosphodiester backbone. The proposed model for circumferential binding of the enzyme at the target site entails protein–phosphate interactions along the minor groove on the side of the helix that includes the scissile bond (8). The amino acid residues that make these contacts have not been identified, although Ser204 has emerged as a candidate based on the effects of alanine substitution, i.e., reduced binding affinity, slowed forward cleavage rate (causing a reduction in the cleavage equilibrium constant), but little comparative effect on strand religation rate (18). Incremental deletion of 10, 15 and 20 aa from the C-terminus of the topoisomerase resulted in reduced DNA binding affinity and a more distributive mode of relaxation (30); however, no specific residues responsible for this effect were identified. It was suggested that the C-terminal end of the protein may comprise part of the circumferential binding surface without making direct contact with DNA (30). The interdomain hinge region is implicated as a DNA contact point, most likely with the scissile phosphate itself (6,17). Yet, mutations of residues in the hinge which have major impact on transesterification chemistry have little or no effect on non-covalent binding to DNA. This suggests that binding and chemistry are mediated by distinct moieties on the enzyme.

The present study provides evidence that specific residues located at the distal end of the N-terminal domain and within the interdomain bridge play a role in site affinity. Crosslinking studies had indicated that Tyr70 and Tyr72 make major groove contacts with the +4C and +3C bases of the CCCTT element (9). Our initial experiments on Y70A and Y72A revealed decreased rates of relaxation and single-turnover cleavage; however, the assays were performed in the presence of salt and/or magnesium (9). Here, by measuring the rates of suicide cleavage under non- stringent reaction conditions, we showed that Y70A and Y72A had no significant effects on the transesterification reaction. This is consistent with our model of the protein–DNA interface, which placed Tyr70 and Tyr72 on the opposite side of the DNA helix from the scissile bond, i.e., away from the active site (Fig. 2).

The expectation is that alterations of Tyr70 or Tyr72 would reduce affinity of the topoisomerase for the CCCTT site, because salt and magnesium are known to influence DNA binding by the topoisomerase, but not reaction chemistry (3), we have assayed solute effects on single-turnover cleavage as an indicator of mutational effects on DNA binding (18,30). Salt and magnesium inhibit single turnover cleavage by the various Y70 and Y72 mutants at concentrations that do not affect the wild-type enzyme. The concentration-dependence of the inhibition curves reflects the magnitude of the binding defect. Several general conclusions can be drawn from our results. First, it is clear that Tyr72 plays a more important role in DNA binding than Tyr70. For example, 0.1 M NaCl reduces Y72A cleavage by 84%, compared to a 10% reduction for Y70A; 2 mM MgCl2 inhibits Y72A cleavage by 77%, compared to 4% for Y70A. Second, structure–function relationships can be gleaned by comparing conservative and non-conservative substitutions. At position 72, the solute effects on cleavage suggested a hierarchy of binding affinities: Tyr > Phe > His > Gln > Ala, Arg. At position 70, the apparent order of affinities was Tyr > Phe, His > Arg, Ala > Gln. The same hierarchies were observed when we examined the effects of Y70 and Y72 mutations on the cleavage of pUC19 DNA (J. Sekiguchi, unpublished). The common thread at both positions is that optimal function depends on a side chain with a planar ring that has resonance structure. Tyr versus Phe comparisons suggest a modest contribution of the tyrosine hydroxyl moiety to DNA binding; effects of Phe replacement were apparent only at high concentrations of salt and magnesium. Tyr70 is strictly conserved

![Figure 10](https://academic.oup.com/nar/article-abstract/25/18/3649/1046341/Mutational-analysis-of-vaccinia-virus/3655)

Figure 10. Mutational effects on cleavage site selection. pUC19 DNA was linearized with XbaI and 3'-end-labeled on both strands with [α-32P]dCMP using Klenow DNA polymerase. Reaction mixtures (20 μl) containing 50 mM Tris–HCl (pH 8.0), 10 ng 3'-end-labeled XbaI-cut pUC19 DNA and 10 ng of wild-type (WT) or mutant topoisomerase were incubated at 37°C for 10 min. Reactions were halted by adding SDS to 0.2% final concentration. The samples were digested with 10 μg proteinase K for 60 min at 37°C. The mixtures were adjusted to 50% formamide and then heated at 95°C for 5 min. The reaction products were analyzed by electrophoresis through a 4% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris–borate, 2.5 mM EDTA). An autoradiograph of the dried gel is shown. A control reaction (lane –) contained no topoisomerase. The positions and sizes (nt) of denatured end-labeled DNA markers are indicated on the right. The products of cleavage at high affinity sites (3,9) are denoted by arrows on the left.
in all poxvirus topoisomerases; Tyr72 is either tyrosine or phenylalanine (Fig. 1).

The solute effects on binding can be invoked to explain the mutational effects on relaxation of supercoiled plasmid DNA. The rate limiting step for wild-type topoisomerase under steady state conditions is product dissociation; this step is accelerated by either NaCl or magnesium (28,29). At 0.1 M NaCl, dissociation of the relaxed product remains rate-limiting for wild-type enzyme, insofar as supplementation with magnesium is still stimulatory. Mutations that lower binding affinity via an increased off-rate may relax supercoils as fast or even faster than wild-type rates (as for R67A, R80A and R84A), provided that the chemical steps are not grossly defective. Some mutations (Y72A) may enhance enzyme dissociation prior to complete relaxation. Increased distributivity is reflected in higher levels of partially relaxed topoisomers. We posit that supplementation of the NaCl containing reactions with magnesium affects substrate binding by the affinity mutants to an extent that binding becomes rate limiting for those proteins; hence the inhibition of relaxation.

Our experiments provide the first functional data on the role of the interdomain bridge in DNA binding. This segment is trypsin-accessible in the free state, but is protected when the interdomain bridge in DNA binding. This segment is limiting for those proteins; hence the inhibition of relaxation.

Arg67, which is implicated in DNA binding, is located within the N-terminal domain on the concave protein surface that includes Tyr70 and Tyr72. Although it is tempting to invoke contacts between this conserved basic side chain and the phosphate backbone of DNA, it should be mentioned that the guanidinium moiety of the arginine side chain is pointing away from the DNA phosphates in the model structure (Fig. 2). However, the crystal structure of the free domain may not accurately reflect the orientation of the side chain in the DNA-bound state and bond rotation within the side chain might permit interaction with the DNA.

In conclusion, we have defined a segment of the vaccinia topoisomerase that participates in DNA binding and have identified six individual amino acid residues that contribute to site affinity. Efforts to crystallize the DNA-bound topoisomerase are in progress.

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REFERENCES