Mutational Analysis of 39 Residues of Vaccinia DNA Topoisomerase Identifies Lys-220, Arg-223, and Asn-228 as Important for Covalent Catalysis*

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Vaccinia DNA topoisomerase, a 314-amino acid type I enzyme, catalyzes the cleavage and rejoining of DNA strands through a DNA-(3'-phosphotyrosyl)-enzyme intermediate. To identify amino acids that participate in the transesterification reaction, we introduced alanine substitutions at 39 positions within a conserved 57-amino acid segment upstream of the active-site tyrosine. Purified wild type and mutant proteins were compared with respect to their activities in relaxing supercoiled DNA. The majority of mutant proteins displayed wild type topoisomerase activity. Mutant enzymes that relaxed DNA at reduced rates were subjected to kinetic analysis of the strand cleavage and religation steps under single-turnover and equilibrium conditions. For the wild type topoisomerase, the observed single-turnover cleavage rate constant (k_{cat}) was 0.29 s⁻¹ and the cleavage-religation equilibrium constant (K_{eq}) was 0.22. The most dramatic mutational effects were seen with R223A; removal of the basic side chain reduced the rates of cleavage and religation by factors of 10⁻⁴ and 10⁻⁵, respectively, and shifted the cleavage-religation equilibrium in favor of the covalently bound state (K_{eq} = 1). Introduction of lysine at position 223 restored the rate of cleavage to 1/10 that of the wild type enzyme. We conclude that a basic residue is essential for covalent catalysis and suggest that Arg-223 is a constituent of the active site. Modest mutational effects were observed at two other positions (Lys-220 and Asn-228), at which alanine substitutions slowed the rates of strand cleavage by 1 order of magnitude and shifted the equilibrium toward the noncovalently bound state. Arg-223 and Lys-220 are conserved in all members of the eukaryotic type I topoisomerase family; Asn-228 is conserved among the poxvirus enzymes.

The eukaryotic type I DNA topoisomerase family includes the nuclear type I enzymes and the topoisomerases encoded by vaccinia and other poxviruses. These proteins relax supercoiled DNA via a common reaction mechanism, which involves noncovalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with concomitant formation of a covalent DNA-(3'-phosphotyrosyl)-protein intermediate, strand passage, and strand religation (1, 2). A shared structural basis for transesterification and strand passage is inferred from the considerable amino acid sequence conservation between the cellular and virus-encoded enzymes (2, 3).

The 314-amino acid vaccinia virus topoisomerase enzyme is the smallest topoisomerase known and likely constitutes the minimal functional unit of a type I enzyme (4). (The cellular type I enzymes vary in size from 765 to 1019 amino acids.) Our aim is to construct a comprehensive structure-function map of the vaccinia topoisomerase by mutagenesis. After performing initial studies via random mutagenesis (5–7), we adopted the alanine-scanning approach to define the amino acid side chains important for covalent catalysis (8, 9). The advantage of this scanning technique is that elimination of an amino acid side chain beyond the β-carbon immediately addresses the essentiality of that particular residue. We previously mutagenized a continuous 41-amino acid segment of the vaccinia topoisomerase extending from residue 126 to residue 167. Fifty-six different mutations were generated, with at least one substitution at each position. Four residues (Arg-130, Gly-132, Tyr-136, and Lys-167) were defined as essential, i.e. substitution by alanine (or in the case of Gly-132, by Ser) either eliminated or severely reduced (by 2 orders of magnitude) activity in DNA relaxation and the formation of the covalent DNA-protein intermediate (5–8). Arg-130, Gly-132, and Lys-167 are strictly conserved in every member of the eukaryotic type I enzyme family. Tyr-136 is conserved among the four known poxvirus-encoded topoisomerases. All other amino acids in the targeted region, including many conserved residues, were nonessential. (We regard as nonessential those residues at which side-chain removal has less than 1 order of magnitude effect on catalysis.)

In the present study, we extend the analysis into a conserved downstream region from residue 213 to residue 269. Thirty-nine of the 57 positions were substituted individually by alanine. After expressing and purifying the mutant proteins, we assessed mutational effects on DNA relaxation and transesterification. Most of the mutations studied had little impact on enzyme activity. Mutations at Lys-220, Arg-223, and Asn-228 elicited significant effects on the rate of DNA cleavage and on the cleavage-religation equilibrium. Replacement of Arg-223 by alanine reduced the rates of cleavage and religation by more than 4 orders of magnitude.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced into the vaccinia virus topoisomerase gene by using the two-stage PCR-based overlap extension method (10). Plasmid pA9topo (11) was the template for the first stage PCR reaction. Gene fragments with overlapping ends obtained from the first-stage reactions were paired and used as templates in the second-stage amplification. Products containing the entire topoisomerase gene were cloned into the T7-based expression vector pET3c as described (7). All mutations were confirmed by dyelex sequencing.

Topoisomerase Expression and Purification—pET-based plasmids were transformed into Escherichia coli BL21. Topoisomerase expression was induced by infection with bacteriophage λC6 (11). Wild type and mutant topoisomerases were purified from soluble bacterial lysates.
FIG. 1. Domain structure and regional mutagenesis of vaccinia topoisomerase. The tripartite domain structure of the 314-amino acid vaccinia topoisomerase is illustrated. The protease-resistant structural domains are punctuated by protease-sensitive interdomain bridge and hinge segments (12). The active-site Tyr-274 is situated within the C-terminal domain. The amino acid sequence of vaccinia virus topoisomerase (vv) from residue 213 to residue 274 is aligned with the homologous segments of the topoisomerases encoded by other members of the poxvirus family: Shope fibroma virus (sf), molluscum contagiosum virus (mc), Orf virus (ov), and fowlpox virus (fp). (For protein sequences, see Refs. 4 and 27–30.) Conserved residues are denoted by asterisks. The two regions targeted for mutagenesis in this study (positions 213–240 and 249–269) are demarcated by the shaded boxes.

by phosphocellulose column chromatography (5). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (Bio-Rad) with bovine serum albumin as the standard.

Suicide Cleavage Assays—An 18-mer CCCTT-containing DNA oligonucleotide was 5' end-labeled by enzymatic phosphorylation in the presence of [γ-32P]ATP and T4 polynucleotide kinase and then gel-purified and hybridized to complementary 30-mer strand (present at a 4-fold molar excess). Cleavage reaction mixtures containing (per 20 μl) 50 μm Tris-HCl (pH 7.5), 0.5 pmol of 18-mer/30-mer DNA, and topoisomerase were incubated at 37 °C. Covalent complexes were denatured by addition of SDS to 1%. The samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Free DNA migrated near the bromphenol blue dye front. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide. The samples were electrophoresed through a 15% polyacrylamide gel containing 7M urea in TBE (90 mM Tris borate, 2.5 mM EDTA). The cleavage product, a 32P-labeled 30-mer strand, was visualized by autoradiography.

The volume was adjusted to 100 μl containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.5 pmol of 18-mer DNA duplex, and topoisomerase were incubated at 37 °C. Covalent complexes were denatured by addition of SDS to 1%. The samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Free DNA migrated near the bromphenol blue dye front. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide. The extent of covalent adduct formation (expressed as the percent cleaved) was determined by the dye-binding method (Bio-Rad) with bovine serum albumin as the standard. The extent of strand cleavage was quantitated by scanning the dried gel using a FUJIX BAS1000 Bio-Imaging Analyzer. A plot of the percent of input DNA cleaved versus time established end point values for cleavage. The data were normalized to the end point values, and kcat was determined by fitting the data to the equation (100 – %Chom) = 100e-kcat.

Equilibrium Cleavage Assays—A 60-mer oligonucleotide containing a centrally placed CCCTT element was 5' end-labeled, gel-purified, and annealed to an unlabeled complementary 60-mer strand. Cleavage reaction mixtures (20 μl) containing 50 μm Tris-HCl (pH 7.5), 0.5 pmol of 60-mer DNA duplex, and topoisomerase were incubated at 37 °C. Covalent complexes were denatured by addition of SDS to 0.2%. The samples were digested for 60 min at 37 °C with 10 μg of proteinase K. The volume was adjusted to 100 μl, and the digests were then extracted with an equal volume of phenol/chloroform. DNA was recovered from the aqueous phase by ethanol precipitation. The pelleted material was dissolved in formamide, and the samples were electrophoresed through a 15% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris borate, 2.5 mM EDTA). The cleavage product, a 32P-labeled 30-mer bound to a short peptide, was well-resolved from the input 60-mer substrate. The extent of strand cleavage was quantitated by scanning the gel with a Bio-Imaging Analyzer.

RESULTS

Mutagenesis Strategy and Production of Mutant Proteins—The 314-amino acid vaccinia topoisomerase consists of three protease-resistant structural domains demarcated by two protease-sensitive segments referred to as the bridge and hinge (Fig. 1) (12, 13). The 9-kDa N-terminal domain (residues 1–80) is implicated in recognizing the 5'-CCCTT target sequence at which vaccinia topoisomerase cleaves duplex DNA (14). The hinge and adjacent C-terminal domain, which includes the active-site nucleophile (Tyr-274), are implicated in the chemistry of transesterification (6–8, 12, 15). The region of vaccinia topoisomerase from position 213 to position 240 is one of the more highly conserved segments of the protein vis à vis three poxvirus-encoded homologues (19 of 28 residues conserved). The sequence of this region is highlighted in the shaded box at the left in Fig. 1. Moreover, the subsegment G/I/LXXKAXRYT is conserved in all cellular type I topoisomerases (2, 3). Prior mutational studies showed that substitutions of invariant residue Arg-223 with Asn, Glu, or Gly inactivated the vaccinia topoisomerase (6, 16), whereas alterations of conserved residues Gly-216 (to Lys) and Ile-217 (to Pro) had little or no effect (16).

These findings prompted us to systematically assess the role of individual amino acid side chains within this region by alanine-scanning mutagenesis. Twenty-one of the 28 positions were substituted singly by alanine. We eschewed mutating Gly-216 and Ile-217 because these had already been found to be nonessential (16). We also demurred on mutating Ile-219, Leu-222, Val-227, Leu-232, and Val-239 because it was felt that aliphatic side-chains were less likely to participate directly in transesterification chemistry. In addition, we replaced Arg-223 with lysine. The 22 mutant alleles of vaccinia topoisomerase were expressed in E. coli. The wild type and mutant proteins were purified from soluble bacterial extracts by phosphocellulose column chromatography. The topoisomerase polypeptide constituted the major species in the protein preparations, as determined by SDS-polyacrylamide gel electrophoresis, and the extents of purification were essentially equivalent (Fig. 2).

Mutational Effects on Relaxation of Supercoiled DNA—To assess the impact of these mutations, all proteins were tested for their ability to relax supercoiled plasmid DNA in vitro. Screening assays were performed in the absence of magnesium. The rate-limiting step under these conditions is the dissociation of topoisomerase from the relaxed plasmid product (17).

Activity was assayed initially by end point dilution. Then, the rates of relaxation were determined at a fixed level of input protein. 2.7 ng of wild type topoisomerase relaxed 0.3 μg of supercoiled pUC19 DNA to completion within 2 min (Fig. 3). We observed that the relaxation rate of 17 of the mutant proteins was equivalent to that of the wild type enzyme. These were K213A, Q214A, F215A, R218A, T224A, Y225A, G226A, Y229A, T230A, F231A, Y233A, N234A, F235A, W236A, T237A, N238A, and K240A. (The results for F215A are shown in Fig. 3; other data are not shown.) In contrast, the R232A mutant catalyzed no detectable relaxation in 15 min (Fig. 3). Conservative substitution of Arg-223 by Lys elicited a milder effect. R232K relaxed DNA at about one-tenth the wild type rate (Fig. 3). Modest rate reductions were also observed for K221A, K220A, and N228A (Fig. 3).
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 same distribution of products was observed for mutants R223K and K220A, which relaxed at reduced rates. However, intermediate topoisomers were prominent during relaxation by D221A and N228A (Fig. 3). This situation could arise if the rate of supercoil release on plasmid DNA were slowed or if the mutant enzyme became more distributive in its action, i.e., dissociating to a new substrate molecule before relaxing to completion. The DNA relaxation assays were also performed in the presence of 5 mM magnesium. (Magnesium enhances product off-rate without affecting the rate of DNA cleavage (17).) Magnesium stimulated the activity of the wild type enzyme such that 2.7 ng of enzyme relaxed all supercoils within 15 s (Fig. 3). None of the mutant proteins shown in Fig. 3 were stimulated by magnesium, suggesting that a step other than product release was rate-limiting.

To determine which component step(s) of the topoisomerase reaction was affected by the engineered mutations, we subjected the K220A, D221A, R223K, R223A, and N228A proteins to a detailed biochemical characterization as described below. The wild type and F215A proteins were analyzed in parallel.

Mutational Effects on DNA Cleavage—A “suicide” substrate containing a single CCCCTT cleavage site for vaccinia topoisomerase (18, 19) was used to examine the transesterification reaction under single-turnover conditions. The substrate consisted of an 18-mer scissile strand annealed to a 30-mer complementary strand (Fig. 4). 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...
value. We used this datum to estimate a wild type rate constant cleavage by wild type enzyme at 5 s was 77% of the end point seen for F215A (R223A—)

acceptor strand (5’-ATTCCGATAGTGACTACA) to a concentration of 25 pmol/20 μl (i.e. a 50-fold molar excess over the input DNA substrate).

Religation to the heterologous acceptor will yield a 5’-32P-labeled 30-mer strand transfer product. Aliquots (20 μl) were withdrawn at the times indicated and quenched immediately. The samples were heat-denatured and then electrophoresed through a 15% polyacrylamide containing 7 μl urea in TBE. The extent of religation (expressed as the percent of the input labeled 18-mer strand recovered as 30-mer) is plotted as a function of reaction time.

(Fig. 4, left panel). The concentration dependence of the F215A, K220A, D221A, R223K, and N228A activity profiles was similar to that of the wild type, with 80–90% of the input substrate becoming covalently bound in 5 min. In contrast, the R223A mutant catalyzed no detectable DNA cleavage in 5 min.

Suicide cleavage by the wild type topoisomerase was nearly complete within 10 s at 37 °C (Fig. 4, right panel). The extent of cleavage by wild type enzyme at 5 s was 77% of the end point value. We used this datum to estimate a wild type rate constant of 0.29 s−1. Relativel mild effects on the rate of cleavage were seen for F215A (kobs = 0.09 s−1) and D221A (kobs = 0.08 s−1). In contrast, R220A (kobs = 0.02 s−1), R223K (kobs = 0.02 s−1), and N228A (kobs = 0.02 s−1) cleaved the DNA 1 order of magnitude slower than did the wild type enzyme (Fig. 4, right panel).

Rates of Single-turnover Cleavage and Religation by R223A—Kinetic analysis of the R223A mutant revealed that this seemingly inactive protein was actually capable of cleaving DNA and that the extent of cleavage increased steadily over 48 h and leveled off at 96–144 h, at which time 38% of the input substrate was covalently bound (Fig. 5A). The observed cleavage rate constant was 1.2 × 10−5 s−1. Hence, the R223A mutation caused a 10−4.4 reduction in the rate of single-turnover cleavage.

The religation reaction of R223A was studied by assaying the ability of a preformed topoisomerase-DNA complex to transfer the covalently held 5’-32P-labeled strand to a heterologous acceptor strand (20). In the experiment shown in Fig. 5B, R223A protein was incubated with the suicide cleavage substrate for 72 h in order to achieve formation of the covalent intermediate. We then added a 50-fold molar excess of a 5’ hydroxyl-terminated 18-mer acceptor strand complementary to the 5’ tail of the covalent donor complex while simultaneously increasing the ionic strength to 0.3 M NaCl. (Addition of NaCl during the religation phase promotes dissociation of the topoisomerase after strand closure and prevents recleavage of the strand transfer product.) Religation to the 18-mer yielded a 32P-labeled 30-mer. The strand transfer product was resolved from the input 32P-labeled 18-mer strand by denaturing gel electrophoresis. Religation by R223A was extremely slow; 30% of the input CCCCTT-containing strand was transferred to the exogenous acceptor over 72 h (Fig. 5B).

The observed rate constant (krel) was 1.4 × 10−5 s−1. The extent of religation by wild type topoisomerase at the earliest time point analyzed (10 s) was >95% of the end point value (data not shown). Thus, the R223A mutation slowed the rate of religation by nearly 5 orders of magnitude relative to the wild type religation rate.

Mutational Effects on Equilibrium DNA Cleavage—We used a 60-mer DNA duplex containing a centrally placed cleavage site with 30 bp upstream and 30 bp downstream of the scissile bond to study topoisomerase cleavage under equilibrium conditions. The wild type topoisomerase cleaved 18% of the 60-mer duplex at saturating levels of input enzyme (Fig. 6). The cleavage equilibrium constant (Kcl = covalent complex/noncovalent complex = kcl/krel) was 0.22. Covalent complex formation by R223K was 24% at saturation; hence, Kcl (0.31) was slightly higher than that of the wild type topoisomerase. Given that the rate of single-turnover cleavage was reduced at least 10-fold by the R223K mutation, we can conclude from the observed Kcl that the rate of religation was reduced to at least an equivalent extent.

All other mutants tested achieved a lower than wild type level of cleavage at saturation. (Increasing the amount of input protein to 370 ng, twice the highest value shown in Fig. 6, did not result in a further increase in the extents of cleavage.) F215A cleaved 10% of the input 60-mer (Kcl = 0.11). K220A achieved 6% cleavage of the 60-mer (Kcl = 0.06); D221A cleaved 5% (Kcl = 0.05); N228A cleaved 3% (Kcl = 0.03). Kinetic analysis of 60-mer cleavage by 93 ng of each protein confirmed that all reactions had achieved equilibrium within 5 min. These effects on the equilibrium constants can be largely accounted for by the mutational effects on the single-turnover cleavage rates.

Equilibrium Cleavage by R223A—The R223A mutant displayed a slow approach to equilibrium over 48 h (Fig. 7A). Remarkably, 50% of the input 60-mer was covalently bound at equilibrium. Hence, the observed equilibrium constant (Kcl = 1) was four times higher than that of the wild type enzyme. The
rate constant \( k_\text{obs} \) for approach to equilibrium by R223A was \( 3.2 \times 10^{-8} \text{ s}^{-1} \). Knowing that \( K_{\text{cl}} = 1 \) and that \( k_\text{obs} = k_{\text{cl}} + k_{\text{rel}} \), we calculated that \( k_{\text{cl}} = 1.6 \times 10^{-5} \text{ s}^{-1} \) and \( k_{\text{rel}} = 1.6 \times 10^{-5} \text{ s}^{-1} \). Because neither the extent of cleavage at equilibrium nor the observed rate constant for approach to equilibrium was affected significantly by a 2-fold variation of the amount of input protein (data not shown), we surmise that the mutation did not affect precleavage binding. (This will be confirmed by additional experiments below.)

We measured single-turnover religation on the 60-mer substrate by allowing the cleavage reaction to reach equilibrium and then adjusting the reaction mixtures to 0.5 M NaCl. This concentration of salt blocks both equilibrium cleavage and single-turnover cleavage by interfering with DNA binding (Refs. 16 and 24 and data not shown). Topoisomerase prebound to an equilibrium cleavage substrate at low ionic strength is dissociated when the salt concentration is raised to \( >0.25 \text{ M} \) (18). Hence, topoisomerase molecules that have catalyzed strand closure on the 60-mer DNA will be dissociated from the DNA by salt and will be unable to rebind and re cleave. The decrease in covalent complex as a function of time after addition of NaCl is plotted in Fig. 7B. R223A covalent complex declined slowly over 72 h (\( k_{\text{rel}} = 1.3 \times 10^{-5} \text{ s}^{-1} \)). Note that the observed rate constant for R223A in single-turnover religation on the 60-mer agreed with the value calculated from the rate of approach to equilibrium on the 60-mer (\( 1.6 \times 10^{-5} \text{ s}^{-1} \)) and with the observed rate constant for single-turnover religation on the 18-mer/30-mer suicide substrate (\( 1.4 \times 10^{-5} \text{ s}^{-1} \)).

**DNA Binding by Catalytically Defective R223 Mutant Topoisomerases—** Gross defects in covalent adduct formation caused by protein mutation either may result from a primary failure to transesterify or may be secondary to an inability to bind DNA noncovalently. A native gel mobility shift assay (6, 21) was used to analyze the DNA binding properties of the R223 mutant proteins. The \(^{32}\text{P}-\)labeled DNA ligand was a 24-bp CCGCTT-containing duplex (Fig. 8). Binding of wild type topoisomerase to the 24-bp DNA resulted in the formation of two discrete protein-DNA complexes of retarded electrophoretic mobility (Fig. 8). The more rapidly migrating species contained topoisomerase bound to the 24-mer duplex, whereas the upper complex contained topoisomerase bound covalently to a 5' tailed DNA cleavage product (6). The upper complex arises via dissociation of the 3' segment of the scissile strand. Mutated versions of topoisomerase that bind noncovalently to DNA but are defective in strand cleavage characteristically form a single protein-DNA complex in the native gel shift assay (6, 7), and this was found to be the case for R223A (Fig. 8). The apparent affinity of R223A for the 24-mer was similar to that of the wild type topoisomerase. The R223K mutant also displayed wild type affinity for the 24-mer CCCTT-containing DNA (Fig. 8).

**Mutational Analysis of the Region from Residue 249 to Residue 269—** The motif, Ser-(Lys/Arg)-X-Tyr, is conserved at the active sites of the eukaryotic type I topoisomerases (2, 3). As expected, replacement of the active-site tyrosine by phenylalanine inactivates the viral and cellular enzymes (15, 22–24). Indeed, alanine replaces serine naturally within the protein encoded by molluscum contagiosum virus (Fig. 1). The sequence of this motif, Ser-270 and Lys-271 of the vaccinia enzyme are well tolerated (18). However, the Ser and Lys residues of this motif are dispensable for topoisomerase activity, insofar as alanine substitutions at Ser-270 and Lys-271 of the vaccinia enzyme are well tolerated (18). Indeed, alanine replaces serine naturally within the protein encoded by molluscum contagiosum virus (Fig. 1). The sequence of this motif, Ser-270 and Lys-271 of the vaccinia enzyme are well tolerated (18).
topoisomerase in relaxing supercoiled plasmid DNA in the absence of magnesium (not shown). Seventeen of the mutants exhibited a typical stimulation of relaxation rate by 5 mM magnesium; the lone exception was I252A, which was not stimulated by divalent cation (not shown). We conclude that none of the amino acid side chains in this region (exclusive of His-265) is important for catalysis.

**DISCUSSION**

In continuing our mutational analysis of the vaccinia DNA topoisomerase, we focused on a 25-amino acid protein segment that is well conserved with the cellular enzymes and an adjacent 21-amino acid segment that is highly conserved among the poxvirus enzymes. The remarkable finding was that only one residue (Arg-223) was essential for catalysis. An essential amino acid side chain was defined as one whose removal, e.g. by alanine replacement, results in complete or drastic (100-fold) loss of function. Arg-223 is conserved in every known eukaryotic type I topoisomerase (Fig. 1); hence, we consider it likely that this residue plays an identical essential role in the chemistry of strand cleavage and religation by other eukaryotic type I topoisomerases. Two other residues, Lys-220 and Asn-228, were identified as important for covalent catalysis, albeit not essential by the above criterion. Alanine substitutions at these positions slowed the rate of strand cleavage by more than a factor of 10. Lys-220 is strictly conserved in all cellular type I topoisomerases, whereas Asn-228 is conserved among the poxvirus enzymes.

Prior mutational studies had suggested an essential role for Arg-223 in strand cleavage (6, 16) but provided no quantitative assessment of the contribution of this residue to the estimated 100-fold enhancement by enzyme of the rate of transesterification (17). This was because only the extents of cleavage by mutant proteins were measured and the reaction times were too short to detect extremely slow rates. In this study, we subjected the wild type and mutant proteins to kinetic analysis of the strand cleavage and religation steps under single-turnover and equilibrium conditions. For the wild type topoisomerase, we observed a single-turnover cleavage rate constant of 0.29 s\(^{-1}\) and a cleavage-religation equilibrium constant of 0.22.

From these values we calculated a religation rate constant of 1.3 s\(^{-1}\). (Note that the rate of single-turnover religation by wild type topoisomerase on the suicide substrate was too rapid for us to measure manually, as was the rate of approach to equilibrium on the 60-mer DNA.) Our values were slightly higher than those reported by Stivers et al. (17)\((k_{cl} = 0.07, k_{rel} = 0.66,\) and \(K_{cl} = 0.12)\) for single-turnover strand cleavage and religation at 20 °C. We suspect the faster cleavage rate and consequently higher equilibrium cleavage value is attributable to our use of a different set of CCCTT-containing substrates and/or the fact that our reactions were performed at 37 °C rather than 20 °C.

The observed rate constant for single-turnover cleavage by R223A on the suicide substrate was \(1.2 \times 10^{-5}\) s\(^{-1}\). The observed rate constant for single-turnover religation by the R223A suicide cleavage complex was \(1.4 \times 10^{-5}\) s\(^{-1}\). The values for \(k_{cl}\) and \(k_{rel}\) determined from the approach to equilibrium cleavage on the 60-mer DNA were \(1.6 \times 10^{-5}\) and \(1.6 \times 10^{-5}\) s\(^{-1}\), respectively. The rate constant for single-turnover religation on the 60-mer DNA was \(3 \times 10^{-5}\) s\(^{-1}\). Thus, there was good agreement between the mutational rate effects gauged under single-turnover and equilibrium reaction conditions using different CCCTT-containing DNAs. Taken together, the results show that removal of the side chain at position 223 resulted in a \(-10^{-4.3}\) effect on strand cleavage and a \(-10^{-5}\) effect on religation. An imbalance in the relative mutational effects on the two chemical steps caused a shift in the cleavage-religation equilibrium in favor of the covalently bound state.

The effects of the R223K mutation were benign compared with R223A. The cleavage rate constant was reduced by 1 order of magnitude. Our finding that the R223K caused only a slight increase in \(K_{cl}\) suggests that lysine substitution exerted comparable effects on the rates of cleavage and religation. These results show that a positive charge at position 223 is critical for transesterification reaction chemistry, while underscoring that arginine per se is important for optimal catalysis. The deleterious effects of the R223A mutation cannot be attributed to nonspecific loss of side chain bulk, because the topoisomerase is also inactivated when Arg-223 is substituted by glutamine (6). Inactivation of topoisomerase by replacement of Arg-223 with glutamic acid (16) is less instructive because this entails charge inversion.

Of the six conserved residues within the Gly-(Ile/Leu)-X-X-Lys-X-X-Arg-Thr-Tyr motif found in all eukaryotic type I topoisomerases, only Arg-223 is essential. Prior mutational analysis of this segment showed that Gly-216 of the vaccinia enzyme could be changed to lysine or glutamate with little effect on topoisomerase activity or DNA cleavage (16). Similarly, Ile-217 tolerated substitution by proline (16). Hence, these residues, although conserved, are noncontributory to catalysis. Earlier studies left unsettled the role of Lys-220. Substitution of Lys-220 by aspartic acid lowered DNA relaxation activity by more than 2 orders of magnitude, yet replacement by asparagine or isoleucine caused only a moderate reduction to 10–20% of the wild type level (16). We have shown here that side chain removal causes a decrement of 1 order of magnitude in the cleavage rate constant. The K220A mutation shifted the equilibrium toward the noncovalently bound state, implying that effect on cleavage rate was ~3-fold more severe than the effect on religation. Cumulatively, these findings suggest that conserved Lys-220 plays a role in catalysis, but its contribution is modest compared with that of Arg-223. Alanine substitutions at residues Thr-224 and Tyr-225 had no apparent effect on topoisomerase activity; hence, these conserved side chains are not critical for reaction chemistry.

Elimination of the side chain at Asn-228 reduced activity in DNA relaxation and slowed the cleavage step by 1 order of magnitude. A similar reduction in \(K_{cl}\) suggested that this mutation had little impact on the religation step. Asn-228 is situated at three residues downstream of the invariant RTY triplet and is found in all poxvirus enzymes. The cellular type I enzymes diverge from the vaccinia sequence immediately following RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY. However, conservation of Asn-228 can be invoked by aligning the vaccinia sequence RTYGVNYT with an inter-acting RTY.
ation under conditions of saturating enzyme is clearly needed to compare the rate constant for supercoil release to those of the component chemical steps.

Intermediate topoisomers were also noted during relaxation by D221A. This mutation (like N228A) elicited a reduction in single-turnover cleavage and a corresponding decrease in $K_{cl}$. Gupta et al. (26) reported that replacement of vaccinia Asp-221 by valine (the residue found at this position in the cellular type I enzymes) resulted in a more drastic (25-fold) reduction in topoisomerase activity. The yield of covalent adduct formed by D221V on a suicide substrate was much lower than that of wild-type enzyme; reaction rates and equilibrium parameters were not reported. We suspect the apparently more severe effects of valine versus alanine substitution for Asp-221 are caused by steric constraints imposed by the bulkier valine side chain.

In summary, we have assessed the effects of alanine substitutions at 39 positions in vaccinia DNA topoisomerase. To our knowledge, this is the most extensive targeted mutational analysis of any topoisomerase reported to date. The results confirm the trend established earlier (7–9) that most residues of the vaccinia protein, even conserved ones, are not essential for catalysis. For those residues that were deemed essential or important for activity, kinetic studies using defined DNA substrates illuminated their contributions to the rate enhancements of DNA cleavage and ligation. Mutational effects ranged from drastic (R223A) to modest (K220A and N228A). We predict that Arg-223 is a constituent of the topoisomerase active site and suggest that this is also the case for the cellular enzymes. Precise knowledge of the disposition of catalytically important side chains awaits a crystal structure of the enzyme bound covalently to DNA. Nonetheless, we suspect that ongoing analysis of vaccinia topoisomerase may define the catalytic center of a eukaryotic type I enzyme in advance of a crystallographic solution.

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