

Replacement of the active site tyrosine of vaccinia DNA topoisomerase by glutamate, cysteine or histidine converts the enzyme into a site-specific endonuclease

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ABSTRACT

Vaccinia topoisomerase forms a covalent protein–DNA intermediate at 5′-CCCTT↓ sites in duplex DNA. The T↓ nucleotide is linked via a 3′-phosphodiester bond to Tyr-274 of the enzyme. Here, we report that mutant enzymes containing glutamate, cysteine or histidine in lieu of Tyr-274 catalyze endonucleolytic cleavage of a 60 bp duplex DNA at the CCCTT↓ site to yield a 3′ phosphate-terminated product. The Cys-274 mutant forms trace levels of a covalent protein–DNA complex, suggesting that the DNA cleavage reaction may proceed through a cysteinyl-phosphate intermediate. However, the His-274 and Glu-274 mutants evince no detectable accumulation of a covalent protein–DNA adduct. Glu-274 is the most active of the mutants tested. The pH dependence of the endonuclease activity of Glu-274 (optimum pH = 6.5) is distinct from that of the wild-type enzyme in hydrolysis of the covalent adduct (optimum pH = 9.5). At pH 6.5, the Glu-274 endonuclease reaction is slower by 5–6 orders of magnitude than the rate of covalent adduct formation by the wild-type topoisomerase, but is ~20 times faster than the rate of hydrolysis by the wild-type covalent adduct. We discuss two potential mechanisms to account for the apparent conversion of a topoisomerase into an endonuclease.

INTRODUCTION

DNA relaxation by the vaccinia virus type I topoisomerase entails a series of partial reactions common to all eukaryotic type IB enzymes. These are: (i) non-covalent binding of the protein to duplex DNA, (ii) cleavage of one DNA strand with formation of a covalent DNA–(3′-phosphotyrosyl)–protein intermediate, (iii) strand passage and (iv) strand religation. A distinctive feature of the vaccinia topoisomerase is that it binds and cleaves duplex DNA at a specific target sequence 5′-(T/C)CCCTT↓ (1). The T↓ nucleotide is linked to Tyr-274 of the enzyme (2). The active site tyrosine of vaccinia topoisomerase is situated near the C-terminus

of the protein within a motif SKxxY that is conserved among all type IB family members (3). Mutations of vaccinia Tyr-274 that eliminate nucleophilicity, e.g., Ala-274 and Phe-274, completely abrogate catalytic activity, but have no apparent effect on the non-covalent interaction of the topoisomerase with duplex DNA (2,4–6). We have also replaced the active site Tyr-274 with serine and threonine, either of which might conceivably serve as an alternative nucleophile. The Ser-274 and Thr-274 mutants were inert in covalent adduct formation on a CCCTT-containing suicide substrate under reaction conditions that could have detected as little as 10^{-7} the activity of the wild-type topoisomerase (7).

The proposed mechanism for covalent adduct formation by vaccinia topoisomerase entails general base catalysis of the attack of Tyr-274 at the scissile phosphate (8). NMR analysis excludes the presence of an ionized tyrosine in the wild-type protein over the pH range 5.1–8.8 (8). The moiety responsible for abstraction of the proton from Tyr-274 remains to be identified. Serine and threonine might be unable to engage in covalent catalysis because they are less prone than tyrosine to deprotonation. Steric issues may also come into play, i.e., the hydroxyl of Ser-274 or Thr-274 may be situated at greater distance from the scissile phosphate or the general base.

Here, we have constructed a new set of topoisomerase mutants in which Tyr-274 is replaced by cysteine, lysine, histidine or glutamate. There is ample precedent in the literature to indicate that these side chains can engage in nucleophilic attack on phosphate esters or phosphoanhydrides. For example, cysteine acts as the nucleophile in formation of a phosphoenzyme intermediate by protein phosphatases (9). Lysine forms a phosphoamide intermediate with a nucleoside monophosphate during catalysis by polynucleotide ligases and mRNA capping enzymes (10,11). Histidine engages in formation of a phosphoamide intermediate during catalysis by hexose-1-phosphate uridylyltransferase (12), nucleoside diphosphate kinase (13) and polyphosphate kinase (14). Acyl-phosphate intermediates have been identified for pyridoxal phosphate and cation transport ATPases (15,16).

We report that the Glu-274, Cys-274 and His-274 mutants of vaccinia topoisomerase have the capacity to cleave CCCTT-containing DNA endonucleolytically. Mutants Lys-274, Ser-274 and Thr-274 have no detectable endonuclease activity.

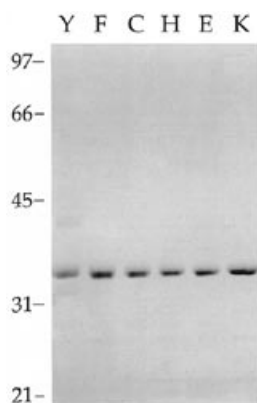


Figure 1. Topoisomerase purification. The phosphocellulose preparations of wild-type topoisomerase (Tyr-274; lane Y), and mutants Cys-274 (lane C), Lys-274-274 (lane K), His-274 (lane H) and Glu-274 (lane E) were analyzed by SDS-PAGE. Protein (5 μ g) was applied to each lane. Polypeptides were visualized by staining the gel with Coomassie Brilliant Blue dye. The positions and molecular weights (in kDa) of co-electrophoresed protein standards are indicated on the left.

MATERIALS AND METHODS

Amino acid substitutions at the active site of vaccinia topoisomerase

Mutations Cys-274, His-274, Lys-274 and Glu-274 were introduced into the vaccinia virus topoisomerase gene by using the two-stage PCR-based overlap extension method (17). 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 pET11b to generate plasmids pET-Y274C, pET-Y274H, pET-Y274K and pET-Y274E. All mutations were confirmed by dideoxy sequencing. The entire coding region was sequenced to exclude the introduction of unwanted changes during amplification and cloning.

Expression and purification of mutant proteins

The pET-based topoisomerase plasmids were transformed into *Escherichia coli* BL21. Topoisomerase expression was induced by infection with bacteriophage λ CE6 as described (18). Mutants Cys-274, His-274, Lys-274 and Glu-274 were purified from soluble bacterial lysates by phosphocellulose column chromatography (18). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (Biorad) with bovine serum albumin as the standard. Topoisomerase purity was assessed by SDS-polyacrylamide gel electrophoresis (Fig. 1). Purification and SDS-PAGE analysis of topoisomerase mutants Ser-274 and Thr-274 has been described (7).

Reaction of topoisomerase with CCCTT-containing duplex DNA

A 60mer CCCTT-containing DNA oligonucleotide was 5'-end labeled by enzymatic phosphorylation in the presence of [γ - 32 P]ATP and T4 polynucleotide kinase, then gel-purified and hybridized to complementary 60mer strand to form the 60 bp

substrate. Reaction mixtures containing (per 20 μ l) 50 mM buffer (as specified), 0.3 pmol of 60 bp DNA, and topoisomerase as specified. The mixture was incubated at 37°C. The reaction was halted by addition of SDS to 0.5%. The denatured samples were digested for 60 min at 45°C with 10 μ g of proteinase K. The volume was adjusted to 50 μ l 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 resuspended in formamide and the samples were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA).

RESULTS

Covalent adduct formation and hydrolysis by wild-type topoisomerase

Wild-type topoisomerase was reacted with a 60 bp duplex DNA containing a 5' 32 P-labeled scissile strand with a centrally placed CCCTT cleavage site. At saturating levels of topoisomerase, 18–20% of the input 60mer is converted to covalent adduct (19). This reflects the establishment of a cleavage-religation equilibrium that favors the non-covalently bound state. Equilibrium is attained within 10 s, which is in keeping with the fast rates of the component cleavage and religation steps ($k_{cl} = 0.28 \text{ s}^{-1}$ and $k_{rel} = 1.2 \text{ s}^{-1}$) (19). The reaction product is a 5' 32 P-labeled 30mer oligonucleotide linked to the enzyme through a 3'-phosphotyrosyl bond. Digestion of the covalent adduct with proteinase K liberates the 5' 32 P-labeled 30mer linked to a short peptide of heterogeneous size. The protease digestion products migrate more rapidly than the input 60mer strand during denaturing gel electrophoresis (Fig. 2). Their apparent size is ~32–35 nt. Omission of the proteinase K digestion step eliminates the prominent cluster of bands derived from the covalent adduct (Fig. 2). However, a discrete 5' 32 P-labeled 30 nt fragment is released very slowly over 72 h at pH 7.5 (Fig. 2). We showed previously that vaccinia topoisomerase catalyzes transfer of the covalently bound CCCTT strand to hydroxyl ion to generate a 3' phosphate-terminated product (7). A second minor species migrating just above the 30mer hydrolysis product results from transfer of the 30mer strand to glycerol present in the reaction buffer (7). A time-dependent increase in the hydrolysis product can also be appreciated in the proteinase K digested samples (Fig. 2).

Site specific endonucleolytic cleavage of DNA by topoisomerase mutant Cys-274

Initial experiments addressed whether cysteine might function in lieu of tyrosine as the active site nucleophile of vaccinia topoisomerase. We thought that cysteine, with an unperturbed pKa of ~8.5, might be more effective than serine in attacking the phosphodiester backbone. Also, cysteine is known to engage in formation of a covalent cysteinyl-phosphate intermediate during hydrolysis of phosphomonoesters by protein phosphatases (9). Stivers *et al.* (8) had previously drawn mechanistic parallels between the reactions catalyzed by type IB topoisomerases and the protein phosphatases, i.e., both enzymes catalyze transesterification to phosphate without a requirement for a divalent cation cofactor. In parallel, we examined the effects of replacing Tyr-274 with lysine, a potential nucleophile with a predicted pKa higher than that of cysteine.

The Cys-274 and Lys-274 mutant proteins were expressed in *E.coli* and purified from soluble bacterial extract by

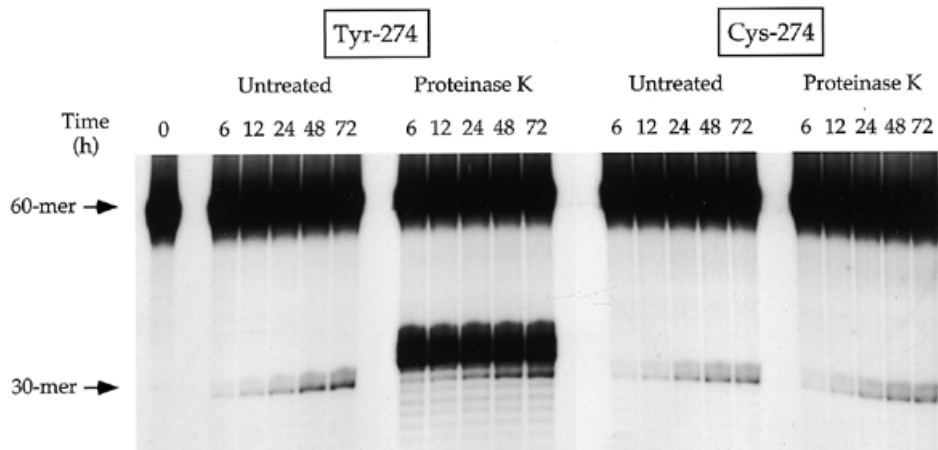


Figure 2. Reaction of wild-type and Cys-274 topoisomerases with a 60 bp CCCTT-containing DNA. Reaction mixtures containing (per 20 μ l) 50 mM Tris-HCl, pH 7.5, 0.3 pmol of 60 bp DNA and 38 ng of wild-type (Tyr-274) or Cys-274 topoisomerase were incubated at 37°C. Aliquots (20 μ l) were withdrawn at the times indicated and the reactions were quenched by adding SDS. The samples were processed as described in the Materials and Methods with or without proteinase K digestion as indicated. The reaction products were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea. An autoradiogram of the gel is shown. The positions of the input 60mer scissile strand and the free 30mer cleavage product are denoted by arrows on the left.

phosphocellulose chromatography. The topoisomerase polypeptide constituted the major species in the protein preparations, as determined by SDS-PAGE, and the extent of Cys-274 and Lys-274 purification was equivalent to that of the wild-type protein (Fig. 1). A native gel mobility shift assay (6,19) was used to analyze the binding of the Cys-274 and Lys-274 mutants to the 32 P-labeled CCCTT-containing 60 bp DNA (Fig. 3). The wild-type topoisomerase (Tyr-274) was analyzed in parallel. Cys-274 and Lys-274 each formed a single discrete protein-DNA complex of retarded mobility (not shown), the yield of which was proportional to the amount of input topoisomerase (Fig. 3). The DNA binding affinities of the Cys-274 and Lys-274 mutants were essentially equivalent to that of the wild-type enzyme.

The mutant proteins were tested for their ability to cleave the 60mer DNA (Fig. 4). The amount of input protein (38 ng/20 μ l) was sufficient to bind nearly all the input DNA as determined by the gel shift assay. We found that Cys-274 cleaved the 32 P-labeled CCCTT strand to form a discrete 30mer product that accumulated steadily over 72 h. Lys-274 was unreactive with the 60mer DNA, as were Thr-274 and Ser-274 (Fig. 4).

It was striking that although the Cys-274 reaction product was digested with proteinase K prior to electrophoresis in the experiment in Figure 4, we did not detect the prominent cluster of peptide-linked 32 P-labeled 30mer formed by wild-type topoisomerase. To clarify the origin of the Cys-274 product, the kinetic analysis of cleavage was repeated with and without the proteinase K treatment (Fig. 2). We found that formation of the 32 P-labeled 30mer was unaffected by omission of the proteolysis step. Moreover, the electrophoretic mobility of the Cys-274 cleavage product was identical to that of the hydrolysis product generated by wild-type topoisomerase (Fig. 2). We surmise that Cys-274 cleaved the 60mer DNA at the CCCTT site to generate a free 3' phosphate terminated 30mer without accumulating significant amounts of a covalent topoisomerase-DNA intermediate. In effect, introduction of cysteine at the active site converted the topoisomerase into an endonuclease, albeit a weak one.

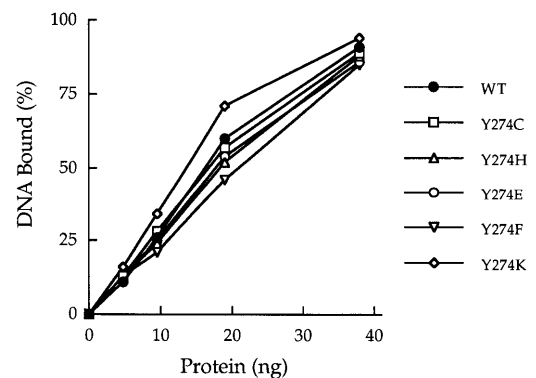


Figure 3. DNA binding by mutant topoisomerases. Reaction mixtures (20 μ l) containing 50 mM Tris-HCl, pH 7.5, 0.3 pmol of 5' 32 P-labeled 60 bp DNA, and increasing amounts of the indicated topoisomerase preparations were incubated for 5 min at 37°C. The mixtures were adjusted to 10% glycerol and then electrophoresed through a native 6% polyacrylamide gel containing 22.5 mM Tris-borate, 0.6 mM EDTA. Free 60 bp DNA and topoisomerase-DNA complexes of retarded electrophoretic mobility were visualized by autoradiographic exposure of the dried gel. The extent of topoisomerase-DNA complex formation (% of input DNA bound) was quantitated after scanning the gel with a FUJIX BAS1000 phosphorimager. DNA binding is plotted as a function of input protein. The structure of the 60 bp DNA ligand is shown; an asterisk denotes the 5'-label on the CCCTT-containing strand.

Cys-274 forms trace levels of a covalent protein-DNA adduct

We were able to detect a covalent interaction between Cys-274 and CCCTT-containing DNA using an alternative assay (4,19) that measures label transfer from a 5' 32 P-labeled 18mer scissile strand to the topoisomerase to form an SDS-stable adduct detectable by SDS-PAGE (Fig. 5). In this electrophoretic system, the labeled DNA strand migrates near the dye front at the bottom of the gel. The electrophoretic mobility of the (Cys-274)-DNA

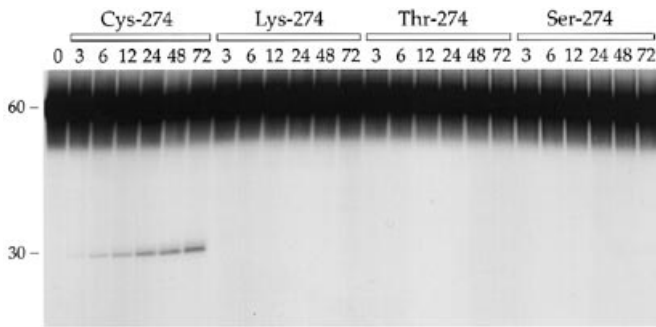


Figure 4. Assay of DNA cleavage by topoisomerase mutants Cys-274, Lys-274, Thr-274 and Ser-274. Reaction mixtures containing (per 20 μ l) 50 mM Tris-HCl, pH 7.5, 0.3 pmol of 60 bp DNA, and 38 ng of Cys-274, Lys-274, Thr-274 or Ser-274 topoisomerase were incubated at 37°C. Aliquots (20 μ l) were withdrawn at the times indicated and the reactions were quenched by adding SDS. The mixtures were digested with proteinase K, then deproteinized and ethanol-precipitated. The reaction products were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea. An autoradiogram of the gel is shown. The positions of the input 60mer scissile strand and the free 30mer cleavage product are denoted by arrows on the left.

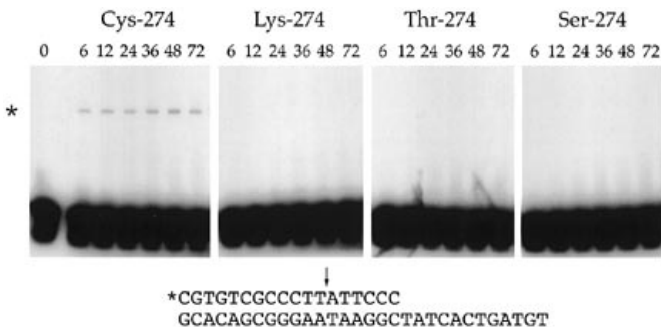


Figure 5. Assay of covalent protein-DNA adduct formation by topoisomerase mutants Cys-274, Lys-274, Thr-274 and Ser-274. Reaction mixtures containing (per 20 μ l) 50 mM Tris-HCl, pH 7.5, 0.3 pmol of 18mer/30mer DNA and 38 ng of Cys-274, Lys-274, Thr-274 or Ser-274 topoisomerase were incubated at 37°C. Aliquots (20 μ l) were withdrawn at the times indicated and the reactions were quenched by adding SDS. The mixtures were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. An autoradiogram of the dried gel is shown. Free DNA migrated near the dye front at the bottom of the gel. The position of the protein-DNA adduct is denoted by asterisk on the left. The structure of the 5' 32 P-labeled 18mer/30mer substrate is depicted at the bottom of the figure.

adduct was identical to that formed by wild-type topoisomerase on the 18mer/30mer DNA (not shown). The level of covalent adduct formed by the Cys-274 enzyme was extremely low; only 0.1–0.3% of the input labeled strand was transferred to the topoisomerase polypeptide. In the case of the wild-type topoisomerase, 95% of the 5' 32 P-labeled strand became covalently linked to the protein (19). The Lys-274 mutant formed no detectable covalent adduct with the 18mer/30mer DNA, nor did mutants Thr-274 and Ser-274 (Fig. 5).

The DNA products formed by the Cys-274 protein on the 18mer/30mer substrate were also analyzed by electrophoresis through a 17% polyacrylamide gel containing 7.5 M urea (Fig. 6). Although unreacted 18mer strand accounted for nearly all of the labeled DNA, we did detect the release of trace amounts of a free 12mer cleavage product over the 72 h incubation at 37°C (not shown). In addition, we detected trace amounts of a labeled

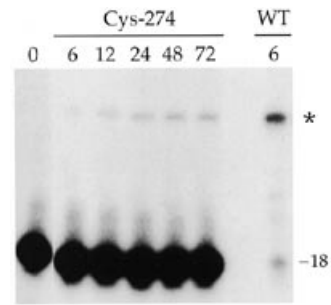


Figure 6. Formation of a hairpin strand transfer product by Cys-274. Reaction mixtures containing (per 20 μ l) 50 mM Tris-HCl, pH 7.5, 0.3 pmol of 18mer/30mer DNA and 38 ng of Cys-274 or WT topoisomerase were incubated at 37°C. Aliquots (20 μ l) were withdrawn at the times (h) indicated above the lanes and the reactions were quenched by adding SDS to 0.5%. The samples were deproteinized by extraction with phenol/chloroform. Aliquots of the aqueous material were adjusted to 50% formamide and then electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea in TBE. An autoradiogram of the gel is shown. The position of the 42 nt hairpin strand transfer product is denoted by an asterisk on the right.

product that was larger than the input 18mer strand and identical in electrophoretic mobility to the 42 nt hairpin DNA molecule formed by wild-type topoisomerase in a parallel reaction with the 18mer/30mer substrate (Fig. 6). The hairpin is formed by enzyme-catalyzed transfer of the covalently held 12mer strand (5'-pCGTGTGCGCCCTT) to the 5' OH-terminus of the 30mer bottom strand (20,21). This implies that the covalent protein-DNA adduct formed by Cys-274 is an intermediate in DNA strand transfer.

Endonuclease activity of mutants His-274 and Glu-274

After detecting DNA cleavage activity by Cys-274, we examined the effects of replacing Tyr-274 by two other nucleophilic amino acids, histidine and glutamate. The purity of the recombinant Glu-274 and His-274 proteins was confirmed by SDS-PAGE (Fig. 1). Both mutants displayed wild-type affinity for the 60mer CCCTT-containing DNA as determined using the gel-shift assay (Fig. 3). Reaction of the 60mer substrate for 72 h at pH 7.5 with 38 ng of the His-274 and Glu-274 proteins resulted in the formation of a free 30mer cleavage product, which comigrated with the 30mer hydrolysis product of the wild-type topoisomerase (Fig. 7). The extent of 30mer formation was unaffected by inclusion or omission of a proteinase K digestion step and no cluster of peptide-linked products was detected when digested and undigested samples were analyzed in parallel (data not shown). We were also unable to detect a covalent adduct by label transfer from a 5' 32 P-labeled 18mer scissile strand to the topoisomerase to form covalent adduct detectable by SDS-PAGE (data not shown).

pH dependence of endonucleolytic cleavage

We showed previously that the rate of hydrolysis of a suicide covalent intermediate by wild-type topoisomerase was pH dependent, with optimal activity seen at pH 9.5 (7). We suggested that the hydrolysis reaction occurs via the attack of a hydroxide ion on the suicide intermediate and that the alkaline pH optimum reflected the dependence of the reaction rate on hydroxide ion concentration in the range of 6.5–9.5. Analysis of the amount of the 30mer hydrolysis product formed by wild-type topoisomerase

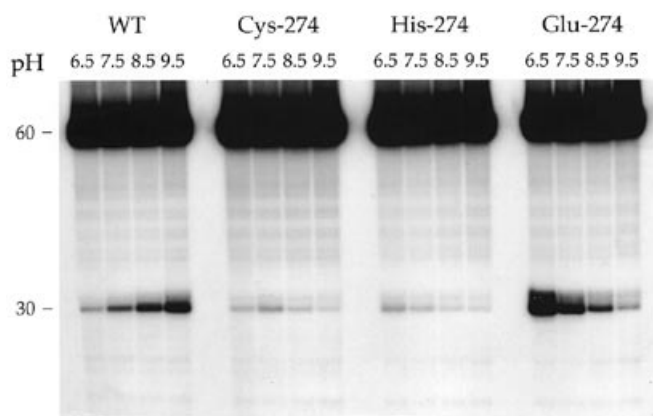


Figure 7. pH-dependent cleavage of the CCCTT-containing DNA strand. Reaction mixtures (20 μ l) containing 50 mM buffer [either sodium 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5; Tris-HCl, pH 7.5 or 8.5; or sodium 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 9.5], 0.3 pmol of 60mer DNA, and 38 ng of the indicated topoisomerase preparation were incubated for 72 h at 37°C. The reaction products were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea. An autoradiogram of the gel is shown.

during a 72 h reaction with the 60mer equilibrium substrate also revealed an alkaline pH optimum (Fig. 7). The extent of hydrolysis at pH 9.5 was 3.7% of the input scissile strand; 0.5% of the substrate was hydrolyzed at pH 6.5.

In stark contrast, the endonuclease activity of Glu-274 was highest at pH 6.5 (9.9% cleavage of the scissile strand) and declined progressively at pH 7.5 (4.2% cleavage), pH 8.5 (1.7% cleavage) and pH 9.5 (0.6% cleavage). At pH 6.5, Glu-274 was 20-fold more effective than the wild-type enzyme in forming the free 30mer product. We surmise that the endonuclease reaction of the Glu-274 mutant occurs via a different kinetic mechanism than the hydrolysis reaction of the wild-type covalent intermediate.

The endonuclease reaction of the His-274 mutant was also optimal at pH 6.5, although the yield of 30mer product (0.5%) was much less than that of Glu-274. The levels of cleavage by Cys-274 at pH 6.5, 7.5 and 8.5 were 0.4, 0.5 and 0.3%, respectively (Fig. 7).

Kinetic analysis of Glu-274

The effects of pH on the rate of cleavage by Glu-274 are shown in Figure 8A. The amount of 30mer product formed at pH 6.5 increased linearly up to five days, at which time 15% of the input DNA was cleaved. (Longer reaction times were not tested.) The rate of DNA hydrolysis at pH 6.5 was 3.4×10^{-5} percent of the input strand converted to 30mer per second. The 30mer product accumulated linearly at pH 6.0 and pH 5.5, albeit more slowly than at pH 6.5 (Fig. 8A). Lower pH values were not tested. Activity declined as pH was increased above pH 6.5. A plot of the reaction rate versus pH is shown in Figure 8B.

Reaction of mutant topoisomerases with supercoiled DNA

The Cys-274, Lys-274, Glu-274 and His-274 proteins were initially tested for their ability to relax a supercoiled plasmid substrate under reaction conditions optimal for wild-type topoisomerase activity. The reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.3 μ g of pUC19 plasmid DNA. Activity was quantitated

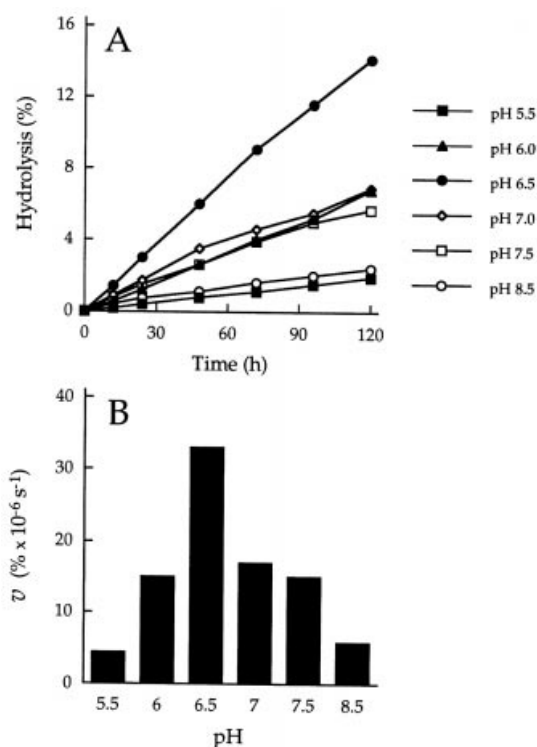


Figure 8. pH-dependence of the rate of cleavage by Glu-274. (A) Reaction mixtures containing (per 20 μ l) 50 mM buffer [either sodium MES, pH 6.0 or 6.5; or Tris-HCl, pH 7.0, 7.5 or 8.5], 0.3 pmol of 60mer DNA and 38 ng of Glu-274 topoisomerase were incubated at 37°C. Aliquots were withdrawn at the times indicated and the reactions were quenched by adding SDS. The reaction products were resolved by gel electrophoresis and the gel was scanned with a phosphorimager. The extent of cleavage (expressed as the percent of the input 60mer strand converted to 30mer) is plotted as a function of time. (B) The rates of hydrolysis (% of input DNA hydrolyzed per second) were determined from the plot in (A) and are plotted as a function of the pH of the reaction buffer.

by end-point dilution, beginning with 100 ng of the phosphocellulose topoisomerase preparation and decreasing by serial 10-fold decrements. Whereas 10 ng of wild-type topoisomerase relaxed the input DNA to completion in 15 min, and 1 ng relaxed about half the DNA, the Cys-274, Lys-274, Glu-274 and His-274 proteins were inactive even at 100 ng of input protein (data not shown). In light of the findings that alternative nucleophiles confer a novel, albeit weak, endonuclease activity on the vaccinia enzyme, we tested the most active of the mutants, Glu-274, for its ability to introduce nicks into closed circular DNA. Glu-274 was reacted with pUC19 DNA for five days at pH 6.5. The products were analyzed by agarose gel electrophoresis in the presence of 0.5 μ g/ml ethidium bromide. The endonuclease activity of Glu-274 was evinced by the time-dependent conversion of the more rapidly migrating closed circular plasmid DNA into more slowly migrating nicked circles (Fig. 9). No endonuclease activity was detected in control reactions containing the Phe-274 mutant of vaccinia topoisomerase.

DISCUSSION

We have shown that the Glu-274, His-274 and Cys-274 mutants of vaccinia topoisomerase catalyze endonucleolytic cleavage of duplex DNA at the same CCCTT target site at which the wild-type topoisomerase transesterifies to form a covalent protein-DNA intermediate. Whereas the wild-type topoisomerase can also

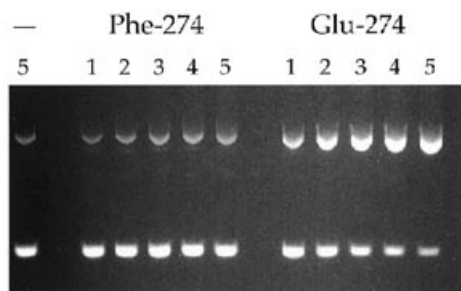


Figure 9. Cleavage of supercoiled plasmid DNA. Reaction mixtures containing (per 20 μ l) 50 mM MES, pH 6.5, 2.5 mM EDTA, 0.3 μ g of pUC19 DNA and 0.5 μ g of Glu-274 or Phe-274 protein were incubated at 37°C. Aliquots (20 μ l) were withdrawn after one to five days and quenched immediately by addition of a solution containing SDS (0.3% final concentration), glycerol, xylene cyanol and bromophenol blue. A control reaction lacking topoisomerase (-) was incubated for five days. Samples were analyzed by electrophoresis through a 1.2% horizontal agarose gel in TBE buffer containing 0.5 μ g/ml of ethidium bromide. The gel was photographed under short wave UV illumination.

generate a free cleavage product at the CCCTT site, the kinetic mechanism of the wild-type reaction is distinct from that of the mutants. Prior work on hydrolysis of CCCTT-containing DNA by the wild-type vaccinia topoisomerase showed a clear precursor-product relationship between the covalent intermediate and the free CCCTTp strand (7). Moreover, a Phe-274 mutant, which lacks an active site nucleophile and is incapable of covalent adduct formation, is inert in forming a free cleavage product (7). This argues that formation of a free CCCTTp strand by the wild-type topoisomerase occurs via hydrolysis of the covalent CCCTTp(Tyr-274) adduct and not via direct attack of water or hydroxyl ion on the scissile phosphate. We speculate that the endonuclease reaction of the Cys-274 mutant occurs via hydrolysis of a covalent CCCTTp(Cys-274) adduct. The mechanism of the endonuclease reaction described for the Glu-274 and His-274 mutants is less certain, particularly in the absence of any direct evidence for accumulation of a covalent intermediate by those enzymes. In

considering possible mechanisms, we will focus primarily on the Glu-274 mutant, which has the most robust endonuclease activity.

Kinetic analysis shows that the Glu-274 endonuclease reaction was linear for at least five days. The rate of DNA cleavage by Glu-274 at pH 6.5 (3.4×10^{-5} percent of the input strand converted to 30mer per second) was about $10^{-5.5}$ the rate of single-turnover covalent adduct formation by the wild-type topoisomerase (19). Yet, viewed relative to the catalytically inert Phe-274 or Ala-274 mutants, glutamate confers considerable activity. Stivers *et al.* (22) estimated that wild-type vaccinia topoisomerase enhances the rate of nucleophilic attack on the DNA backbone by $\sim 10^9$ -fold. Our limits of detection indicate that the Phe-274 and Ala-274 mutants have $<10^{-7}$ the activity of wild-type enzyme. Assuming that loss of a nucleophile renders the enzyme truly inert, we infer that the Glu-274 enzyme enhances the rate of attack on the scissile phosphate by about a factor of 10^3 - 10^4 .

We considered two potential reaction pathways by which Glu-274 might cleave DNA: (i) enzyme-catalyzed attack by water directly on the phosphodiester backbone of DNA (Fig. 10A) or (ii) attack by glutamate at the scissile phosphate to form an acyl phosphate linkage to DNA, followed by hydrolysis of the covalent intermediate (Fig. 10B). Reaction A is distinct from the normal topoisomerase reaction in that it does not involve transesterification. Instead, Glu-274 acts as a general base to catalyze the attack of water on the scissile phosphate. Although not illustrated in the figure, this would presumably involve the participation of a general acid to promote expulsion of the 5' OH terminated leaving group. General acid catalysis of this step has been proposed for the transesterification reaction of wild-type topoisomerase (8); hence, we imagine that the same moiety on the enzyme (as yet unidentified) would serve as proton donor in the hydrolysis reaction in Figure 10A. The merit of this model is that it mandates no explanation of the inability to detect a covalent protein-DNA adduct. However, the model does have some weakness in accounting for the spectrum of alternative nucleophiles that support endonuclease activity. Glutamate and histidine fit insofar as both can serve as proton acceptors. However,

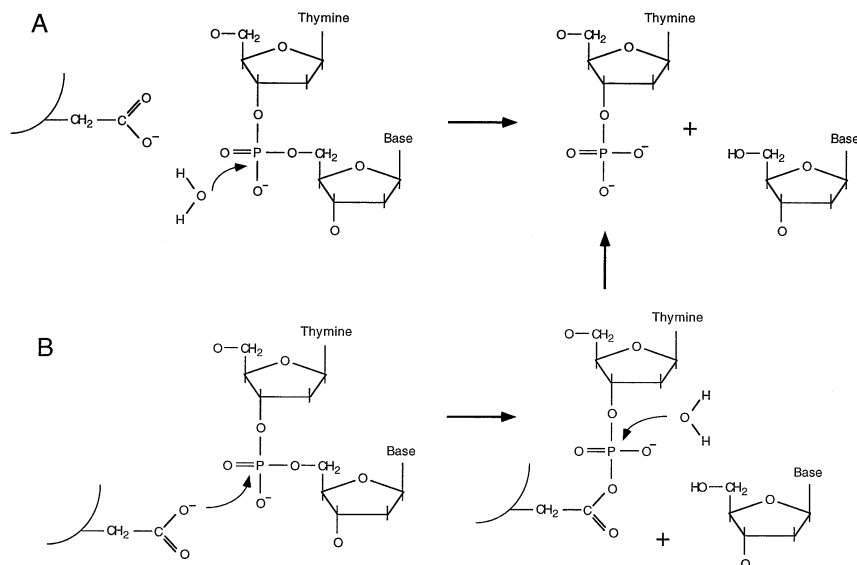


Figure 10. Possible mechanisms of DNA cleavage by topoisomerase mutants with alternative active site nucleophiles. (A) Cleavage by enzyme catalyzed attack of water on the DNA phosphodiester; (B) Cleavage by transesterification to form an acylphosphate intermediate followed by hydrolysis of the covalent adduct.

cysteine is a very poor proton acceptor, except when ionized, in which case we might have expected the Cys-274 mutant to evince an increase in activity at higher pH values (which favor ionization), instead of the relatively broad optimum observed.

Mechanism B is analogous to the wild-type hydrolysis reaction, insofar as it posits the enzyme-mediated attack of water on a covalent protein–DNA intermediate. In the wild-type reaction, the hydrolysis of the tyrosyl-phosphate linkage is extremely slow compared to the rate of attack by Tyr-274 on the DNA backbone to form the covalent adduct (7). The apparent absence of a covalent intermediate in our experiments involving the Glu-274 and His-274 mutants, and the minuscule levels of covalent intermediate formed by Cys-274, can be rationalized if the rates of hydrolysis of their respective acylphosphate, phosphoramidate and phosphorothioate intermediates are significantly greater than the respective rates of intermediate formation. Clearly, there is an enormous catalytic penalty when non-tyrosine side chains serve as nucleophiles in this scheme. Yet, if mechanism B applies, it is also clear that the rate of topoisomerase-mediated hydrolysis of the putative Glu-274 acylphosphate intermediate at pH 6.5 is 20-fold faster than the rate of hydrolysis of the phosphotyrosine adduct by the wild-type enzyme, presumably because glutamate is a better leaving group than tyrosine.

At present, we cannot choose with certainty between the two models, or even exclude the possibility that some of the alternative nucleophiles act via pathway A and others via pathway B. The observed formation of a covalent protein–DNA adduct by Cys-274 on the 18mer scissile strand, and the formation of a hairpin strand transfer product, are at least consistent with a transesterification-based pathway for the Cys-274 mutant. The extremely low enzymatic activity of Cys-274 is consistent with non-enzymatic studies by Dantzman and Kiessling (23) on the chemical reactivity of a 2' thiol at a 3' nucleoside phosphodiester. They demonstrate that the sulfur can transesterify to form a cyclic phosphorothioate, but note that the rate of thiolate attack on the adjacent phosphodiester bond is 10^{-7} slower than that of an alkoxide moiety, perhaps because sulfur is less inclined than oxygen to occupy the apical position of the presumptive pentacoordinate phosphorane transition state (23). Our findings suggest that cysteine can engage in enzyme-catalyzed transesterification at a DNA phosphodiester.

Our findings concerning the use of alternative nucleophiles by a eukaryotic type IB topoisomerase are in keeping with recent studies of the RepA protein of plasmid pC194, which initiates rolling circle replication via site-specific transesterification to DNA to form a DNA–(5'-phosphotyrosyl)–protein adduct (24). The initiation step resembles that of bacteriophage phiX174 A protein, but the RepA and A proteins differ with respect to the termination reaction. The termination step carried out by the phiX174 A protein involves transesterification by a second tyrosine side chain at the newly synthesized origin site and attack by the resulting 3' OH-terminus on the initial phosphotyrosyl linkage (25,26). pC194 RepA uses glutamate in lieu of a second tyrosine to achieve termination by endonucleolytic cleavage of the new initiation sequence. This is occurs via hydrolysis instead of transesterification (24). The proposed mechanism of catalysis by glutamate is analogous to that depicted in Figure 10A (except that a 3' OH is the leaving group). Replacing the glutamate of pC194 RepA by tyrosine effects a switch in the termination reaction mechanism from hydrolysis to transesterification (24). A pC194 RepA mutant containing glutamate at both active site

positions retained a low level of replication activity *in vivo*, suggesting that glutamate may function in place of tyrosine during the initiation step, albeit inefficiently.

Yokochi *et al.* (27) reported recently that histidine substitution for the active site tyrosine of *E. coli* topoisomerase IV converted the enzyme into an endonuclease. Topo IV is a type II enzyme that normally transesterifies to both strands of DNA to form DNA–(5'-phosphotyrosyl)–enzyme adducts at sites staggered by 4 nt. The His mutant of topo IV introduced single-strand nicks into supercoiled plasmid DNA to yield open circles that were apparently not linked to the enzyme. Although the authors suggest a mechanism whereby the histidine side chain facilitates direct attack by a water molecule on the DNA backbone (again analogous to Fig. 10A), the experiments do not exclude the formation and hydrolysis of a phosphoamide intermediate.

In conclusion, this and other recent studies underscore the evolutionary links between enzymes that break the phosphodiester backbone of DNA by hydrolysis and those that act through a covalent DNA–enzyme intermediate.

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