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DNA strand transfer reactions catalyzed by vaccinia topoisomerase: hydrolysis and glycerololysis of the covalent protein–DNA intermediate

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ABSTRACT

Vaccinia topoisomerase forms a covalent protein–DNA intermediate at sites containing the sequence 5′-CCCTT↓. The T↓ nucleotide is linked via a 3′-phosphodiester bond to Tyr-274 of the enzyme. Here, we report that the enzyme catalyzes hydrolysis of the covalent intermediate, resulting in formation of a 3′-phosphate-terminated DNA cleavage product. The hydrolysis reaction is pH-dependent (optimum pH = 9.5) and is slower, by a factor of 10^{-5}, than the rate of topoisomerase-catalyzed strand transfer to a 5′-OH terminated DNA acceptor strand. Mutants of vaccinia topoisomerase containing serine or threonine in lieu of the active site Tyr-274 form no detectable covalent intermediate and catalyze no detectable DNA hydrolysis. This suggests that hydrolysis occurs subsequent to formation of the covalent protein–DNA adduct and not via direct attack by water on DNA. Vaccinia topoisomerase also catalyzes glycerololysis of the covalent intermediate. The rate of glycerololysis is proportional to glycerol concentration and is optimal at pH 9.5.

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) noncovalent 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)CCTT↓ (1). The T↓ nucleotide (designated position +1) is linked to Tyr-274 of the enzyme (2).

Duplex DNA substrates containing a single cleavage site have been used to study individual steps of the vaccinia topoisomerase reaction (3–10). ‘Suicide’ substrates have been especially useful; these are CCCTT-containing DNAs that contain ≤6 bp 3′ of the scissile bond. An example of a suicide substrate is shown in Figure 1A. Attack by the active site tyrosine of topoisomerase on the suicide substrate results in formation of a covalent intermediate and this is accompanied by dissociation of the 6 nt leaving group, ATTCCC. With no readily available DNA acceptor for religation, the enzyme is essentially trapped on the DNA. The structure of this suicide intermediate in shown in Figure 1B. In the presence of a molar excess of topoisomerase, >90% of the CCCTT-containing DNA strand becomes covalently bound to protein. The rate constant for single-turnover strand cleavage of the suicide substrate at 37°C is 0.28/s (11).

Topoisomerase bound covalently to the suicide substrate can transfer the incised DNA strand to a DNA acceptor strand containing a 5′-OH terminus (4,5). Religation occurs rapidly when the suicide intermediate is provided with an exogenous 5′-OH terminated acceptor strand, the sequence of which is complementary to the single strand tail of the noncleaved strand in the immediate vicinity of the scissile phosphate. In this reaction, the 5′-OH of the acceptor strand attacks the 3′-phosphotyrosyl bond and expels Tyr-274 as the leaving group. The rate constant for strand religation under these circumstances is ~1.3/s (6,11). Religation is a topoisomerase-catalyzed event, in so far as amino acid substitutions on the enzyme can reduce the rate of single-turnover DNA religation by two to four orders of magnitude (11,12). Strand transfer can also occur in the absence of an exogenous acceptor when the 5′-OH end of the noncleaved strand of the suicide intermediate attacks the 3′ phosphotyrosyl bond. This results in formation of a hairpin DNA loop. The rate constant for hairpin formation by the suicide intermediate shown in Figure 1B is 5.7×10^{-4}/s (5).

Here, we report that the vaccinia suicide intermediate catalyzes transfer of the covalently held strand to water, resulting in formation of a 3′-phosphate-terminated cleavage product (Fig. 1C). The rate of hydrolytic cleavage by vaccinia topoisomerase is slower, by a factor of 10^{-5}, than the rate of religation to a DNA acceptor strand. The pH-dependence of the rate of hydrolysis is distinctly different from that of the transesterification reaction to DNA (13). The vaccinia topoisomerase can also catalyze strand transfer to glycerol. These reactions are analogous to the DNA hydrolysis and glycerololysis reactions of human DNA topoisomerase I, which were described by Christiansen *et al.* (14).

MATERIALS AND METHODS

Preparation of the suicide cleavage intermediate

An 18mer 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

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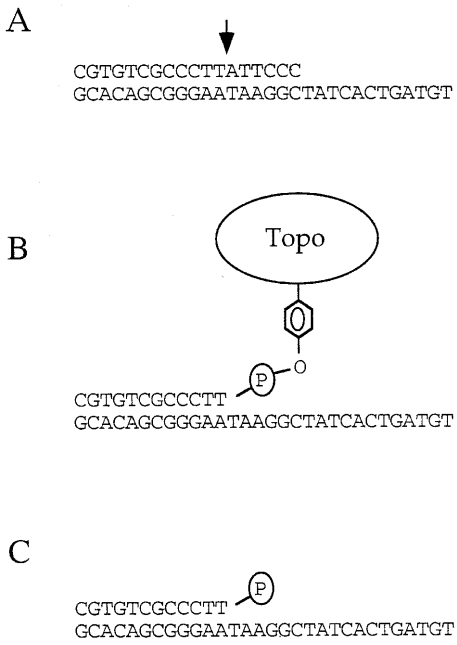

Figure 1. Structure of the DNA suicide substrate for vaccinia topoisomerase (**A**), the cleaved suicide intermediate (**B**) and the hydrolysis reaction product (**C**). See text for details.

hybridized to complementary 30mer strand to form the suicide substrate (Fig. 1A). Recombinant vaccinia topoisomerase was expressed in bacteria and purified as described (15). The phosphocellulose enzyme fraction was used in all experiments. Covalent topoisomerase–DNA complexes were formed in a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 3 pmol 18mer/30mer DNA and 10 pmol vaccinia topoisomerase (per 20 µl). The mixture was incubated for 5 min at 37°C, then processed as indicated.

Hydrolysis of the cleavage intermediate

Aliquots (4 µl) of a suicide cleavage reaction mixture were pipetted into 36 µl of a 50 mM buffer solution, either sodium acetate pH 4.6; sodium 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.6 or 6.5; Tris–HCl pH 7.5 or 8.5; sodium 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 9.5 or 10.5. The samples were incubated for 72 h at 37°C. The reactions were then quenched by adding SDS to 0.5%. The samples were deproteinized by serial extraction with phenol–chloroform and chloroform–isoamyl alcohol. The aqueous material was adjusted to 50% formamide and then heated at 95°C for 5 min. The samples were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA).

To determine the rates of hydrolysis of the suicide cleavage intermediate, 20 µl aliquots of the cleavage reaction mixture were pipetted into 180 µl of a 50 mM solution of either sodium acetate pH 4.6; MES pH 5.6 or 6.5; Tris–HCl pH 7.5 or 8.5; CAPS pH 9.5 or 10.5. Aliquots (20 µl) were withdrawn at 3, 6, 12, 24, 48 and 72 h. The samples were quenched immediately by adding SDS, then deproteinized, and analyzed by gel electrophoresis as described in the preceding paragraph. The extent of hydrolysis was quantitated by scanning the wet gel using a FUJIX BAS1000 Bio-Imaging Analyzer.

Glycerololysis of the cleavage intermediate

Aliquots (20 µl) of a suicide cleavage reaction mixture were pipetted into 180 µl of a 50 mM solution of either Tris–HCl (pH 7.5 or 8.5) or CAPS (pH 9.5) containing either no added glycerol or glycerol added to a final concentration of 1, 5, 10 or 20% (v/v). The samples were incubated for 72 h at 37°C, then processed as described above and electrophoresed through a 17% polyacrylamide gel.

Amino acid substitutions at the active site of vaccinia topoisomerase

Mutations Y274S and Y274T were introduced into the vaccinia virus topoisomerase gene by using the two-stage PCR-based overlap extension method (16). Plasmid pA9topo (15) was used as the template for the first stage PCR reaction. Gene fragments with overlapping ends obtained from the first stage reactions were paired and used as template in the second stage amplification. Products containing the entire topoisomerase gene were cloned into the T7-based expression vector pET3c as described (17) to generate plasmids pET-Y274S and pET-Y274T. All mutations were confirmed by dideoxy sequencing.

Expression and purification of mutant proteins

The pET-Y274S and pET-Y274T plasmids were transformed into *Escherichia coli* BL21. Topoisomerase expression was induced by infection with bacteriophage λCE6 as described (15). Mutant topoisomerases were purified from soluble bacterial lysates by phosphocellulose column chromatography (15). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (BioRad) with bovine serum albumin as the standard.

RESULTS

Hydrolysis of the topoisomerase–DNA intermediate

Incubation of vaccinia topoisomerase with an 18mer/30mer suicide substrate that has been 5′ ^{32}P-labeled on the CCCTT-containing strand results in formation of a covalent intermediate in which a 12mer oligonucleotide 5′ [^{32}P]CGTGTCGCCCTT is linked to the enzyme through a 3′-phosphotyrosyl bond (Fig. 1B). This bond is chemically stable provided the enzyme has been denatured or proteolyzed before it is subjected to analysis. For example, a DNA-(3-phosphotyrosine) linkage is resistant to treatment with 1 M HCl or 1 M NaOH for 5 h at 37°C (18). In contrast, the native topoisomerase–DNA intermediate reacts readily to transfer the covalently bound 12mer to a suitable 5′ OH-terminated DNA acceptor strand (4). This religation reaction proceeds to completion within 5–10 s after adding the acceptor DNA (11). In the absence of an acceptor strand, the 12mer remains covalently bound to the enzyme, i.e., we previously found no evidence for release of the 12mer from the topoisomerase during incubation for up to 60 min at neutral pH (11).

A more sensitive assay of the stability of the covalent intermediate now reveals that a discrete 5′ ^{32}P-labeled CCCTT-containing DNA oligonucleotide was liberated after incubation of the suicide intermediate for 72 h at pH 7.5 (Fig. 2, lane 3). This

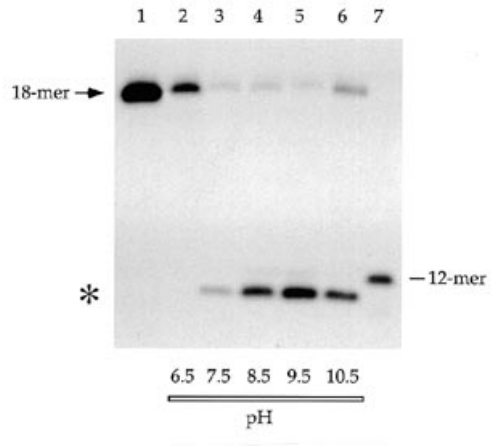

Figure 2. pH-dependent hydrolysis of the CCCTT-containing DNA strand. Suicide cleavage intermediates were incubated for 72 h at 37°C in 50 mM buffer at the indicated pH (lanes 2–6). A control reaction containing the 5′ ^{32}P-labeled suicide substrate but lacking topoisomerase was processed in parallel (lane 1). The reaction products were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea. An autoradiogram of the gel is shown. Lane 7 contains a 5′ ^{32}P-labeled 12mer oligonucleotide (5′-CGTGTC-GCCCTT).

product migrated a half-nucleotide step faster during denaturing polyacrylamide gel electrophoresis than did a 12mer oligonucleotide 5′ [^{32}P]CGTGTCGCCCTT containing a 3′-OH terminus (Fig. 2, lane 7). We surmise that the reaction product is the 3′ phosphate-terminated species 5′-[^{32}P]CGTGTCGCCCTTp and that it arises via transfer of the covalently bound strand to water or hydroxyl ion rather than to a DNA nucleophile.

pH-dependence of hydrolysis of the suicide intermediate

The amount of the 12mer product formed depended on the pH of the hydrolysis reaction mixture (Fig. 2). Little or no 12mer was detected when the suicide intermediate (which was formed during a 5 min incubation at pH 7.5) was adjusted to pH 6.5 and incubated for an additional 72 h (Fig. 2, lane 2), nor was any 12mer formed during a 72 h incubation at pH 5.6 or 4.6 (data not shown). The yield of the hydrolysis product increased at alkaline pH, with maximal hydrolysis observed at pH 9.5 (lane 5). The extent of hydrolysis declined as pH was increased further to 10.5 (lane 6).

The effects of pH on the rate of hydrolysis by the suicide intermediate are shown in Figure 3. In this experiment, 95% of the input CCCTT-containing strand was covalently bound to the topoisomerase after the 5 min suicide cleavage reaction and no hydrolysis product was detected at this point (time zero). The hydrolysis time course was initiated by dilution of the suicide intermediate into reaction buffers with pH values between 4.6 and 10.5. Aliquots were withdrawn at 3, 6, 12, 24, 48 and 72 h, and the products were analyzed by gel electrophoresis. The amount of the of the hydrolysis product formed at pH 9.5 (expressed as the % of the input 5′-end-labeled 18mer strand converted to 12mer) increased steadily with time; 15% of the input DNA was hydrolyzed in 72 h. The initial rate of DNA hydrolysis at pH 9.5 was 0.31% of input DNA hydrolyzed/h. The 12mer product accumulated linearly over 72 h at pH 8.5 and 7.5, albeit more slowly than at pH 9.5 (Fig. 3A). At pH 10.5, the 12mer accumulated linearly for 12 h, at which time 1.5% of the input

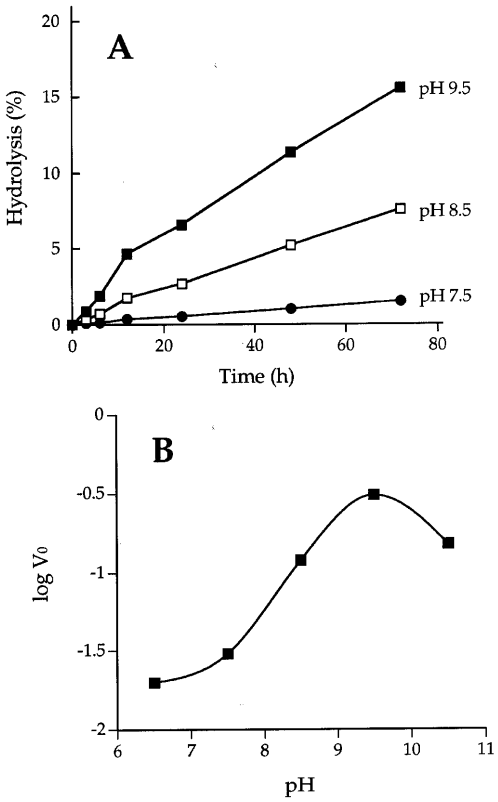

Figure 3. pH-dependence of the rate of hydrolysis. (**A**) Suicide cleavage intermediates were incubated at 37°C in 50 mM buffer. Aliquots were withdrawn after 3, 6, 12, 24, 48 and 72 h and the reactions were quenched by adding SDS. The deproteinized reaction products were resolved by gel electrophoresis and the gel was scanned with a phosphorimager. The extent of hydrolysis (expressed as the % of the input 18mer strand converted to 12mer) is plotted as a function of time. (**B**) The initial rates of hydrolysis (% of input DNA hydrolyzed/h) were determined from the plot in (A) and other data not shown. The logarithm of the initial rate is plotted as a function of the pH of the hydrolysis reaction buffer.

DNA was hydrolyzed; the reaction progressed slowly thereafter with only 1.9% hydrolyzed at 72 h (not shown). At pH 6.5, the reaction was near-linear for 24 h, at which time the reaction plateaued with 0.2% of input DNA hydrolyzed (not shown). No hydrolysis product was detected over the course of a 72 h incubation at either pH 5.6 or 4.6 (not shown). This behavior was likely caused by inactivation of the topoisomerase during the long incubations at pH 4.6–6.5 and 10.5. It was noted previously that vaccinia topoisomerase is inactivated in a time-dependent fashion ($t_{1/2}$ ~ 20 min) during incubation at pH ≤5.0 (6).

The dependence of the logarithm of the initial rate of hydrolysis as a function of pH in the range 6.5–10.5 is shown in Figure 3B. The rate of hydrolysis peaks at pH 9.5 and declines at lower values. In contrast, the rate constant for transfer of the cleaved strand from the covalent intermediate to a complementary DNA strand is independent of pH from 4.6 to 9.8 (13).

That the hydrolysis reaction was indeed enzyme-catalyzed was confirmed by performing a control experiment in which the covalently bound topoisomerase was inactivated by adding 0.5% SDS to the suicide intermediate prior to incubation for 72 h at pH 7.5, 8.5 and 9.5. Treatment with SDS prevented the appearance of the 12mer hydrolysis product (data not shown).

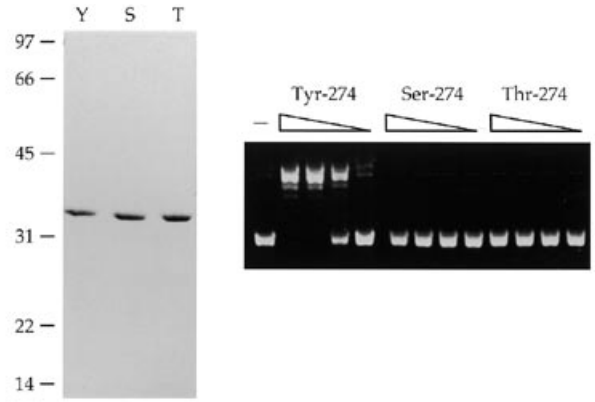

Figure 4. Characterization of mutants Ser-274 and Thr-274. (**Left**) Protein purification. The phosphocellulose preparations of wild-type topoisomerase (Tyr-274; lane Y), and mutants Ser-274 (lane S) and Thr-274 (lane T) were analyzed by SDS–PAGE. An aliquot of 5 µg of protein was applied to each lane. 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. (**Right**) DNA relaxation. Reaction mixtures (20 µl) contained 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 2.5 mM EDTA, 0.3 µg of pUC19 DNA and serial 10-fold dilutions of the indicated phosphocellulose enzyme preparations (100, 10, 1, 0.1 ng of protein, proceeding from left to right within each titration series). Enzyme was omitted from a control reaction (–). After incubation for 15 min at 37°C, the reactions were quenched by addition of a solution containing SDS (0.3% final concentration), glycerol, xylene cyanol and bromophenol blue. Samples were analyzed by electrophoresis through a 1.2% horizontal agarose gel in TBE buffer. The gels were stained in a 0.5 µg/ml ethidium bromide solution, destained in water, and photographed under short wave UV illumination.

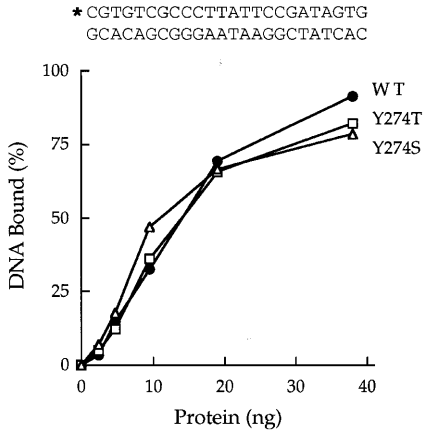

Figure 5. DNA binding by catalytically defective topoisomerase mutants. Reaction mixtures (20 µl) containing 50 mM Tris–HCl, pH 7.5, 0.3 pmol of 5′ ^{32}P-labeled 24-bp CCCTT-containing DNA and increasing amounts of the indicated topoisomerase preparations, were incubated for 5 min at 37°C. The mixtures 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 24-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 phosphorimager. DNA binding is plotted as a function of input protein. The structure of the DNA ligand is shown; an asterisk denotes the 5′-label on the CCCTT-containing strand.

Tyrosine-274 is essential for both suicide cleavage and hydrolysis

There are two potential reaction pathways by which vaccinia topoisomerase might hydrolyze DNA: (i) enzyme-catalyzed attack by water or hydroxyl ion on the DNA-(3′-phosphotyrosyl) linkage of the suicide intermediate, or (ii) enzyme-catalyzed attack by water or hydroxyl ion directly on the phosphodiester backbone of DNA. We gave serious consideration to the second pathway in light of recent studies of FLP recombinase (19). FLP, like vaccinia topoisomerase, catalyzes DNA strand cleavage and rejoining reactions via a covalent DNA–(3′-phosphotyrosyl) intermediate. Lee and Jayaram have reported that FLP mutants lacking the active site tyrosine are inert in forming the covalent protein–DNA adduct, but are nonetheless capable of site-specific hydrolysis of DNA in the presence of a strong nucleophile, e.g., hydrogen peroxide (19). Hence, FLP can catalyze direct attack on the scissile phosphate.

In order to address whether this is the case for the hydrolysis reaction of the vaccinia topoisomerase, we 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 proteins were expressed in *E.coli* and purified from soluble bacterial extracts by phosphocellulose chromatography. The topoisomerase polypeptide constituted the major species in the protein preparations, as determined by SDS–PAGE, and the extents of purification of the mutants were equivalent to that of the wild-type protein (Fig. 4). The mutant proteins were tested for their ability to relax a supercoiled plasmid substrate. Activity was quantitated by end-point dilution, beginning with 100 ng of the phosphocellulose topoisomerase preparation and decreasing by serial 10-fold decrements to 0.1 ng. 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 Ser-274 and Thr-274 proteins were inactive even at 100 ng of input protein (Fig. 4). We further characterized these two mutants with respect to their ability to form a covalent adduct on the 18mer/30mer suicide substrate. Suicide cleavage reactions containing 50 nM topoisomerase and 15 nM DNA were incubated for up to 5 days at 37°C at pH 7.5. The wild-type topoisomerase (Tyr-274) cleaved 74% of the input scissile strand in 5 s, whereas Ser-274 and Thr-274 catalyzed no detectable cleavage in 5 days (data not shown). We also detected no suicide cleavage in 5 days when we assayed the activity of the Ala-274 mutant described previously (17). Hence, any alterations of the tyrosine nucleophile, even ones that preserve the nucleophilic hydroxyl moiety, reduced the rate of DNA cleavage by a factor of at least 10^{-7}.

A native gel mobility shift assay (20) was used to analyze the DNA-binding properties of these mutants. The ^{32}P-labeled DNA ligand was a 24-bp CCCTT-containing DNA (Fig. 5). The Ser-274 and Thr-274 proteins each formed a single discrete protein–DNA complex of retarded mobility, the yield of which was proportional to the amount of input topoisomerase (Fig. 5). The DNA binding affinities of the Ser-274 and Thr-274 mutants were equivalent to that of the wild-type protein.

Having established that the Ser-274 and Thr-274 mutant proteins were defective in covalent catalysis, but not DNA binding, we assayed their ability to form the 12mer hydrolysis product during a 72 h continuous incubation at either pH 7.5, 8.5 or 9.5. No hydrolysis was detected (data not shown). We conclude that formation of the covalent protein–DNA intermediate is a prerequisite for site-specific DNA hydrolysis by vaccinia

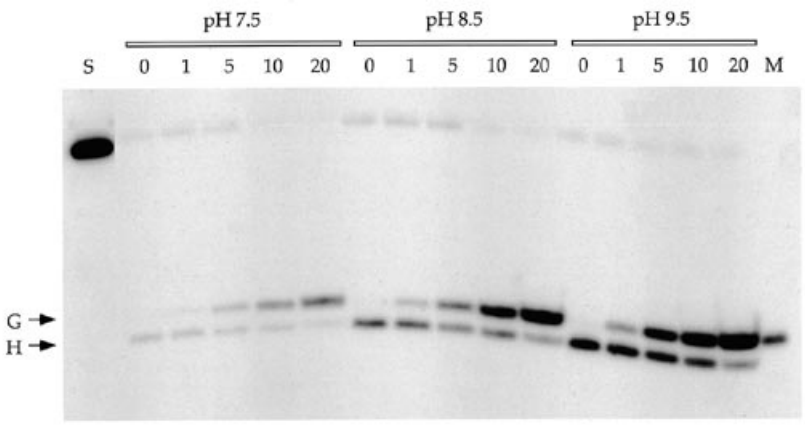

Figure 6. pH-dependent glycerololysis of the CCCTT-containing DNA strand. Suicide cleavage intermediates were incubated for 72 h at 37°C in 50 mM buffer (pH 7.5, 8.5 or 9.5) containing 0, 1, 5, 10 or 20% glycerol. The concentration of glycerol is indicated above each lane. A control reaction containing the 5′ ^{32}P-labeled suicide substrate but lacking topoisomerase was processed in parallel (lane S). The reaction products were electrophoresed through a 17% polyacrylamide gel containing 7.5 M urea. An autoradiogram of the gel is shown. Lane M contains a 5′ ^{32}P-labeled 12mer oligonucleotide (5′-CGTGTCGCCCTT). The products of hydrolysis (H) and glycerololysis (G) are indicated on the left by arrows.

topoisomerase and that the enzyme does not catalyze the direct attack of water on DNA.

Glycerololysis of the topoisomerase–DNA intermediate

A single reaction product was liberated upon hydrolysis of the covalent topoisomerase–DNA intermediate at pH 7.5, 8.5 or 9.5 (Fig. 6, denoted by H). However, a novel reaction product was detected when the intermediate was incubated in the presence of glycerol. This product (G in Fig. 6) migrated about a half-nucleotide step more slowly than the hydrolysis product. The amount of G formed was directly proportional to the concentration of glycerol (Figs 6 and 7A). Forty-two percent of the input 18mer strand was converted to G during incubation at pH 9.5 in 20% glycerol (Fig. 7A). Indeed, the increase in the amount of G at high glycerol concentrations occurred at the expense of the hydrolysis product H (Fig. 6), suggesting that glycerol is competing with water in attacking the suicide intermediate. Product G is presumed to be the 3′ phosphoglycerol-terminated species 5′-[^{32}P]CGTGTCGCC-CTTp(glycerol). To confirm that formation of product G was enzyme-catalyzed, we performed a control experiment in which the covalently bound topoisomerase was inactivated by adding 0.5% SDS to the suicide intermediate prior to incubation with 20% glycerol for 72 h at pH 7.5, 8.5 and 9.5. Treatment with SDS prevented the appearance of the glycerololysis product (data not shown).

Glycerololysis, like hydrolysis, was optimal at pH 9.5 over the entire range of glycerol concentrations tested (Fig. 7A). The kinetics of DNA strand transfer to glycerol were examined as a function of pH in the presence of 20% glycerol (Fig. 7B). The amount of product G formed increased steadily with time over 72 h. The initial rate of glycerololysis at pH 9.5 was 0.83% of input DNA converted to product G/h (Fig. 7B). The rates of glycerololysis at pH 8.5 and 7.5 were 0.46 and 0.13%/h, respectively. The rates of glycerololysis were two to four times faster than the rates of hydrolysis of the suicide intermediate in the range of pH 7.5–9.5 (compare Figs 3A and 7B).

Decay of the topoisomerase–DNA intermediate during hydrolysis and glycerololysis

Kinetic evidence that the products of hydrolysis and glycerololysis were derived from the covalent topoisomerase–DNA adduct is shown in Figure 8. In this experiment, the suicide intermediate was incubated at pH 9.5 with or without 20% glycerol. Aliquots were withdrawn at various times and the level of the covalent intermediate was measured by subjecting the samples to SDS–PAGE (3). Ninety-one percent of the input CCCTT-containing strand was bound covalently to the topoisomerase after the 5 min suicide cleavage reaction (time zero). Subsequent incubation at pH 9.5 in the absence of glycerol resulted in a time-dependent decrease in the level of the covalent intermediate (i.e., to 72% after 72 h at 37°C) (Fig. 8). The rate and extent of decay of the covalent topoisomerase–DNA intermediate at pH 9.5 were in good agreement with the rate and extent of formation of the hydrolysis product in the experiment shown in Figure 3. This implies a precursor–product relationship between the covalent topoisomerase–DNA complex and 3′ phosphate-terminated oligonucleotide.

Inclusion of 20% glycerol during the incubation at pH 9.5 accelerated the rate of decay of the covalent intermediate by 3–4-fold (Fig. 8). After 72 h, 31% of the CCCTT strand remained bound covalently to protein. The amount of DNA released from the covalent intermediate (60% of the input labeled strand) was consistent with the sum of glycerololysis and hydrolysis reaction products formed during incubation at pH 9.5 (i.e., as seen in the experiments shown in Figs 6 and 7).

DISCUSSION

We have shown that vaccinia topoisomerase catalyzes hydrolysis of the DNA-(-3′-phosphotyrosyl) linkage of the covalent intermediate to liberate a 3′-phosphate-terminated DNA. Kinetic analysis illuminates significant differences between hydrolysis and DNA transesterification by the vaccinia covalent intermediate. First, the rate of hydrolysis is extremely slow compared with strand transfer

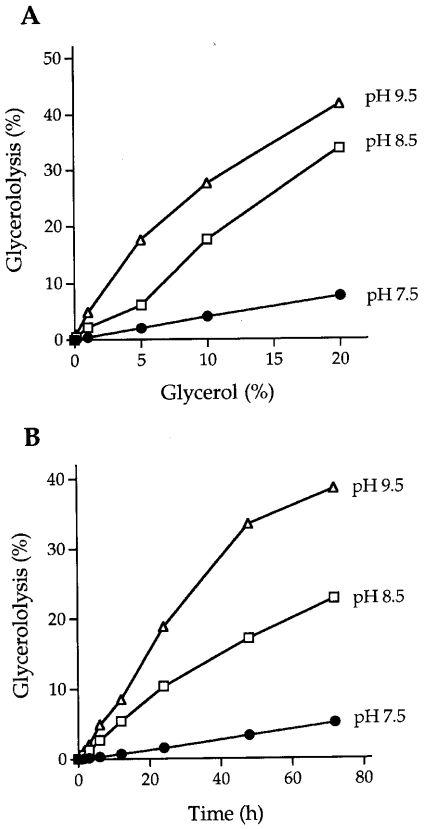

Figure 7. Glycerol concentration-dependence and pH rate-dependence of glycerololysis. (**A**) Glycerol concentration-dependence. The gel in Figure 6 was scanned with a phosphorimager. The amount of product G formed (expressed as the % of the input 18mer converted to G) is plotted as a function of glycerol concentration. (**B**) Kinetics of glycerololysis. Suicide cleavage intermediates were incubated at 37°C in 50 mM buffer (pH 7.5, 8.5 or 9.5) containing 20% glycerol. Aliquots were withdrawn after 1.5, 3, 6, 12, 24, 48 and 72 h and the reactions were quenched by adding SDS. The extent of glycerololysis (expressed as the % of the input 18mer strand converted to product G) is plotted as a function of time.

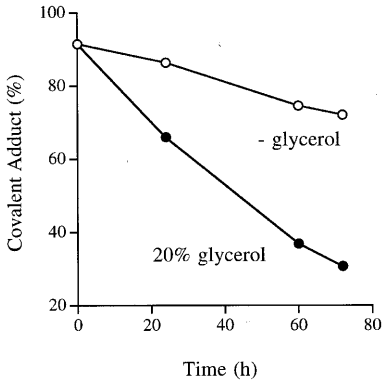

Figure 8. Decay of the topoisomerase–DNA intermediate during hydrolysis and glycerololysis. Suicide cleavage intermediates were incubated at 37°C in 50 mM CAPS buffer (pH 9.5) in the presence or absence of added glycerol. Aliquots were withdrawn at the times indicated and the reactions were quenched with SDS. The samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. The radiolabeled protein–DNA adduct was detected by autoradiographic exposure of the dried gel. The level of covalent adduct (expressed as the % of the input 5′ ^{32}P-labeled oligonucleotide that was covalently transferred to the topoisomerase polypeptide) was quantitated by scanning the gel with a phosphorimager.

to DNA. The hydrolysis reaction did not reach an endpoint, even after 72 h. By comparing the initial rate of hydrolysis at pH 9.5 (0.31% of covalent–intermediate hydrolyzed/h) to the initial rate of intermolecular DNA religation by the 12mer/30mer suicide intermediate [90% of covalent–intermediate religated in 5 s (5)], we conclude that strand transfer to water is ~$10^{-5.3}$ slower than religation to DNA.

Second, the pH-dependence of hydrolysis is markedly different from the pH-dependence of DNA religation (and, for that matter, from the pH-dependence of covalent adduct formation). The pH-dependence of the rate constant for cleavage of a CCCTT-containing suicide substrate is bell-shaped, with optimal cleavage at neutral pH and apparent pK_a values of 6.3 and 8.4 (13). The dependence of the cleavage–religation equilibrium constant reveals similar pK_a values of 5.8 and 8.6. Stivers *et al.* (13) proposed a mechanism for cleavage of the DNA phosphodiester backbone involving general base catalysis of the attack of Tyr-274 at the scissile phosphorus and general acid catalysis of the expulsion of the 5′-ribose hydroxyl group of the leaving DNA strand. It is presumed that the religation reaction proceeds by the same mechanism, i.e., general base catalysis of the attack of a DNA 5′-hydroxyl end on the phosphorus of the DNA-(3-phosphotyrosyl) linkage and general acid catalysis of expulsion of Tyr-274. However, the experiments of Stivers *et al.* (13) indicate that the rate of DNA religation by the covalent intermediate is independent of pH. They suggest that the pK_a values of the catalytic groups on the enzyme are masked by a rate-limiting pH-independent conformational step which occurs prior to the chemical step of religation.

If hydrolysis of the covalent intermediate is mechanistically analogous to DNA strand transfer, then why is the pH-dependence of hydrolysis so distinct from the pH-independent DNA religation reaction? We suggest that the hydrolysis reaction occurs via the attack of a hydroxide ion on the suicide intermediate and that the alkaline pH optimum reflects the dependence of the reaction rate on hydroxide ion concentration in the range of 6.5–9.5. Note that DNA strand transfer is assayed by adding a molar excess of the acceptor oligonucleotide to the suicide intermediate and the reaction rate is zero order with respect to acceptor concentration (6). Note also that the DNA acceptor strand is held in position at the active site of the topoisomerase because it is base-paired to the 18mer single-strand tail of the noncleaved strand. In this state, the 5′-OH terminus would be positioned optimally to attack the phosphorus. Free hydroxide ion (or any other small non-DNA nucleophile) would not be so constrained. This may account, in part, for the extremely slow rate of hydrolysis compared with DNA religation. Another possible explanation for the slow rate of hydrolysis is that the active site of the covalently bound enzyme is not very accessible to solvent. However, the observation that the +1 thymine base of the covalently bound CCCTT strand is hypersensitive to oxidation by permanganate (10) militates against exclusion of small molecules from the active site.

The finding that replacement of the active site Tyr-274 by serine or threonine abrogates the transesterification and hydrolysis reactions (without apparent effect on noncovalent DNA binding) argues that the vaccinia topoisomerase does not catalyze direct attack of water on the DNA backbone. The Ser-274 and Thr-274 mutants were impaired in transesterification by at least seven orders of magnitude compared with the wild type topoisomerase.

Note that a serine does serve as the nucleophile in site-specific DNA transesterification reactions catalyzed by resolvases and invertases (21). Hence, the vaccinia virus topoisomerase displays an extremely stringent requirement for tyrosine at the active site. This may be a general property of the eukaryotic type I enzyme family, insofar as serine substitution at the active site tyrosine of *Saccharomyces cerevisiae* topoisomerase I resulted in an apparent loss of DNA relaxation activity (22); however, the effects of serine substitution on yeast topoisomerase reaction chemistry have not been quantitated.

Vaccinia topoisomerase, like the human type I enzyme (14), can catalyze strand transfer to glycerol. It remains to be established whether the 3′ phosphate of the glycerololysis product is linked exclusively to the α or β carbon of glycerol or if the product is a mixture of α and β derivatives. Given the yield of glycerololysis product (40% of input DNA), the relative ease of obtaining large quantities of recombinant vaccinia topoisomerase, and the likelihood that other non-DNA nucleophiles will attack the covalent intermediate, this reaction pathway may prove useful for the synthesis of novel 3′ DNA phosphodiesters.

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