DNA Strand Transfer Catalyzed by Vaccinia Topoisomerase: Peroxidolysis and Hydroxylaminolysis of the Covalent Protein−DNA Intermediate†

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Received January 31, 2000; Revised Manuscript Received March 14, 2000

ABSTRACT: Vaccinia topoisomerase forms a covalent DNA−(3′-phosphotyrosyl)−enzyme intermediate at sites containing the sequence 5′-CCCTT↓. The covalently bound topoisomerase can religate the CCCTT strand to a 5′-OH-terminated polynucleotide or else transfer the strand to a non-DNA nucleophile such a water or glycerol. Here, we report that vaccinia topoisomerase also catalyzes strand transfer to hydrogen peroxide. The observed alkaline pH-dependence of peroxidolysis is consistent with enzyme-mediated attack by peroxide anion on the covalent intermediate. The reaction displays apparent first-order kinetics. From a double-reciprocal plot of k_{obs} versus [H_2O_2] at pH 10, we determined a rate constant for peroxidolysis of 6.3×10^{-3} s^{-1}. This rate is slower by a factor of 200 than the rate of topoisomerase-catalyzed strand transfer to a perfectly aligned 5′-OH DNA strand but is comparable to the rate of DNA strand transfer across a 1-nucleotide gap. Strand transfer to 2% hydrogen peroxide is 300 times faster than strand transfer to 20% glycerol and ∼2000 times faster than topoisomerase-catalyzed hydrolysis of the covalent intermediate. Hydroxylamine is also an effective nucleophile in topoisomerase-mediated strand transfer ($k_{obs} = 6.4 \times 10^{-4}$ s^{-1}). The rates of the peroxidolysis, hydroxylaminolysis, glycerololysis, and hydrolysis reactions catalyzed by the mutant enzyme H265A were reduced by factors of 100−700, in accordance with the 100- to 400-fold rate decrements in DNA cleavage and religation by H265A. We surmise that vaccinia topoisomerase catalyzes strand transfer to DNA and non-DNA nucleophiles via a common reaction pathway in which His-265 stabilizes the scissile phosphate in the transition state rather than acting as a general acid or base.

DNA relaxation by vaccinia virus topoisomerase entails a series of partial reactions common to all eukaryotic type IB topoisomerases. These are the following: (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; (iv) strand religation (*1*). A distinctive feature of the vaccinia topoisomerase is that it binds and cleaves duplex DNA at a specific target sequence 5′-(T/C)CCTT↓ (*2*).

DNA substrates containing a single cleavage site have been used to study the cleavage and religation transesterification reactions of vaccinia topoisomerase (Figure 1A). "Suicide" substrates have been especially useful; these are CCCTT-containing DNAs that contain six or fewer base pairs 3′ of the scissile bond. (An example of a suicide substrate is shown in Figure 2.) Attack by the active site Tyr-274 of vaccinia topoisomerase on the suicide substrate results in formation of a covalent intermediate and dissociation of the 6-nucleotide leaving group, ATTCCC. In the presence of a molar excess of topoisomerase, >90% of the CCCTT-containing DNA strand is covalently bound to protein. The rate constant for single-turnover strand cleavage of the suicide substrate at 37 °C is ∼0.3 s^{-1} (*3*). Topoisomerase bound covalently to the suicide substrate can transfer the incised DNA strand to a DNA acceptor strand containing a 5′-OH terminus (*4*). 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 (Figure 1A). The rate constant for strand religation under these circumstances is ∼1.2 s^{-1} (*5*, *6*).

The cleavage−religation equilibrium of type IB topoisomerase on duplex DNA favors the noncovalently bound state. However, the equilibrium can be strongly skewed toward covalent adduct formation in vitro by modifications in the DNA target site and by drugs such as camptothecin that selectively impede religation (*7*−*9*). DNA structural alterations that induce high levels of covalent adduct formation by topoisomerase IB include naturally occurring lesions such as nicks, gaps, base mismatches, abasic sites, UV-induced thymine dimers, deoxyuridine incorporation, and ribonucleotide incorporation immediately 3′ of the scissile phosphate and nicks on the complementary strand immediately flanking the scissile phosphate (*6*, *10*−*13*). Thus, topoisomerase will catalyze suicide cleavage reactions in vivo in response to natural and pharmacological poisons. Covalent trapping of topoisomerase IB on DNA by camptothecin is cytotoxic.

† This work was supported by Grant GM46330 from the National Institutes of Health (to S. S.). B.O.K. is a Ph.D. candidate at the University of Aarhus and is supported in part by the Danish Cancer Society and the Danish Center for Molecular Gerontology.

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10.1021/bi000184u CCC: $19.00
Published on Web 04/29/2000

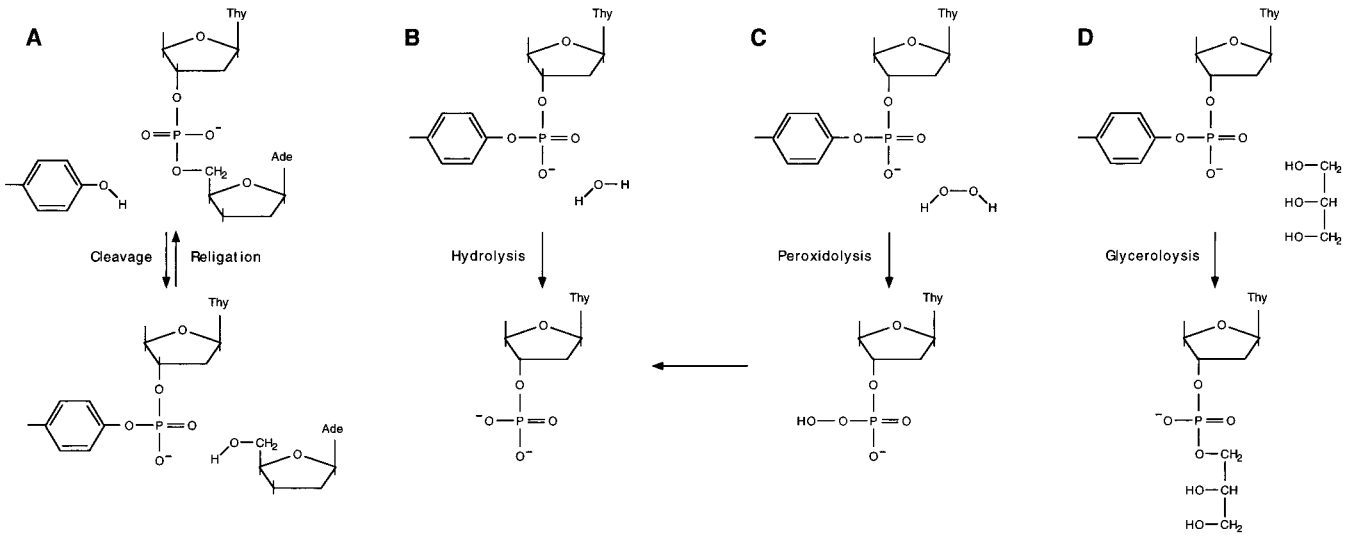

FIGURE 1: Strand transfer reactions with DNA and non-nucleic acid nucleophiles. (A) Reversible transesterification. In the forward cleavage reaction, attack by Tyr-274 on the scissile phosphate results in formation of a covalent DNA−(3′-phosphotyrosyl-)−enzyme intermediate and expulsion of a 5′-OH DNA leaving group. Religation entails attack by the 5′-OH DNA terminus to restore the DNA phosphodiester backbone and expel the tyrosine. (B) Hydrolysis of the covalent intermediate to yield a DNA−3′-phosphate. (C) Proposed mechanism of peroxidolysis. (D) Strand transfer to glycerol.

In principle, there are multiple pathways to remove topoisomerase I when it binds covalently to DNA and the original 5′-OH leaving strand is not available for religation. These pathways can be classified as extrinsic (i.e., catalyzed by enzymes other than topoisomerase) or intrinsic (i.e., catalyzed by topoisomerase per se). An example of an extrinsic repair factor is the 3′-tyrosyl phosphodiesterase discovered by Nash and colleagues; this enzyme, the product of a single gene, can hydrolytically resolve DNA−3′-pTyr to yield DNA−3′-phosphate and Tyr (*14*). Topoisomerase itself is capable of transferring the covalently held DNA strand to a nucleic acid acceptor other than the strand originally cleaved. The acceptor can be 5′-OH DNA, in which case the repair process is recombinogenic (*4*, *6*, *15*−*18*). The acceptor can also be 5′-OH RNA (*6*).

Recent studies indicate that topoisomerase IB also transfers its covalently held DNA strand to non-nucleic acid nucleophiles. Topoisomerase-catalyzed attack by water or hydroxide ion on the covalent intermediate releases the topoisomerase and leaves a DNA−3′-phosphate product (Figure 1B) (*19*, *20*). Reaction with glycerol or its anion results in formation of a DNA−3′-phosphoglycerol product (Figure 1D) (*19*, *20*). Other polyalcohols can serve as nucleophiles with varying efficacy (*20*). The hydrolysis and glycerololysis reactions catalyzed by vaccinia topoisomerase are extremely slow compared to DNA strand transfer, but the extents of the reactions can be quite high (*20*). Lisby et al. (*21*) reported that hydrogen peroxide is a more effective nucleophile that water or glycerol in resolving the covalent intermediate formed by human DNA topoisomerase I.

Hydrogen peroxide is generated in cells at low levels as a byproduct of normal metabolism and at higher levels as a consequence of oxidative stress or in response to external cues (*22*). Thus peroxide may well contribute to a physiological response to topoisomerase IB poisons (said poisons may include free radicals derived from hydrogen peroxide that damage DNA). In addition, the analysis of reactions of topoisomerase IB with efficient non-nucleic acid nucleophiles such as peroxide can provide instructive insights into enzyme mechanism. Here we study in depth the peroxidolysis reaction catalyzed by vaccinia topoisomerase. Near-quantitative liberation of a DNA−3-phosphate product is achieved at alkaline pH with a rate constant of 6.3×10^{-3} s^{-1}. Hydroxylamine is also an effective nucleophile in resolving the covalent topoisomerase−DNA intermediate. We show that strand transfer to the non-DNA nucleophiles is dependent on His-265, which interacts with the scissile phosphate. Mechanistic implications of the findings are discussed.

METHODS AND MATERIALS

Preparation of the Suicide Cleavage Intermediate. An 18-mer CCCTT-containing DNA oligonucleotide was 5′ end-labeled by enzymatic phosphorylation in the presence of [γ^{32}P]ATP and T4 polynucleotide kinase and then gel-purified and hybridized to an unlabeled complementary 30-mer strand to form the suicide substrate (Figure 2). A 5′-phosphate was added to the 30-mer strand during chemical synthesis to preclude intramolecular hairpin formation (*4*). Recombinant wild-type vaccinia topoisomerase and mutant enzyme H265A were expressed in bacteria and purified as described (*23*). The phosphocellulose enzyme fractions were used in all experiments. Covalent topoisomerase−DNA complexes were formed in reaction mixtures containing (per 5 μL) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer/30-mer DNA, and 2 pmol of wild-type vaccinia topoisomerase or mutant H265A. The mixtures were incubated for 5 min (wild-type topoisomerase) or 2 h (H265A) at 37 °C and then processed as indicated.

Peroxidolysis of the Covalent Intermediate. Aliquots (5 μL) of a suicide cleavage reaction mixture were pipetted into 45 μL of a 50 mM buffer solution of either bis-trispropane (BTP) or sodium 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and hydrogen peroxide as specified. The samples were incubated at 37 °C. The reactions were quenched by adding an equal volume of stop solution containing 95% formamide, 1% SDS, 20 mM EDTA, and 0.05% bromphenol blue. The samples were heated at 95 °C

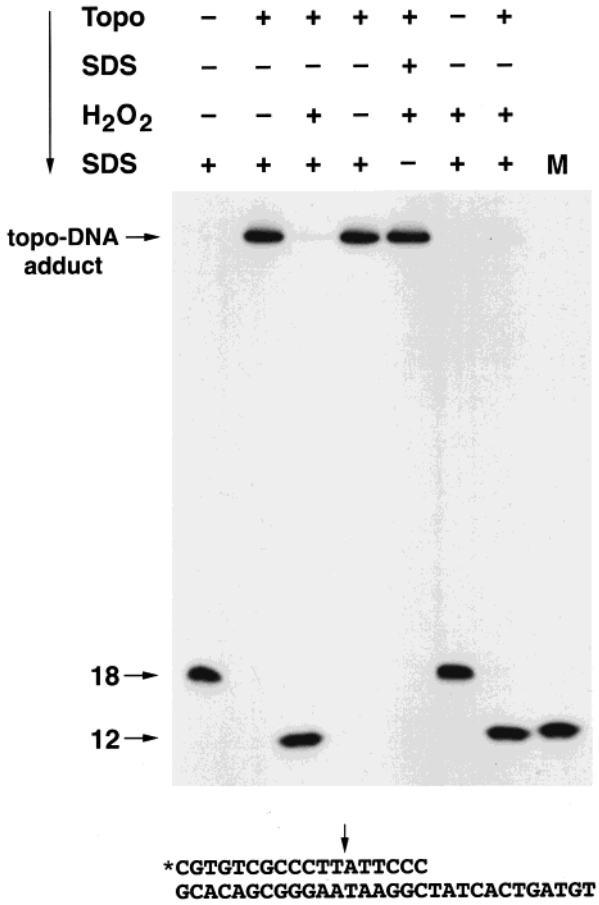

FIGURE 2: Topoisomerase-catalyzed peroxidolysis of the suicide covalent intermediate. The 18-mer/30-mer suicide substrate is shown at the bottom of the figure with the cleavage site indicated by the arrow and the 5′-^{32}P-label denoted by an asterisk. Aliquots of preformed topoisomerase−DNA complexes (Topo +) or the DNA substrate alone (Topo −) were incubated for 1 h at 37 °C in 50 mM BTP buffer (pH 10) with 0.5% H_2O_2 (H_2O_2 +) or with buffer lacking peroxide (H_2O_2 −). The reactions were quenched with SDS and analyzed by gel electrophoresis. An autoradiograph of the gel is shown. A control reaction (SDS + above H_2O_2 +) contained topoisomerase−DNA complexes that were adjusted to 1% SDS prior to incubation for 1 h with 0.5% H_2O_2 in 50 mM BTP (pH 10) and 0.5% SDS. The arrow to the left of the lane headings specifies the order of addition of SDS relative to hydrogen peroxide. A 5′-^{32}P-labeled 12-mer d(CGTGTCGCCCTT) is analyzed in lane M. The positions of the 18-mer scissile strand, the topoisomerase−DNA adduct, and the 12-mer peroxidolysis product are denoted by arrows on the left.

for 5 min. Aliquots (40 μL) were electrophoresed through a 20% polyacrylamide gel containing 7.5 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA).

To determine the rates of peroxidolysis of the suicide cleavage intermediate, aliquots of a cleavage reaction mixture were pipetted into a 9× volume of a 50 mM solution of either BTP or CAPS buffer containing hydrogen peroxide as specified. Aliquots (50 μL) were withdrawn after various intervals of incubation at 37 °C, and the reactions were quenched immediately by adding an equal volume of stop solution. Samples were analyzed by gel electrophoresis as described above. The extent of peroxidolysis was quantitated by scanning the wet gel using a Fujix BAS2500 phosphorimager. A plot of the percent of input labeled DNA converted to free 12-mer product versus time established end point values for peroxidolysis. The data were normalized to the

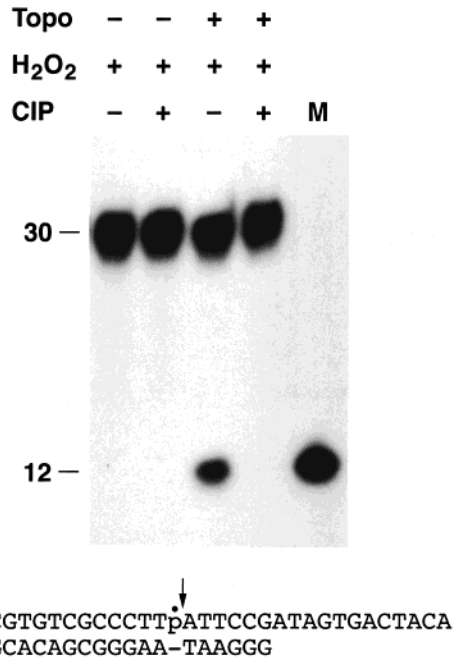

FIGURE 3: Peroxidolysis of a covalent intermediate labeled at the scissile phosphate. The 30-mer/18-mer substrate is shown at the bottom of the figure with the cleavage site indicated by the arrow and the internal ^{32}P-label denoted by a dot. Reaction mixtures containing (per 5 μL) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 30-mer/18-mer DNA, and either 2 pmol of topoisomerase (Topo +) or no added enzyme (Topo −) were incubated for 5 min at 37 °C. Aliquots (5 μL) were diluted 1:9 into 50 mM BTP buffer (pH 10) at a final concentration of 0.5% H_2O_2. The reactions were quenched after 1 h at 37 °C by addition of SDS to 1%. The samples were ethanol precipitated, and the insoluble material was resuspended in 20 μL of Tris-HCl (pH 7.5). The samples were split into two aliquots, one of which was incubated for 1 h at 37 °C with 1 unit of calf intestine alkaline phosphatase (CIP +), while the other was incubated without phosphatase (CIP −). The reaction products were denatured in formamide and then analyzed by electrophoresis through a 20% polyacrylamide gel. An autoradiograph of the gel is shown. The positions of the 30-mer scissile strand and the 12-mer peroxidolysis product are denoted on the left. A 5′-^{32}P-labeled 12-mer d(CGTGTCGCCCTT) is analyzed in lane M.

end point values, and the apparent rate constant k_{obs} was determined by fitting the data to the equation $(100 - \%Cl_{norm}) = 100e^{-kt}$.

Preparation of a Cleavage Substrate Radiolabeled Uniquely at the Scissile Phosphate. An 18-mer DNA oligonucleotide d(ATTCCGATAGTGACTACA) was 5′ end-labeled by enzymatic phosphorylation in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase and then gel-purified. The labeled 18-mer and an unlabeled 12-mer oligonucleotide d(CGTGTCGCCCTT) were annealed to a complementary 36-mer DNA strand (at a molar ratios of 18-mer/12-mer/36-mer of 1:3:2) to form a nicked duplex molecule radiolabeled at the 5′-phosphate of the nick. The nick was sealed by reaction of the DNA with *Chlorella* virus DNA ligase (*24*). The ligation product, a 30-mer labeled internally at CCCTTpA, was gel-purified and then hybridized to a complementary 18-mer oligonucleotide (in 4-fold molar excess) to form the 30-mer/18-mer topoisomerase cleavage substrate depicted in Figure 3.

Effects of Transient Exposure to Hydrogen Peroxide on Topoisomerase Activity. Vaccinia topoisomerase (7.5 μg) was preincubated at 37 °C in a 10 μL solution of 4% H_2O_2 in

buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.5 mM DTT, 10% glycerol, 0.1% Triton X-100). Aliquots of the mixture were withdrawn after 5 or 45 min exposure to peroxide and then diluted 1:9 in buffer A to attain a protein concentration of 75 ng/μL. The diluted enzyme was immediately assayed for transesterification activity on the 5′-^{32}P-labeled 18-mer/30-mer suicide substrate. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18/30-mer DNA, 2 pmol of topoisomerase (1 μL of diluted peroxide-treated protein), and 0.02% H_2O_2 (contributed by the peroxide-treated enzyme) were incubated at 37 °C. A control reaction mixture contained 0.02% H_2O_2 plus 2 pmol of topoisomerase that had not been preincubated with peroxide. The reactions were initiated by addition of a prewarmed DNA solution to the prewarmed enzyme. Aliquots (20 μL) were withdrawn at the times specified, and the reaction was quenched by adding 5 μL of SDS sample buffer (0.25 mM Tris-HCl (pH 6.8), 5% SDS, 50% glycerol, and 0.5% bromphenol blue. The samples were analyzed by SDS−PAGE. Covalent complex formation was revealed by the transfer of 5′-^{32}P-labeled oligonucleotide to the topoisomerase polypeptide. The extent of covalent adduct formation (i.e., the percent of the input 5′-^{32}P-label that was covalently transferred to protein) was quantitated by scanning the gel with a phosphorimager.

Hydroxylaminolysis. Aliquots of a suicide cleavage reaction mixture were pipetted into a 9× volume of a 100 mM solution of BTP buffer (pH 9) containing hydroxylamine as specified. The hydroxylamine stock solution was also pH 9. Aliquots (50 μL) were withdrawn after incubation at 37 °C, and the reactions were quenched by adding SDS to 1% final concentration. The samples were ethanol precipitated in the presence of 20 μg of glycogen, resuspended in a solution of 50% formamide, 10 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol, and then electrophoresed through a 20% polyacrylamide gel containing 7.5 M urea in TBE.

Materials. Hydrogen peroxide (30% w/w solution) was purchased from Sigma. Hydroxylamine (50% w/w solution) was from Aldrich. Glycerol was purchased from Fisher. Alkaline phosphatase from calf intestine was purchased from Boehringer Mannheim.

RESULTS

Peroxidolysis of the Topoisomerase−DNA Intermediate. Incubation of vaccinia topoisomerase with an 18-mer/30-mer suicide substrate that was 5′-^{32}P-labeled on the CCCTT-containing strand resulted in formation of a covalent intermediate in which a 12-mer oligonucleotide 5′-^{32}P-CGTGTCGCCCTT was linked to the enzyme through a 3′-phosphotyrosyl bond. The bond was stable in SDS, and the covalent intermediate remained trapped at the sample origin when the reaction products were electrophoresed through a polyacrylamide/urea gel (Figure 2). Nearly all (94%) of the input 18-mer strand became covalently bound to the topoisomerase. Incubation of the covalent intermediate with 0.5% H_2O_2 at pH 10 for 60 min resulted in the liberation of a discrete 5′-^{32}P-labeled 12-mer oligonucleotide at the expense of the labeled covalent intermediate (Figure 2). Incubation of the labeled DNA substrate with hydrogen peroxide in the absence of topoisomerase resulted in no change in its electrophoretic mobility or release of shorter cleavage products. Denaturation of the covalent intermediate with SDS prior to the addition of hydrogen peroxide completely prevented release of the labeled 12-mer (Figure 2).

The free ^{32}P-labeled product of the reaction with peroxide migrated about a half-nucleotide step faster during denaturing polyacrylamide gel electrophoresis than did the 12-mer oligonucleotide 5′-^{32}P-CGTGTCGCCCTT containing a 3′-OH terminus (Figure 2, lane M). We surmised that the reaction product was a 3′-phosphate-terminated species 5′-^{32}P-CGTGTCGCCCTTp and that it arose via topoisomerase-catalyzed transfer of the covalently bound strand to hydrogen peroxide.

Peroxidolysis of a Covalent Intermediate Labeled at the Scissile Phosphate. Further evidence that the peroxidolysis reaction generated a 3′-phosphate-terminated product is shown in Figure 3. Here, we used DNA ligase to prepare a 30-nucleotide scissile strand that was internally ^{32}P-labeled uniquely at the scissile phosphate. This strand was annealed to a complementary 18-mer strand to form an 18-bp duplex with a 12-nucleotide 3′ tail (Figure 3). Reaction of topoisomerase with this substrate resulted in covalent attachment of Tyr-274 to the labeled phosphate. Twenty percent of the input substrate was covalent bound to the enzyme in this experiment. Subsequent reaction of the topoisomerase−DNA complex with hydrogen peroxide resulted in the release of a ^{32}P-labeled 12-mer oligonucleotide. The instructive finding was that the ^{32}P-label in the 12-mer peroxidolysis product was eliminated completely by treatment with alkaline phosphatase (CIP), whereas the labeled phosphodiester of the 30-mer strand was unaffected by alkaline phosphatase (Figure 3). This result, together with the prior analysis of the 5′-labeled reaction product, proved that peroxidolysis entails attack on the DNA−3′-phosphotyrosine bond to form a 3′-phosphomonoester. We suggest a reaction scheme in Figure 1, initially proposed by Lisby et al. (*21*) for human topoisomerase I, whereby the immediate reaction product, a DNA−3′-peroxyphosphate, spontaneously decays to yield a 3′-phosphate end.

Alkaline pH Dependence of the Peroxidolysis Reaction. The effects of pH on peroxidolysis of the 5′-^{32}P-labeled covalent intermediate are shown in Figure 4A. In this experiment, 95% of the input CCCTT-containing strand was covalently bound to the topoisomerase after the 5 min cleavage reaction. The peroxidolysis reaction was initiated by dilution of the covalent intermediate into BTP buffers, with pH values between 6.5 and 10, and simultaneous adjustment of the mixtures to 0.5% H_2O_2. The reactions were quenched after 30 min and the products analyzed by gel electrophoresis. Little 12-mer was formed at pH 6.5 and 7.5, but the yield of the 12-mer product increased progressively to 80% of total labeled DNA as the pH was increased from 8 to 10 (Figure 4A). A plot of the log of the extent of peroxidolysis versus pH yielded a straight line with a slope of 0.55 (not shown).

We propose, in agreement with Lisby et al. (*21*), that the peroxidolysis reaction occurs via attack of the peroxide anion on the covalent intermediate and that the alkaline pH optimum reflects the increased concentration of the reactive anionic species at progressively alkaline pH.

Kinetic Analysis of the Peroxidolysis Reaction. The extent of peroxidolysis of the covalent intermediate in a 5 min

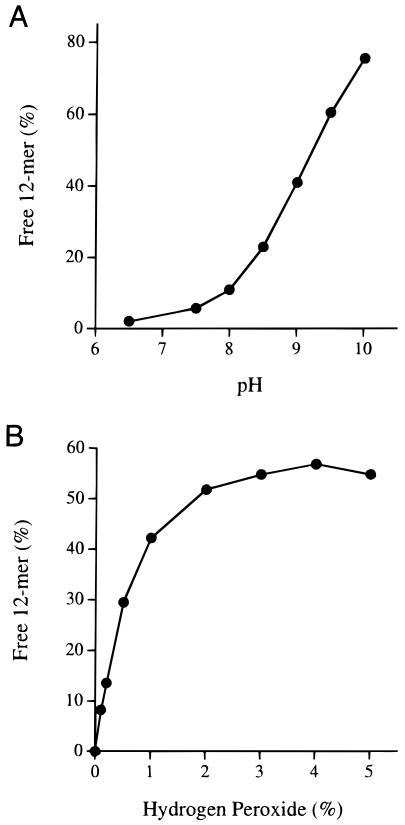

FIGURE 4: pH-dependence and H_2O_2 concentration dependence of peroxidolysis. (A) Suicide covalent complexes were incubated for 30 min at 37 °C in 50 mM BTP buffer (at pH 6.5, 7.5, 8, 8.5, 9, 9.5, or 10) with 0.5% H_2O_2. The yield of free ^{32}P-labeled 12-mer product (expressed a percent of total radioactivity) is plotted as a function of pH. (B) Suicide cleavage complexes were incubated for 5 min at 37 °C in 50 mM BTP buffer (pH 10) with 0, 0.1, 0.2, 0.5, 1, 2, 3, 4, or 5% H_2O_2. The yield of free ^{32}P-labeled 12-mer product is plotted as a function of hydrogen peroxide concentration.

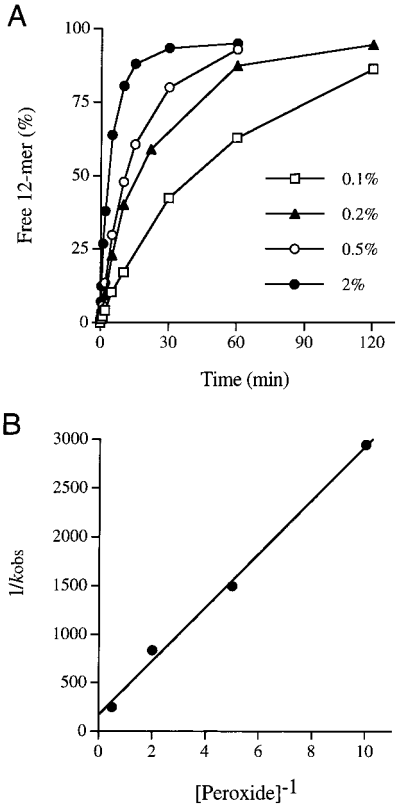

FIGURE 5: Kinetics of peroxidolysis. (A) Suicide covalent complexes were incubated at 37 °C in 50 mM BTP buffer (pH 10) with 0.1, 0.2, 0.5, or 2% H_2O_2. Aliquots were withdrawn at the times indicated and quenched immediately with stop-solution containing SDS. The yield of free ^{32}P-labeled 12-mer product (expressed a percent of total radioactivity) is plotted as a function of time for each peroxide concentration. (B) The data in part A were normalized to the end point values and the apparent rate constant for each reaction (k_{obs}) was determined by fitting the data to the equation $(100 - \%Cl_{norm}) = 100e^{-kt}$. A double-reciprocal plot of $1/k_{obs}$ (s) versus $1/[\%\ H_2O_2]$ is shown.

reaction at pH 10 was proportional to H_2O_2 concentration up to 1% and plateaued at 2−5% H_2O_2 with approximately 55% of the input scissile strand converted to free 12-mer (Figure 4B). The rate of peroxidolysis was measured at pH 10 at four different H_2O_2 concentrations. At the optimal concentration of 2% H_2O_2, the 12-mer product accumulated steadily to an end point at which it comprised >90% of the total labeled DNA. The reaction was complete in 20−30 min (Figure 5A). The data fit well to a single exponential with an apparent rate constant (k_{obs}) of 4×10^{-3} s^{-1}. Decreasing the H_2O_2 concentration to 0.5, 0.2, and 0.1% progressively reduced the rates of the reaction without affecting the extents of peroxidolysis at the reaction end points (Figure 5A). The apparent rate constants were 1.2×10^{-3} s^{-1} (0.5% H_2O_2), 6.7×10^{-4} s^{-1} (0.2% H_2O_2), and 3.4×10^{-4} s^{-1} (0.1% H_2O_2). From a double-reciprocal plot of k_{obs} versus H_2O_2 concentration (Figure 5B), we calculated a rate constant k_{perox} of 6.3×10^{-3} s^{-1} extrapolated to infinite peroxide concentration. The apparent binding constant for hydrogen peroxide was 0.47 M (or 1.6%) at pH 10. On the basis of a pK_a of 11.6 for hydrogen peroxide and the assumption that peroxide anion is the reactive nucleophile, we calculated an apparent binding constant of 11 mM for the peroxide anion.

The kinetics of peroxidolysis were also measured in CAPS buffer at pH 10.5 in the presence of 2% H_2O_2. The reaction attained an end point at which 96% of the input scissile strand was converted to free 12-mer (not shown). The apparent rate constant was 7×10^{-3} s^{-1}.

Peroxidolysis of an Equilibrium Cleavage Substrate. The peroxidolysis experiments described above were conducted using a suicide covalent intermediate from which the 5′-OH portion of the scissile strand had dissociated. Thus, in strand transfer reactions catalyzed by the suicide intermediate, access of the incoming nucleophile to a putative "acceptor site" is unimpeded by the 5′-OH of the original leaving group. To evaluate if peroxidolysis occurs under conditions in which the leaving strand remains in place, we tested the reactivity of hydrogen peroxide with the equilibrium cleavage complex of topoisomerase bound to a 60-bp duplex substrate (Figure 6). At saturating levels of topoisomerase at pH 7.5, 20% of the input 60-mer was converted to a covalent intermediate. At pH 10, 15% of the scissile strand was covalently bound. The low extent of transesterification on the 60-bp substrate reflects the establishment of a cleavage−religation equilibrium that favors the noncovalently bound state. The reaction product is a 5′-^{32}P-labeled 30-mer oligonucleotide linked to the enzyme through a 3′-phosphotyrosyl bond. The unlabeled 30-mer 5′-OH leaving strand remains stably bound to the covalent complex because of extensive base pairing to the nonscissile strand. Successful attack by an exogenous nucleophile on the equilibrium

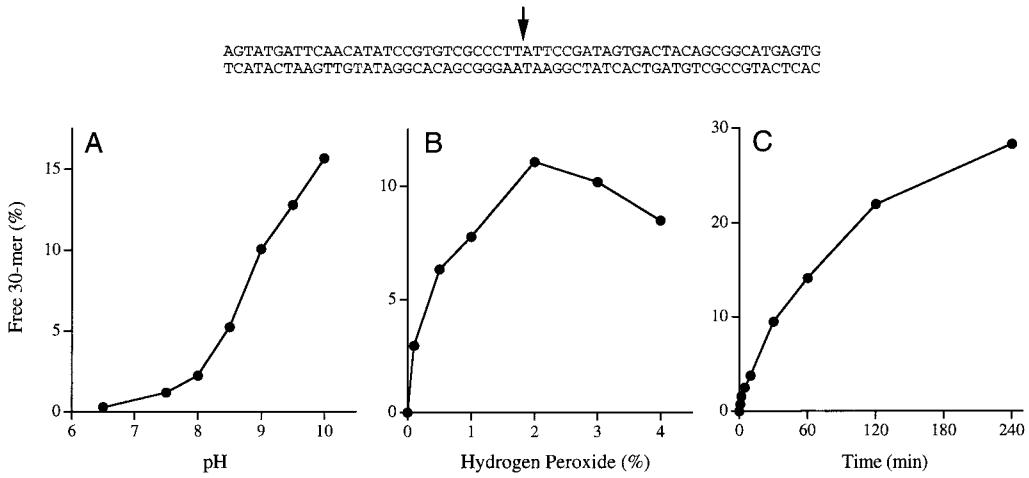

FIGURE 6: Peroxidolysis of an equilibrium cleavage complex. The 60-bp substrate is shown at the top of the figure with the cleavage site indicated by the arrow. Reaction mixtures containing (per 5 μL) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 60-bp DNA substrate, and 2 pmol of topoisomerase were incubated for 5 min at 37 °C and then processed as indicated. (A) Aliquots (5 μL) of the equilibrium cleavage reaction mixtures were mixed with 45 μL of BTP buffer (50 mM final concentration at pH 6.5, 7.5, 8, 8.5, 9, 9.5, or 10) containing H_2O_2 (0.5% final concentration) and incubated for 2 h at 37 °C. The yield of free ^{32}P-labeled 30-mer product (expressed a percent of total radioactivity) is plotted as a function of pH. (B) Equilibrium cleavage complexes were incubated for 30 min at 37 °C in 50 mM BTP buffer (pH 10) with 0, 0.1, 0.5, 1, 2, 3, or 4% H_2O_2. The yield of free ^{32}P-labeled 30-mer product is plotted as a function of H_2O_2 concentration. (C) Equilibrium cleavage complexes were incubated at 37 °C in 50 mM BTP buffer (pH 10) with 2% H_2O_2. Aliquots were withdrawn at the times indicated and quenched immediately with SDS. Release of ^{32}P-labeled 30-mer is plotted as a function of time.

cleavage complex necessarily entails competition for the acceptor site occupied by the 5′-OH end of the downstream 30-mer.

Incubation of preformed equilibrium cleavage complexes with 2% H_2O_2 resulted in the appearance of a free 5′-^{32}P-labeled 30-mer product (not shown). Control experiments confirmed that (i) formation of the free 30-mer required incubation of the 60-mer duplex with topoisomerase prior to treatment with hydrogen peroxide, i.e., peroxide alone did not cleave the DNA, and (ii) denaturation of the topoisomerase−DNA complex with SDS prior to treatment with peroxide blocked formation of the free 30-mer (not shown).

The alkaline pH dependence of the peroxidolysis reaction of the equilibrium cleavage complex (Figure 6A) was similar to that of the suicide intermediate. The yield of the free 30-mer at pH 10 varied with peroxide concentration and was optimal at 2% H_2O_2 (Figure 6B). The 30-mer product accumulated steadily during the reaction with 2% H_2O_2 at pH 10, reaching an apparent end point after 4 h at which 30% of the input 60-mer strand had been converted to free 30-mer (Figure 6C). The initial rate of 30-mer formation during the first 5 min (0.8% per min) was 3% of the initial rate of formation of free 12-mer by the suicide intermediate (27% per min) under the same reaction conditions. Correcting for the 6-fold greater abundance of reactive covalent complex at the onset of the peroxidolysis reaction on the suicide intermediate at pH 10, we see that the adjusted peroxidolysis reaction rate of the equilibrium complex was still only 18% of that of the suicide intermediate. We surmise that peroxide and the 5′-OH of the downstream DNA compete for a single acceptor site from which nucleophilic attack on the covalent intermediate can occur. The fact that peroxidolysis occurred at all on the equilibrium substrate implies conformation flexibility of the DNA duplex downstream of the scissile phosphate, whereby the 5′-OH of the noncovalently held segment of the cleaved strand moves out of the acceptor site. Said flexibility corresponds in all likelihood to the strand rotation step of the topoisomerase catalytic cycle (see Discussion).

Peroxidolysis of a Duplex Suicide Substrate. A better appreciation of the competition between DNA and peroxide for the acceptor site was obtained by analyzing the rate of peroxidolysis by topoisomerase bound covalently to a duplex suicide substrate that retained the downstream portion of the incised strand. Quantitative transesterification without dissociation of the DNA leaving strand can be achieved by introducing a 5′-bridging phosphorothiolate Tp↓(s)A at the cleavage site (*25*). The phosphorothiolate is situated within a 5′-^{32}P-labeled 34-mer such that transesterification results in covalent attachment of a labeled 12-mer to the topoisomerase; the 22-nucleotide 5′-SH leaving strand is held in place by base-pairing for its entire length to the noncleaved strand (Figure 7). Whereas the cleavage equilibrium constant (K_{cl}) for the 34-mer substrate with a standard Tp↓A phosphodiester is 0.35, the Tp↓(s)A modification shifts K_{cl} to >30 (*25*). At saturating enzyme, ≥97% of the input labeled strand is covalently bound to topoisomerase. The altered equilibrium is the result of the extremely poor reactivity of the 5′-sulfhydryl DNA terminus in the religation step (*25*).

Reaction of the duplex suicide intermediate with 2% H_2O_2 at pH 10 resulted in the release of 92% of the total labeled DNA as free 12-mer (Figure 7). The reaction was nearly complete in 60 min. The data fit well to a single exponential with an apparent rate constant of 1×10^{-3} s^{-1}. The rate constant for peroxidolysis of the suicide 34-mer duplex was one-fourth the rate constant for peroxidolysis of the suicide 12-mer/18-mer complex that had no DNA strand occupying the acceptor site (4×10^{-3} s^{-1}). The 4-fold difference in these two rate constants agrees quite well with the ∼5-fold difference in the normalized initial rates of peroxidolysis by the equilibrium 60-mer duplex versus the suicide 12-mer/18-mer (see above).

Pretreatment of Topoisomerase with Hydrogen Peroxide Inhibits Transesterification. The observation that the extent of peroxidolysis on the equilibrium substrate was limited to

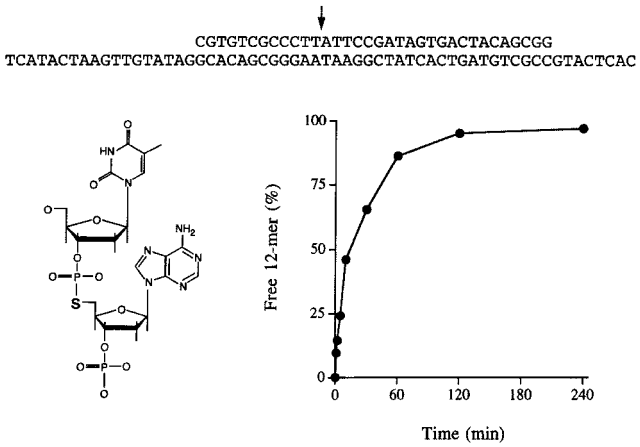

FIGURE 7: Peroxidolysis of a duplex suicide substrate. The 34-mer/60-mer duplex suicide substrate is shown with the cleavage site indicated by the arrow. The 5′-^{32}P-labeled scissile strand, containing a 5′-bridging phosphorothiolate Tpϟ(s)A, was hybridized to a 4-fold molar excess of the complementary 60-mer strand. The structure of the Tpϟ(s)A dinucleotide is illustrated on the left. A cleavage reaction mixture containing (per 5 μL) 50 mM Tris HCl (pH 7.5), 0.3 pmol of 34-mer/60-mer, and 2 pmol of topoisomerase was incubated for 5 min at 37 °C. The mixture was diluted 1:9 with BTP buffer (pH 10; 50 mM final concentration) containing H_2O_2 (2% final concentration) and incubation was continued at 37 °C. Aliquots were withdrawn at the times indicated and quenched immediately with SDS. The yield of free ^{32}P-labeled 12-mer product (expressed a percent of total radioactivity) is plotted as a function of time.

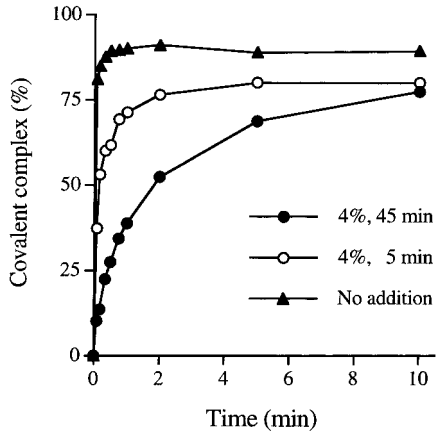

FIGURE 8: Effects of transient exposure to hydrogen peroxide on topoisomerase activity. Vaccinia topoisomerase was preincubated for 5 or 45 min in 4% H_2O_2 and then assayed for transesterification activity on the 18-mer/30-mer suicide substrate. The kinetics of covalent complex formation by peroxide-treated and control (untreated) enzyme preparations are shown.

30% of the input substrate led us to suspect that prolonged incubation in high concentrations of hydrogen peroxide might inactivate vaccinia topoisomerase. To test this, we transiently exposed concentrated aliquots of topoisomerase to 4% H_2O_2 for 5 or 45 min at 37 °C and then diluted the protein with peroxide-free buffer and assayed the treated protein for transesterification to the 18-mer/30-mer suicide substrate. The transesterification reaction mixtures contained a residual peroxide concentration of 0.02% contributed by the enzyme solution. The control transesterification reaction of topoisomerase that was not exposed to peroxide was therefore supplemented with 0.02% H_2O_2.

Kinetic analysis of the cleavage reactions showed that 4% H_2O_2 inactivated vaccinia topoisomerase in a time-dependent manner (Figure 8). The untreated control enzyme cleaved 90% of the input substrate, and the reaction was complete in 10 s. An apparent cleavage constant (k_{cl}) calculated from the extent of cleavage at the earliest time point (5 s) was 0.4 s^{-1}. (This value is similar to the rate constant for suicide cleavage by topoisomerase in reaction mixtures lacking 0.02% H_2O_2; thus, the residual peroxide had no effect on enzyme activity.) Topoisomerase pretreated with 4% H_2O_2 attained a similar reaction end point, but k_{cl} was slowed to 0.1 s^{-1} by 5 min exposure to 4% H_2O_2 or to 0.02 s^{-1} by 45 min in 4% H_2O_2.

Hydrogen peroxide can affect enzyme function by inducing conformational changes as a consequence of the oxidation by peroxide of cysteine side chains (*26*). Although neither of the two cysteines of vaccinia topoisomerase (Cys-100 or Cys-211) is essential for catalysis (*3*), it was conceivable that conformation effects of their oxidation might contribute to the observed peroxide inhibition of transesterification. Our finding that transient exposure to 4% H_2O_2 inhibited the transesterification activities of mutant proteins C100A and C211A and of the double-mutant C100A−C211A to the same extent as it inhibited wild-type topoisomerase (not shown) suggested that the effects of 4% H_2O_2 on vaccinia topoisomerase are not caused by cysteine oxidation.

The extent of peroxide damage to vaccinia topoisomerase depended on peroxide concentration. Transient exposure to 0.5% H_2O_2 had relatively little effect on strand cleavage; k_{cl} was 0.17 s^{-1} after 5 min exposure to 0.5% H_2O_2 and 0.11 s^{-1} after 45 min exposure. Treatment with 2% H_2O_2 resulted in a k_{cl} of 0.17 s^{-1} after 5 min exposure and 0.06 s^{-1} after 45 min exposure (data not shown).

Catalytic Role of Histidine-265 in Strand Transfer to Non-Nucleic Acid Nucleophiles. His-265 is one of four amino acid side chains of vaccinia topoisomerase other than Tyr-274 that are essential for DNA transesterification chemistry (*3*, *5*, *27*). (The other essential residues are Arg-130, Lys-167, and Arg-223.) We define essential residues as those at which an alanine substitution slows the rate of single-turnover DNA ligation (k_{rel}) by at least 2 orders of magnitude. Transesterification was restored to nearly wild-type rates when His-265 was substituted by Asn or Gln (*3*). These and other results argued that His-265 is not functioning as a general acid−base catalyst but rather engages in a hydrogen bond with the scissile phosphate that helps stabilize the transition state (*3*, *28*).

Is the peroxidolysis reaction catalyzed by the same functional groups on the topoisomerase that mediate the standard transesterification reactions of DNA cleavage and ligation? To address this question, we assayed peroxidolysis of the covalent intermediate of mutant protein H265A on the suicide substrate. H265A was reacted with the 18-mer/30-mer cleavage substrate for 2 h, at which time 95% of the CCCTT strand was covalently bound to the topoisomerase. The covalent intermediate was catalytically active in transferring the 5′-labeled CCCTT strand to an exogenous 18-mer 5′-OH DNA acceptor to form a 30-mer strand transfer product. Virtually all of the covalently bound 12-mer was transferred to the 18-mer DNA acceptor, and the apparent first-order rate constant (k_{rel}) was 3×10^{-3} s^{-1} (not shown). The religation rate constant for wild-type topoisomerase is ~1.2 s^{-1} (*5*).

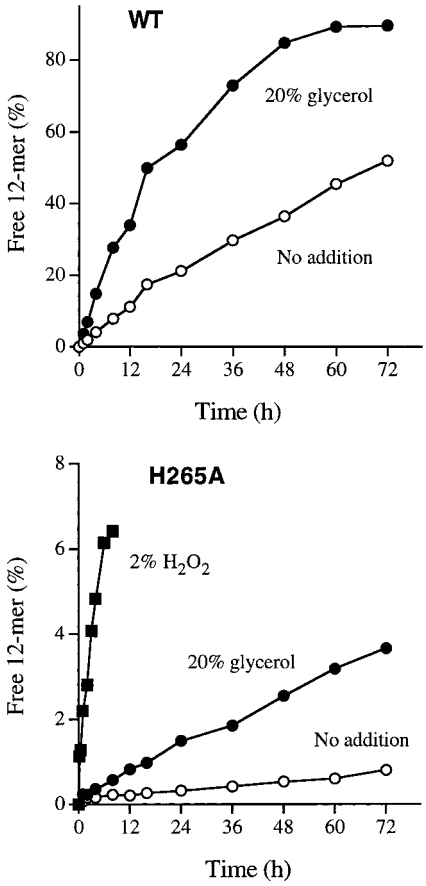

FIGURE 9: Catalytic role of His-265 in strand transfer to non-nucleic acid nucleophiles. Top panel: Glycerololysis and hydrolysis by wild-type topoisomerase. Suicide covalent complexes were incubated at 37 °C in 50 mM BTP buffer (pH 10) with either no added nucleophile (hydrolysis reaction) or with 20% glycerol (glycerololysis reaction). Bottom panel: Peroxidolysis, glycerololysis, and hydrolysis by H265A. Suicide cleavage complexes were incubated at 37 °C in 50 mM BTP buffer (pH 10) with either no added nucleophile, with 20% glycerol, or with 2% H_2O_2 (peroxidolysis reaction). Aliquots were withdrawn at the times specified, and the reactions were quenched with stop-solution containing SDS. The release of free ^{32}P-labeled oligonucleotide product (expressed a percent of total radioactivity) is plotted as a function of time.

The H265A covalent complexes were reacted with 2% H_2O_2 at pH 10, and release of the free 12-mer peroxidolysis product was measured as a function of time (Figure 9, bottom panel). The 12-mer accumulated slowly over 8 h to the extent that 6% of the input labeled strand was released from the covalent complex. Longer incubations resulted in no further product accumulation; we presume that the enzyme was ultimately inactivated by prolonged exposure to 2% H_2O_2 (see above). Although we could not calculate a peroxidolysis rate constant for the mutant protein, a comparison of the initial rate of peroxidolysis by H265A (0.037% 12-mer released/min) with the initial rate for the wild type covalent intermediate (27% 12-mer released per minute) indicated that the H265A mutation slowed the peroxidolysis reaction by a factor of approximately 700. This rate decrement for peroxidolysis is in accord with the approximately 400-fold reduction in the rate of reaction of covalently bound H265A with a 5′-OH DNA nucleophile.

Vaccinia topoisomerase also catalyzes glycerololysis of the covalent intermediate. The reaction is extremely slow, but yield of free product is quite high. For example, incubation of the wild-type suicide intermediate with 20% glycerol at pH 10 results in near-quantitative release of a free 12-mer product (DNA−3′-phosphoglycerol) over 60 h (Figure 9, top panel). The kinetic data fit to a single exponential with an apparent rate constant for glycerololysis of 1.3×10^{-5} s^{-1}. Thus, glycerololysis is 5 orders of magnitude slower than DNA religation (1.2 s^{-1}) and 300-fold slower than peroxidolysis in 2% H_2O_2 at pH 10 (4×10^{-3} s^{-1}). The instructive finding was that the H265A mutation significantly reduced the rate of glycerololysis (Figure 9, bottom panel) The free product accumulated steadily over a 72 h incubation in 20% glycerol to an extent of less than 4% of the input scissile strand being released from the covalent complex. Comparison of the initial rates of glycerololysis by H265A and wild type topoisomerase showed that the alanine substitution slowed the reaction by nearly 2 orders of magnitude.

We assayed the hydrolysis reactions of the wild-type and H265A covalent intermediates. The wild-type complex catalyzed the steady release of a free 12-mer strand to an extent of 50% of the total labeled DNA during a 72 h reaction at pH 10 (Figure 9, top panel; no addition). The hydrolysis reaction of H265A was quite feeble; >1% of the DNA was released as 12-mer in 72 h (Figure 9, bottom panel). Comparison of the initial rates of hydrolysis indicated that the H265A mutation elicited a rate decrement of nearly 2 orders of magnitude.

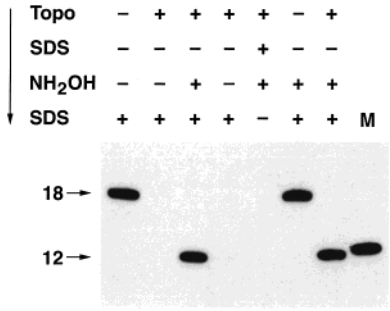

FIGURE 10: Hydroxylaminolysis of the covalent topoisomerase−DNA intermediate. Aliquots of topoisomerase−DNA complexes formed on the 18-mer/30-mer suicide substrate (Topo +) or the DNA substrate alone (Topo −) were incubated for 1.5 h at 37 °C in 100 mM BTP buffer with 1.2 M hydroxylamine at pH 9 (NH_2OH +) or with buffer lacking hydroxylamine (NH_2OH −). The reactions were quenched with SDS, the DNA was ethanol-precipitated, and the samples were analyzed by gel electrophoresis. An autoradiograph of the gel is shown. A control reaction (SDS + above NH_2OH +) contained topoisomerase−DNA complexes that were adjusted to 1% SDS prior to incubation for 1.5 h with 1.2 M hydroxylamine in 100 mM BTP (pH 9) and 0.5% SDS. The arrow to the left of the lane headings specifies the order of addition of SDS relative to hydroxylamine. A 5′-^{32}P-labeled 12-mer d(CGTGTCGCCCTT) is analyzed in lane M. The positions of the 18-mer scissile strand and the 12-mer peroxidolysis product are denoted by arrows on the left.

Hydroxylaminolysis of the Covalent Intermediate. We tested the reactivity of the covalent topoisomerase−DNA intermediate with hydroxylamine. Incubation for 1.5 h with 1.2 M hydroxylamine at pH 9 resulted in the release of a free 5′-^{32}P-labeled 12-mer oligonucleotide (Figure 10). Formation of this species required incubation of the labeled DNA with topoisomerase prior to treatment with hydroxylamine; i.e., hydroxylamine by itself did not cleave the DNA. The hydroxylaminolysis reaction was abrogated by dena-

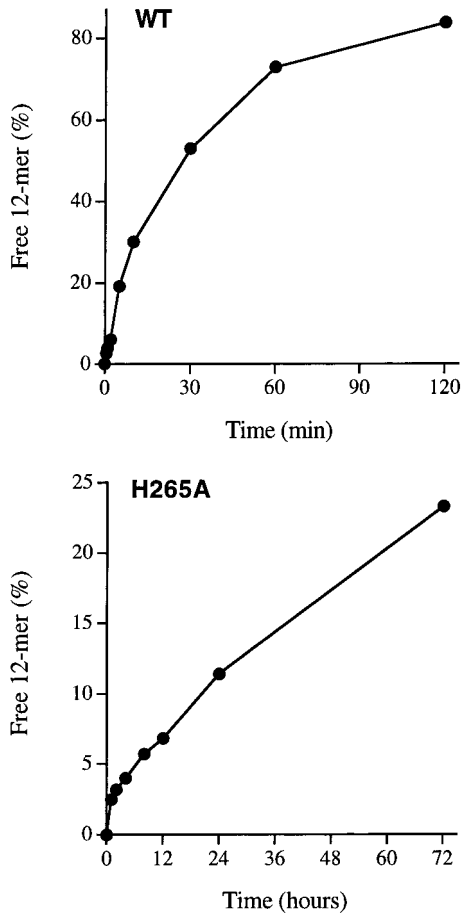

FIGURE 11: Kinetics of hydroxylaminolysis. Suicide covalent complexes formed by wild-type topoisomerase (top panel) or H265A (bottom panel) were incubated at 37 °C with 1.2 M hydroxylamine (pH 9). Aliquots were withdrawn at the times indicated and quenched immediately with SDS. The yield of free ^{32}P-labeled 12-mer product (expressed a percent of total radioactivity) is plotted as a function of time.

turation of the covalent intermediate with SDS prior to treatment with hydroxylamine (Figure 10). Thus, the reaction was topoisomerase-catalyzed.

Kinetic analysis of the reaction in 1.2 M hydroxylamine at pH 9 showed an apparent first-order approach to an end point at which the 12-mer product comprised 84% of the total labeled DNA (Figure 11A). The apparent rate constant for hydroxylaminolysis was 6.4×10^{-4} s^{-1}.

The H265A covalent intermediate was quite feeble in its reaction with 1.2 M hydroxylamine at pH 9. Incubation for 72 h resulted in 25% conversion of the scissile strand to a free 12-mer product (Figure 11B). Comparison of rates of hydroxylaminolysis by the wild-type and H265A enzymes showed that the histidine enhances the rate by a factor of 600.

To test the effect of hydroxylamine on topoisomerase activity, we transiently exposed concentrated aliquots of topoisomerase to 1.2 M hydroxylamine for 45 min at 37 °C and then diluted the protein with hydroxylamine-free buffer and assayed the treated protein for transesterification to the 18-mer/30-mer suicide substrate. The observed cleavage rate constant of the hydroxylamine-treated enzyme was 0.3 s^{-1}.

DISCUSSION

DNA strand transfer to non-nucleic acid nucleophiles by the type IB topoisomerases has been studied in this laboratory and by Westergaard and colleagues. Qualitatively similar findings have been made for the vaccinia virus and human enzymes, to wit that (i) eukaryotic topoisomerase I can utilize a wide array of chemical nucleophiles to attack the DNA−(3′-phosphotyrosyl)−enzyme bond and thereby liberate a free DNA−3′-phosphoryl product; (ii) hydrogen peroxide is the most effective non-nucleic acid nucleophile identified thus far, and (iii) chemical nucleophiles compete for the same site on the enzyme that accepts the 5′-OH of an attacking DNA strand. Our quantitative kinetic analyses of simple nucleophiles as substrates for vaccinia topoisomerase, when integrated with prior kinetic characterization of strand transfer reaction with nucleic acid nucleophiles, provides insights into the underlying mechanism of transesterification and the flexibility of the active site. (The equivalent transesterification reactions of cellular topoisomerase I have not been analyzed kinetically.) The findings also raise the possibility that topoisomerase can exploit non-nucleic acid nucleophiles to rectify suicidal cleavage events in vivo.

The reaction of hydrogen peroxide with the covalent intermediate frees the CCCTT strand from its connection to Tyr-274. By specifically labeling the scissile phosphodiester of the transesterification substrate, we show unambiguously that the scissile phosphate is present in the peroxidolysis product and that it is accessible to hydrolysis by alkaline phosphatase. These results support the peroxidolysis reaction scheme shown in Figure 1. The critical dependence of peroxidolysis on alkaline pH suggests that the peroxide anion is the relevant nucleophile. Similarly, the reactions of the topoisomerase−DNA complex with glycerol and water are also optimal at alkaline pH, implying that the glycerol anion and hydroxide ion are the reactive nucleophiles, respectively.

Hydrogen peroxide and hydroxylamine exhibit more robust activity in topoisomerase-catalyzed nucleophilic attack on the topoisomerase−DNA intermediate than do glycerol and water. This may be a manifestation of the "alpha effect" seen with nonenzymatic reactions of peroxide anion and hydroxylamine, whereby the presence of an electronegative atom with a free electron pair adjacent to the nucleophilic atom confers unusually high nucleophilic reactivity (*29*). Although peroxide and hydroxylamine reacted readily with the covalent topoisomerase−DNA intermediate, neither compound could replace Tyr-274 as the nucleophile in the forward DNA cleavage reaction. Incubation of the Y274F mutant protein with a CCCTT-containing 60-bp duplex equilibrium substrate for 2 h at 37 °C in the presence of 0.1 to 2% H_2O_2 or 0.15−2 M hydroxylamine resulted in no detectable cleavage at the CCCTT site or anywhere else in the 5′-labeled scissile strand (data not shown). Thus, vaccinia topoisomerase does not catalyze direct attack of peroxide or hydroxylamine on the scissile DNA phosphodiester.

The peroxidolysis rate constant of 0.0063 s^{-1} is merely 200-fold slower than the religation rate constant for strand transfer to a perfectly annealed 5′-OH DNA nucleophile (1.2 s^{-1}). Peroxidolysis is only 15-fold slower than the rate of strand transfer to a perfectly annealed 5′-OH RNA nucleophile (0.1 s^{-1}) (*6*). Indeed, the rate of peroxidolysis is comparable to the rate of topoisomerase-catalyzed DNA

strand transfer across a 1-nucleotide gap (0.005 s^{-1}) and is faster than the rates of transfer across a 2-nucleotide gap or to a DNA strand with an unpaired 5′-dinucleotide insertion (*6*). These comparisons underscore that an especially reactive non-nucleic acid acceptor like peroxide is a plausible nucleophile for self-catalyzed release of topoisomerase I from DNA in situations where it becomes covalently trapped, especially when no competing DNA strand is present in cis. The reaction occurs at mildly alkaline pH and proceeds to a significant extent at suboptimal peroxide concentrations over a time scale of minutes to hours that is compatible, in principle, with damage correction within a single cell cycle (abetted perhaps by damage-induced cell cycle delays or checkpoints). Peroxide or other reactive species generated in response to oxidative stress might achieve local concentrations that suffice for self-catalyzed release of topoisomerase.

The transesterification reaction of vaccinia topoisomerase is postulated to proceed through a pentacoordinate phosphorane transition state in which the attacking and leaving groups (i.e., the phenolic oxygen of Tyr-274 and the 5′-deoxyribose oxygen of DNA in the cleavage step and vice versa in the religation step) are positioned apically (*1*). It is likely that topoisomerase-catalyzed reactions of non-nucleic acid nucleophiles with the covalent intermediate occur via the same mechanism, in which case the non-nucleic acid nucleophile would occupy the same apical site as the 5′-deoxyribose oxygen of DNA. The finding that a fully annealed downstream DNA strand offers a relatively modest impediment (4-fold) to the rate of attack by hydrogen peroxide on the covalent complex implies that the noncovalently held DNA end is fairly mobile, i.e., that it spontaneously departs from the acceptor site while still tethered to the complex by the nonscissile strand. In catalyzing the relaxation of supercoiled DNA, vaccinia topoisomerase releases its grip on the downstream duplex and permits rotation of the duplex around the phosphodiester opposite the scissile phosphate. Kinetic analysis of the relaxation of negatively supercoiled plasmid DNA indicates that an average of 5 supercoils are removed for each cleavage event (*30*). Studies of the DNA cleavage and religation reactions using linear substrates do not take into account any DNA rotation events, because there are no measurable changes in topology. We surmise that the vacation of the DNA acceptor site that permits peroxidolysis by topoisomerase covalently bound at a nick is a manifestation of the rotational freedom required for DNA relaxation.

The likely source of the estimated 10^9-10^{12} enhancement of the rate transesterification by vaccinia topoisomerase (*31*) is a stabilization of the transition state via bivalent and monovalent interactions of the guanidinium nitrogens of Arg-130 and Arg-223 with the oxygens of the scissile phosphate (*1*, *5*, *27*). These two residues are conserved in the tertiary structures of all type IB topoisomerases and in the λ-integrase family of site-specific recombinases (*28*). Indeed, they make direct contact with the nonbridging oxygens of the scissile phosphate in the noncovalent and covalent states in the topoisomerase−DNA and recombinase−DNA cocrystals (*32*−*34*). Arg-130 and Arg-223 each contribute a 10^5 rate enhancement of transesterification by vaccinia topoisomerase (*5*, *27*). Note that the extremely slow rates of DNA cleavage and religation by the R130A and R223A mutants make it practically difficult to analyze the effects of the arginine mutations on the even slower rates of transesterification to non-DNA nucleophiles. Conserved residue His-265 enhances the rate of forward and reverse DNA transesterification by 100- to 400-fold (*3*); hence the H265A mutant was better suited to address the question of whether transesterification to non-DNA nucleophiles relied on the same catalytic residues of the topoisomerase. The observation that the covalent intermediate of the H265A mutant was impaired in peroxidolysis, hydroxylaminolysis, glycerololysis, and hydrolysis provides clear proof that strand transfer to non-nucleic acid nucleophiles is topoisomerase-catalyzed and that the underlying mechanism is similar to that of DNA transesterification.

What do the present results tell us about the catalytic role of His-265? It was proposed, on the basis of the mutational effects and the crystal structure of the vaccinia topoisomerase catalytic domain, that His-265 engages in a hydrogen bond with a scissile phosphate oxygen and thereby contributes to transition state stabilization (*3*, *28*). Our mutational data showing regain of function by substitution with Asn or Gln appeared to exclude a role for His-265 as a general acid−base catalyst (*3*). The equivalent histidine of human topoisomerase I was found to hydrogen bond to a *nonbridging* oxygen of the scissile phosphate in the noncovalent enzyme−DNA cocrystal (*33*, *34*). Despite the mutational evidence available for the vaccinia topoisomerase, Stewart et al. (*34*) proposed a mechanism whereby the conserved histidine acts as a general acid catalyst, donating its proton to the 5′-bridging oxygen of the leaving DNA strand during the DNA cleavage reaction. If this model is correct, and the prevailing assumption holds that religation is the microscopic reverse of cleavage, then the same histidine should in turn serve as a general base during DNA strand transfer by abstracting a proton from the attacking 5′-OH DNA strand. According to the mechanism proposed here and by Lisby et al. (*21*), attack by peroxide anion at alkaline pH should not require a general base because deprotonation of the nucleophile is not dependent on the topoisomerase. If the histidine acts principally as a general base, then we would have expected to find relatively little catalytic impairment for H265A in strand transfer to hydrogen peroxide compared to its defect in DNA religation. The fact that peroxidolysis still requires His-265 is further evidence that this residue does not serve as a general acid−base catalyst but rather stabilizes the transition state. The putative general acid and general base catalysts of transesterification by the type IB topoisomerases remain to be identified.

REFERENCES

1. Shuman, S. (1998) *Biochim. Biophys. Acta 1400*, 321−337.
2. Shuman, S., and Prescott, J. (1990) *J. Biol. Chem. 265*, 17826−17836.
3. Petersen, B. Ø., and Shuman, S. (1997) *J. Biol. Chem. 272*, 3891−3896.
4. Shuman, S. (1992) *J. Biol. Chem. 267*, 8620−8627.
5. Wittschieben, J., and Shuman, S. (1997) *Nucleic Acids Res. 25*, 3001−3008.
6. Sekiguchi, J., Cheng, C., and Shuman, S. (1997) *J. Biol. Chem. 272*, 15721−15728.
7. Burgin, A. B., Huizenga, B. N., and Nash, H. A. (1995) *Nucleic Acids Res. 15*, 2973−2979.
8. Kingma, P. S., and Osheroff, N. (1998) *Biochim. Biophys. Acta 1400*, 223−232.
9. Pommier, Y., Pourquier, P., Fan, Y., and Strumberg, D. (1998) *Biochim. Biophys. Acta 1400*, 83−105.

10. Lanza, A., Tornaletti, S., Rodolfo, C., Scanavini, M. C., and Pedrini, A. M. (1996) *J. Biol. Chem. 271*, 6978−6986.
11. Pourquier, P., Ueng, L. M, Kohlhagen, G., Mazumder, A., Gupta, M., Kohn, K. W., and Pommier, Y. (1997) *J. Biol. Chem. 272*, 7792−7796.
12. Pourquier, P., Pilon, A. A., Kohlhagen, G., Mazumder, A., Sharma, A., and Pommier, Y. (1997) *J. Biol. Chem. 272*, 26441−26447.
13. Cheng, C., and Shuman, S. (1999) *Biochemistry 38*, 16599−16612.
14. Pouliot, J. J., Yao, K. C., Robertson, C. A., and Nash, H. A. (1999) *Science 286*, 552−555.
15. Shuman, S. (1992) *J. Biol. Chem. 267*, 16755−16758.
16. Shuman, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10104−10108.
17. Shuman, S. (1994) *J. Biol. Chem. 269*, 32678−32684.
18. Christiansen K., and Westergaard, O. (1994) *J. Biol. Chem. 269*, 721−729.
19. Christiansen, K., Knudsen, B. R., and Westergaard, O. (1994) *J. Biol. Chem. 269*, 11367−11373.
20. Petersen, B. Ø., and Shuman, S. (1997) *Nucleic Acids Res. 25*, 2091−2097.
21. Lisby, M., Krogh, B. O., Boege, F., Westergaard, O., and Knudsen, B. R. (1998) *Biochemistry 37*, 10815−10827.
22. Ames, B. N, Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915−7922.
23. Shuman, S., Golder, M., and Moss, B. (1988) *J. Biol. Chem. 263*, 16401−16407.
24. Ho, C. K., Van Etten, J. L., and Shuman, S. (1997) *J. Virol. 71*, 1931−1937.
25. Krogh, B. O., Cheng, C., Burgin, A., and Shuman, S. (1999) *Virology 264*, 441−451.
26. Caselli, A., Marzocchini, R., Camici, G., Manao, G., Moneti, G., Pieraccini, G., and Ramponi, G. (1998) *J. Biol. Chem. 273*, 32554−32560.
27. Cheng, C., Wang, L. K., Sekiguchi, J., and Shuman, S. (1997) *J. Biol. Chem. 272*, 8263−8269.
28. Cheng, C., Kussie, P., Pavletich, N., and Shuman, S. (1998) *Cell 92*, 841−850.
29. Jencks, W. P. (1987) *Catalysis in Chemistry and Enzymology*, Dover Publications, New York.
30. Stivers, J. T., Harris, T. K., and Mildvan, A. S. (1997) *Biochemistry 36*, 5212−5222.
31. Stivers, J. T., Shuman, S., and Mildvan, A. S. (1994) *Biochemistry 33*, 327−339.
32. Guo, F., Gopaul, D. N., and Van Duyne, G. D. (1997) *Nature 389*, 40−46.
33. Redinbo, M. R., Stewart, L., Kuhn, P., Champoux, J. J., and Hol, W. G. J. (1998) *Science 279*, 1504−1513.
34. Stewart, L., Redinbo, M. R., Qiu, X., Hol, W. G. J., and Champoux, J. J. (1998) *Science 279*, 1534−1541.

BI000184U