Vaccinia DNA Topoisomerase I: Kinetic Evidence for General Acid−Base Catalysis and a Conformational Step†

James T. Stivers,‡,§ Stewart Shuman,|| and Albert S. Mildvan*,‡

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, and Program in Molecular Biology, Sloan-Kettering Institute, New York, New York 10021

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ABSTRACT: The pH dependences of the internal equilibrium (K_{cl}) and rate constants for site-specific DNA strand cleavage (k_{cl}) and resealing (k_r) catalyzed by Vaccinia DNA topoisomerase I have been investigated using single-turnover conditions in the pH range 4.6−9.8 at 20 °C. The pH dependence of the rate constant for strand cleavage (k_{cl}) shows a bell-shaped profile with apparent pK_a values of 6.3 ± 0.2 and 8.4 ± 0.2, suggesting base catalysis of the attack of the active site Tyr-274 on the phosphodiester phosphorus, and acid catalysis of the expulsion of the 5′-deoxyribose oxygen. A low pK_a (*i.e.*, 6.3) for Tyr-274 in the free enzyme is ruled out by NMR titration from pH 5.1 to 8.8 monitoring the C-ζ chemical shift of [ζ-^{13}C]-tyrosine-enriched topoisomerase. The dependence of the internal equilibrium constant (K_{cl}) on pH reveals very similar pK_a values as k_{cl} (5.8 ± 0.2 and 8.6 ± 0.2). However, k_r is found to be independent of pH. The differing response of k_{cl} and k_r to pH rules out a simple two-state internal cleavage equilibrium and suggests that a conformational change occurs following formation of the covalent complex which retains the correct protonation state for strand religation. A conformation step is further indicated by a 4.6-fold "thio effect" on k_{cl} upon substitution of the nonbridging oxygen atom of the attacked phosphoryl group by sulfur [Stivers, J. T., Shuman, S., & Mildvan, A. S. (1994) *Biochemistry 33*, 327], and the absence of such an effect on k_r, ($k_r^{phos}/k_r^{thio} = 0.9 \pm 0.2$), indicating the rates of cleavage and religation to be limited by covalent chemistry and a conformational step, respectively. The rate constant of this conformational change in the direction of religation agrees with the average rate constant for supercoil release from plasmid substrates, suggesting this conformational change to be a part of the topoisomerization step. Although the general acid and general base catalysts have not yet been identified, the quantitative roles of these and other residues in catalysis are discussed.

The reactions catalyzed by the eukaryotic type I topoisomerases involve the reversible cleavage and rejoining of one strand of duplex DNA with the transient formation of a covalent 3′-phosphotyrosyl intermediate involving the DNA and a conserved active site tyrosine (Champoux, 1990). Apparently, the biological function of these enzymes is to maintain the topological state of cellular DNA by removing supercoils generated during DNA replication and/or transcription. Topoisomerase I from Vaccinia has been shown to have sequence homologies and mechanistic similarities to eukaryotic type I topoisomerases (Shuman & Moss, 1987; Shuman *et al.*, 1988).

The rate constants for DNA strand cleavage and religation reactions, as well as for substrate binding and product dissociation from the Vaccinia type I topoisomerase, have been determined (Figure 1A) by taking advantage of the sequence-specific DNA cleavage catalyzed by this enzyme (Stivers *et al.*, 1994a). This approach has permitted a detailed kinetic and thermodynamic description of the reaction of this enzyme with small linear duplex DNA substrates containing the preferred cleavage sequence (5′-CCCTT↓-3′) (Shuman *et al.*, 1989; Shuman & Prescott, 1990) and has revealed the rate-limiting steps under single-turnover and steady-state conditions (Stivers *et al.*, 1994a).

Here we describe the effects of pH on the rate constants for the cleavage and religation of enzyme-bound DNA and the pH dependence of the internal equilibrium for DNA strand cleavage and religation. The results provide evidence for general acid−base catalysis of the strand cleavage reaction and reveal an additional conformational step which is rate-limiting in the religation reaction (Figure 1B). A preliminary abstract of this work has been published (Stivers *et al.*, 1994b).

MATERIALS AND METHODS

Materials. The radionucleotides [γ-^{32}P]ATP and [α-^{32}P]-dTTP were from New England Nuclear. 4-^{13}C-ring-labeled tyrosine (99%) was obtained from Cambridge Isotope Labs (Woburn, MA). All unlabeled dNTPs of the highest purity available were obtained from Pharmacia. Oligonucleotides (phosphodiester and phosphorothioate) were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by reverse-phase high-performance liquid chromatography (HPLC).[1] The sulfurizing reagent 3*H*-1,2-benzodithiole-3-one 1,1-dioxide was from Glen Research, Sterling, VA. Oligonucleotide concentrations were determined by ultraviolet absorbance at 260 nm using the extinction coefficients for the component nucleotides (Maniatis *et al.*, 1982).

Enzymes. Wild-type Vaccinia topoisomerase was overexpressed in *Escherichia coli* and purified to >95% homo-

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* To whom correspondence should be addressed. Phone: 410-955-2038. FAX: 410-955-5759.

‡ The Johns Hopkins University School of Medicine.

§ American Cancer Society Postdoctoral Fellow.

|| Sloan-Kettering Institute.

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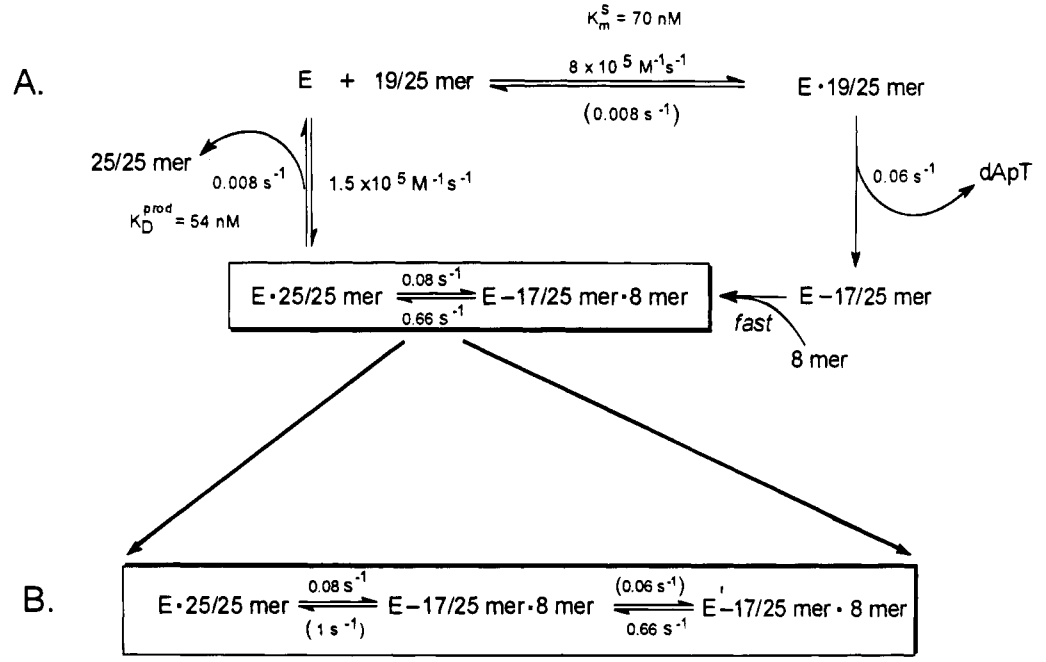


FIGURE 1: Kinetic and thermodynamic description of the reactions of Vaccinia topoisomerase (E) with linear duplex DNA (19/25-mer) containing the preferred cleavage sequence 5′-CCCTT↓-3′ (pH 7.5, 50 mM Tris-HCl, 20 °C). (A) General scheme based on the DNA binding studies, as well as single-turnover and steady-state kinetic analyses of Stivers *et al.* (1994). The enzyme initially binds the 19/25-mer cleavage substrate at a rate 10^2-10^3-fold less than diffusional encounter, suggesting a multistep binding process which may be important in site-specific recognition and cleavage. After DNA binding, the site-specific cleavage occurs, yielding a covalent adduct (E−17/25-mer) between Tyr-274 of the enzyme and the phosphodiester backbone of DNA; the dinucleotide leaving group (dApT) is rapidly and irreversibly released into solution. In the kinetically transparent step which follows, the incoming 8-mer strand, which is complementary to the single-strand overhang of the E−17/25-mer complex, binds rapidly to the cleaved complex. Following 8-mer binding, religation occurs to form bound product (25/25-mer). Slow product release, which limits the rate under steady-state conditions, regenerates free enzyme in the final step. On the basis of results presented herein, the single cleavage/religation step highlighted by the box is shown to consist of at least two steps, covalent chemistry and a conformational step (see Figure 1B). (B) Expansion of the boxed cleavage/religation step in Figure 1A showing the discrete steps of covalent chemistry and a conformational change from E to E′ which are supported by the results presented herein (see text and Figure 6A,B). The rate constants in parentheses are estimates based on the available kinetic and thermodynamic constants (see Discussion).

geneity as described (Shuman *et al.*, 1988; Morham & Shuman, 1992). [ζ-^{13}C]Tyrosine-enriched wild-type topoisomerase was overexpressed from *E. coli* strain BL21(DE3) transformed with the T7-based expression plasmid pET21-Topo. The pET21 plasmid was obtained from Novagen, Madison, WI. This vector places the topoisomerase gene under tighter negative control than in previously used plasmids because the construct contains a lac operator upstream of the gene. Selective ^{13}C enrichment of tyrosine residues was accomplished by growing the bacteria at 37 °C in M9 minimal medium containing 4 g/L glucose and 300 μg/mL ampicillin. The minimal medium was supplemented with 200 mg/mL each of the unlabeled amino acids (except tyrosine) and 100 mg/mL ^{13}C-ζ-enriched tyrosine. When the culture reached an absorbance of 1 OD (600 nm), topoisomerase was induced by adding IPTG to 1 mM final concentration. The cells were harvested after an additional 3 h and purified as described above for the unlabeled wild-type enzyme. A total of ~10 mg of purified ^{13}C-labeled topoisomerase was obtained from 1 L of cell culture. The H150A, R130A, and R130K mutant topoisomerases were generated by standard protocols for site-directed mutagenesis as previously described (Shuman *et al.*, 1989).[2] The mutations were confirmed by dideoxynucleotide sequencing, and the mutant enzymes were purified as described for the unlabeled wild-type topoisomerase. Topoisomerase concentrations were determined by ultraviolet absorbance at 280 nm; a 1 mg/mL solution gave an A_{280} = 1.08 at pH 7.5. T4 polynucleotide kinase was from Pharmacia, and 3′,5′-exonuclease-deficient Klenow DNA polymerase was from U.S. Biochemical Corp.

Radiolabeling of Oligonucleotide Substrates. The duplex DNAs and oligonucleotides used in these experiments are shown in Figure 2; the radiolabeling and hybridization of these substrates have been described previously (Stivers *et al.*, 1994a). Briefly, the 19-mer or 25-mer oligonucleotide containing the 5′-CCCTT-3′ cleavage motif was labeled at the 5′-end by enzymatic phosphorylation in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase and then purified by centrifugal gel filtration before hybridization to the complementary 25-mer stand. The 19/25-mer cleavage substrate 3′-^{32}P-labeled on the leaving group of the 19-mer strand was prepared from the duplex unlabeled 18/25-mer precursor by primer extension using Klenow DNA polymerase and [α-^{32}P]dTTP and purified as above.

pH Dependence of K_{cl} and Noncovalent DNA Binding to Enzyme. The pH dependence for the apparent cleavage

[1] Abbreviations: HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; 2D-NOESY, two-dimensional nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation; TOCSY, total correlation spectroscopy; TSP, sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4; pH*, pH meter reading in ^2H_2O.

[2] J. Wittschieben and S. Shuman, submitted for publication (1994).

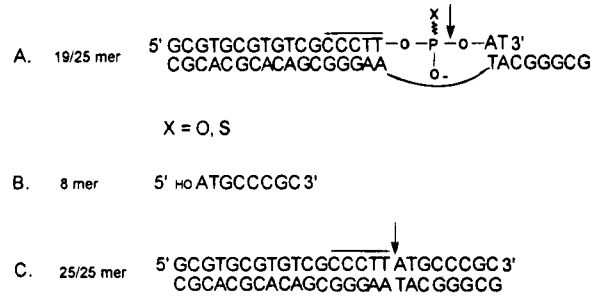


FIGURE 2: DNA substrates used in the experiments. The preferred cleavage site (↓) and pentameric consensus sequence (—) are indicated. (A) 19/25-mer used in the cleavage experiments. Cleavage at the indicated position (↓) produces the radiolabeled dinucleotide product dApT, which can be resolved from uncleaved 19/25-mer by TLC. (B) 5′-HO-8-mer acceptor strand used in the ligation experiments. This sequence is complementary to the eight-nucleotide single strand overhang produced by cleavage of the 19/25-mer. (C) 25/25-mer product of the coupled cleavage−ligation reaction between the 19/25-mer and 8-mer.

equilibrium constant (K_{cl}) on topoisomerase was determined using conditions of excess enzyme such that all the duplex DNA is enzyme-bound over the entire pH range studied (see binding studies below). The equilibrium constant K_{cl} measures the ratio of the sum of all enzyme−DNA covalent complexes to the sum of all noncovalently bound enzyme−DNA complexes. Since measurement of K_{cl} would be compromised by dissociation of the cleaved leaving strand, these experiments were performed in the presence of a large excess of 8-mer single-stranded DNA. In this experiment, a solution of 5′-^{32}P-labeled 25/25-mer and 8-mer was mixed with topoisomerase to give final concentrations of 15 nM 25/25-mer, 18 μM 8-mer, and 2 μM topoisomerase in 50 mM buffer of appropriate pH and ionic strength equal to 0.1 M with a final volume of 20 μL. The reactions were incubated at 20 °C for 5−10 min and then quenched by the addition of 20 μL of 2% SDS. Control experiments demonstrated that there was no difference in the measured K_{cl} value for reactions that were quenched at 5 or 10 min, indicating that the reactions had reached equilibrium. The radiolabeled E−17/25-mer complex, which is trapped by the SDS quench, was resolved from free DNA by electrophoresis on a 13% SDS−polyacrylamide gel at 200 V for 2 h. The counts present in the complex and free DNA were separately determined (after autoradiography) by liquid scintillation counting of the excised gel slices. Since some of the reactions required the quantitation of very small amounts of the covalent complex, it was important to carefully correct for background counts. This was accomplished by excising a piece of the gel equivalent in size and midway between the excised slices for complexed and free DNA, and subtracting the counts in this slice from the radioactivity present in the complex. The internal equilibrium constant at each pH value was calculated from the ratio of the radioactivity present in the covalent complex to the radioactivity present in DNA according to eq 1:

$$K_{cl} = \frac{\text{cpm in covalent complex}}{\text{cpm in uncleaved DNA}} \quad (1)$$

The validity of the above treatment requires that all of the duplex DNA is enzyme-bound at all pH values. Otherwise, the observed equilibrium would partially reflect an equilibrium between free and bound DNA. To confirm this point, nitrocellulose filter binding studies were performed. In this assay, free duplex DNA will pass through the nitrocellulose filter while DNA bound to enzyme is retained (Riggs *et al.*, 1970). The binding reactions were performed in a 25 μL volume using identical reaction conditions as described above for the determination of K_{cl}. Duplicate samples (10 μL) from the binding reactions were applied to nitrocellulose (0.2 μm pore size, 25 mm diameter, Whatman) on a vacuum apparatus and aspirated. Filters were washed under vacuum with five 1 mL aliquots of the appropriate reaction buffer, and the retained radioactivity was determined by liquid scintillation counting. Ten microliters of each reaction mixture was spotted on nitrocellulose without aspiration to determine the total counts in the reaction. The nonspecific binding of free duplex DNA to the nitrocellulose membrane in the absence of enzyme was ≤5% over the entire pH range studied. The results from these experiments (see Figure 3) confirmed that all of the duplex DNA was enzyme-bound under the conditions of these reactions.

pH Dependence of Strand Cleavage. Cleavage experiments were performed using single-turnover conditions (with manual mixing) at 20 °C with the following buffers (50 mM): sodium acetate, pH 4.6−5.0; NaMES, pH 5.0−6.5; NaTES, pH 7.0−8.0; Tris-HCl, pH 7.5−9.0; NaCAPS, pH 9.5−10.2. The ionic strength was maintained at 0.1 M with the addition of NaCl, and the pH was measured at 20 °C at the conclusion of each reaction. Topoisomerase (3 μM) and 3′-^{32}P-labeled 19/25-mer (210 nM) were separately preincubated in buffer for 2 min prior to initiating the reaction by mixing the enzyme with the DNA to give final concentrations of 2 μM topoisomerase and 70 nM DNA. Control experiments demonstrated that under these conditions, the enzyme was saturated with DNA, and the rate was independent of topoisomerase concentration over the entire pH range studied. Aliquots (9) of 10 μL were removed at specified times and quenched in 10 μL of 2% SDS. Unreacted 19/25-mer substrate was then resolved from the radiolabeled dinucleotide cleavage product (dApT) by spotting 1 μL of the quenched reaction on a Whatman poly(ethylenimine)−cellulose TLC (PEI-TLC) plate. The plates were then developed with 0.3 M potassium phosphate, pH 8.0. The sections corresponding to substrate and product were excised and mixed with 5 mL of Ecolite (+) (ICN Research Products) scintillation cocktail. The ratio of product counts to total counts at each time point was quantitated by liquid scintillation spectrometry using a Beckman LS-6000 scintillation counter.

Single-turnover cleavage reactions were first-order for ~3 half-lives, and the first-order rate constants for cleavage at each pH value were determined from a nonlinear least-squares fit of the data to a single exponential (Grafit, Erithacus Software Ltd., Staines, U.K.). The observed end points for the cleavage reaction were independent of pH in the range 5.1−9.8, with typical end point values varying randomly between 60 and 85%. This incomplete and variable fractional conversion was previously reported and was found to be due to an unreactive form of the duplex substrate (Stivers *et al.*, 1994a).

At pH values ≤5.0, a time-dependent *reversible* inactivation of the enzyme was observed ($t_{1/2}$ ~ 20 min) which resulted in abnormally low end points (20−40% conversion). Therefore, in this pH range, the rate constants for cleavage were estimated from the initial linear rates (≤10% conversion) of product formation. We estimate the errors for the rate constants obtained at pH values <5.1 in this manner to

be ≤50% on the basis of the uncertainty in the rate of inactivation. Although this procedure was necessary to extend the pH−rate profile to cover the maximum possible range, none of our conclusions are affected by the uncertainty in these rate constants at pH values below 5.1.

pH Dependence of Strand Religation. The rates of 8-mer strand religation to the preformed 5′-^{32}P-labeled enzyme-17/25-mer covalent complex (E−17/25-mer) in the pH range 4.6−9.8 were determined using single-turnover conditions. The E−17/25-mer complex was formed by incubating 3.4 μM topoisomerase with 288 nM 5′-^{32}P-labeled 19/25-mer in 5 mM Tris-HCl, pH 7.5, for 15−30 min at 37 °C. Typically, 75−90% of the input DNA was covalently bound to the enzyme after this incubation. The covalent complex was then diluted 2-fold in 5 mM Tris, pH 7.5, after which 6 μL aliquots (5−9) were placed in wells of a 96-well microtiter plate and mixed with 4 μL of a 250 mM buffer stock of appropriate pH and 0.1 M ionic strength. The religation reactions were initiated by the rapid addition (by manual pipeting) of 20 μL of a 4 μM solution of 8-mer strand in distilled, deionized H_2O. Samples at various times (0.5−30 s) were rapidly quenched in 20 μL of 10% SDS dispensed from a second manual pipetor. For these reactions, accurate quench timing was achieved through the use of a metronome. Although a rapid quench flow apparatus was previously used for the strand religation reaction (Stivers *et al.*, 1994a), the method described above provides identical rate constants, within experimental error. Previously (Stivers *et al.*, 1994a), the forward rate constant for religation (k_r) was obtained from the observed first-order rate constant for approach to equilibrium (k_{obsd}) by solving the simultaneous equations $k_{obsd} = k_{cl} + k_r$ and, from the equilibrium end point, $K_{cl} = k_{cl}/k_r$, assuming an equilibrium between two states. Since $k_{cl} \ll k_r$, the value of k_{obsd} closely approximates k_r. The results obtained in the present work provide evidence for an intermediate in the cleavage religation reaction, indicating that k_{obsd} is a more complex function of four rate constants (see below). To simplify the analysis, the k_{obsd} values for religation are not corrected for the slight reversibility of the reaction. This treatment is valid because the approach to equilibrium correction is quite small and the religation reactions proceed to at least 92% completion depending on the pH of the reaction (see Results). The uncorrected k_r values obtained here differ on average by less than 10% from those values reported previously using the approach to equilibrium correction, a difference which is no greater than the estimated errors in the measurements.

Thio Effect on Strand Religation. The rate of religation of the 8-mer strand to the enzyme−17/25-mer covalent complex containing a nonbridging sulfur atom at the reactive phosphoryl group was obtained as follows. The covalent complex was formed from the thio-substituted 19/25-mer as described above for the phosphodiester 19/25-mer. However, only 45% of the input DNA was covalently attached to the enzyme after a 15 min incubation. Since this substrate contains a mixture of the R_p and S_p thio isomers, and only one isomer is cleaved to a significant extent during this 15 min incubation (Stivers *et al.*, 1994a), then the 45% conversion indicates that the complex is enriched in the more reactive thio isomer. The religation reaction with the 8-mer strand was performed as described above for the 19/25-mer substrate. The rate constant for religation was obtained directly from k_{obsd} because the 4.6-fold slowing of the cleavage reaction as a result of the thio effect (Stivers *et al.*, 1994a) makes the religation reaction essentially irreversible.

NMR Spectroscopy. One-dimensional ^{13}C-NMR experiments were performed at 32 °C with a Bruker AM-600 NMR spectrometer at a ^{13}C frequency of 150 MHz using a 10 mm broad-band probe. The ^{13}C spectra were recorded with the following acquisition parameters: a 60° radio frequency pulse; spectral width, 5000 Hz; carrier frequency, 159.76 ppm; an acquisition time of 0.512 s; and a relaxation delay of 0.488 s. FIDs of 2048 data points were processed by zero-filling to 4096 data points and applying 20 Hz line broadening. Carbon chemical shifts were referenced to TSP.

pH Titration by ^{13}C-NMR. The NMR sample was prepared by dissolving the selectively [ζ-^{13}C]tyrosine-labeled enzyme in 99.9% ^2H_2O and lyophilizing. This process was performed a total of 3 times to remove exchangeable protons from the enzyme. The final lyophilized enzyme was dissolved in 2 mL of 99.9% ^2H_2O in a 10 mm NMR tube to give a final enzyme concentration of 0.1 mM (pH* 7.1). Titrations were performed by the addition of small aliquots of either 0.1 M DCl or KOD. The pH* values reported are not corrected for deuterium isotope effects.

Data Modeling. The pH dependencies of k_{cl} and K_{cl} were well fit to eq 2 ($C = k_{cl}$ or K_{cl}) which assumes two titratable groups on the enzyme giving a bell-shaped pH response with a maximum (C_{max}) at pH = (pK_{a_1} + pK_{a_2})/2:

$$\log C = \log C_{max} - \log\left(\frac{10^{-pK_{a_1}}}{10^{-pH}} + \frac{10^{-pH}}{10^{-pK_{a_2}}} + 1\right) \quad (2)$$

RESULTS

pH Dependence of K_{cl}. The apparent equilibrium constant (K_{cl}) for cleavage of the 25/25-mer (S), which represents the ratio of the sum of all enzyme−DNA covalent complexes to the sum of all noncovalent complexes (eq 3), is shown as

$$K_{cl} = \frac{[\text{covalent complexes}]}{[E_H^H{\cdot}S] + [E^H{\cdot}S] + [E{\cdot}S]} \quad (3)$$

a function of pH in Figure 3. The data show a bell-shaped dependence of log K_{cl} on pH with limiting slopes of unity on either side of the pH maximum, indicating that two ionizable groups (Scheme 1) on the enzyme influence this equilibrium constant and that the $E^H{\cdot}S$ form of the enzyme is catalytically active. The pK_a values determined from a nonlinear least-squares fit of the data (Figure 3) to eq 2 are 5.8 (±0.2) and 8.6 (±0.2). It is unlikely that these pK_a values represent ionizations of the DNA substrate because the titratable groups of the DNA bases, sugar, and phosphate have pK_a values of <∼4 and >9 (Saenger, 1984).

Figure 3 shows that the 25/25-mer is fully bound to the enzyme over the entire pH range studied as determined by nitrocellulose filter binding. This confirms that the pH dependence of K_{cl} is due solely to ionizations involved in the internal equilibrium and not in DNA binding.

pH Dependence of k_{cl} and k_r. The first-order rate constants k_{cl} and k_r, which are defined in eqs 4 and 5, respectively,were

$$E{\cdot}19/25\text{-mer} \xrightarrow{k_{cl}} E-17/25\text{-mer}{\cdot}dApT \quad (4)$$

$$E-17/25\text{-mer}{\cdot}8\text{-mer} \xrightarrow{k_r} E{\cdot}25/25\text{-mer} \quad (5)$$

obtained using single-turnover conditions as described under

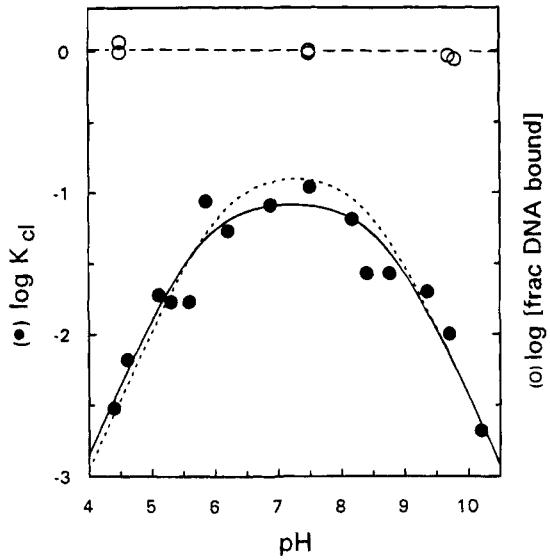
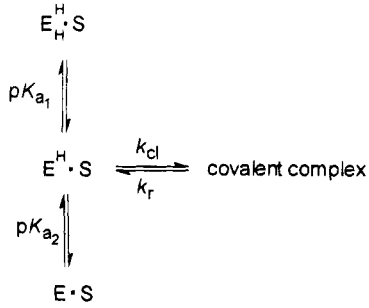


FIGURE 3: pH dependence of the internal equilibrium constant (K_{cl}) for cleavage (closed circles) and extent of DNA binding (open circles) to topoisomerase. Reactions contained 15 nM 5′-^{32}P-labeled 25/25-mer, 18 μM 8-mer, and 2 μM topoisomerase in 50 mM buffer at 20 °C with the ionic strength adjusted to 0.1 M with NaCl. The 8-mer strand is provided in large excess to prevent dissociation of the cleaved leaving strand. The solid curve is the nonlinear least-squares fit to the data using eq 2, which gives pK_a values of 5.8 ± 0.2 and 8.6 ± 0.2. The dashed curve is the calculated curve for the pH dependence of K_{cl} using the best-fit pK_a values for the pH dependence of k_{cl} (see text and Figure 4), indicating that K_{cl} and k_{cl} have indistinguishable pH dependencies, within experimental error.

Scheme 1



Materials and Methods and are shown as a function of pH in Figure 4. The pH dependence of k_{cl} (Figure 4) gives a bell-shaped curve revealing two titratable groups on the enzyme with pK_a values of 6.3 ± 0.2 and 8.4 ± 0.2. In Figure 3, the calculated curve for the pH dependence of K_{cl} using the best-fit pK_a values for the pH dependence of k_{cl} is shown. This line visually demonstrates that K_{cl} and k_{cl} have indistinguishable pH dependences, within experimental error. In sharp contrast, k_r (Figure 4) was found to be essentially independent of pH over the range 4.5−9.5. However, at pH values >9.5, the measured k_r value consistently deviates positively from the calculated value, suggesting a change in the reaction pathway (Jencks, 1987).

The following observations argue against reversible or irreversible inactivation of the enzyme in the pH range 5.2−9.7 and indicate that the pH dependences of k_{cl} and K_{cl} are due solely to the titration of residues on the enzyme which are required for catalysis.

(i) Topoisomerase which was incubated at pH 5.2 or 9.7 for 30 min and then returned to pH 7.5 retained ≥90% of its initial DNA cleavage activity.

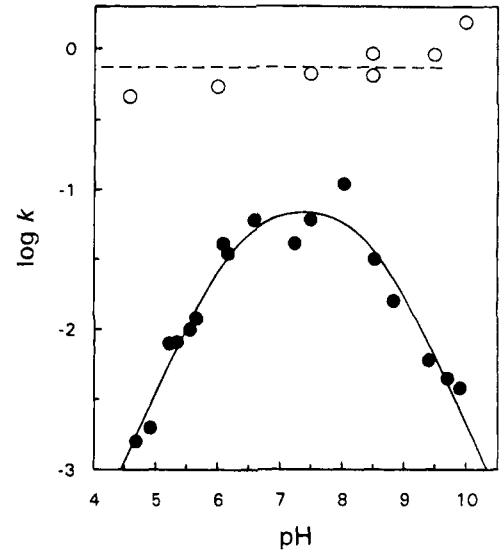


FIGURE 4: pH dependence of the first-order rate constants for strand cleavage (k_{cl}, closed circles) and religation (k_r, open circles) obtained using single-turnover conditions [50 mM buffer, ionic strength = 0.1 M (NaCl), 20 °C]. Cleavage reactions contained 2 μM topoisomerase and 70 nM 3′-^{32}P-labeled 19/25-mer; the religation reactions contained ~52 nM 5′-^{32}P-labeled E−17/25-mer complex and 2.7 μM 8-mer strand. The solid curve is the nonlinear least-squares fit to the cleavage data using eq 2, which gives pK_a values of 6.3 ± 0.2 and 8.4 ± 0.2.

(ii) Topoisomerase which had been preincubated at pH 5.2 or 9.7 for 30 s or 5 min, prior to initiating the cleavage reaction at the same pH, gave the same k_{cl} values, providing evidence against time-dependent enzyme inactivation in the pH range 5.2−9.7. Also, the observed end points for the cleavage reaction were pH-independent in this same range.

(iii) No obvious changes in the resolved aromatic, Cα-H, or upfield methyl resonances in the 1D ^{1}H-NMR spectra of topoisomerase or in the 2D-NOESY, TOCSY, and ^{15}N-HSQC spectra in the pH range 5.1−7.5 were observed (data not shown).

(iv) The rate of religation and the extent of DNA binding are independent of pH over the pH range 4.6−9.7 (Figures 3 and 4).

Thio Effect on Strand Religation. Substitution of a nonbridging oxygen atom of a phosphoryl group by sulfur would be expected to decrease the chemical reactivity of the phosphoryl group to nucleophilic attack by 4−11-fold (Herschlag *et al.*, 1991) although more complex steric explanations for thio effects are sometimes required (Polesky *et al.*, 1992). The absence of thio effects on enzyme-catalyzed nucleophilic substitution reactions at phosphorus provides evidence for a rate-limiting step other than covalent chemistry [see Stivers *et al.* (1994a) and references cited therein]. Previously we reported thio effects for the single-turnover cleavage reaction ($=k_{cl}^{phos}/k_{cl}^{thio}$) of 4.6- and 115-fold, and concluded that the chemical step was rate-limiting and that the two thio isomers of the racemic phosphorothioate-substituted 19/25-mer substrate were cleaved at significantly different rates.

Figure 5 shows the single-turnover religation reaction (eq 5) between 8-mer and the preformed E−17/25-mer covalent complex with an oxygen atom or sulfur at the nonbridging position of the reactive phosphorus. For this reaction, the covalent complex with the thio-substituted substrate was enriched in the more reactive thio isomer (see Materials and Methods). The data show clearly that there is no significant

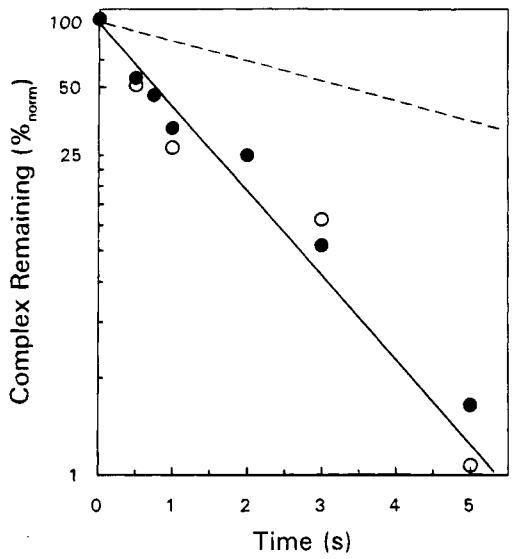


FIGURE 5: Thio effect ($=k_r^{phos}/k_r^{thio}$) on single-turnover strand religation (50 mM Tris-HCl, pH 7.5, and 20 °C). Religation reactions were performed as described under Materials and Methods and the legend to Figure 3. A semilogarithmic plot of the data is shown, and the solid line is the nonlinear least-squares fit to the data for the reaction of the 5′-^{32}P-labeled E−17^{thio}/25-mer (closed circles) with the 8-mer strand. The data for the analogous reaction of the phosphodiester complex (open circles) are also shown. For comparison, the theoretical line (dashed) for a thio effect of 4.6, which would be expected if covalent chemistry were rate-limiting, is also shown.

thio effect for the religation reaction with $k_r^{phos}/k_r^{thio} = 0.9 \pm 0.2$ and provide evidence that the rate-limiting step for religation is not covalent chemistry. For comparison, Figure 5 shows the calculated rate for a thio effect of 4.6, which would be expected if covalent chemistry were rate-limiting.

The rate-limiting step observed for religation cannot be the binding of the entering 8-mer because this reactant is provided in large excess (*i.e*, k_{on}[8-mer] $\gg k_r$). Furthermore, it is unlikely that a concentration-independent unimolecular event such as nucleation and annealing of the 8-mer to the single-strand overhang of the E−17/25-mer is kinetically important, because an identical value for k_r was determined using an approach-to-equilibrium method with the 25/25-mer substrate (Stivers *et al.*, 1994a). This is significant because in the approach-to-equilibrium method the 8-mer strand is already annealed to the duplex and does not have to bind from solution.

Expanded Kinetic Scheme for Religation. The data of Figures 4 and 5 show respectively that the religation reaction is pH-independent in the range 4.5−9.5 and that the substitution of a sulfur atom for a nonbridging phosphoryl oxygen at the site of cleavage of the 19/25-mer has no significant effect on the rate of strand religation. These results contrast sharply with the bell-shape pH dependence of k_{cl} (Figure 4) and the substantial thio effect of 4.6-fold which is observed for this reaction (Stivers *et al.*, 1994a). The different responses of k_r and k_{cl} to pH and to a thio-substituted substrate strongly indicate that a different rate-limiting transition state is being observed for each reaction.

The simplest model which explains the observed pH dependences and thio effects on these reactions is shown in Figure 1B and as a chemical mechanism and free energy profile in Figure 6. In this model, the cleavage step and K_{cl} are pH-dependent, whereas the *observed* religation rate (k_r^{conf}) shows no pH dependence because this reaction is limited by a separate pH-insensitive step. The absence of a thio effect on religation strongly suggests that this step does not involve covalent chemistry but is, rather, a conformational change in the enzyme−DNA complex. This model makes the reasonable assumption that the actual chemical step of strand resealing (k_r^{chem}) would be the microscopic reverse of the cleavage step, and would show the same pH dependence and thio effect as k_{cl} (Figures 1B and 6).

An alternative model that could explain the pH dependences of k_{cl} and k_r^{conf} involving a rapid pH-dependent conformational preequilibrium preceding a single rate-limiting transition state for DNA cleavage and religation, is unlikely because the thio effects indicate two different rate-limiting transition states for k_{cl} and k_r^{conf}.

Estimation of k_r^{chem} and k_{conf}. As shown in Figures 1B and 6, and as discussed above, the cleavage of enzyme-bound DNA (k_{cl}) has been expanded into two steps: a pH-dependent chemical step with a thio effect of 4.6-fold (k_{cl}), followed by a conformational step (k_{conf}) which is not directly observed. Similarly, the religation of enzyme-bound DNA (k_r) also consists of two steps: a rate-limiting reversal of the conformational step (k_r^{conf}) followed by a more rapid chemical step (k_r^{chem}) which is not directly observed. The rate constants k_{conf} and k_r^{chem} for the steps which are not directly observed can be estimated from the measured values of k_{cl}, k_r^{conf}, and the overall equilibrium constant for DNA cleavage on the enzyme ($K_{cl} = 0.12 \pm 0.02$) (Stivers *et al.*, 1994a) as follows. From the assumptions that k_r^{chem} has a 4.6-fold thio effect (*i.e.*, identical to that observed for k_{cl}) and that a 1.2-fold thio effect on religation could have been detected, we obtained a lower limit partitioning ratio $k_r^{chem}/k_{conf} = 17$ from consideration of the kinetic equations and forward commitment factor (k_r^{chem}/k_{conf}) for the DNA religation reaction (Northrop, 1982) (Figure 6A). From the conservation of mass and from the definitions of the equilibrium constants for chemical cleavage ($K_{chem} = k_{cl}/k_r^{chem}$), for the conformational change ($K_{conf} = k_{conf}/k_r^{conf}$), and for the overall cleavage of enzyme-bound DNA at pH 7.5 [$K_{cl}' = K_{chem}(K_{conf} + 1)$] which was experimentally determined[3] as 0.10, a set of four simultaneous equations with four unknowns was obtained: the concentrations of three enzyme-bound species in Figure 1B and the value of k_{conf}. Solution of these equations led to $k_{conf} = 0.06 \pm 0.02$ s^{-1} and $k_r^{chem} = 1.0 \pm 0.3$ s^{-1} (Figure 1B). From this analysis, $K_{chem} = 0.08$ and $K_{conf} = 0.09$, indicating that on the enzyme both the chemical cleavage of DNA and the subsequent conformational change to form E′−S·L are thermodynamically unfavorable by only 1.46 and 1.40 kcal mol^{-1}, respectively (Figure 6). From K_{chem} and K_{conf}, the distribution of enzyme−DNA complexes at pH 7.5 is 92% E^H·S, 7.3% E^H−S·L, and 0.7% $E^{H'}$−S·L.

Catalytic Residues. Although pK_a values alone are insufficient to diagnose specific groups, the kinetically determined pK_a values of 6.3 and 8.4 suggest unperturbed His and Cys residues, respectively (Fersht, 1985). Experiments to identify the enzyme residues responsible for these pK_a values have been initiated using the methods of site-directed mutagenesis and NMR titration. At present, the residues have not been

[3] The equilibrium constant $K_{cl}' = 0.10$, which describes the equilibrium between all enzyme−DNA complexes in the active protonation state (E^H) at pH 7.5, was obtained by multiplying the measured value of $K_{cl} = 0.12$ by 0.84, the fraction of the total enzyme in the E^H protonation state at pH 7.5.

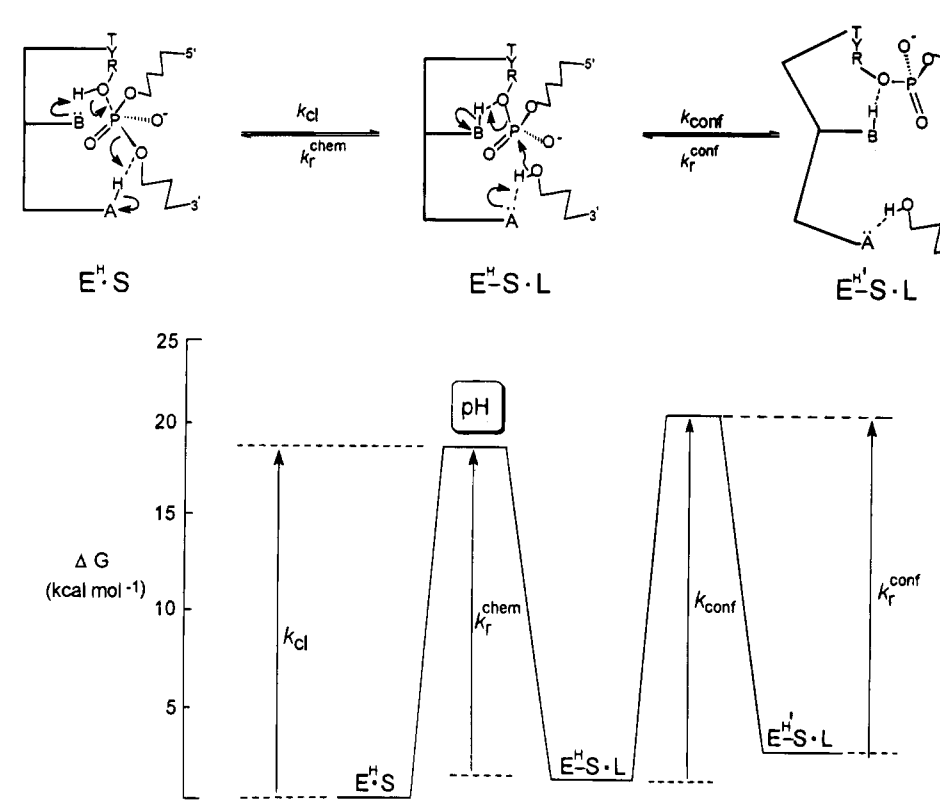


FIGURE 6: Chemical mechanism and free energy diagram of the internal reactions on topoisomerase I. (A) Mechanism for topoisomerase (E^H) catalysis of nucleophilic substitution at the phosphorus backbone of DNA consistent with the pH dependencies of K_{cl}, k_r, and k_{cl}. For clarity, only a single strand of the DNA duplex is drawn. For cleavage (k_{cl}), an enzymic general-base (B) is proposed to remove the hydroxyl proton of Tyr-274, and a separate enzyme group (AH) assists in protonation of the leaving 5′-ribose oxygen. The chemical religation step (k_r^{chem}) is reasonably assumed to proceed by the microscopic reverse of cleavage. In addition to the step involving covalent chemistry, a conformational step (k_{conf}) is indicated, the reverse of which is observed to be rate-limiting for single-turnover religation (k_r^{conf}). (B) Free energy diagram consistent with the observed pH dependencies and thio effects on the strand cleavage and religation reactions catalyzed by Vaccinia topoisomerase (E). In this diagram, the observed single-turnover cleavage reaction (E^H•S → E^H–S•L, where L is the leaving DNA strand) reflects a step which is sensitive to pH and thio substitution. The internal equilibrium constant (K_{cl}) shows the same pH dependence as k_{cl} because only one ionization state of all the noncovalent enzyme–DNA complexes (E^H•S, eq 3) is competent to catalyze DNA strand cleavage. However, the *observed* rate constant for religation ($E^{H'}$–S•L → E^H•S) is limited by a second step which is insensitive to pH and does not involve covalent chemistry. These results are consistent with a conformational change in the covalent enzyme–DNA complex (E^H–S•L ⇌ $E^{H'}$–S•L).

identified, but the following important negative results have been obtained. We have considered (and eliminated) two potential candidate residues for the pK_a = 6.3, His-152 and the active site tyrosine (Tyr-274). His-152 of the Vaccinia enzyme is the most conserved histidine in eukaryotic topoisomerases (Lynn *et al.*, 1989) and seemed a reasonable candidate for the general-base. However, the H152A mutant topoisomerase generated by site-directed mutagenesis retained wild-type topoisomerase activity when assayed for relaxation of supercoiled pUC19 DNA at pH 7.5.[4] Mutations of the remaining seven histidines and the two nonconserved cysteines are in progress.[4]

While a tyrosine residue in the free Vaccinia enzyme would not be expected to have a pK_a = 6.3, it remained an attractive possibility that the enzyme might catalyze the reaction by decreasing the pK_a of the nucleophilic hydroxyl group of Tyr-274. Lowering the pK_a of a nucleophilic residue increases its concentration and provides a catalytic advantage to an enzyme near neutral pH (Hershlag & Jencks, 1990). For example, the active site nucleophile Cys-403 of the *Yersinia* protein tyrosine phosphatase has a pK_a = 4.67 in the free enzyme (Zhang & Dixon, 1993) which increases the relative concentration of the nucleophilic thioate >10^3-fold at pH 5 compared with the thiol group of an unperturbed cysteine residue (pK_a = 8.35; Fersht, 1985).

Figure 7 shows representative ^{13}C-NMR spectra of [ζ-^{13}C]-tyrosine-enriched topoisomerase at pH 5.10, 7.10, and 8.42. The Vaccinia enzyme has 14 tyrosine residues which, with the exception of one resolved resonance (see pH 7.10 spectrum), showed extensive resonance overlap in the pH range studied (5.1–8.8). However, despite the significant overlap, it would have been possible to detect an ionization of a single low pK_a tyrosine in this experiment because the ^{13}C-ζ chemical shift of ionized tyrosine is shifted downfield by ~11 ppm from protonated tyrosine (Richarz & Wüthrich, 1978). No [^{13}C]tyrosine resonances were observed to shift over the pH range 5.1–8.8 (not shown). Assuming that 20% deprotonation would have been detected, all tyrosines of topoisomerase I therefore have pK_a values exceeding 9.5. Hence, this experiment excludes a mechanism of catalysis involving a low pK_a for Tyr-274 in the *free* enzyme.

[4] B. Østergaard-Petersen and S. Shuman, unpublished results (1994). Preliminary studies indicate that the C100A and C211A mutations retain wild-type activity in relaxing supercoiled pUC19 DNA at pH 7.5.

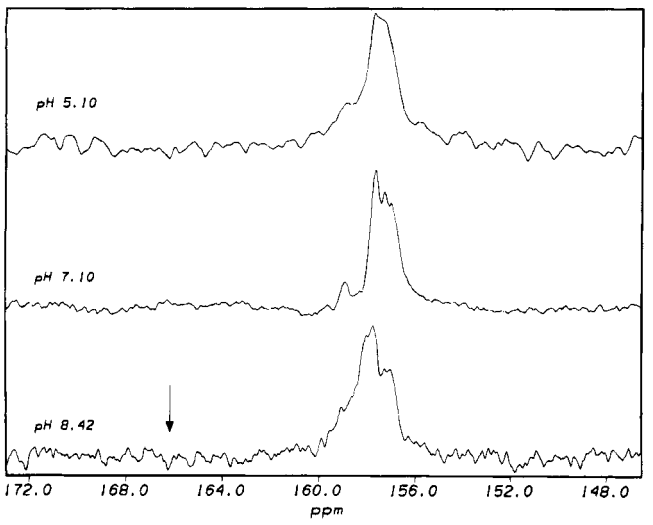


FIGURE 7: Representative ^{13}C-NMR spectra (150 MHz) of ^{13}C-ζ-ring-labeled tyrosine-enriched topoisomerase (0.1 mM) in ^2H_2O at pH* 5.93, 7.10, and 8.42 at 32 °C. The arrow marks the expected ^{13}C chemical shift for the ζ-ring carbon of ionized tyrosine in model peptides (see text).

Mutation of the conserved Arg-130 has been shown to profoundly decrease the activity of Vaccinia topoisomerase in relaxing supercoiled pUC19 DNA, but not to significantly alter DNA binding at pH 7.5.[2] Single-turnover cleavage assays of the R130A and R130K mutants at pH 7.5, as described under Materials and Methods, reveal $10^{5.0}$ and $10^{4.3}$-fold decreases in k_{cl}, respectively, confirming the importance of this catalytic residue (data not shown). Attempts to measure the pH dependences of k_{cl} with these mutants were unsuccessful due to the low rates of cleavage.

DISCUSSION

Chemical Mechanism of Strand Cleavage. Direct experimental evidence for the chemical mechanism(s) by which eukaryotic type I topoisomerases catalyze nucleophilic substitution at the phosphorus backbone of DNA has been largely absent in the literature, although it has been clear for some time that the mechanisms of the eukaryotic enzymes are distinct from those of the bacterial type I topoisomerases because the former have no absolute divalent cation requirement (McConaughty *et al.*, 1981; Goto *et al.*, 1984; Shuman *et al.*, 1988). Therefore, the estimated rate enhancements of at least 10^9- and 10^{12}-fold for the DNA strand cleavage and religation reactions catalyzed by the Vaccinia enzyme (Stivers *et al.*, 1994a), which are very similar to the eukaryotic enzymes, must be achieved without the considerable catalytic contributions conferred by a divalent metal ion (Herschlag & Jencks, 1990; Chaffee *et al.*, 1973).

The comparatively simple bell-shaped pH dependence of the strand cleavage reaction (Figure 4) indicates the presence of two essential titratable groups on the enzyme over the pH range 4.6−9.7. One enzyme group ($pK_a = 6.3$) must be unprotonated, and the second group ($pK_a = 8.4$) protonated to give maximal activity. This pH dependence suggests a mechanism for cleavage of the phosphodiester backbone involving general-base catalysis ($pK_a = 6.3$) of the attack of Tyr-274 at phosphorus and general-acid catalysis ($pK_a = 8.4$) of the expulsion of the 5′-ribose hydroxyl group (Figure 6A). Presumably, the religation reaction proceeds by the same mechanism, but the pK_a values of the catalytic groups are "masked" by a pH-independent step which occurs prior to covalent chemistry (see Figures 1B, 6B).

The pH dependence of the DNA strand cleavage reaction and the chemical mechanism suggested in Figure 6A are reminiscent of bovine pancreatic ribonuclease catalyzed cleavage of RNA (Herries *et al.*, 1962) and the catalytic mechanism of the protein-tyrosine phosphatases (Zhang *et al.*, 1994). Detailed kinetic studies of these enzymes provided evidence for two titratable groups important for activity, which were later assigned by X-ray crystallography or site-directed mutagenesis (Zhang *et al.*, 1994; Barford *et al.*, 1994; Wlodawer & Sjölin, 1983). Like the eukaryotic type I topoisomerases, the protein-tyrosine phosphatases and ribonuclease do not require divalent cations for activity, and $\sim 10^5-10^6$ of the 10^{11}- and 10^{17}-fold rate enhancements achieved by these enzymes, respectively, can reasonably be attributed to combined general acid−base catalysis (Zhang *et al.*, 1994; del Cardayre & Raines, 1994).[5] A catalytic contribution of $\sim 10^6$-fold has also been attributed to general acid−base catalysis of DNA hydrolysis by staphylococcal nuclease on the basis of double mutagenesis of the acid (Arg-87) and base (Glu-43) residues involved (Weber *et al.*, 1991). These comparisons suggest that, at most, 10^6 of the 10^9- and 10^{12}-fold rate enhancements of the Vaccinia topoisomerase can be attributed to general acid−base catalysis.

The remaining contribution may involve transition state stabilization (Jencks, 1981; Fersht, 1985). Such effects on the order of 10^3-10^5-fold have been observed with other enzymes and with catalytic antibodies (Carter & Wells, 1988; Martin *et al.*, 1991; Gibbs *et al.*, 1992; Janda *et al.*, 1993). With Vaccinia topoisomerase, transition state stabilization by Arg-130 is suggested by the 10^5- and $10^{4.3}$-fold decreases in cleavage activity of the R130A and R130K mutations, respectively. Effects of similar magnitude are found with staphylococcal nuclease upon mutation of Arg-35 and Arg-87, residues known to contribute to transition state stabilization [see Weber *et al.* (1992) and references cited therein]. The failure of Lys to substitute for Arg in staphylococcal nuclease was explained by bifunctional hydrogen bonding of the pentacoordinate phosphorane transition state, and monofunctional hydrogen bonding of the phosphodiester ground state (Weber *et al.*, 1992). Arg-130 of topoisomerase, which does not contribute to DNA binding in the ground state, may interact bifunctionally with the transition state. Arg-223, which is highly conserved, has also been shown to be a catalytically important residue in topoisomerase because marked decreases in DNA cleavage and DNA relaxation activity but no significant decrease in DNA binding have been found with the R223G, R223E, and R223Q mutant enzymes (Morham & Shuman, 1990; Klemperer & Traktman, 1993). However, the R223K mutation shows little loss in activity, indicating that a monofunctional cationic residue suffices at this position (Klemperer & Traktman, 1993). As previously pointed out (Stivers *et al.*, 1994a), the supercoil release step need not be catalyzed by

[5] The contributions of general acid−base catalysis to the rate accelerations achieved by the *Yersinia* protein-tyrosine phosphatase were determined by single and double mutagenesis of the general acid (Asp-356) and general base (Glu-290) residues involved (Zhang *et al.*, 1994). The overall rate enhancement for ribonuclease was estimated from the k_{cat} value of 1400 s^{-1} (pH 6, 25 °C) for cleavage of UpA (del Cardayre & Raines, 1994) and the spontaneous rate of hydrolysis of phosphodiesters of 10^{-14} s^{-1} extrapolated to pH 7.5, 25 °C (Serpersu *et al.*, 1987; Mildvan & Serpersu, 1989).

topoisomerase.

It should be noted that in a recent publication, Christiansen *et al.* (1994) reported the pH dependence of the *extent* of strand cleavage and religation reactions, hydrolysis and alcohololysis reactions catalyzed by the human type I topoisomerase. It is not possible to compare our results with those obtained with the human enzyme because in the work of Christiansen *et al.* (1994) *end points* rather than *rates* of reaction were measured. A model for eukaryotic topoisomerase I-catalyzed DNA cleavage and religation involving enzymic general acid−base catalysis was also proposed by these workers; however, no kinetic evidence was presented.

A Conformational Step on the Reaction Pathway. The absence of a rate decrease in an enzymatic reaction upon "thio substitution" can provide evidence for a rate-limiting step other than covalent chemistry, such as a conformational change [see Stivers *et al.* (1994a) and references cited therein]. The thio effect of 4.6-fold we previously reported for the single-turnover strand cleavage reaction provided evidence for a rate-limiting bond-breaking event, indicating that there is no kinetically significant conformational change prior to the cleavage step (Stivers *et al.*, 1994a). In contrast, herein we report that the rate of religation shows no thio effect, providing evidence that a conformational step prior to the chemical religation step is rate-limiting (Figures 1B, 6). These two results are most simply explained by placing a conformational step after the cleavage step in the forward cleavage reaction (Figures 1B, 6). The estimated rate constant for the forward conformational change ($k_{conf} = 0.06$ s^{-1}) is similar to the single-turnover cleavage rate ($k_{cl} = 0.08$ s^{-1}), indicating that both steps are partially rate-limiting under steady-state conditions when product release is fast (*i.e.*, high [salt]) (Stivers *et al.*, 1994a). Conformational changes at comparably slow rates have been detected in *cis*−*trans*-proline isomerization during the folding of barnase (Matouschek *et al.*, 1990) and in dye binding to α-chymotrypsin (Fersht & Requena, 1971). Both k_{cl} and k_{conf} are similar to the overall rate constant for relaxation of supercoiled plasmid DNA, $k_{relax} = 0.035$ s^{-1}, which was obtained under single-turnover (processive) conditions at 20 °C and pH 7.9 with several eukaryotic type I topoisomerases (Caserta *et al.*, 1990).

Independent evidence for a significant conformational change in the enzyme upon binding and cleavage of linear duplex DNA has recently been obtained by evaluating the products of partial proteolysis of the free enzyme and the enzyme−DNA covalent complex. A protease-sensitive site that is present in the free enzyme becomes refractile to proteolysis after formation of the enzyme−DNA covalent complex, and an additional protease-sensitive site appears.[6]

Another consequence of this conformational change is that the active protonation state of the enzyme (E^H) is preserved, as indicated by the pH independence of the rate constant for religation (Figure 4). This rate constant shows less than a 2-fold variation over the entire pH range studied, in spite of the fact that only ~4% and 7% of the enzyme is in the active protonation state at pH 4.5 and 9.5, respectively. Since the same acid and base residues on the enzyme are presumed to catalyze the chemical steps of cleavage and religation (Figure 6A), then religation should have shown a pH dependence over the range studied unless the irreversible cleavage reaction had trapped the enzyme−DNA covalent complex in the active protonation state. Alternatively, and less likely, the pK_a values of residues involved in religation lie well outside the range 4.5−10.

Implications. The physical mechanism by which type I DNA topoisomerases remove supercoils, and generate topological isomers of DNA differing in linking number by steps of one, is unknown. Two general mechanisms for supercoil release have been proposed [see Champoux (1990) and references cited therein]; the first mechanism involves free rotation of the DNA around the uncleaved strand, while the second mechanism envisions passage of the unbroken strand of the DNA through the cleaved strand. A critical unknown in either of these two general mechanisms is the extent to which the chemical steps of cleavage and religation are coupled to supercoil release. That is, how many supercoils are released for each cleavage/religation cycle? It is attractive to suggest that the conformational change detected here during the religation of linear DNA substrates reflects a conformational step which occurs during the relaxation of supercoiled DNA molecules, as has been suggested from the three-dimensional structure of topoisomerase I from *E. coli* (Lima *et al.*, 1994). Assuming this interpretation with Vaccinia topoisomerase I, only 0.7% of the enzyme-bound DNA is in the active $E^{H'}$−S·L conformation, poised for supercoil release (Figure 6). Once this active conformation has been reached, then partitioning between supercoil release and reversion to the inactive E^H−S·L conformation ($k_r^{conf} = 0.66$ s^{-1}) occurs. If the average rate of supercoil release measured with plasmid substrates under steady-state conditions [~1 supercoil released s^{-1} at 20 °C; see Stivers *et al.* (1994a) and references cited therein] reflects the microscopic rate of supercoil release on the enzyme (k_{relax}), then the conformational step (k_r^{conf}) will compete effectively with that for the supercoil release step, resulting in a partition ratio (k_{relax}/k_r^{conf}) near unity. If the partition ratio is significantly greater than unity, then more than one supercoil will be removed each time the active $E^{H'}$−S·L conformation is attained, resulting in an uncoupling between the chemical cleavage−religation step and supercoil release. Experiments are currently in progress to directly measure k_{relax} on a supercoiled substrate.

[6] J. Sekiguchi and S. Shuman, unpublished results (1994).

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