Fluorescence Spectroscopy Studies of Vaccinia Type IB DNA Topoisomerase

CLOSING OF THE ENZYME CLAMP ISFASTER THAN DNA CLEAVAGE

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The prototypic type IB topoisomerase isolated from vaccinia virus cleaves the phosphodiester backbone of duplex DNA at the sequence 5'-G(T)CCCT, forming a covalent 3'-phosphotyrosyl adduct. A precleavage conformational change in which the enzyme clamps circumferentially around the DNA has been implicated on the basis of structural and biochemical studies. However, no direct measurements to elucidate this key step have been obtained to date. To address this shortcoming we have developed two new fluorescence assays that allow detection of conformational changes in both the enzyme and substrate DNA, and allow determination of the thermodynamic and kinetic mechanism for noncovalent DNA binding and phosphodiester cleavage. The results indicate that clamp closing occurs in a rapid step (>25 s⁻¹) that is at least 14-fold faster than the maximal rate of DNA cleavage. Opening of the clamp to release the noncovalently bound substrate is also 5–8-fold more rapid than DNA cleavage. We propose a model in which DNA cleavage and religation are connected through a single high energy transition state involving covalent bond breaking. Alternative models that involve a slow precleavage conformational step are not easily reconciled with the available data.

A prototypic eukaryotic type IB topoisomerase, the vaccinia virus topoisomerase, catalyzes reversible site-specific cleavage and religation of the phosphodiester backbone of duplex DNA at 5'-G(T)CCCT sites (1). The formation of a covalent 3'-phosphotyrosyl intermediate with the conserved active site tyrosine (Tyr-274) is nature's elegant solution to two problems, preserving the energy of the phosphodiester linkage so that the DNA can be religated and providing a swivel point for relaxation of DNA supercoils (2). Although one of the most fascinating aspects of type IB topoisomerase action is the dynamic motion of the enzyme and DNA, little is known about the conformational fluctuations that occur before and after the cleavage step or the role these changes may play in site-specific binding and in controlling the rate of DNA relaxation. Because of their essential role in maintaining the superhelical density of DNA in the cell, topoisomerases have attracted much attention as targets for antiviral, antibacterial, and anticancer drugs.

Importantly, many of these drugs act to trap the enzyme in nonproductive conformations that are incapable of religating after the initial cleavage step. Accordingly, an understanding of topoisomerase conformational dynamics is of great interest. Structural and biochemical studies on the 314-residue vaccinia topoisomerase have revealed that the enzyme consists of two domains with two flexible loop regions that become protected resistance upon DNA binding, suggesting an induced fit conformational change in the enzyme (3–6). The amino-termini domain consists of residues 1–80 and contributes to site-specific DNA binding (6). The carboxyl-terminal or catalytic domain (residues 81–314) contains the active site tyrosine and a constellation of basic groups that form direct interactions with the scissile phosphodiester that have been shown to be essential for efficient catalysis (Arg-130, Lys-167, Arg-223, and His-265) (7–9). The crystal structure of the free catalytic domain has also provided strong evidence for a precleavage conformational change, because the essential residues Tyr-274, Arg-130, and Lys-167 were not in positions that would allow direct interaction of these groups with the scissile phosphodiester. Although a conformational step is strongly implicated, there have been differing viewpoints as to whether this step is faster or slower than the chemical step of DNA cleavage (Fig. 1) (1, 8).

In principle, if one could develop approaches in which the conformational steps could be directly monitored, the overall rate-limiting step could be ascertained.

In this study we report the first continuous fluorescence kinetic measurements of a type IB DNA topoisomerase. Incorporation of the fluorescent adenine analogue, 2-aminopurine, 3' to the DNA cleavage site afforded a strong increase in fluorescence intensity of the DNA upon noncovalent binding by the Y274F mutant and during site-specific cleavage by the wild-type enzyme. This signal from the DNA was used to measure the association rate, dissociation rate, equilibrium constant for noncovalent DNA binding, and the DNA cleavage rate. In addition, the tryptophan fluorescence intensity of the topoisomerase was found to decrease about 50% upon noncovalent DNA binding, providing an independent probe of the conformational changes in the enzyme. Combining these fluorescence results with detailed kinetic measurements of DNA cleavage and religation...
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FIG. 1. Free energy reaction coordinate diagrams of two models for the formation of the covalent complex between topo-isomerase I and its DNA substrate. A, the proposed clamp closing step (↓) is rate-limiting. B, the phosphodiester cleavage step (↓) is rate-limiting. In both models the open and closed conformations of the enzyme are indicated by E and E′, respectively. Both models also depict a conformational change in the DNA substrate (S′) that is detected in 19F NMR experiments described in the accompanying article (28). In models A and B, the conformational changes in the DNA and enzyme are depicted as simultaneous events. However, an alternative model (depicted in inset) in which the DNA conformational change occurs as a separate event within the closed form of the enzyme-substrate complex cannot be excluded (see the accompanying article (28)). The hydroxyl group in the diagram represents the active site Tyr-274 that forms the chemical step of cleavage (P). The open and closed conformations of the enzyme are indicated by E and E′, respectively. Both models also depict a conformational change in the DNA substrate (S′) that is detected in 19F NMR experiments described in the accompanying article (28).

FIG. 2. Sequence of the DNA substrates used in these studies. All of the substrates contain the conserved pentapyrimidine sequence (5′-CCCTT-3′). The substrates designated with 2-AP contain the fluorescent reporter group 2-aminopurine.

Single Turnover Cleavage Kinetics—The 18-mer DNA strand containing the CCCTT motif was 3′ end-labeled with [α-32P]ATP using terminal deoxynucleotidyltransferase (Amersham Biosciences), and the labeled 18-mer was hybridized with a 1.1-fold molar excess of 24-mer (Fig. 2). The labeled 18/24-mer duplex DNA was then purified using Bio-Gel P-6 spin column (Bio-Rad). The cleavage experiments were performed using Buffer A. The final concentration of DNA was 2.3 nM, and wild-type topoisomerase was varied in the range 50–1000 nM. One μl of the quenched reactions was spotted on polyethyleneimine-cellulose TLC plates (EM Science) and developed with a mobile phase containing 0.15 M potassium phosphate, pH 7.5, for 15 min to separate the labeled 6-mer product (P₁ = 0.6) from the substrate (R₀ = 0). The TLC plates were imaged using a Storm 640 PhosphorImager (Molecular Dynamics) and the fractional extent of reaction at a given time (frac P = cpm 6-mer/total cpm), normalized to the observed end point (frac P_end = cpm P_end/frac P), was quantified using the ImageQuant software (i.e. frac Pnorm = frac P/frac P_end). The measured pseudo-first-order rate constants for cleavage at each concentration of topoisomerase (k_cleavage) were plotted as a function of enzyme concentration to obtain the maximal cleavage rate (k_max) and K_int. The data were fitted to a single exponential rate equation to follow the changes in 2-AP fluorescence using an Applied Photophysics instrument in the two-syringe mode. The excitation wavelength was 315 nm, and emission was monitored using a 360 nm cut-off filter. The cleavage rate measurements were made using pseudo-first-order conditions, where the concentration of the 18AP/24-mer was 10-fold less than the enzyme. For these measurements, the labeled DNA concentration was 10 or 25 nM, and the concentration of topoisomerase was varied in the range 0.1 to 1.2 μM. The data were fitted to a single exponential rate equation to obtain the observed rate constants, which were then plotted against the topoisomerase concentration and fitted to Equation 1 to obtain k_max and K_int.

k_max = k_0[E] / K_int + [E] (Eq. 1)

The maximal cleavage rate (k_max) will be equal to the intrinsic rate constant for cleavage (k_cleavage, Fig. 1) when there are no internal equilibrium steps (K_int) before cleavage, otherwise k_max = k_0[k_cleavage, assuming the equilibrium steps are rapid compared with k_cleavage. The cleavage kinetics for the 18AP/24-mer substrate (Fig. 2) were determined using stopped-flow fluorescence measurements by following the changes in 2-AP fluorescence using an Applied Photophysics instrument in the two-syringe mode. The excitation wavelength was 315 nm, and emission was monitored using a 360 nm cut-off filter. The cleavage rate measurements were made using pseudo-first-order conditions, where the concentration of the 18AP/24-mer was 10-fold less than the enzyme. For these measurements, the labeled DNA concentration was 10 or 25 nM, and the concentration of topoisomerase was varied in the range 0.1 to 1.2 μM. The data were fitted to a single exponential rate equation to obtain the observed rate constants, which were then plotted against the topoisomerase concentration and fitted to Equation 1 to obtain k_max and K_int.

Single Turnover Religation Kinetics—Religation experiments were performed by rapidly mixing the preformed covalent complex with an excess of the 12-mer ligation strand. The covalent complex was first formed by incubating topoisomerase (330 nM) with 5′-32P-labeled 18/24-mer (300 nM) for 30 min to quantitatively form the E-12/24-mer covalent complex. The religation reactions were initiated by rapid mixing of equal volumes of the covalent complex with the complementary 12-mer (Fig. 2). The reactions were quenched by the addition of 10% SDS. The final concentrations of the covalent complex and 12-mer were 20 nM and 2 μM, respectively. The E-12/24-mer covalent complex and the 24/24-mer religation product were separated by electrophoresis using a 15% (w/v) polyacrylamide gel containing SDS. Under these dilute conditions we would expect that ~90% of the covalently bound DNA would religate. However, only 70–80% of the DNA in the covalent complex was rapidly converted to full-length ligation product, possibly due to some nonproductive binding of the 12-mer strand. The fractional extent of covalent complex remaining at a given time (frac complex = cpm complex/total cpm) was quantified using the ImageReader version 1.75 software and then normalized to the observed end point (frac complex_end).

Thus in Equation 2, Frac complex = frac complex/Frac complex_end, where k_r is the religation rate constant.

The cloning and purification of wild-type topoisomerase Enzymes—The cloning and purification of wild-type topoisomerase I and the 2-AP mutant have been described previously (10, 11). The enzyme concentrations were determined by UV absorbance using an extinction coefficient of 28,140 M⁻¹ cm⁻¹ in a buffer containing 20 mM sodium phosphate, pH 6.0, and 6 μg guanidinium hydrochloride (11).

DNA Substrates—The sequences of DNA substrates used in these studies are shown in Fig. 2. The oligonucleotides were synthesized using an ABI 380B synthesizer. The phosphoramidite reagent of the 18/24-mer was 10-fold less than the enzyme. The sequences of DNA substrates used in these studies are shown in Fig. 2. The oligonucleotides were synthesized using an ABI 380B synthesizer. The phosphoramidite reagent of the 18/24-mer was 10-fold less than the enzyme. All of the substrates contain the conserved pentapyrimidine sequence (5′-CCCTT-3′). The substrates designated with 2-AP contain the fluorescent reporter group 2-aminopurine.

Experimental Procedures

Enzymes—The cloning and purification of wild-type topoisomerase I and the Y274F mutant have been described previously (10, 11). The enzyme concentrations were determined by UV absorbance using an extinction coefficient of 28,140 M⁻¹ cm⁻¹ in a buffer containing 20 mM sodium phosphate, pH 6.0, and 6 μg guanidinium hydrochloride (11).

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Equilibrium Cleavage Measurements—The equilibrium cleavage measurements were performed using Buffer A by titrating 5'-2-AP-labeled 40/40-mer or 24/24-mer (30 – 100 nM) with increasing concentrations of wild-type topoisomerase (10 – 1500 nM). In these titrations it was found that high concentrations of covalent complex (>0.6 µM) inhibited the further formation of covalent complex. Thus the end points (K$_d$) were determined by small extrapolation using concentrations of topoisomerase less than 0.6 µM. These extrapolations were typically quite small, and do not appreciably influence the certainty in the reported K$_d$ values. The covalent complex was trapped by the rapid addition of 1 volume of 10% SDS, and the free and covalently bound DNA were separated by electrophoresis using an 18% polyacrylamide gel containing 0.2% SDS. The fraction covalent complex (Frac complex = counts covalent complex/counts covalent complex + counts free DNA) was plotted against the total topoisomerase concentration and fitted to Equation 3, which describes the reversible two step binding and cleavage mechanism shown in Equation 4.

\[ \text{Frac complex} = \frac{b - 4 \theta + a[E][S]}{2a^2[S]} \]  
\[ F + S \rightleftharpoons K_{d0} \rightleftharpoons E^* + P \]  

In this analysis, the counts that migrate as free DNA represent the sum of the counts for DNA that was bound noncovalently through the magnesium ion to the DNA that was free. In Equation 3, $a = 1 + 1/K_{d0}$ and $b = a[E] + a[S] + K_{d0}/K_{d}$, where [E] and [S] are the total enzyme and substrate concentrations.

Tryptophan Fluorescence Measurements of Noncovalent DNA Binding—Equilibrium titrations of the Y274F mutant with the 18/24-mer or 40/40-mer DNA were performed using the same conditions as described above, although the concentration ranges were 0.8 to 100 µM. The excitation wavelength was 295 nm, and the emission was monitored from 315 to 450 nm. Corrections for the inner filter effect of substrate DNA were performed using Equations 5 and 6.

\[ A_{295} = A_{295}^0 + A_{295}^c \]  
\[ F_{295} = F_{295}^c \times 10^{\frac{b}{50}} \]  

where $A_{295}^0$, $A_{295}^c$, $l$, $c_{DNA}$, and $F$ are the absorbance of the DNA, the extinction coefficient at 295 nm, the DNA concentration, and fluorescence intensity, respectively. The corrected fluorescence spectra were used to determine $K_{d0}$ using Equation 7, where $b = K_{d0} + [E]_{tot} + [S]_{tot}$, and $F_0$ and $F_E$ are the initial and final corrected fluorescence intensities at 335 nm.

\[ F = F_0 - (F_0 - F_E^c) / 2 [DNA]_{tot} (b - \theta^2 - 4[E]_{tot}[DNA]_{tot}) \]  

In order to fit the data, the concentration dependence of the observed binding rates were fitted to Equation 9, from which the dissociation rates ($k_{d}$) were also measured using a trapping protocol. Preformed complexes of Y274F and the 18AP/24-mer or 40AP/40-mer DNA were rapidly mixed in a 1:1 ratio with a large molar excess of competing unlabeled DNA (10 µM), and the time-dependent decrease in 2-AP fluorescence was monitored. The observed off-rate was obtained directly from a fit of the observed fluorescence change to a first-order decay equation (Equation 8). The concentration of the competing trapping DNA was sufficiently high such that the rate-limiting step was dissociation of the complex (not shown).

RESULTS

DNA Cleavage and Religation Using High Salt Conditions—Previous studies of the vaccinia topoisomerase have always been performed using low salt concentrations (50 mM Tris-HCl, pH 7.5 or pH 8.0). Under these nonphysiological conditions, the enzyme-DNA complex is extremely stable ($K_{D} <$ 5 nM) making it difficult to study DNA binding and the concentration dependence of the kinetic parameters. Therefore, to enable the use of fluorescence methods, we investigated the kinetic activity of topoisomerase using higher salt concentrations with the goal of increasing the $K_{D}$ value by ionic screening.

By using substrates such as the 18/24-mer allows the cleavage reaction to be studied in irreversible conditions because the 6*-mer product spontaneously dissociates making religation extremely inefficient (10, 11). Under the new high salt conditions (20 mM Tris-HCl, 200 mM NaCl at pH 8.0 at 25 °C), the maximal single turnover cleavage rate for the 18/24-mer was identical to that measured at low salt concentrations (Fig. 3, $k_c = 0.62$ s$^{-1}$) (11). However, the presence of 200 mM NaCl was found to increase the apparent $K_{D}$ for single turnover DNA cleavage by over 50-fold (Fig. 4A, $K_{D}^{calc} = 220 ± 60$ nM). As shown in Fig. 4B, the observed rate of religation ($k_r = 0.64$ s$^{-1}$) was somewhat smaller than that measured previously using the low salt buffer ($k_r = 0.90$ s$^{-1}$). Assuming that cleavage and religation may be approximated by a simple two-state equilibrium as observed previously at low salt concentrations (11), the ratio of $k_c/k_r$ gives the cleavage equilibrium constant $K_{D}^{calc} = 1.02 ± 0.26$. This calculated value is within error of the $K_{D}^{calc}$ value determined from equilibrium quench experiments (see below), indicating that the two-state approximation applies at high salt concentration as well. The complete kinetic parameters for cleavage and religation of the 18/24-mer using these high salt conditions are listed in Table I.

Equilibrium DNA Binding and Cleavage—To confirm the calculated value for $K_{D}$ obtained from the kinetic measurements, we also performed equilibrium quench experiments using the 40/40-mer and 24/24-mer substrates. These substrates show reversible cleavage and religation because the leaving
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The parameters were determined at 25 °C in Buffer A containing 20 mM Tris-HCl, 200 mM NaCl, pH 8.0, at 25 °C.

| Substrate | $k_d$ | $k_r$ | $K_{diss}^{obs}$ | $K_{emb}^{app}$ |
|-----------|------|------|----------------|---------------|
| 18/24     | 0.62 ± 0.06 | 0.6 ± 0.2 | 1.0 ± 0.3 | 220 ± 60 |
| 18AP/24   | 1.8 ± 0.2 | 1.0 ± 0.2 | 1.8 ± 0.4 | 160 ± 60 |

These values were obtained from the ratio $k_d/k_r$. The apparent dissociation constant was determined from the concentration dependence of $k_{emb}$ for single turnover cleavage (Equation 1). This value was calculated from the equation $k_r = k_{emb}/K_{diss}$.

Fluorescence Measurements of Noncovalent DNA Binding—To determine the noncovalent binding affinity of Y274F for the DNA, we performed titrations in which the changes in 2-AP or tryptophan fluorescence of the DNA or enzyme were followed. Titration of the 40AP/40-mer or 18AP/24-mer with increasing concentrations of Y274F results in a 2- and 1.8-fold increase in fluorescence intensity at 370 nm (Fig. 5, A and B), which could be fit to Equation 4 to obtain $K_D$ values of 240 and 200 nM, respectively. A similar $K_D = 250 ± 60$ nM was measured for the 18/24-mer when quenching of the tryptophan fluorescence signal was being followed (Fig. 6 and Table III). The increase in 2-AP fluorescence intensity suggests that the 2-AP base becomes less stacked upon noncovalent binding, because 2-AP fluorescence increases greatly when its stacking interactions with other bases are diminished (see Refs. 13–15 and references therein). To establish that no gross enzyme aggregation was occurring during these titrations, dynamic light scattering experiments were performed on the free Y274F and its complex with the 18/24-mer in the concentration range 1–10 μM. These data were analyzed with respect to the hydrodynamic radius and polydispersity of the solute. In this concentration range the results were consistent with a monomeric enzyme, or a 1:1 complex of enzyme and DNA (data not shown).

Real Time Fluorescence Measurements of DNA Binding and Disassociation—Stopped-flow fluorescence kinetic experiments indicate that Y274F binding to the 18AP/24-mer or 40AP/40-mer occurs in a single kinetic phase, which could be analyzed as a single exponential process (Fig. 7A). Similar single exponential time courses were measured at other concentrations of the enzyme in the range 0.2–1.5 μM. The observed rate constant for noncovalent binding showed a linear dependence on Y274F concentration, suggesting that a single rate-limiting transition state links the free enzyme and substrate with the ES complex.
TABLE II
Cleavage equilibrium constants and equilibrium dissociation constants for wild-type topoisomerase determined from equilibrium quench experimentsa

| Substrate | \( K_d \) | \( K_D \) |
|-----------|----------|----------|
| 24/24     | 0.9 ± 0.1| 160 ± 70 |
| 40/40     | 1.0 ± 0.1| 190 ± 50 |
| 40AP/40\(^*\) | 1.9 ± 0.4| 290 ± 120 |

a The measurements were performed at 25 °C in Buffer A. The \( K_d \) and \( K_D \) values were determined from equilibrium quench experiments using Equation 3.

b The titration exhibited inhibition of covalent complex formation at total enzyme concentrations less than required to determine accurately the end point in Equation 3. Thus, the \( K_d \) value was obtained by fixing the \( K_d \) value at 290 nM, the average value determined for the 40AP/40-mer using the Y274F mutant (Table III).

c \( K_D \) values were obtained from equilibrium titrations using the Y274F mutant. For the 18/24-mer, tryptophan fluorescence measurements were used.

TABLE III
Kinetic parameters for binding of Y274F to DNA substratesa

| Substrate | \( k_{on} \) | \( k_{off} \) | \( K_{on}^{calc} \) | \( K_{off} \) |
|-----------|-------------|-------------|-----------------|------------|
| 18AP/24   | (1.5 ± 0.2) \( \times 10^7 \) | 3 ± 1 | 190 ± 70 | 200 ± 20 |
| 40AP/40   | (1.5 ± 0.2) \( \times 10^7 \) | 5 ± 2 | 340 ± 140 | 240 ± 20 |
| 18/24     | 1.5 \( \times 10^7 \) | 1.9 ± 0.4 |           |            |

a The on-rate \( (k_{on}) \) and off-rate \( (k_{off}) \) were determined using Equation 9. The \( k_{off} \) values in parentheses are from the irreversible dissociation rate experiments (Fig. 9).

b \( K_{on}^{calc} = k_{on}/k_{off} \).

c These \( K_D \) values were obtained from equilibrium titrations using the Y274F mutant. For the 18/24-mer, tryptophan fluorescence measurements were used.

FIG. 5. Binding affinity of Y274F for the 18AP/24-mer and 40AP/40-mer DNA substrates. A, changes in the 2-AP fluorescence emission spectra of 200 nM 40AP/40-mer upon sequential addition of Y274F. The final concentration range of the topoisomerase mutant was 0–1.2 μM. B, the solid lines are the nonlinear least squares best fits to Equation 7. In these experiments, [40AP/40-mer] (□) = 200 nM and [18AP/24-mer] (○) = 150 nM. The equilibrium dissociation constants \( (K_D) \) for the binding of Y274F to the 18AP/24-mer and 40AP/40-mer were 200 ± 20 and 240 ± 20 nM, respectively. These values were obtained from global analysis of three data sets obtained using fixed DNA concentrations in the range 150–600 nM.

FIG. 6. Intrinsic tryptophan fluorescence measurements of noncovalent DNA binding by Y274F. A, changes in the tryptophan fluorescence spectrum of 0.4 μM Y274F upon sequential addition of 0–3 μM 18AP/24-mer. B, the corrected fluorescence intensity at 335 nm is plotted as a function of the substrate DNA concentration. The solid line is the nonlinear least squares best fit to Equation 7. A \( K_D = 250 ± 60 \) nM was obtained from the global analysis of 3 titrations in which the fixed enzyme concentration was varied in the range 0.4–1.4 μM (Table III).

FIG. 7. Stopped-flow fluorescence measurements of the association rates of Y274F with the 18AP/24-mer and 18/24-mer substrates. A, the time course for the increase in 2-AP fluorescence of the 18AP/24-mer DNA substrate (50 nM final concentration) was monitored after rapidly mixing with a solution of Y274F (200 nM final concentration). The solid line is the best fit to Equation 8. The inset shows an identical experiment except that the Tyt-274 concentration was 800 nM. B, the time course for the decrease in tryptophan fluorescence of Y274F (200 nM) upon binding to the 18/24-mer (750 nM).

For a simple two-state binding mechanism, the slope of a linear plot of \( k_{obs} \) against enzyme concentration provides the on-rate \( (k_{on}) \); the y intercept gives an estimate of the off-rate \( (k_{off}) \), and the ratio \( k_{off}/k_{on} \) should be equal to the dissociation constant \( (K_D^{calc}) \). As shown in Table III, \( k_{on} = 1.5 \times 10^7 \) M\(^{-1}\) s\(^{-1}\) for both the 18AP/24-mer and the 40AP/40-mer and \( k_{off} \) is in the range 9–5 s\(^{-1}\). The ratio \( k_{off}/k_{on} \) for both substrates agrees well with their respective \( K_D \) values (Table III), providing independent validation of the kinetic measurements. The association rates for the 18/24-mer and Y274F were also measured by monitoring the time-dependent decrease in tryptophan fluorescence at two concentrations of DNA (Fig. 7B and 8). These \( k_{obs} \) values are within error of the association rates determined using 2-AP fluorescence (see symbol, Fig. 8) indicating that the conformational change in the DNA and enzyme are likely to occur simultaneously. A further study of the concentration dependence of the binding rates using changes in tryptophan
fluorescence as the spectroscopic signal was pre-empted by signal-to-noise limitations.

To confirm the off-rates that were extrapolated from the concentration dependence of the association rates (Fig. 8), $k_{\text{off}}$ was also determined directly using a trapping experiment. In this approach (Fig. 9), a preformed complex between Y274F and 18AP/24-mer was rapidly mixed with a large excess of 18/24-mer competing DNA, such that 18AP/24-mer dissociation was irreversible. The kinetic traces were analyzed using a single exponential model to obtain the unimolecular dissociation constant, $k_{\text{off}} = 2 \text{ s}^{-1}$. This $k_{\text{off}}$ is within error of the y intercept extrapolated from the linear fit of $k_{\text{obs}}$ against Y274F concentration (Fig. 8).

**Stopped-flow Fluorescence Measurements of 18AP/24-mer Cleavage**—Because 2-AP fluorescence proved to be a useful probe for the noncovalent binding step using the Y274F mutant (see above), we speculated that 2-AP might also serve as a useful probe for the cleavage step if all steps before and after cleavage were fast. In this experiment, several predictions would hold true if the cleavage step were slow compared with the formation of the noncovalent complex. 1) The observed rate constants obtained from stopped-flow fluorescence measurements using the 18AP/24-mer (see Fig. 4A) are within error of the $k_{\text{obs}}$ determined in binding studies using the Y274F mutant (Fig. 5B).

Preliminary experiments showed that incubation of a limiting amount of 18AP/24-mer with a stoichiometric amount of wild-type topoisomerase resulted in a 3-fold increase in fluorescence intensity at 370 nm (not shown). This increase is 1.7-fold greater than that observed upon noncovalent binding of the Y274F mutant (Fig. 5B), because irreversible cleavage of the 18AP/24-mer releases the 6-mer (2-AP/TTCCC) to solution where it is highly fluorescent. We then measured the kinetics of this 2-AP fluorescence change in stopped-flow experiments with the wild-type topoisomerase (Fig. 10A). All of the kinetic traces followed a first-order rate equation with no lags. As expected for rate-limiting cleavage, the observed rate constants showed a hyperbolic dependence on the enzyme concentration, and the $K_{D}^{*\text{pp}} = 160 \pm 60$ (Fig. 10B) was within error of the binding constant for the 18AP/24-mer ($K_D = 200 \pm 20$, Fig. 5).

These are the expected results if all steps leading to the observed fluorescence change are rapid. However, for the 18AP/24-mer, the plateau value of the hyperbolic fit using Equation 1 obtained for the 18/24-mer (see Fig. 4) shows the best fit to Equation 1, and the dashed line shows the best fit to Equation 1 obtained for the 18/24-mer (see Fig. 4A).
as compared with the 18/24-mer. To confirm this, we also measured the maximal cleavage rate for the \(^{32}\)P-labeled 18AP/24-mer using a KinTek rapid mix-chemical quench device (open circles, Fig. 10A). These cleavage data perfectly overlaid that of the fluorescence results, confirming that the 18AP/24-mer is cleaved 3-fold faster than that of the 18/24-mer (\(k_{\text{off}} = 1.8 \, \text{s}^{-1}\)). This larger \(k_{\text{off}}\) value suggested that the cleavage equilibrium constant might also be larger for the 40AP/40-mer. This prediction was confirmed in equilibrium quench measurements, where it was found that \(K_D = 1.8 \pm 0.4\) for the 40AP/40-mer, from which a \(k_{\text{f}} = 1.0 \pm 0.2\) may be calculated using the relationship \(k_{\text{f}} = k_{\text{off}}/K_D\). Thus, although the cleavage rates of the 18/24 and 18AP/24-mers differ by 3-fold, topoisomerase has a similar noncovalent binding affinity for these substrates (see \(K_D^{\text{ssp}}\) values in Table 1) and similar rates for religation. We conclude that substitution of 2-AP for adenine selectively decreases the kinetic barrier for the cleavage step, with little effect on noncovalent DNA binding or religation (see “Discussion”). It should be pointed out that although cleavage is faster with the 18AP/24-mer, it is still 14-fold slower than the rate of formation of the noncovalent complex at the highest concentration of Y274F investigated in Fig. 8 (\(k_{\text{obs}} = 25 \, \text{s}^{-1}\)). Thus, for either substrate, the precleavage conformational change is much faster than cleavage.

**DISCUSSION**

In general, detailed biophysical studies of type IB topoisomerases have been problematic because the enzymes express poorly in bacterial expression systems, and the DNA binding, cleavage, and religation steps are difficult to quantify in a rigorous manner. The vaccinia enzyme has been the preferred system to pursue such quantitative studies because it is easily overexpressed in bacteria, and its site-specific cleavage and religation activities allow thermodynamic and kinetic measurements at a single defined site (10, 11). Despite the useful insights obtained from previous studies of the vaccinia enzyme, a fuller understanding of the topoisomerase mechanism has been hampered by the lack of spectroscopic assays to probe kinetic steps and intermediates that are transparent when measuring the rate of DNA cleavage or religation alone.

**Topoisomerase Is Fully Active Using High Salt Concentrations**—Although previous studies of the vaccinia topoisomerase have always used low salt concentrations, we have shown here that increasing the NaCl concentration to 200 mM has no effect on the maximal rate of cleavage and only serves to decrease the binding affinity of the enzyme for substrate DNA by about 50-fold. The latter effect explains a previous report (16) that high concentrations of NaCl inhibited the enzymatic activity. This concentration of NaCl increases the cleavage equilibrium constant \(K_{\text{eq}} = k_{\text{f}}/k_{\text{off}}\) from 0.6 to 0.1 to near unity. This small increase in \(K_{\text{eq}}\) arises mostly from a decrease in \(k_{\text{off}}\) from 0.9 \, \text{s}^{-1} using the low salt conditions to 0.6 \, \text{s}^{-1} in this study (Table 1). This salt effect on \(K_{\text{eq}}\) adds to the increasing list of factors that affect this parameter as follows: DNA sequence effects (17), nonbridging phosphorothioate substitutions (11), and mutagenesis of the enzyme (18, 19).

**Closing of the Enzyme Clamp Is Faster Than DNA Cleavage**—The stopped-flow fluorescence and single turnover kinetic studies reported here provide a surprisingly simple picture of DNA binding by the vaccinia topoisomerase. First, all of the kinetic processes measured in this work were analyzed quite well using a single exponential rate equation. In no case was there any evidence for another significant kinetic barrier during binding that would require the use of a second exponential to fit the data adequately. Second, the observed association rate constant showed a linear concentration dependence on Y274F concentration, with no evidence for curvature (Fig. 8). If a second step involving isomerization of the enzyme were to become kinetically important, the observed rates would reach a limiting plateau value corresponding to a change in rate-limiting step from bimolecular association to isomerization. Thus, the maximum observed rate constant for association of 25 \, \text{s}^{-1} sets a conservative lower limit value for any isomerization process that may occur after formation of the initial collision complex and before cleavage (Fig. 8). We conclude from these considerations that any step leading to formation of the noncovalent complex must occur with a rate constant greater than 25 \, \text{s}^{-1}. This lower limit value is about 14- and 40-fold greater than the maximal rate of strand cleavage for the 18AP/24-mer and 18/24-mer (Figs. 4A and 10A).

The dissociation rate kinetic experiments also suggest a simple binding mechanism. As observed with the association rate measurements, \(k_{\text{off}}\) could be analyzed as a single exponential process (Fig. 9), suggesting that dissociation involves a single rate-limiting transition state, or if two or more forms of the ES complex exist (as depicted in Fig. 1), they are in rapid equilibrium as compared with the final dissociation step (\(k_{\text{off}}\) in Fig. 1B). The magnitude of \(k_{\text{off}}\) (3–5 \, \text{s}^{-1}, Table III) indicates that reopening of the clamp to release the bound substrate is at least 5–8-fold faster than cleavage of the 18/24-mer. In addition to the kinetic evidence presented above, the excellent agreement between the ratio \(k_{\text{off}}/k_{\text{f}}\) and the equilibrium constant \(K_{\text{eq}}\) makes a rate-limiting clamp closing step highly unlikely.

The above experimental facts are consistent with two kinetic mechanisms for noncovalent DNA binding and cleavage. In the first mechanism (Scheme 1), bimolecular association and the detected conformational changes in the enzyme and DNA occur simultaneously. In this case, \(k_{\text{on}} = k_{1}\) and \(k_{\text{off}} = k_{-1}\) for a simple two-state equilibrium, the ratio \(k_{\text{off}}/k_{\text{on}} = k_{-1}/k_{1} = K_{D}\), and \(k_{\text{chem}} = k_{2}\) (12).

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}\text{]}{k_{1}} E^*S \\
& \xrightarrow{k_{\text{chem}}} E^* + P
\end{align*}
\]

**Scheme 1**

In the second mechanism (Scheme 2), bimolecular association leads to the formation of a weak collision complex (ES) that then rapidly isomerizes to form \(E^*S\), the closed form of the enzyme-DNA complex. In this case, \(k_{\text{on}}\) is still equal to \(k_{1}\), but \(k_{\text{off}} = k_{-2}k_{-3}/k_{2}\), \(K_{D}^{\text{ssp}} = k_{\text{off}}/k_{1}\), and \(k_{\text{chem}} = k_{2}\) (12).

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}\text{]}{k_{1}} ES \\
& \xrightarrow{k_{2}} E^*S \\
& \xrightarrow[k_{-2}\text{]}{k_{3}} E^* + P
\end{align*}
\]

**Scheme 2**

Although the choice of mechanism has no effect on the conclusion that all steps leading up to the cleavage step are fast, we favor the two-step binding mechanism depicted in Scheme 2, which is also shown as a free energy reaction coordinate diagram in Fig. 1B. This mechanism is consistent with the structural information that requires a significant conformational change in the topoisomerase and is also compatible with the observation that the enzyme forms a weak nonspecific complex with DNA that lacks the pentamer consensus sequence (20). The present data provide little information on the absolute magnitudes of the rate constants \(k_{1}, k_{2}\), and \(k_{3}\); except that \(k_{2} > k_{-2}\) and \(k_{3} > k_{-3}\). Thus, taken in their entirety, the collective data are strongly consistent with a free energy profile resembling that shown in Fig. 1B. The 13\(^{\text{FP}}\) NMR studies presented in the accompanying article (28) provide additional evidence for a rapid and reversible conformational fluctuation...
of the +1T base between an intrahelical and extrahelical state within the closed form of the enzyme-DNA complex (Fig. 1F, inset), suggesting an enzyme-induced destabilization of the DNA duplex in this region.

Why Is the Cleavage Rate Faster for the 2-AP Substrate?—The stopped-flow fluorescence and rapid mix-chemical quench results in Fig. 10 clearly demonstrate that the incorporation of 2-AP in the −1 position of the 18AP/24-mer and 40AP/40-mer substrates results in a 3-fold increase in kcat, yet no significant change in koff. One possible interpretation of this result is that the 0.5 kcal/mol weaker Watson-Crick hydrogen bonding energy of the 2-AP−T base pair facilitates distortion of the DNA duplex near the scissile phosphodiester (21). An alternative explanation would be that the 2-amino group of 2-AP leads to a duplex near the scissile phosphodiester (21). An alternative explanation would be that the 2-amino group of 2-AP leads to a decrease in tryptophan fluorescence arises from a change in the environment of Trp-236, which is located at the carboxyl-terminal region of helix 6 (3). This residue may sense the movement of helix 8, thereby providing a valuable spectroscopic signal for the induced formation of the activated enzyme-DNA complex. The only other tryptophan residue of topoisomerase (Trp-50) is solvent-exposed and is located in the amino-terminal domain quite removed from the DNA-binding interface and catalytic domain. The environment and location of Trp-50 makes this residue less likely to be responsible for the observed fluorescence changes, although it is likely to contribute to the total observed fluorescence intensity.

As discussed above and in the accompanying article (28), the 2-AP fluorescence increase likely reports on an enzyme-induced distortion of the DNA that leads to dynamic unpairing of the +1T. This DNA distortion may play a role in allowing access of catalytic groups to the scissile phosphodiester, in facilitating strand transfer reactions catalyzed by topoisomerase (25–27), or to perhaps allow unhindered rotation of the DNA duplex during supercoiling relaxation (2).

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Fluorescence Spectroscopy Studies of Vaccinia Type IB DNA Topoisomerase: CLOSING OF THE ENZYME CLAMP IS FASTER THAN DNA CLEAVAGE

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