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Steady-state and Rapid Kinetic Analysis of Topoisomerase II Trapped as the Closed-clamp Intermediate by ICRF-193*

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DNA topoisomerase II uses a complex, sequential mechanism of ATP hydrolysis to catalyze the transport of one DNA duplex through a transient break in another. ICRF-193 is a catalytic inhibitor of topoisomerase II that is known to trap a closed-clamp intermediate form of the enzyme. Using steady-state and rapid kinetic ATPase and DNA transport assays, we have analyzed how trapping this intermediate by the drug perturbs the topoisomerase II mechanism. The drug has no effect on the rate of the first turnover of decatenation but potently inhibits subsequent turnovers with an IC_{50} of 6.5 ± 1 μM for the *Saccharomyces cerevisiae* enzyme. This drug inhibits the ATPase activity of topoisomerase II by an unusual, mixed-type mechanism; the drug is not a competitive inhibitor of ATP, and even at saturating concentrations of drug, the enzyme continues to hydrolyze ATP, albeit at a reduced rate. Topoisomerase II that was specifically isolated in the drug-bound, closed-clamp form continues to hydrolyze ATP, indicating that the enzyme clamp does not need to re-open to bind and hydrolyze ATP. When rapid-quench ATPase assays were initiated by the addition of ATP, the drug had no effect on the sequential hydrolysis of either the first or second ATP. By contrast, when the drug was prebound, the enzyme hydrolyzed one labeled ATP at the uninhibited rate but did not hydrolyze a second ATP. These results are interpreted in terms of the catalytic mechanism for topoisomerase II and suggest that ICRF-193 interacts with the enzyme bound to one ADP.

Type II DNA topoisomerases are essential and ubiquitous enzymes that catalyze the ATP-dependent transport of one double-stranded DNA through an enzyme-mediated break in another. These enzymes are the targets of numerous antibiotic and anticancer drugs (1–4), and their mechanisms have been well studied (for recent reviews, see Refs. 5 and 6). The eukaryotic enzymes are homodimers (7–10) whose primary dimer interface is near the COOH terminus (11). The enzyme cleaves one segment of DNA, the gated or G segment,[1] through the transient covalent attachment with a pair of active site tyrosines (12, 13). The enzyme traps a second segment of DNA, the transported or T segment, when ATP binding causes the NH_2-terminal ATPase domains to dimerize (14–17). This form of the enzyme has been called the closed-clamp intermediate (16). The enzyme rapidly hydrolyzes one of its two bound ATP molecules (18) and then transports the T segment through the cleaved G segment (19). Only after the first ADP has been released from the enzyme does the topoisomerase hydrolyze the second ATP (20). This unusual type of sequential ATP hydrolysis mechanism is presently only known to be shared with the DNA polymerase clamp-loading enzymes (21).

There are two very different types of drugs that inhibit topoisomerase II: the DNA-cleaved complex-stabilizing drugs exemplified by etoposide and the catalytic inhibitors including the bisdioxopiperazines (*e.g.* ICRF-193, -187, and -159) (22, 23). The *in vivo* target of ICRF-193 and related compounds was shown to be topoisomerase II using a yeast genetic system in which the levels of the enzyme could be modulated (24). *In vitro* studies of these drugs have shown that they function by trapping the enzyme as the closed-clamp intermediate in the presence of ATP, inhibiting the ATPase activity and catalytic turnover of the topoisomerase (25–27). Point mutations, causing resistance to these drugs, map to the ATPase domains of topoisomerase II (28, 29). Additionally, ICRF-193 has been shown to directly bind to the dimerized ATPase domains of the yeast enzyme (17).

Biochemical studies of the topoisomerase II-targeting drugs are useful both for probing the mechanism of the enzyme and for understanding the drug interactions. Recently we analyzed the effect of etoposide on the ATPase and DNA transport activities of yeast topoisomerase II using steady-state and rapid kinetic techniques (30). By preventing G segment religation, etoposide inhibits topoisomerase II after the first ATP has been hydrolyzed and the T segment has been transported and before the second ADP is released. These results explain why this drug is a hyperbolic noncompetitive inhibitor of the ATPase activity. Although ICRF-193 is known to inhibit the ATPase and DNA transport activities of topoisomerase II by stabilizing the closed clamp (26), its precise mechanism of inhibition remains obscure. In the present study, steady-state and pre-steady-state kinetics were used to probe the effects of stabilizing the closed-clamp intermediate on the topoisomerase II reaction mechanism.

EXPERIMENTAL PROCEDURES

Materials and Buffers—Reagents used for ATPase and decatenation assays were previously described (30). ICRF-193 was a kind gift of S. Olland and J. C. Wang (Harvard University). ICRF-193 stock solutions (40 mM) were prepared in dimethyl sulfoxide; all reactions lacking the drug contained 1% dimethyl sulfoxide. Wild type *Saccharomyces cerevisiae* topoisomerase II was expressed and purified as before (31); the concentrations given are for the enzyme dimer. The reaction buffer used throughout contained 50 mM HEPES-KOH (pH 7.5), 150 mM potassium acetate, and 10 mM magnesium acetate.

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[1] The abbreviations used are: G segment, gated DNA segment; T segment, transported DNA segment; AMPPNP, 5′-adenylyl-β-γ-imidodiphosphate; kDNA, kinetoplast DNA.

The biotin-labeled circular DNA substrate was produced by a coupled nick translation/religation reaction. Purified plasmid DNA (10 kb) was nicked using DNase I in the presence of excess ethidium bromide as described previously (32). Biotinylated DNA was produced by reacting 15 μg of nicked plasmid DNA, 10 units of *Escherichia coli* DNA polymerase I, 0.3 mM dTTP, dGTP, and dATP, and 0.3 mM biotinylated-dCTP/dCTP in 100 μl using the BioProbe nick translation DNA-labeling kit (Sigma). After 20 s at 20 °C, *E. coli* DNA ligase (25 units, New England Biolabs) and 10 μl of 10× reaction buffer containing NAD^+ were added, and incubation was continued for 30 min at 16 °C. The reaction was stopped with 50 mM EDTA, and the biotinylated DNA was purified using a TE-100 spin column (CLONTECH).

Decatenation Assays—Each decatenation reaction contained 50 nM topoisomerase II and 20 μg/ml kinetoplast DNA (kDNA) in reaction buffer plus 250 μg/ml bovine serum albumin and ICRF-193. ATP (1 mM) was added, and the reactions were held at 30 °C for 20 min before being quenched with 50 mM EDTA and 0.5% SDS. To determine the concentration required to reduce topoisomerase II decatenation activity by 50% (IC_{50}), free DNA circles were quantified from reactions containing 8 different ICRF-193 concentrations (0.5 μM to 50 μM). Gels were quantified, and IC_{50} values were determined as described previously (30). Rates of multiple turnover and single turnover decatenation were analyzed as described previously (30) in the presence and absence of 150 μM and 500 μM ICRF-193, respectively.

ATPase Assays—Steady-state and pre-steady-state ATPase assays were done as described previously (18, 30, 33). Control reactions showed that neither ICRF-193 nor dimethyl sulfoxide inhibited the coupling system used for the steady-state assays. Reactions were initiated by the addition of ATP to mixtures of preincubated topoisomerase II, DNA, buffer, and ICRF-193 at the indicated concentrations. For the pulse-chase pre-steady-state ATPase assays, the delay time was increased to 2.0 s. Pre-steady-state experiments with pre-bound ICRF-193 were performed as follows. Topoisomerase II (30 μM) was mixed with 5 mM DNA base pairs and 300 μM ATP with and without 500 μM ICRF-193 in 150-μl final volume. The reactions were incubated for 30 min and then diluted 10-fold with reaction buffer plus 500 μM ICRF-193 or dimethyl sulfoxide. These diluted solutions were then used for chemical quench and pulse-chase reactions as described above.

Topoisomerase II-DNA Clamp Isolation and Analysis—Topoisomerase II (500 nM) was incubated with biotinylated circular DNA (50 nM plasmid) in reaction buffer for 30 min. Reactions contained either ATP (1 mM) or ICRF-193 (500 μM) and ATP (1 mM) or AMPPNP (1 mM) in a final volume of 100 μl; the same nucleotide and drug present in these initial incubations were continued in later washes and reaction buffer. Following this incubation, 10 μl of immobilized streptavidin beads (Pierce) were added, and each reaction was incubated for an additional 30 min at 4 °C with slow rotation. The beads were washed 15 times over several hours with 1 ml of 2 M KCl plus either 1 mM ATP, 1 mM ATP, and 150 μM ICRF-193 or 1 mM AMPPNP to isolate topologically trapped topoisomerase II-DNA complexes. Finally, the streptavidin beads from each reaction were resuspended in 50 μl of reaction buffer with or without 500 μM ICRF-193. One-half of each mixture was analyzed for the presence of topoisomerase II by Western blotting using polyclonal anti-topoisomerase II antibodies. The other half of each reaction was assayed for ATPase activity using a discontinuous TLC method (33) with 1 mM [α-^{32}P]ATP (0.05 μCi/μl).

RESULTS

ICRF-193 Inhibits Multiple Turnover Decatenation by Topoisomerase II—As a prelude to these studies, inhibition of topoisomerase II-catalyzed DNA transport by ICRF-193 was analyzed for the yeast enzyme under the present reaction conditions. kDNA, a network of approximately 5,000 multiply catenated 2.5-kilobase circles, was used for the multiple turnover decatenation assays. Fig. 1*A* shows a time course of kDNA decatenation in the absence and presence of the drug; the catenated substrate remains in the wells, whereas the unlinked products migrate with the expected mobility. In the absence of drug, topoisomerase II releases increasing concentrations of free DNA circles with time. In the presence of high concentrations of drug, the amount of free circle released remains constant from 15 s to 30 min. Additionally, the amount of free circle released in the presence of ICRF-193 and ATP is similar to the amount released when the reaction contained the nonhydrolyzable ATP analog AMPPNP (compare the first *lane* with the last seven *lanes*). These results indicate that ICRF-193 potently inhibits DNA transport after at least one turnover has already occurred. To determine the IC_{50} for ICRF-193 and yeast topoisomerase II, the relative inhibition of kDNA decatenation after 20 min of incubation was determined for eight different drug concentrations (Fig. 1*B*). The IC_{50} value was determined to be 6.5 ± 1 μM. Previously determined IC_{50} values for ICRF-193 have ranged from 0.9 to 12.6 μM for type II topoisomerases isolated from various eukaryotes (34), excluding *S. cerevisiae*.

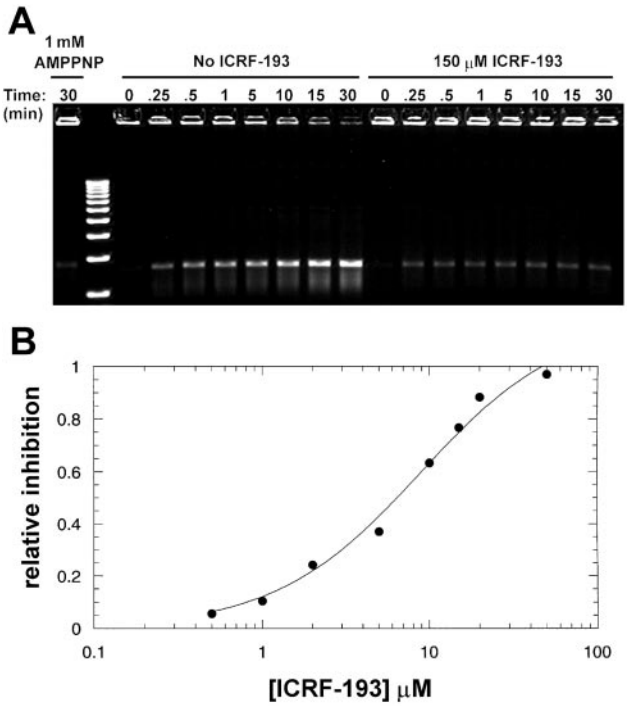


FIG. 1. **ICRF-193 inhibited the decatenation of kinetoplast DNA.** All reactions contained 50 nM topoisomerase II, 20 μg/ml kDNA, and 1 mM ATP. *A*, agarose gel analysis of kDNA decatenation reaction time courses in the absence and presence of 150 μM ICRF-193 is shown. In the first *lane*, a similar reaction containing 1 mM AMPPNP in place of ATP was analyzed as a control. *B*, the relative inhibition of kDNA decatenation activity at eight different ICRF-193 concentrations was determined using methods described previously (30). The data were fit to the equation relative inhibition = [ICRF-193]/([ICRF-193] + IC_{50}); the IC_{50} was determined to be 6.5 ± 1 μM.

Steady-state Analysis Shows That ICRF-193 Is an Atypical Inhibitor of the ATPase Activity of Topoisomerase II—ICRF-193 has been shown to inhibit the ATPase activity of topoisomerase II (17, 26), but this inhibition has never been characterized. The progress curves for the ATP hydrolysis reaction showed significant time-dependent nonlinearity in the presence of low drug (0.5–20 μM) and ATP (50–350 μM) concentrations (not shown). Nonlinearity in ATPase progress curves was previously seen in studies with NH_2-terminal fragments of yeast topoisomerase II in the presence of the same drug (17). Although this curvature indicates that ICRF-193 binds slowly to topoisomerase II, the drug is not a typical slow-binding inhibitor. Slow-binding inhibitors generally bind competitively with the substrate, and preincubation of the enzyme with the inhibitor abolishes the curvature of the progress curves (35). The inhibition of ATPase activity by ICRF-193 shows neither of these features. An explanation for this time-dependent inhibition will be presented under "Discussion."

To analyze the steady-state inhibition, the final inhibited rates of ATP hydrolysis by topoisomerase II bound to DNA at five ATP concentrations and nine ICRF-193 concentrations were determined by fitting the progress curves to [ADP] = $v_s t$ −

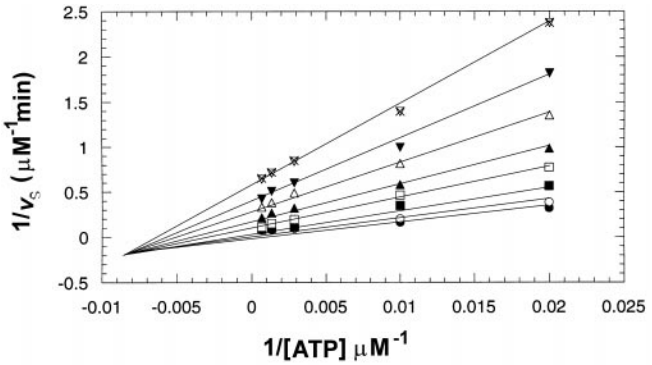
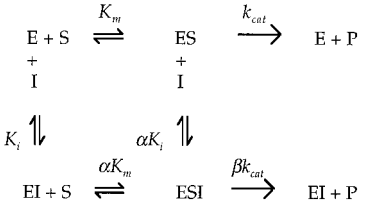


FIG. 2. **Double-reciprocal analysis of the inhibition of ATPase activity by ICRF-193.** The final inhibited velocities of reactions containing 50 nM topoisomerase II, 10 μM base pairs of DNA, and 0 μM (●), 0.5 μM (○), 1 μM (■), 2 μM (□), 5 μM (▲), 10 μM (△), 20 μM (▼), 50 μM (▽), or 500 μM (X) ICRF-193 are shown. The data were fit to the equation describing mixed-type inhibition (41), $1/v_s = (K_{m,app}(1 + I/K_i))/(SV_{max}(1 + \beta I/\alpha K_i)) + (1 + I/\alpha K_i)/(V_{max}(1 + \beta I/\alpha K_i))$ using Grafit (Erithicus Software), where I is the ICRF-193 concentration, and S is the ATP concentration.



SCHEME I.

$(v_s - v_0)/k$, where v_s is the final inhibited rate, v_0 is the initial inhibited rate, t is the time, and k is the observed rate constant for conversion between the initial and final rates (36). Since a coupled ATPase assay was used in which ATP is constantly being regenerated, the ATP concentration did not change during the reactions. A double-reciprocal plot of these data is shown in Fig. 2. These data clearly indicate that ICRF-193 is not a competitive inhibitor of ATP, since the curves do not converge on the y axis (*i.e.* $V_{max,app}$ continues to decrease at increasing inhibitor concentrations at infinite [ATP]. Note that the borders of this graph do not correspond to the axes.) The data also show that the $K_{m,app}$ decreases at increasing drug concentrations (the x intercept is $-1/K_{m,app}$), which suggests that ICRF-193 binds the enzyme-ATP or enzyme-ADP complex. These data are not well fit by standard uncompetitive or noncompetitive inhibition models but can be fit by a hyperbolic mixed-type model, as shown in Fig. 2. The mechanism for this type of model is shown in Scheme 1.

The fit to this model gives values of 22 μM and 2 μM for K_i and αK_i, respectively, indicating that ICRF-193 binds poorly to the free enzyme. The determined values of k_{cat} and βk_{cat} are 4.9 s^{-1} and 1.0 s^{-1}, respectively, suggesting that the enzyme can continue to hydrolyze ATP even with the drug bound. However, since the ATPase mechanism for topoisomerase II is considerably more complex than that shown in Scheme 1, these values should be taken as a rough approximation of the true values. In the absence of DNA, the ATPase data show similar characteristics but with altered values for the kinetic parameters (not shown).

Topoisomerase II Locked in the Closed-clamp Conformation by ICRF-193 Continues to Hydrolyze ATP—The steady-state inhibition data shown in Fig. 2 suggest that topoisomerase II can continue to slowly hydrolyze ATP when ICRF-193 is bound. Previous studies showed that when this drug binds to the enzyme in the presence of ATP, the drug locks it into the "closed-clamp conformation," in which the ATPase domains are

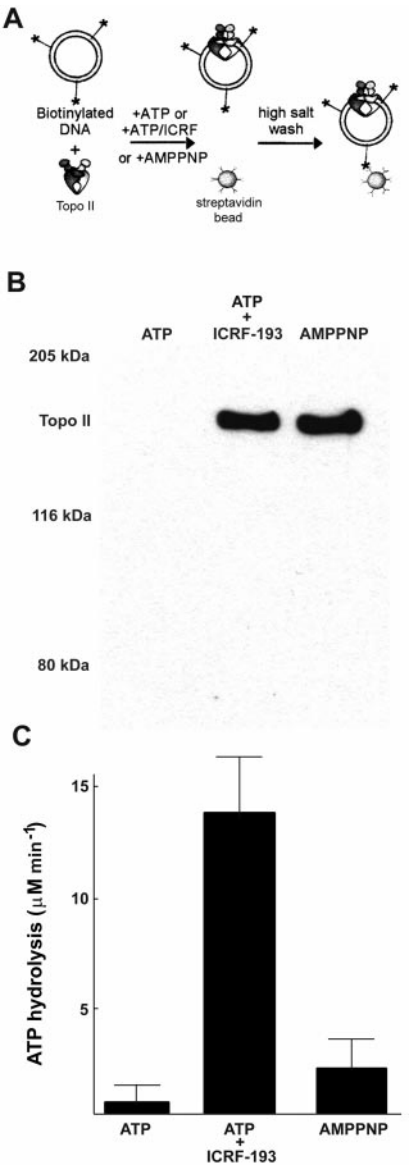


FIG. 3. **Topoisomerase II trapped in the closed clamp conformation by ICRF-193 continues to hydrolyze ATP.** *A*, schematic illustrating how the trapped topoisomerase was isolated. *B*, Western blot analysis of the beads from three separate reactions after extensive washing. The positions of molecular weight markers and topoisomerase (*Topo*) II are shown. *C*, the rates of ATP hydrolysis catalyzed by topoisomerase isolated on the beads after extensive washing was determined as described under "Experimental Procedures." *Error bars* were determined by repeating the entire experiment three separate times.

dimerized and circular DNA can be topologically trapped within the enzyme dimer (26). Together, these data suggest that the enzyme may continue to hydrolyze ATP even when it is locked in the closed conformation. However, another possibility is that a separate population of the enzyme remains free of drug and contributes all of the measured ATPase activity. To distinguish between these possibilities, the topoisomerase II complexes that were bound to ICRF-193 in the closed-clamp conformation were specifically isolated as illustrated in Fig. 3*A* and tested for ATPase activity. Circular DNA that was nick-translated to contain a small number of biotin adducts was incubated with topoisomerase II. The three sets of reactions contained either 1 mM ATP, 1 mM ATP and 500 μM ICRF-193, or 1 mM AMPPNP. Streptavidin beads were added, allowed to bind the biotin-DNA, and washed extensively in 2 M KCl with or without ICRF-193 to isolate only the topoisomerase II

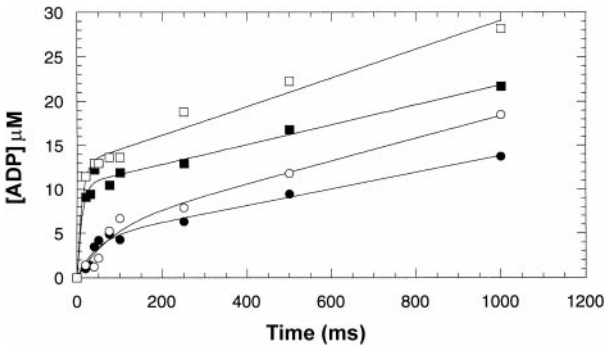


FIG. 4. **Rapid kinetic analysis of ATP hydrolysis in the absence and presence ICRF-193.** The concentrations of [α-^{32}P]ADP produced during chemical quench and pulse-chase reactions containing 7.3 μM topoisomerase II dimer, 1.75 mM DNA base pairs, and 350 μM [α-^{32}P]ATP were determined by TLC analysis. Chemical quench (○, ●) and pulse-chase (□, ■) data are shown for reactions without and with 500 μM ICRF-193, respectively. The data are shown fit to the burst equation ([ADP] = $A(1 - e^{-Bt}) + Ct$), where A is the burst amplitude, B is the burst rate constant, and C is the steady-state term. In the absence of drug, the values for the chemical quench reaction are A = 6.5 ± 1.6 μM, B = 23 ± 8 s^{-1}, C = 14 ± 1 μM s^{-1} and for the pulse-chase reaction are A = 13.8 ± 0.5 μM, B = 97 ± 20 s^{-1}, C = 17 ± 1 μM s^{-1}. In the presence of drug, the values for the chemical quench reaction are A = 5.5 ± 0.8 μM, B = 22 ± 8 s^{-1}, C = 7 ± 1 μM s^{-1} and for the pulse-chase reaction are A = 11.6 ± 0.5 μM, B = 91 ± 17 s^{-1}, C = 9 ± 1 μM s^{-1}.

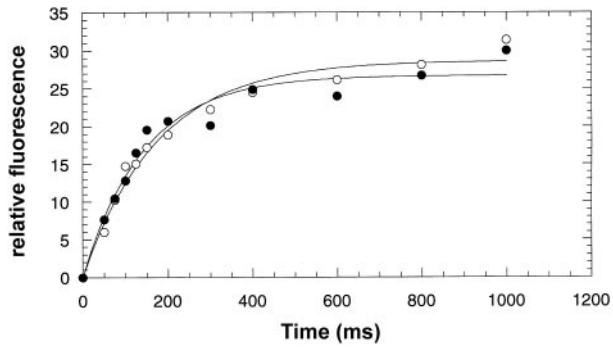


FIG. 5. **Single turnover decatenation measured in the millisecond time scale.** Topoisomerase II (50 nM) was preincubated with singly catenated DNA circles (25 nM) in the presence (●) or absence (○) of 500 μM ICRF-193 before rapid mixing with 350 μM ATP. The reactions were quenched at the indicated time points with 50 mM EDTA and analyzed by agarose gel electrophoresis as described previously (19, 30). The data are shown fit to a single exponential equation (relative fluorescence = $A(1 - e^{-kt})$), where A is the burst amplitude, and k = 6.9 ± 0.8 s^{-1} and 5.9 ± 0.5 s^{-1} in the presence and absence of drug, respectively.

dimers that were topologically trapped around the DNA; topoisomerase II does not remain covalently attached to cleaved DNA under these conditions, and it has been well demonstrated that ICRF-193 traps the enzyme in a topological linkage with circular DNA (26). Aliquots of the beads were boiled and analyzed by Western blot analysis, probing with anti-topoisomerase II antibodies, as shown in Fig. 3*B*. These results show that a significant portion of the topoisomerase II was stably trapped around the isolated DNA in the presence of AMPPNP or ATP plus ICRF-193 but not in the presence of ATP alone. Separate aliquots of the beads were analyzed for ATPase activity, as shown in Fig. 3*C*. A significant level of ATPase activity was only seen for the beads from the reaction that contained ATP and ICRF-193. This level of drug-inhibited ATPase activity represents the amount expected for ~60% of the input topoisomerase II. Together these results directly show that topoisomerase II trapped by ICRF-193 in the closed-clamp conformation continues to hydrolyze ATP.

Pre-steady-state Analysis of ICRF-193 Inhibition—To further characterize how ICRF-193 inhibits the topoisomerase II reaction, saturating concentrations of the drug were included in pre-steady-state ATPase and decatenation experiments. Two types of pre-steady-state ATPase experiments, pulse-chase and chemical quench, have been used to determine the ATPase mechanism of topoisomerase II, as described previously (18, 20, 30, 31). The pulse-chase burst amplitude and burst rate indicate the concentration of ATP that is bound and hydrolyzed within a single turnover of the enzyme and the rate at which that ATP is bound, respectively. The chemical quench burst amplitude and rate indicate the concentration and rate of the ATP hydrolyzed before the rate-determining step in the mechanism, respectively. The results of chemical quench and pulse-chase experiments performed with 7.3 μM topoisomerase II dimer with and without 500 μM ICRF-193 are shown in Fig. 4. In the absence of drug, the results are the same as those previously seen; the pulse-chase burst amplitude equals the ATP active-site concentration present, and the chemical quench burst amplitude is approximately half that concentration. The results in the presence of the drug indicate that ICRF-193 has little effect on the first turnover of ATP hydrolysis by topoisomerase II. The burst rates are unaffected by the drug, whereas both burst amplitudes are reduced by 15%. These results are in agreement with the interpretation of the steady-state results, indicating that ICRF-193 may bind and inhibit a small fraction of the enzyme unbound to ATP; however, the majority of inhibition is detected after the first turnover.

The results shown in Fig. 1 indicate that ICRF-193 inhibits multiple turnover decatenation. To determine if any inhibition is seen in the first turnover, rapid kinetic analysis of single turnover decatenation reactions was performed. The rates of single turnover decatenation by topoisomerase II in the presence of ATP have previously been measured using singly catenated DNA circles and a rapid quench apparatus (19, 30). The results of similar experiments performed in the presence and absence of ICRF-193 are shown in Fig. 5. The drug has no effect on the rate of single turnover decatenation, indicating that inhibition only occurs after the first turnover.

The results from above pre-steady-state experiments indicate that ICRF-193 does not significantly inhibit topoisomerase II during the first turnover. However, it is also apparent that this drug inhibits soon after the first turnover. To further characterize how it inhibits the topoisomerase, a pre-steady-state experiment in which the drug was pre-bound to the enzyme was performed. Since the drug did not appreciably bind in the absence of ATP, high concentrations of enzyme, DNA, and ICRF-193 were preincubated with 300 μM unlabeled ATP for 30 min. This reaction mixture was then diluted 10-fold into buffer containing ICRF-193 before performing pre-steady-state analysis. An identical experiment was performed in the absence of drug to serve as a control. The results of these chemical quench and pulse-chase reactions, in which the final topoisomerase II concentration was 1.5 μM dimer, are shown in Fig. 6. In the absence of drug the results are the same as seen previously where the pulse-chase burst amplitude is twice the chemical quench burst amplitude. However, in the presence of ICRF-193, the chemical quench and pulse-chase burst amplitudes are the same, indicating that when the drug is bound, topoisomerase II only hydrolyzes one ATP. These results are interpreted in terms of a model for the enzyme mechanism under "Discussion."

DISCUSSION

ICRF-193 inhibits topoisomerase II by an unusual mechanism. Although this drug had no effect on the rate of first turnover decatenation, it potently inhibited later turnovers. At

low drug concentrations, steady-state ATPase progress curves were nonlinear; however, the drug was not a typical slow-binding inhibitor. This drug did not bind competitively with ATP, and the steady-state inhibition is best explained by a mixed-type inhibition model. As was previously seen, ICRF-193 locked topoisomerase II into the closed-clamp conformation that surprisingly continued to hydrolyze ATP with a rate approximately 4-fold lower than the uninhibited rate. Pre-steady-state ATPase assays performed on the millisecond time scale showed that this drug did not inhibit the rate of hydrolysis of either the first or second ATP. However, once the drug had been allowed to pre-bind in the presence of ATP, it again had no effect on hydrolysis of the first labeled ATP, but it prevented hydrolysis of a second labeled ATP.

Together, all of these results suggest a consistent model for how ICRF-193 inhibits topoisomerase II. To understand how this drug inhibits the enzyme, these results must be analyzed in the context of the topoisomerase II reaction mechanism. Previous studies showed that this enzyme, bound to DNA, hydrolyzes two ATP molecules in a sequential fashion (20). Transport of the T DNA segment through the G segment occurs after hydrolysis of the first ATP and before hydrolysis of the second ATP (19). A new mechanism based on these results has been previously described in detail (19). A schematic representation of this mechanism as well as a proposed mechanism for ICRF-193 inhibition is shown in Fig. 7. Since reactions initiated by the addition of ATP showed no inhibition by ICRF-193 in the first turnover of either ATP hydrolysis or DNA transport, the drug did not significantly bind to the free enzyme or to one of the enzyme-ATP complexes (*i.e.* ICRF-193 did not bind to any of the complexes before complex *E* in Fig. 7). However, since both the ATPase and DNA transport reactions were inhibited immediately after the first turnover products were seen, we predict that this drug binds to the enzyme-ADP complex (complex *E*). When the topoisomerase II dimer is bound to only one ATP, the closed-clamp form of the enzyme is known to be stabilized (37). Therefore, it is not unreasonable to predict that the enzyme bound to one ADP has a similar conformation, as shown for complex *E*. The drug etoposide was previously shown to inhibit the reaction at one of the steps converting complex *D* to complex *E*, either release of the first ADP or hydrolysis of the second ATP (30). Since etoposide is known to inhibit DNA religation (38, 39), it follows that G segment religation may occur at one of these two steps.

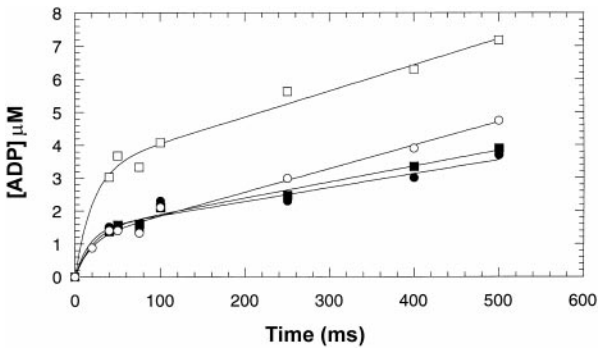


FIG. 6. **Rapid kinetic analysis of ATP hydrolysis after pre-binding of ICRF-193.** ICRF-193 (500 μM) was pre-bound to topoisomerase II as described under "Experimental Procedures." Chemical quench (○, ●) and pulse-chase (□, ■) experiments were performed in the absence and presence of drug, respectively. The final concentrations of topoisomerase II dimer, DNA base pairs, [α-^{32}P]ATP, and ICRF-193 were 1.5 μM, 300 μM, 350 μM, and 500 μM, respectively.

ICRF-193 binding to complex *E*, converting it to complex *F*, would explain many of the results reported in this and previous studies. Only one labeled ATP can bind and be hydrolyzed by complex *F*, exactly what is seen in the pre-steady-state results shown in Fig. 6. Complex *F* is predicted to be locked in the closed clamp conformation and to hydrolyze ATP with a reduced rate, as is seen in the results shown in Fig. 3. Previously, ICRF-193 was shown to bind to the closed-clamp form of the enzyme and prevent its conversion to the open-clamp form (26). This proposed mechanism also predicts the nonlinear progress curves seen at low drug concentrations in the present and previous studies (17); partitioning of the reaction between release of ADP (complex *E* → complex *A*) and binding of ICRF-193 (complex *E* → complex *F*) was shown by computer modeling to result in slow inhibition (not shown). Studies on the antagonism of etoposide-stabilized DNA cleavage by ICRF-193-type inhibitors suggested that etoposide acts before ICRF-193 in the mechanism (22, 40), again in agreement with the model shown in Fig. 7.

When the ICRF-193 inhibition was analyzed in terms of the sequential mechanism for ATP hydrolysis by topoisomerase II, it became clear why the closed-clamp form of the enzyme can continue to hydrolyze ATP. Topoisomerase II rapidly hydrolyzes only one of its two bound ATP and releases the P_i and

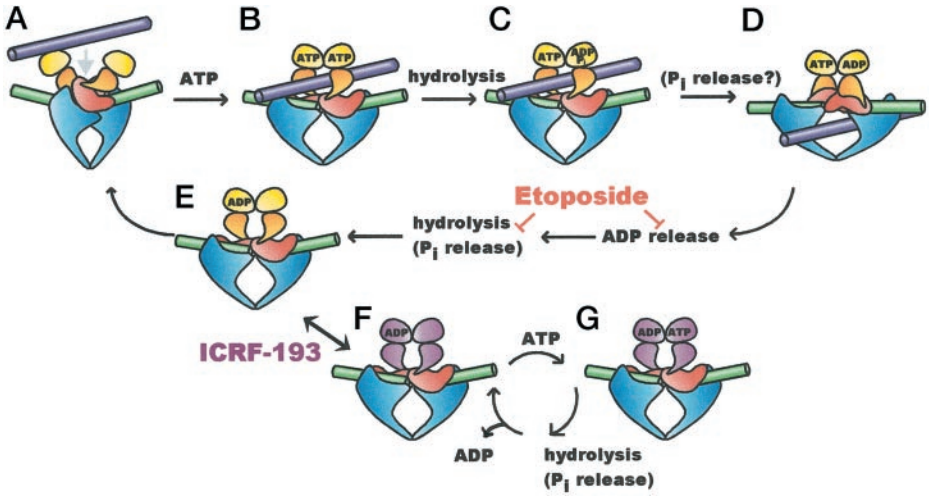


FIG. 7. **A schematic model for the inhibition of topoisomerase II by ICRF-193.** The various domains of the topoisomerase II dimer are shown in different colors, and the G and T segments of DNA are shown as *green* and *purple rods*, respectively. Since it is unknown at which step the T DNA segment exits from the enzyme-G DNA segment complex, this part of the mechanism is left unshown. ICRF-193 is known to bind to the NH_2-terminal ATPase domains (17), and these domains are shown in *violet* when the drug is bound. Inhibition of DNA religation by etoposide was previously shown to perturb the topoisomerase II mechanism either at release of the first ADP or hydrolysis of the second ATP (30).

ADP before the second ATP can be hydrolyzed (20). Since ICRF-193 apparently binds and locks the enzyme in the closed-clamp conformation after the first ADP has been released and the second ATP has been hydrolyzed, the resulting complex has one open ATP binding site. Our model predicts that ATP can bind to this site, as shown in complex *G* of Fig. 7 and be hydrolyzed by the enzyme. These results demonstrate the utility of incorporating pre-steady-state analysis in the study of enzyme-drug interactions.

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