DNA topoisomerase II catalyzes two different chemical reactions as part of its DNA transport cycle: ATP hydrolysis and DNA breakage/religation. The coordination between these reactions was studied using mutants of yeast topoisomerase II that are unable to covalently cleave DNA. In the absence of DNA, the ATPase activities of these mutant enzymes are identical to the wild type activity. DNA binding stimulates the ATPase activity of the mutant enzymes, but with steady-state parameters different from those of the wild type enzyme. These differences were examined through DNA binding experiments and pre-steady-state ATPase assays. One mutant protein, Y782F, binds DNA with the same affinity as wild type protein. This mutant topologically traps one DNA circle in the presence of a nonhydrolyzable ATP analog under the same conditions that the wild type protein catenates two circles. Rapid chemical quench and pulse-chase ATPase experiments reveal that the mutant proteins bound to DNA have the same sequential hydrolysis reaction cycle as the wild type enzyme. Binding of ATP to the mutants is not notably impaired, but hydrolysis of the first ATP is slower than for the wild type enzyme. Models to explain these results in the context of the entire DNA topoisomerase II reaction cycle are discussed.

Type II DNA topoisomerases catalyze the ATP-dependent transport of one duplex DNA segment through a transient break in another (for recent reviews, see Refs. 1–3). One essential function of this class of enzymes is to separate intertwined daughter chromosomes (4). Additionally, due to their ability to change DNA topology, these enzymes exert a global influence on DNA metabolism (2). Clinically these enzymes are of interest because they are targets of a diverse group of anticancer and antibiotic drugs (5–7).

Type II topoisomerases catalyze two types of chemical reactions: ATP hydrolysis and DNA breakage/religation. These are large homodimeric enzymes with an ATPase domain at the amino terminus of each monomer. Several types of experiments have indicated that when ATP binds, the ATPase domains dimerize (8, 9). Hydrolysis is thought to occur by the direct in-line attack of an activated water molecule on the γ-phosphate of ATP (10). The steady-state ATPase rates of enzymes purified from several organisms are DNA-stimulated (11–13). Pre-steady-state ATPase experiments show a rapid burst in ATP hydrolysis, but only when the topoisomerase is bound to DNA (14). These results indicate that DNA binding stimulates the rate of ATP binding and/or the rate of ATP hydrolysis. Analysis of pH rate profiles suggests that binding of DNA primarily stimulates ATP binding (15). Additionally, the pre-steady-state results, in conjunction with results of inhibitor studies, show that although the topoisomerase binds two ATPs, it hydrolyzes only one rapidly (15). Following the first hydrolysis, the enzyme apparently releases the P1 and ADP produced before hydrolyzing the second ATP.

The second chemical reaction, double strand DNA breakage, is catalyzed by the nucleophilic attack from the hydroxyl groups of two active site tyrosines, one in each monomer, on a staggered pair of phosphodiester bonds in the DNA (reviewed in Ref. 2). This attack results in the transient covalent attachment of each monomer to the 5′-end of one strand of DNA. Religation of the DNA occurs by essentially the reverse reaction in which the 3′-OH ends of DNA attack the pair of phosphotyrosyl linkages. The equilibrium between DNA cleavage and religation in a topoisomerase II-DNA complex normally lies strongly toward religation (16). This equilibrium is somewhat perturbed by ATP binding (17, 18) and is strongly perturbed by the presence of anticancer drugs, such as etoposide, amsacrine, and mitoxantrone (5–7).

These two chemical reactions, ATP hydrolysis and DNA breakage/religation, can occur independently. However, in order to understand how the enzyme can transport one segment of DNA (termed the transported (T) segment) through a protein-mediated gate in another segment (termed the gated (G) segment) while using the energy available from ATP binding and hydrolysis, it is essential to understand how these two reactions are coordinated. In the present study, we begin to probe the interactions between the ATPase and DNA breakage reactions. Without the hydroxyl group of the active site tyrosine, the topoisomerase cannot cleave DNA through covalent attachment. Without covalent attachment to the G segment of DNA, the topoisomerase cannot undergo the conformational changes normally associated with opening of the gate without first breaking its noncovalent DNA binding interactions. To study how the inability to cleave DNA and related disturbances in protein conformational changes affect the ATPase reaction cycle, the active site tyrosine, amino acid 782 in the S. cerevisiae enzyme, 2 has been mutated to phenylalanine. The DNA-
stimulated ATPase activity of this mutant is shown to differ from the wild type activity. The difference in steady-state ATPase rates for the mutant and wild type enzyme could be due to differences in DNA binding, the rates of ATP binding/dissociation, the rates of ATP hydrolysis/synthesis, or the rates of ADP/Pi dissociation. To interpret how these differences in ATPase rate may provide clues to the overall topoisomerase II mechanism, it is necessary to understand precisely which of these parameters is affected. DNA binding and pre-steady-state ATPase experiments are described that help to define the differences between the wild type and mutant enzymes. To ensure that this perturbation in ATPase activity is solely due to a lack in DNA breakage ability by this mutant enzyme, and not some unrelated consequence of mutation, a second DNA cleavage-mutant 19 is also studied.

**EXPERIMENTAL PROCEDURES**

*Materials—* Standard reagents were purchased from the following commercial resources: ATP, Amesherm Pharmacia Biotech; [α-32P]dCTP (3000 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol), NEN Life Science Products; 5′-adenyl-γ,δ-imidodiphosphate, AMPPNP, and ultra-pure HEPES, Boehringer Mannheim; NADH, phospho(enol)pyruvate (trisodium salt hydrate), and pyruvate kinase (700 units/ml) lactate dehydrogenase (100 units/ml) mixture from rabbit muscle, Sigma. All buffers were filtered (0.45 μm). The sheared salmon sperm DNA used for ATPase assays was prepared as described previously (14).

**Expression and Purification of Saccharomyces cerevisiae Topoisomerase II**—A new method of topoisomerase II purification based on the IMPACT system (New England Biolabs) was used for these studies. The expression plasmid used for the wild type enzyme, pJEL236, is essentially YEpTOP2-2-PGAL1 (20) with the insertion of the sequences coding for the modified intein and chitin binding domains (intein/CBD) from pCYB2 (New England Biolabs) just prior to the stop codon. This vector expresses a fusion of yeast topoisomerase II with the intein/CBD that allows purification. The constructs were made using polymerase chain reactions that replaced the final aspartic acid codon and stop codon of the TOP2 gene with a unique blunt cutting EcoRI restriction site that could be ligated to a Sma1 site at the 5′-end of the intein/CBD coding sequences. The construct was designed such that the only difference between wild type *S. cerevisiae* topoisomerase II and the final purified wild type topoisomerase is that the final aspartic acid has been changed to glycine. The purified protein with this one change has two identifiable biochemical characteristics to the fully wild type enzyme, and for the purposes of these studies, it will be referred to as wild type topoisomerase II.

The expression plasmid pSKM1 for the mutant in which the active site tyrosine has been changed to phenylalanine, Y782F, was made from plasmid YEpTOP2-2-PGAL1-Y782F (a kindgift of Brian Davis, United States Department of Agriculture). The topoisomerase II coding sequence was verified by sequencing, which showed that the only alteration was a change from TAT to TTT at codon 782. The intein/CBD coding sequences were then inserted into this plasmid to make pSKM1 as described above for the wild type expression plasmid. The expression plasmid for the mutant R690A fused to the intein/CBD was derived from the plasmid pSW201-R690A (a kind gift of Qiyong Liu and James C. Wang, Harvard University). pSW201-R690A was cut with Kpn1 and AvrII, and the fragment encoding the mutated portion of topoisomerase II was swapped with the similar fragment from YEpTOP2-2-PGAL1 to make pSKM2. The final expression plasmid (pSKM3) was made by inserting the intein/CBD sequences as above. Both of the final purified mutant proteins, Y782F and R690A, also have the final aspartic acid replaced by glycine.

The enzyme-intein/CBD fusions were expressed to very high levels from the plasmids pJEL236, pSKM1, and pSKM3 in the yeast strain BCY123 as described previously for the wild type enzyme (21). The basic buffer used throughout purification, Buffer I, contained 50 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 10% glycerol. The cells (36 g) were washed and cracked as described previously (22). The cracked cells were diluted to 200 ml with load buffer (Buffer I + 500 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 150 μg/ml benzamidine) and centrifuged for 30 min at 45,000 × g. The supernatant was passed onto a 50-ml (5 cm in diameter) chitin bead (New England Biolabs) column by gravity. The column was washed with 5 volumes of load buffer to 2 ml/min followed by 10 volumes of wash buffer (Buffer I + 1 x KCl + 0.1% Triton X-100) at 3 ml/min. Cleavage of the intein/CBD from topoisomerase II was induced by washing the column with four volumes of Buffer I + 500 mM KCl + 30 mM dithiothreitol. The released topoisomerase II was eluted 12 h later. The eluant was diluted 2 × in Buffer I prior to loading onto a phosphocellulose column equilibrated with Buffer I + 150 mM KCl. The phosphocellulose column was determined by the amount of protein eluted from the chitin column; 1 ml of phosphocellulose was used for each 3 mg of protein. The column was washed with three volumes of Buffer I + 300 mM KCl, and the topoisomerase II was eluted with a minimal volume of Buffer I + 1 × M KCl. The peak fractions were combined, frozen in liquid nitrogen, and stored at −70 °C. For DNA binding assays, the protein was dialyzed extensively in Buffer I + 250 mM KCl prior to freezing. This method provided 45 mg of highly purified wild type or 15 mg of mutant topoisomerase II from 12 liters of cell growth. These large quantities of protein were required because 15–20 mg of enzyme is needed for each pre-steady-state ATPase assay.

**Steady-state ATPase Assays**—A coupled assay using pyruvate kinase, phospho(enol)pyruvate, lactate dehydrogenase, and NADH was used as described previously (13). Reactions were performed at 30 °C in ATPase reaction buffer (50 mM HEPES-KOH (pH 7.5), 150 mM KCl, and 10 mM Mg(OAc)2) at 10 different ATP concentrations (25 μM to 1 mM). Purified and sheared salmon sperm DNA was used at a ratio of 200 bp/enzyme dimer; this DNA was shown to stimulate the ATPase activity identically to supercoiled plasmid DNA (14). Topoisomerase II binds specifically to supercoiled DNA, as revealed by Michalski and Menten plots with slight sigmoidal character at the lowest ATP concentrations. However, in the present analysis, this cooperativity was ignored and the data were fit to the standard Michaelis-Menten equation as follows using KaleidaGraph 3.0.

**DNA Binding Assays**—The binding affinities of wild type and Y782F topoisomerase II for DNA were measured using a double-filter method developed by Wong and Lohman (23). The DNA used was a 3′-labeled 46-mer duplex, similar in sequence to oligonucleotides used previously for DNA binding and cleavage studies with type II topoisomerases isolated from various organisms (24, 25). The substrate was made by annealing complementary 43-mer oligonucleotides (5′-[GGTGAATACATTGACCGGCGCAAGGCGCCAGTTCG] and 5′-GGACGGCGGCCAGGATGACGATTGTCG) such that the duplex had a 3-guanine overhang on each 5′-end. Equal concentrations of the oligonucleotides (final concentration, 41 μM) were mixed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM KCl, heated to 98 °C, and allowed to cool to room temperature over 4 h. The overhangs were filled in with Klenow fragment, 100 μCi of [α-32P]dCTP (3000 Ci/mmol), and 200 μM each of dTTP, dGTP, and dATP. After 15 min, the reaction was chased with 200 μM unlabeled dCTP for 1 min. The substrate was purified away from free nucleotides using a Chroma spin TE-10 column (CLONTECH). Following phenol/chloroform extraction and ethanol precipitation, the substrate was resuspended in 10 mM Tris-HCl, 1 mM EDTA, and its concentration was determined by absorbance at 260 nm ($e_{260} = 6.16 \times 10^3$ M$^{-1}$ cm$^{-1}$).

The double-filter method employed uses a nitrocellulose membrane to trap labeled DNA bound to protein on top of a DEAE membrane that traps free DNA (23). This method allows one to measure both protein-bound DNA and free DNA counts, improving precision and accuracy over conventional binding methods. We used the same membranes, prepared in the same fashion, and a similarly altered dot blot apparatus, as described previously (23). Topoisomerase II and the labeled DNA substrate were mixed at room temperature such that the final DNA concentration was 2 nM and the final enzyme dimer concentration ranged from 0.5 to 400 nM. The reactions performed in the absence of Mg$^{2+}$ also contained 85 mM KCl, 25 mM HEPES-KOH (pH 7.5), 5–7% glycerol, and 250 μg/ml bovine serum albumin. The reactions that contained 5 mM MgCl$_2$ were similar except that the KCl concentration was lowered to 45–60 mM to maintain a similar overall ionic strength between experiments. Using the optimal ATPase reaction buffer including 150 mM KCl, the maximum percentage of DNA bound was only 10–20% (not shown). The concentrations of KCl were therefore dropped to 50 mM to see if binding near naive or Kd values. The slight range in glycerol and KCl concentrations was inevitable due to the different amounts of enzyme added to the binding reactions. The binding reactions were allowed to equilibrate for 10–30 min at room temperature prior to filtration through the nitrocellulose and DEAE membranes at 6 ml/min. Aliquots (25 μl) of each reaction were filtered in triplicate and rinsed with 150 μl of cold
binding buffer. DNA counts on the dried membranes were determined at each spot by phosphorimage analysis (Molecular Dynamics PhosphorImager TM model 400 with ImageQuant (version 3.3) software). Control reactions lacking topoisomerase were also filtered as described to determine the nonspecific DNA counts trapped by the nitrocellulose. For binding reaction, the ratio of bound to total DNA was determined as indicated below and plotted versus the topoisomerase II concentration.

\[
\left[ \text{DNA}_{\text{bound}} \right] / \left[ \text{DNA}_{\text{total}} \right] = \frac{T}{D} \frac{K_s}{T + D} (\text{Eq. 2})
\]

The data were fit to the binding mechanism,

\[
T + D \underset{K_s}{\overset{T}{\rightleftharpoons}} TD (\text{Eq. 3})
\]

where \( T \) represents the topoisomerase II dimer, \( D \) represents the duplex DNA oligomer, and \( K_s \) represents the equilibrium dissociation constant. The values for \( K_s \) were determined by fitting the data to the equation describing this mechanism,

\[
\left[ \text{DNA}_{\text{bound}} \right] / \left[ \text{DNA}_{\text{total}} \right] = \frac{[T]C}{K_s + [T]} (\text{Eq. 4})
\]

where \( C \) is a constant that approaches 1 as the asymptote approaches 1, using KaleidaGraph 3.0.

Trapping of Circular DNA Analyzed by CsCl Density Gradient Ultracentrifugation—The technique used is essentially that described previously (26). Reactions of 30 \( \mu \text{L} \) containing 150 \( \mu \text{M} \) topoisomerase II dimer, 250 nm relaxed or linearized pBluescript plasmid DNA (3 kilobase pairs), 50 mm Tris-HCl, pH 7.5, 80 mm KOAc, and 8 mm Mg(OAc)_2 were incubated for 10 min at 30 °C. AMPPNP was added to a final concentration of 1 mM, and incubation was continued an additional 30 min. To an aliquot of 20 \( \mu \text{L} \) of each reaction, 130 \( \mu \text{L} \) of buffer (50 mm Tris-HCl, pH 7.5, 80 mm KOAc, and 8 mm Mg(OAc)_2) and 334 \( \mu \text{L} \) of saturated CsCl were added. These samples were spun at 40,000 rpm in an analytical ultracentrifuge (XL-A ultracentrifuge, Beckman Instruments) with scans at 260 and 280 nm taken at 36 and 40 h. Data plots were made using Microcal Origin 3.7.

Pre-steady-state ATPase Assays—Rapid chemical quench and pulse-chase experiments were performed as described previously (14) using the KinTek Model RQF-3 rapid quench apparatus (27). Briefly, topoisomerase II bound to DNA was rapidly mixed with [\( \alpha^{-32}\text{P} \)]ATP (0.01 \( \mu \text{Ci/\muL} \) ATPase reaction buffer. The final topoisomerase II dimer concentration in the reactions ranged from 5.5 to 7.5 mm (see legends to Figs. 3 and 4), whereas the DNA concentration was kept at a constant ratio of 200:1 base pairs:enzyme dimer. The final labeled ATP concentration was typically 350 \( \mu \text{M} \), a concentration previously shown to essentially saturate the ATP active sites while still providing a good signal to noise ratio (14). The enzyme and substrate were allowed to react for 20 ms to 1.5 s. For chemical quench experiments, the reaction was rapidly quenched by addition of 250 mm EDTA in 100 mm Tris base (pH 10) at the indicated time points. SDS (final concentration, 1%) was present in the sample collection tubes to ensure that no residual enzyme activity persisted. For pulse-chase experiments, 10.6 mm unlabeled ATP was mixed with the reaction at the indicated time points. After time periods for multiple enzyme turnovers, 1.5 s for the wild type enzyme and 3 s for the mutant enzymes, the reactions were chemically quenched as indicated above. The concentrations of [\( \alpha^{-32}\text{P} \)]ADP produced at each time point were determined in quintuplicate as described previously (14). The data were all fit to a single exponential equation with a linear term called the burst equation, \( A(1 - e^{-Bt}) + Ct \), using KaleidaGraph 3.0. The value \( A \) is the burst amplitude, \( B \) is the burst rate constant, and \( C \) is the steady-state term.

RESULTS

Steady-state ATPase Analysis—As a first step to understanding the relationship between ATP hydrolysis and DNA cleavage by topoisomerase II, steady-state ATPase parameters were determined for wild type and Y782F enzymes. This mutant cannot cleave DNA through covalent attachment because it lacks the active site hydroxyl nucleophiles that normally would attack a pair of staggered 5’ phosphates. The results of the steady-state ATPase assays are given in Table I. Although the results shown are from a single set of experiments, the same relative values have been found a minimum of four times using four different sets of protein preparations. In the absence of DNA, \( k_{cat} \) and \( k_{cat}/K_m \) values are identical for the wild type and mutant enzymes. This is expected if the mutation only affects interactions with DNA. It has been previously shown for several type II topoisomerases that DNA stimulates the steady-state ATPase rates (11–13). The present study is in agreement and shows that when either the wild type or mutant enzyme is bound to DNA, \( k_{cat} \) and \( k_{cat}/K_m \) values increase. Whereas \( k_{cat}/K_m \) values increase to the same extent for the two proteins, the \( k_{cat} \) value for Y782F is reproducibly 4-fold lower than that for wild type. Results of previous studies indicate that \( k_{cat}/K_m \) for the mechanism of ATP hydrolysis by topoisomerase II essentially includes only rate constants involved in ATP binding, whereas \( k_{cat} \) reflects the rate-determining steps (15). These present data are consistent with the Y782F mutant enzyme-DNA complex binding ATP in a similar fashion to wild type-DNA complex. However, why is the \( k_{cat} \) of ATP hydrolysis lower for a mutant that cannot covalently cleave DNA? The following experiments were performed to address this question.

DNA Binding—Because topoisomerase II is a DNA-stimulated ATPase, any perturbation in DNA binding could affect the ATPase activity of the enzyme. Therefore, to determine whether the decreased \( k_{cat} \) value for the Y782F protein is due to altered noncovalent interactions with DNA, DNA binding studies were performed. A double-filter binding method (23) was used to measure the affinity of wild type and Y782F enzymes for a 46-bp duplex oligonucleotide substrate. Wild type topoisomerase II requires a divalent cation for covalent attachment to DNA. To ensure that only noncovalent interactions were being measured in the first binding experiment, reaction and wash buffers lacked Mg\(^{2+}\). The results of these binding studies are shown in Fig. 1A. The data fit well to an equation describing a single DNA molecule binding per enzyme dimer, with \( K_d \) values of 29 nm for both wild type and Y782F mutant enzymes.

The presence of Mg\(^{2+}\) not only allows the wild type enzyme to covalently cleave DNA but may affect important noncovalent interactions as well. Therefore, the binding experiments were repeated in the presence of 5 mm MgCl\(_2\) (Fig. 1B). In the presence of Mg\(^{2+}\), both enzymes bound DNA more tightly than in its absence, with \( K_d \) values of 11 nm for both wild type and Y782F. Again, the enzymes bound DNA with equal affinities, even though under these conditions, the wild type can covalently attach to the DNA. These results are in agreement with many previous results indicating that the DNA cleavage-religation equilibrium for topoisomerase II strongly favors religation (16).

Topologic Trapping of Circular DNA—Topoisomerase II can topologically trap circular DNA if it subsequently binds a non-hydrolyzable ATP analog (18, 28, 29). If a high concentration of DNA circles is used in such an experiment, the wild type enzyme can catenate two circles; the enzyme remains directly topologically linked to one circle, which is catenated to the
other circle (28). Given that the buoyant densities of free topoisomerase II and *Escherichia coli* plasmid DNA are approximately 1.37 g/ml (21) and 1.71 g/ml (30), respectively, complexes between the two will have intermediate densities depending on the ratios of DNA to enzyme. This property has been used previously to analyze topoisomerase II-circular DNA complexes by equilibrium sedimentation in a CsCl gradient (26, 31). The results of similar experiments performed with wild type and Y782F topoisomerase II and a molar excess of 3 kilobase pairs relaxed circular or linearized DNA are shown in Fig. 2. In each absorbance trace, free DNA is seen as a large peak at the bottom of the density gradient. Two peaks of intermediate density are seen for reactions with wild type enzyme bound to circular DNA and AMPPNP (Fig. 2A). When the binding reactions were analyzed by agarose gel electrophoresis, a band migrating at the position expected for catenated circles was detected for only the wild type reaction (not shown). The most reasonable interpretation of these data is that the intermediate peak of higher density represents the enzyme linked to a catenane it has produced. The lower density peak indicates the enzyme linked to a single DNA circle. Because the DNA:topoisomerase ratio is high in these experiments, no peaks caused by multiple topoisomerase dimers per DNA circle are seen. In contrast, the reaction with the Y782F mutant produced only the lower density intermediate peak (Fig. 2D). Because this mutant cannot perform a catenation reaction, it is not surprising that the higher density intermediate is missing. However, these experiments do confirm that a mutant unable to transport one DNA through another can still only topologically trap one DNA circle (3). Even under conditions where the wild type enzyme can catenate two circles, Y782F apparently cannot trap both the G and T segments of DNA.

Control reactions were performed to ensure that the intermediate peaks that were seen result from the topologic trapping of circular DNA by topoisomerase II forming an AMPPNP-induced clamp around the DNA. When linear DNA was used instead of circular, no peaks of intermediate density were seen (Fig. 2, B and E). Additionally, in the absence of AMPPNP, no intermediate peaks were detected (Fig. 2, C and F).

**Pre-steady-state ATPase Assays**—Because the difference in $k_{cat}$ values for ATP hydrolysis by the wild type and Y782F mutant enzymes is apparently not due to differences in DNA affinities, pre-steady-state chemical quench and pulse-chase experiments were used to further explore the differences. In the pre-steady state, information about the rates of individual steps in the reaction pathway can be obtained; this information

![Fig. 1. Affinity of wild type and Y782F topoisomerase II for DNA as measured by double-filter nitrocellulose binding.](image1.png)

**FIG. 1.** Affinity of wild type and Y782F topoisomerase II for DNA as measured by double-filter nitrocellulose binding. The ratios of bound DNA to total DNA were determined in the absence (A) and presence (B) of 5 mM MgCl₂ in reactions including varying concentrations of wild type (●) or Y782F (○) topoisomerase II, as described under "Experimental Procedures." The data were fit to Eq. 4, which resulted in identical $K_d$ values for the wild type and Y782F mutant of 29 and 11 nM in A and B, respectively. The error bars represent the deviation from mean of data collected in triplicate.

![Fig. 2. AMPPNP-induced trapping of DNA circles by wild type and Y782F topoisomerase II observed by analytical ultracentrifugation.](image2.png)

**FIG. 2.** AMPPNP-induced trapping of DNA circles by wild type and Y782F topoisomerase II observed by analytical ultracentrifugation. Absorbance traces of the CsCl density gradients taken at 40 h and 260 nm are shown for each reaction. The large peak seen at the right end of each trace is from the absorbance of free DNA running at the bottom of each gradient.
concentrations of 6.7 μM enzyme dimer, 1.6 mM DNA bp, and 350 μM ATP. The data were fit as described under “Experimental Procedures” with values for the chemical quench reaction of $A = 6.0 \pm 0.6 \mu M$, $B = 25 \pm 5 \text{s}^{-1}$, and $C = 14 \pm 1 \mu M \text{s}^{-1}$, and for the pulse-chase reaction of $A = 14 \pm 1 \mu M$, $B = 50 \pm 10 \text{s}^{-1}$, and $C = 14 \pm 1 \mu M \text{s}^{-1}$. Biological chemical quench (●) and pulse-chase (○) time courses were performed with wild type topoisomerase II at final concentrations of 7.5 μM enzyme dimer, 1.8 mM DNA bp, and 350 μM ATP. The parameters obtained by fitting the data are $A = 8 \pm 1 \mu M$, $B = 6 \pm 1 \text{s}^{-1}$, and $C = 6 \pm 1 \mu M \text{s}^{-1}$ for $A = 15 \pm 1 \mu M$, $B = 50 \pm 10 \text{s}^{-1}$, and $C = 6 \pm 1 \mu M \text{s}^{-1}$ for the chemical quench and pulse-chase reactions, respectively.

is obscured in steady-state kinetics. The ATPase activity of wild type topoisomerase II has been previously studied extensively using these rapid quench methods (14, 15). New results of chemical quench and pulse-chase time courses for the wild type enzyme bound to DNA are shown in Fig. 3A. The results of these present time courses are essentially the same as those reported previously. The chemical quench time course shows a rapid burst of ATP hydrolysis, followed by the steady-state rate of enzyme turnover. The burst amplitude ($6.0 \pm 0.6 \mu M$) approximately equals the enzyme dimer concentration (6.7 μM), or half the concentration of ATP active sites.

Fig. 3A also shows the results of a pulse-chase experiment performed with the same enzyme-DNA sample. If instead of chemically quenching the ATPase reaction, a large excess of unlabeled ATP is added at the indicated time points, the enzyme can either continue to hydrolyze any labeled ATP that it had bound prior to the chase or release the unhydrolyzed ATP. Any new ATP bound will be unlabeled and therefore undetectable in the assay system. The reaction is allowed to continue for several turnovers before it is chemically quenched. A burst in ADP production is again seen in the pulse-chase results, but this time the burst amplitude ($14 \pm 1 \mu M$) approximately equals the enzyme active site concentration (13.4 μM). This experiment shows that at 350 μM ATP, the enzyme active sites are essentially saturated with ATP, and once the two ATP are bound, they are hydrolyzed faster than they are released. In conjunction with inhibitor studies, these results showing a doubling of the burst amplitude from one half the ATP active site concentration in chemical quench experiments to essentially equal the ATP active site concentration in pulse-chase experiments have been used to define the ATPase reaction mechanism for the DNA-bound topoisomerase II (15). The simplest mechanism in agreement with all of these data is that the enzyme binds two ATP, hydrolyzes one rapidly, releases the products of the first hydrolysis, and then hydrolyzes the second ATP and releases those products. The clearly defined burst in hydrolysis of one ATP shows that a rate-determining step occurs after hydrolysis of the first ATP and before hydrolysis of the second ATP. A reaction mechanism describing this pathway is shown in Scheme 1, where $E_2$ represents dimeric topoisomerase II bound to DNA, $S$ represents ATP, $P$ represents ADP, and $E_S$ represents a state of topoisomerase II bound to a single ATP that differs from $E_S$.

Similar chemical quench and pulse-chase reaction time courses were performed with the Y782F mutant enzyme (Fig. 3B). The results of the chemical quench experiment again show a burst in ATP hydrolysis, with the burst amplitude ($8 \pm 1 \mu M$) approximating the dimeric enzyme concentration (7.5 μM). As expected, the calculated steady-state rate is also slower for Y782F (6 μM s$^{-1}$) as compared with the wild type (14 μM s$^{-1}$). The same chemical quench results were obtained when the ATP concentration was increased to 1 mM, confirming that the low value for the burst rate constant is not a result of saturation of the ATP active sites at 350 μM ATP (data not shown). The pulse-chase results show a burst amplitude ($15 \pm 1 \mu M$) equal to the enzyme active site concentration (15 μM) and a burst rate constant equal to that seen for the wild type enzyme ($50 \pm 10 \text{s}^{-1}$). A comparison of the chemical quench and pulse-chase results indicates that Y782F binds two ATP with a high commitment for catalysis, just as the wild type enzyme. Y782F had bound ATP at slower rates or lower affinities than the wild type enzyme, the pulse-chase burst rate constant and amplitude would have been reduced. This is not seen. Additionally, the identical $k_{cat}/K_m$ values support the conclusion that there is no significant decrease in DNA-stimulated ATP binding by Y782F compared with wild type topoisomerase II.

In order to determine which rate constants are decreased for the mutant enzyme, further analysis of the data is required. For the wild type enzyme, the clear distinction between the burst and steady-state phases of the data allowed the use of singular perturbation theory to determine rate constants for each step of the pathway shown in Scheme 1 (15). This distinction no longer exists for the data obtained with Y782F. Therefore, the data were analyzed using a simplified reaction pathway shown in Scheme 2, where $k_{cat} = k_{cat}[S]$.
hydrolysis of the first ATP occurs at a slower rate for the mutant enzyme. The fact that both DNA cleavage mutants have similar ATPase kinetics indicates that it is the inability to cleave DNA that causes a perturbation in ATP hydrolysis by topoisomerase II.

**DISCUSSION**

Topoisomerase II mutants that cannot covalently cleave DNA retain a DNA-stimulated ATPase activity but hydrolyze ATP with a slower maximum turnover rate than the wild type enzyme. Wild type and Y782F mutant proteins bind a single segment of DNA with equal affinities. Y782F can topologically trap a single circle of DNA in the presence of AMPPNP under conditions where the wild type protein can catenate two circles. Pre-steady-state ATPase kinetics show that the Y782F and R690A mutants bind two ATPs with a high commitment for catalysis and without a significant decrease in ATP affinity or binding rates. However, the noncleaving mutants hydrolyze the first ATP 3–4-fold slower than the wild type enzyme. A step or steps later in the reaction pathway, potentially steps associated with ADP or P, release or hydrolysis of the second ATP, also occur more slowly for these mutant enzymes.

There are several potential mechanistic implications of these results. The two mutations studied, Y782F and R690A, are not in the ATPase domain, and it is unlikely that these residues come within ~20 Å of the ATP. Therefore, it is doubtful that these mutations directly affect the rate of the chemical step of ATP hydrolysis. One possibility is that the ATP hydrolysis rates of these mutant proteins are perturbed because they cannot undergo the normal pathway of conformational changes. In order to allow transport of the T segment of DNA through the G segment, the active site tyrosines and attached 5’ phosphorolyl ends of the G segment must move 35–40 Å relative to each other (33). However, the DNA must be cleaved first for the enzyme-DNA complex to undergo this conformational change. Neither Y782F nor R690A is capable of cleaving DNA and cannot undergo this conformational change unless their noncovalent interactions with DNA are broken. Because the rates and affinities of ATP binding are not appreciably lower for the mutant proteins, this conformational change is probably not coupled to the steps of ATP binding. However, the rate of hydrolysis of the first ATP is measurably reduced in the mutants. This suggests that either a DNA cleavage-dependent conformational change occurs after ATP binding but before hydrolysis or that hydrolysis is directly coupled to the conformational change. The rates of product release, or conformational changes associated with product release, are also decreased severalfold for these mutant proteins.

An alternative hypothesis involves altered binding of the T segment of DNA to the mutant proteins. When wild type topoisomerase II binds circular DNA, followed by AMPPNP, it can catenate two circles and remain directly topologically linked to one of them (28). The circle that remains directly linked to the enzyme presumably contains the G segment cleaved during the reaction. The other circle in the catenane is not directly linked to the topoisomerase and contains the T segment that was transported through the cleaved DNA and the enzyme. It is apparently the G segment that binds to the topoisomerase with sufficient affinity to be measured by the double-filter binding assay described above. Interactions between the T segment and the enzyme are not well understood. It has been postulated that the T segment binds within the cavity formed by the enzyme. It is unlikely that these residues come within ~20 Å of the ATP. Therefore, it is doubtful that these mutations directly affect the rate of the chemical step of ATP hydrolysis. One possibility is that the ATP hydrolysis rates of these mutant proteins are perturbed because they cannot undergo the normal pathway of conformational changes. In order to allow transport of the T segment of DNA through the G segment, the active site tyrosines and attached 5’ phosphorolyl ends of the G segment must move 35–40 Å relative to each other (33). However, the DNA must be cleaved first for the enzyme-DNA complex to undergo this conformational change. Neither Y782F nor R690A is capable of cleaving DNA and cannot undergo this conformational change unless their noncovalent interactions with DNA are broken. Because the rates and affinities of ATP binding are not appreciably lower for the mutant proteins, this conformational change is probably not coupled to the steps of ATP binding. However, the rate of hydrolysis of the first ATP is measurably reduced in the mutants. This suggests that either a DNA cleavage-dependent conformational change occurs after ATP binding but before hydrolysis or that hydrolysis is directly coupled to the conformational change. The rates of product release, or conformational changes associated with product release, are also decreased severalfold for these mutant proteins.

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Analysis of Topoisomerase II DNA Cleavage Mutants

Y782F can topologically trap only one circle. Similar conclusions have been reported elsewhere as unpublished results (3). These results are interpreted as showing that Y782F binds the G segment of DNA normally but fails to productively bind the T segment. Results of previous kinetic studies suggested that DNA stimulates the ATPase activity of topoisomerase II by increasing the rates or affinities of ATP binding and potentially the rate of hydrolyzing the first ATP (15). Y782F binds ATP normally but hydrolyzes at least the first ATP slowly. Therefore, a second simplified hypothesis to explain these results is that binding of the G segment of DNA primarily stimulates ATP binding, whereas binding of the T segment stimulates hydrolysis of at least the first ATP. These two proposed hypotheses are not mutually exclusive; the topoisomerase may need to undergo the conformational change to open the G segment in order to accommodate productive binding of the T segment. Either the conformational change or the T segment binding may be the direct cause of increased ATP hydrolysis.

A double-filter binding method has been used to quantitatively determine the binding parameters of wild type and Y782F mutant topoisomerase II to DNA. In the absence of Mg2+, when neither enzyme can covalently attach to the DNA, they have identical Kd values of 29 nM. In the presence of 5 mM Mg2+, the Kd value for each protein decreases to 11 nM. The ionic strengths were kept constant between the plus and minus reactions, so that the 2–3-fold decrease in Kd values should be specifically due to the presence of the divalent cation. Previous binding studies with type II topoisomerasers and DNA have provided varied results on the effects of Mg2+. Tight binding of E. coli DNA gyrase (35) and yeast topoisomerase II (36) were only detected in the presence of Mg2+, whereas binding of the Drosophila enzyme was minimally affected by Mg2+ (37, 38). Because noncovalent and potential covalent interactions can be clearly distinguished in the present study with the Y782F mutant, these data can unambiguously be interpreted to show that Mg2+ increases the noncovalent binding affinity of yeast topoisomerase II 2–3-fold for DNA. Additionally, because under both conditions the wild type and mutant have identical dissociation constants, only a negligible fraction of wild type topoisomerase II is covalently attached to DNA at equilibrium in the presence of Mg2+. These results are in agreement with quenched DNA cleavage results reported from many laboratories (see ref. 16 for a review). One potential concern with the cleavage reactions is that the quenches used could drive the enzyme-DNA cleavage-religation equilibrium toward religation. The present equilibrium binding studies do not use a quench and support the idea that the true DNA cleavage-religation equilibrium strongly favors religation.

Previous studies to determine the effects of perturbing DNA cleavage and religation on the ATPase activity of topoisomerase II have produced a variety of results. Preliminary data for the yeast Y782F mutant enzyme (referred to in a previous study as Y783F in accordance with the older sequence information) have been described as showing that this mutant has no DNA-stimulated ATPase activity (39). In the present results, the kcat for ATP hydrolysis of this mutant is shown to increase 2–3-fold in the presence of DNA, whereas the kcat decreases almost 10-fold. Depending on how the previous experiments were done, the rather subtle effect on kcat might have been missed. One result of the DNA stimulation on the ATPase activity is an easily detectable burst in hydrolysis equal to half the ATP active site concentration; no burst is seen in the absence of DNA (14). Both the Y782F and R690A cleavage mutants show a similar burst in ATPase activity when bound to DNA, indicative of DNA stimulating the rates of ATP binding and/or hydrolysis of the first ATP. A variety of drugs perturb the DNA cleavage/religation equilibrium catalyzed by topoisomerase II. Fortune and Osheroff (40) found that merbarone blocks DNA cleavage yet has no effect on the ATPase activity of human topoisomerase IIa. It is presently unclear why a drug that prevents DNA cleavage would have such a different effect on the ATPase activity than the DNA cleavage mutants. Etoposide, a drug that is thought to slow the rate of DNA religation by topoisomerase II, was shown to have essentially no effect on the ATPase activity of Drosophila topoisomerase II (41), whereas it dramatically inhibited the ATPase activity of human topoisomerase IIa (39). The mechanistic implications of the differences in these results are presently unclear. It should be interesting to determine the pre-steady-state effects of these drugs to more clearly define where they perturb the mechanistic pathway of DNA topoisomerase II.

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