Stability of the Topoisomerase II Closed Clamp Conformation May Influence DNA-stimulated ATP Hydrolysis*

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Type II DNA topoisomerases catalyze changes in DNA topology and use nucleotide binding and hydrolysis to control conformational changes required for the enzyme reaction. We examined the ATP hydrolysis activity of a bisdioxopiperazine-resistant mutant of human topoisomerase IIα with phenylalanine substituted for tyrosine at residue 50 in the ATP hydrolysis domain of the enzyme. This substitution reduced the DNA-dependent ATP hydrolysis activity of the mutant protein without affecting the relaxation activity of the enzyme. A similar but stronger effect was seen when the homologous mutation (Tyr28 → Phe) was introduced in yeast Top2. The ATPase activities of human TOP2α(Tyr50 → Phe) and yeast Top2(Tyr28 → Phe) were resistant to both bisdioxopiperazines and the ATPase inhibitor sodium orthovanadate. Like bisdioxopiperazines, vanadate traps the enzyme in a salt-stable closed conformation termed the closed clamp, which can be detected in the presence of circular DNA substrates. Consistent with the vanadate-resistant ATPase activity, salt-stable closed clamps were not detected in reactions containing the yeast or human mutant protein, vanadate, and ATP. Similarly, ADP trapped wild-type topoisomerase II as a closed clamp, but could not trap either the human or yeast mutant enzymes. Our results demonstrate that bisdioxopiperazine-resistant clamps are catalytic inhibitors of type II topoisomerases with an N-terminal domain capable of binding and hydrolyzing ATP; a central domain critical for DNA breakage and rejoining; and a C-terminal domain that is not essential for enzyme activity, but that is thought to be involved in regulation of enzyme activity and localization (2). DNA topoisomerase II interacts with two double-stranded DNA segments, a segment that will be cleaved by the enzyme (the G-segment) and a strand that will be passed through the cleaved strand (the T-segment). In the normal reaction cycle, the enzyme binds the G-segment; and upon ATP binding, the N-terminal domains of the subunits dimerize, which can lead to the capture of the T-segment, which passes through a double strand break in the G-segment and ultimately exits the protein clamp through a C-terminal protein “gate” (6–8). Following religation of the DNA strand break, ATP hydrolysis leads to the dissociation of the N-terminal dimerization, allowing release of the G-segment or initiation of another catalytic cycle (9). Structural studies by Berger and co-workers (10) have confirmed the importance of ATP binding in maintaining N-terminal dimerization, in agreement with earlier structural studies with the prokaryotic DNA gyrase (11, 12). Lindsley and co-workers (13, 14) have suggested from kinetic studies of ATP hydrolysis by yeast Top2 that hydrolysis of the two ATP molecules in the reaction cycle occurs sequentially rather than simultaneously. This suggests that ATP hydrolysis may play additional roles in enzyme dynamics beside controlling the N-terminal dimerization.

Topoisomerases are targeted by two classes of inhibitors. The first class of inhibitors, which include topoisomerase poisons such as etoposide, stabilize the covalent intermediate formed when the enzyme generates DNA strand breaks (15). The second class of inhibitors, termed catalytic inhibitors, do not lead to elevated levels of protein-DNA covalent complexes. Bisdioxopiperazines are catalytic inhibitors of type II topoisomerases that stabilize the closed clamp form of the enzyme, an intermediate in which the N-terminal domains of the protein have dimerized (16–19). Although earlier studies suggested that bisdioxopiperazines are cytotoxic solely due to reducing in vivo enzyme activity, recent studies have suggested that the closed clamp form of the enzyme may also interfere with DNA metabolism (20). In addition to stabilizing the closed clamp form of topoisomerase II, bisdioxopiperazines inhibit the ATP hydrolysis activity of the eukaryotic enzymes (17, 21). In studies with N-terminal fragments of human topoisomerase IIα, bisdioxopiperazines inhibited the ATP hydrolysis activity of these truncated proteins, although the extent of inhibition was less.

DNA topoisomerases change DNA topology by introducing transient strand breaks in DNA. Regulated changes in DNA topology are critical for many cellular processes, including DNA replication, transcription, chromosome condensation, and chromosome separation prior to cell division (1, 2). Topoisomerases are divided into two major classes: type I enzymes, which introduce transient single strand breaks in DNA, and type II enzymes, which introduce double strand breaks.

Structural and biochemical studies have led to a detailed picture of how type II enzymes carry out topological changes in DNA (3–5). Eukaryotic type II topoisomerases are homodimeric enzymes with an N-terminal domain capable of binding and hydrolyzing ATP; a central domain critical for DNA breakage and rejoining; and a C-terminal domain that is not essential for enzyme activity, but that is thought to be involved in regulation of enzyme activity and localization (2). DNA topoisomerase II interacts with two double-stranded DNA segments, a segment that will be cleaved by the enzyme (the G-segment) and a strand that will be passed through the cleaved strand (the T-segment). In the normal reaction cycle, the enzyme binds the G-segment; and upon ATP binding, the N-terminal domains of the subunits dimerize, which can lead to the capture of the T-segment, which passes through a double strand break in the G-segment and ultimately exits the protein clamp through a C-terminal protein “gate” (6–8). Following religation of the DNA strand break, ATP hydrolysis leads to the dissociation of the N-terminal dimerization, allowing release of the G-segment or initiation of another catalytic cycle (9). Structural studies by Berger and co-workers (10) have confirmed the importance of ATP binding in maintaining N-terminal dimerization, in agreement with earlier structural studies with the prokaryotic DNA gyrase (11, 12). Lindsley and co-workers (13, 14) have suggested from kinetic studies of ATP hydrolysis by yeast Top2 that hydrolysis of the two ATP molecules in the reaction cycle occurs sequentially rather than simultaneously. This suggests that ATP hydrolysis may play additional roles in enzyme dynamics beside controlling the N-terminal dimerization.

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than that observed with the full-length protein, suggesting that protein domains outside of the N terminus influence bisdioxopiperazine inhibition (22, 23).

Recently, Berger and co-workers (10) reported the first x-ray crystal structure of the ATP hydrolysis domain of a eukaryotic type II topoisomerase. The 1.9-Å structure of the ATPase domain of yeast topoisomerase II in complex with the bisdioxopiperazine ICRF-187 identified residues involved in the enzyme-drug interaction. The crystal structure of a bisdioxopiperazine bound to the ATPase domain provided a rationale for many of the bisdioxopiperazine-resistant mutations in human topoisomerase IIα that had been identified previously (24–26). Although some of the mutations clearly have the potential to alter drug binding, others also affect nucleotide binding and may alter the stability of the N-terminal dimerization.

To understand in greater biochemical detail the action of bisdioxopiperazines on eukaryotic topoisomerase IIα, we began to characterize the ATP hydrolysis activity of the bisdioxopiperazine-resistant mutants. In this study, we describe the ATPase activity of HTOP2α(Tyr50Phe) and its yeast counterpart, yTOP2(Tyr28Phe). Although the ATPase activity is bisdioxopiperazine-resistant, as expected, we also found that both yeast mutants have other alterations in their ATPase activity. Notably, both proteins have greatly diminished DNA-stimulated ATPase activity. In addition, we found that the mutant proteins are also resistant to other conditions that lead to the formation of a salt-stable N-terminal clamp. Our results indicate that the Tyr50Phe mutation alters the stability of the N-terminal clamp independent of effects due to bisdioxopiperazine binding and suggest that the stability of the clamp is important for the ability of the TOP2 ATPase activity to be stimulated by DNA.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—ICRF-193 was synthesized by Dr. Donald Witaik (University of Wisconsin, Madison, WI) as described previously (27). ICRF-187 was obtained from Chiron. ATP was purchased from Amersham Biosciences. HEPEs, NaDH, phosphoenolpyruvate (trisodium salt hydrate), pyruvate kinase (5000 units/1.9 ml), and lactate dehydrogenase (from rabbit muscle, 8333 units/ml) were purchased from Sigma. Sodium orthovanadate and 3-bromopyruvate were obtained from Calbiochem.

**Plasmids**—Plasmid pCM1 was used for the expression of human topoisomerase IIα (28). Mutations were introduced into this plasmid by oligonucleotide-directed mutagenesis to express mutant forms of human topoisomerase IIα. Yeast Top2 and the yTop2(Tyr50Phe) mutant were expressed using YEPTOP2-pGAL1 or YEptop2(Y28F)-pGAL1. Assessment of DNA-stimulated ATPase activity with closed circular DNA was carried out using plasmid pUC18. Plasmid DNA was routinely purified from bacterial cells using Qiagen kits.

**Oligonucleotide-directed Mutagenesis**—The Tyr50Phe mutation was introduced into YEPTOP2-pGAL1 using the QuikChange mutagenesis kit. The two mutagenic primers used were TTCACAGAACAGCGAATGGCTCTCTTTTTGCATCTCCTGTGGAA and AAAAGACAGACCATTATTCTGCTGTGGA, where the underlined nucleotides indicate nucleotides that differ from the yeast wild-type sequence. The Tyr50Phe mutation was introduced into pCM1 using the mutagenic primers described previously (20). All mutations generated by oligonucleotide-directed mutagenesis were confirmed by DNA sequencing.

**Purification of Topoisomerase II from Yeast Cells**—Human wild-type topoisomerase IIα, yeast wild-type topoisomerase II, and the mutant proteins HTOP2α(Tyr50Phe) and yTOP2(Tyr28Phe) were purified from the protease-deficient strain JEL1 (trp1, leu2, ura3-52, pbr1-1122, pep4-3, his3:pGAL10GAL4, top1-10001) as described previously (29, 30). Enzymes were >90% pure as assessed by SDS-PAGE (data not shown; see Ref. 28 for purity of enzymes obtained using the same expression vector and purification procedure).

**Measurement of Topoisomerase II Relaxation Activity**—DNA topoisomerase II activity assays were carried out as described previously (31). Enzyme was added to initiate reactions containing 200 ng of supercoiled pUC18 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, and 2 mM ATP. The reaction was carried out at 37 °C for 30 min when using human topoisomerase IIα or at 30 °C with yeast topoisomerase II. One microliter of 10× DNA loading buffer containing 0.25% orange-G dye, 0.1 mM EDTA, and 30% glycerol was added to stop the reaction. The samples were then loaded onto a 1% agarose gel, and electrophoresis was carried out using Tris acetate/EDTA buffer. The gel was stained with ethidium bromide and visualized under UV light.

**ATP Hydrolysis Assay for Topoisomerase II**—The rate of enzyme-catalyzed ATP hydrolysis was measured using a coupled enzyme assay as described by Harkins and Lindsley (32) with minor modifications. Briefly, the 1-ml reaction mixture contained 6.3 units of pyruvate kinase, 8.8 units of l-lactate dehydrogenase, 50 mM HEPEs (pH 7.7), 8 mM Mg(OAc)2, 150 mM KOAc, 3.3 mM phosphoenolpyruvate (trisodium salt), 160 μM NaDH, 10–30 mM topoisomerase II, and ATP in the presence or absence of DNA. Reactions contained DNA from either salmon sperm DNA (Sigma) or pUC18 prepared using Qiagen kits. Where indicated, sodium orthovanadate or ICRF-187 was also added to the reaction mixture. Reaction mixtures were equilibrated for 2 min at the appropriate temperature, and the reactions were then initiated by the addition of ATP. The reactions were carried out at 37 °C for human topoisomerase IIα or at 30 °C for yeast topoisomerase II. The ATP hydrolysis was measured spectrophotometrically for 10 min by the decrease in NADH absorbance at λ = 340 nm, and rates were calculated using the extinction coefficient for NADH of ε = 6.22 mM cm−1.

Assays for Closed Clamp Formation by Topoisomerase II—Assessment of the salt-stable closed clamp conformation of topoisomerase II was carried out as described previously (6, 28) with several modifications. The reaction buffer for filter binding assays with the yeast enzyme contained 150 mM KCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 8 mM MgCl2, 7 mM β-mercaptoethanol, and 0.1 mM acetylated bovine serum albumin. The reaction conditions for the human enzyme were identical, except that the KCl concentration was 100 mM. All reactions also contained 500 ng of pUC18 and 500 ng of yeast or human topoisomerase IIα. Where indicated, reactions contained 2 mM ATP, 4 mM ADPNNP, 5 mM ADP, 300 mM sodium orthovanadate, and/or 50 μM ICRF-187 in a final volume of 10 μl. Samples were incubated at 30 °C (for yTop2) or 37 °C (for hTOP2) for 10 min before loading onto the filters. Centrex MF glass microfiber centrifugal filters (catalog number 02200, Schleicher & Schuell) were preincubated with 200 μl of reaction buffer containing 0.1 mg/ml sonicated salmon sperm DNA for 5–10 min at room temperature. The columns were centrifuged at 40,000 g for 5 min and washed twice with reaction buffer before the addition of the samples. Samples were added to the filters and incubated for 2 min before centrifugation at 40 × g for 5 min. The filters were washed three times with 200 μl of reaction buffer (low salt wash), and the eluent and low salt filtrates were combined. The filters were then washed three times with 200 μl of high salt buffer (reaction buffer containing 1 M NaCl) and three times with 200 μl of SDS wash buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% SDS) that has been preheated to 65 °C. The nucleic acids in each sample were precipitated with 0.7 volume of 1 M sodium isopropyl alcohol, resuspended in 10 μl of 1× loading dye, and run on a 1% agarose gel at 95 V for 45 min. Gels were then stained with ethidium bromide, and nucleic acids were visualized under UV light.

**RESULTS**

**Stimulation of the DNA-dependent ATP Hydrolysis Activity of Human Wild-type Topoisomerase IIα by Linear and Covalently Closed DNA**—Eukaryotic type II DNA topoisomerases exhibit ATP hydrolysis activity that is stimulated 3–17-fold by the addition of DNA (22, 33–35). The extent of stimulation depends on both the DNA concentration and the source of DNA. The rate of ATP hydrolysis catalyzed by human topoisomerase IIα as a function of DNA concentration using either supercoiled closed circular plasmid or heterogeneous double-stranded linear DNA (Fig. 1). The addition of either closed circular or linear DNA led to an 10-fold enhancement of the rate of ATP hydrolysis. With linear DNA (Fig. 1A), the rate increased from 41 ± 16 nmol/min/mg to a maximum of 435 ± 36 nmol/min/mg at a base pair/TOP2 holoenzyme ratio of 2500. A similar result was observed using the supercoiled

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1 The abbreviation used is: hTOP2, human TOP2; yTop2, yeast Top2; ADPNNP, adenosine 5′-[(3′,5′-iminothiophosphate)].
The analogous mutation in the gene encoding yTop2 and overexpressed DNA-stimulated ATP hydrolysis, we constructed the homology—

To assess the effect of substituting Phe for Tyr50 on the activity of wild-type yeast topoisomerase II unless otherwise indicated. The highest rate of topoisomerase II ATP hydrolysis occurred at a base pair:TOP2 holoenzyme ratio of 2500. DNA was used in subsequent experiments to maintain a base pair:TOP2 holoenzyme ratio at 2500 when examining the DNA-dependent rate of ATP hydrolysis.

The hTOP2α(Tyr50 → Phe) Mutant and Its Yeast Counterpart Have Reduced Levels of DNA-dependent ATP Hydrolysis Activity—To assess the effect of substituting Phe for Tyr50 on the ATP hydrolysis activity, we compared the rates of DNA-independent and DNA-dependent ATP hydrolysis catalyzed by the wild-type and mutant enzymes as a function of ATP concentration (Fig. 2). Human wild-type topoisomerase IIα in the absence of DNA (Fig. 2A, open circles) and the hTOP2α(Tyr50 → Phe) mutant (Fig. 2B, open circles) displayed similar levels of ATP hydrolysis activity. However, although DNA stimulated the ATP hydrolysis activity of the hTOP2α(Tyr50 → Phe) mutant, the stimulation was lower than that seen with the wild-type enzyme. At 1 mM ATP, the addition of DNA to the wild-type enzyme produced an ~7-fold stimulation of the rate of ATP hydrolysis, whereas the hTOP2α(Tyr50 → Phe) mutant showed an ~4-fold stimulation. These results are consistent with our analysis of other N-terminal domain mutants of human topoisomerase IIα that also displayed a reduction in DNA-stimulated ATP hydrolysis activity (28).

To further examine the effect of the Tyr50 → Phe mutation on DNA-stimulated ATP hydrolysis, we constructed the homologous mutation in the gene encoding yTop2 and overexpressed and purified the mutant protein. As shown in Fig. 3A, the purified yTop2(Tyr28 → Phe) protein had similar DNA relaxation activity compared with wild-type yTop2. This result is similar to what we observed previously for hTOP2α(Tyr50 → Phe) (24).

Because the results suggested a possible defect in ATP utilization by the yTop2(Tyr28 → Phe) protein, we also examined relaxation at low ATP concentrations. Fig. 3B shows DNA relaxation carried out by the yTop2(Tyr28 → Phe) protein under the same conditions as described for Fig. 3A, but with varying ATP concentrations, and Fig. 3C shows the results obtained with wild-type yTop2. The relaxation activities of the two proteins at suboptimal ATP concentrations were not significantly different, with complete relaxation requiring an ATP concentration of ~100 μM. The yTop2(Tyr28 → Phe) protein may be slightly more active at low ATP concentrations than the wild-type protein since 50 μM ATP in the reaction led to nearly complete relaxation by the yTop2(Tyr28 → Phe) protein and clearly less relaxation by the wild-type protein.

We then examined the DNA concentration required for maximum stimulation of the ATP hydrolysis activity of the yeast wild-type protein by titration with heterogeneous double-stranded linear DNA (Fig. 4A). We determined a maximum level of DNA stimulation using a base pair:yTop2 holoenzyme ratio of 2500 (a ratio used in subsequent experiments). Yeast wild-type topoisomerase II and the yTop2(Tyr28 → Phe) mutant exhibited similar levels of DNA-independent ATP hydrolysis, as shown in Fig. 4 (B and C). However, the addition of DNA to the yTop2(Tyr28 → Phe) mutant generated only a 1.5-fold stimulation of the rate of ATP hydrolysis at 1 mM ATP compared with the wild-type enzyme, which was stimulated ~5-fold under the same conditions.

To further characterize the effects of the Tyr50 → Phe mutation, we next determined the kinetic parameters $K_{\text{app}}$, $k_{\text{cat}}$, and $V_{\text{max}}$ for the wild-type and mutant enzymes. Alterations in the binding of the nucleotide substrate may be reflected as changes in the Michaelis binding constant $K_m$ for ATP, whereas changes in other steps in the reaction could result in changes in the kinetic parameters $V_{\text{max}}$ and $k_{\text{cat}}$. The rates of ATP hydrolysis as a function of ATP concentration were used to construct Lineweaver-Burk plots and to obtain values for $k_{\text{cat}}$, $K_m$, and $V_{\text{max}}$ (Table I). In the absence of DNA, human wild-
DNA-stimulated ATPase of Eukaryotic Top2

Fig. 2, hTOP2α(Tyr50 → Phe) shows reduced DNA-stimulated ATPase activity. The ATP hydrolysis activities of wild-type hTOP2α (A) and hTOP2α(Tyr50 → Phe) (B) were determined as a function of ATP concentration using a coupled enzyme assay. ○, ATP hydrolysis in the absence of DNA; ●, ATP hydrolysis in the presence of 50 μg/ml heterogeneous double-stranded linear DNA. ATP hydrolysis is expressed as nanomoles of ATP hydrolyzed per min/mg of protein. Error bars indicate the S.D. calculated from a minimum of three independent experiments.

Fig. 3. yTop2(Tyr28 → Phe) shows wild-type levels of DNA relaxation activity. The catalytic activities of wild-type yTop2 and the yTop2(Tyr28 → Phe) mutant were determined using ATP-dependent relaxation of pUC18 under the conditions described under “Experimental Procedures.” A, activity of the wild-type and mutant proteins as a function of added protein. The quantities of protein added are indicated above each lane. The λ lane in A and the L lane in B and C contain the λHindIII marker, and the C lane (control) contains the substrate without added protein. B and C, relaxation carried out under the same conditions as described for A, with 20 ng of added protein and varying ATP concentrations as indicated. B shows results obtained with yTop2(Tyr28 → Phe), and C shows results obtained with the wild-type protein.

Type topoisomerase IIα and the hTOP2α(Tyr50 → Phe) mutant show no significant alterations in \( k_{cat} \), \( K_m \) and \( V_{max} \). In the presence of DNA, the \( k_{cat} \) for the wild-type enzyme was 3.6 ± 1.1 s\(^{-1}\), a 7-fold difference above the level seen without DNA. These values agree reasonably with values determined previously for topoisomerase II purified from HeLa cells (33). The \( k_{cat} \) for hTOP2α(Tyr50 → Phe) in the presence of DNA was 1.5 ± 0.01 s\(^{-1}\), significantly lower than that of the wild-type enzyme. Although there was a significant reduction in the \( k_{cat} \) for hTOP2α(Tyr50 → Phe) in the presence of DNA, the \( K_{m(app)} \) for the two proteins was similar whether DNA was present or absent.

Similar results were obtained with yeast topoisomerase II and the yTop2(Tyr28 → Phe) mutant. For wild-type yTop2, the \( k_{cat} \) in the absence of DNA was 1.1 ± 0.2 s\(^{-1}\) compared with 1.0 ± 0.1 s\(^{-1}\) for yTop2(Tyr28 → Phe). These values are slightly higher than the value of 0.4 s\(^{-1}\) obtained by Lindasdale and Wang (12). However, it is clear that the DNA-independent hydrolysis is not altered in the yTop2(Tyr28 → Phe) mutant. In the presence of DNA, the \( k_{cat} \) for the yeast wild-type enzyme was 3.6 ± 0.1 s\(^{-1}\) compared with 1.2 ± 0.03 s\(^{-1}\) for yTop2(Tyr28 → Phe).

As was seen with the human enzyme, the \( K_{m(app)} \) did not change significantly between the wild-type and mutant enzymes, nor did the addition of DNA significantly alter the \( K_{m(app)} \). Taken together, our results indicate that the mutation Tyr50 → Phe and its yeast counterpart do not change the apparent affinity of the enzyme for ATP, but likely change the rates of other steps of the reaction that influence the rate of ATP hydrolysis.

The ATP Hydrolysis Activities of hTOP2α(Tyr50 → Phe) and Its Yeast Counterpart Are Bisdioxopiperazine-resistant—We demonstrated previously that the hTOP2α(Tyr50 → Phe) mutant has bisdioxopiperazine-resistant relaxation activity (20, 24). We assessed the effect of bisdioxopiperazines on the ATP hydrolysis activity of the mutant and wild-type enzymes by examining the rate of ATP hydrolysis in the presence of increasing concentrations of the bisdioxopiperazine ICRF-187 at 1 mM ATP and 50 μg/ml linear DNA (Fig. 5). Under these conditions, human wild-type topoisomerase IIα had an almost 50% decrease in ATP hydrolysis activity with the addition of 5 μM ICRF-187. Higher concentrations of ICRF-187 continued to reduce the ATP hydrolysis activity of human wild-type topoisomerase IIα to a rate similar to that of DNA-independent ATP hydrolysis (Fig. 5A). The addition of up to 60 μM ICRF-187 to the ATP hydrolysis reaction catalyzed by the hTOP2α(Tyr50 → Phe) mutant caused no significant change in the ATPase activity of the enzyme (Fig. 5B). Yeast wild-type topoisomerase II ATPase appeared to be more resistant to ICRF-187, similar to what we reported for inhibition of relaxation of yeast topoisomerase II by bisdiox-
opiperazines (20). There was a 50% reduction in ATP hydrolysis activity with the addition of 20 \( \mu \)M ICRF-187 (Fig. 5C). However, similar to the hTOP2\(\alpha\)(Tyr\(^{50}\) → Phe) mutant, the ATP hydrolysis activity of yTop2(Tyr\(^{28}\) → Phe) was unaffected by the addition of up to 60 \( \mu \)M ICRF-187 (Fig. 5D). Combined with our earlier findings of bisdioxopiperazine-resistant relaxation activity, these
results demonstrate that both the hTOP2α(Tyr50 → Phe) and yTop2(Tyr28 → Phe) mutants have catalytic activities that are resistant to bisdioxopiperazines.

Vanadate Inhibits the ATP Hydrolysis and Relaxation Activities of Wild-type Topoisomerase II, but Not the hTOP2α(Tyr50 → Phe) Mutant—Vanadate (VO$_4^{3-}$) inhibits P-type ATPases through the formation of a ternary enzyme-ADP-V$_i$ complex that presumably mimics the enzyme-ATP transition state. Previous studies with vanadate demonstrated noncompetitive inhibition of yeast topoisomerase II after hydrolysis of the first ATP molecule (35). Kinetically, topoisomerase II inhibition by vanadate parallels inhibition by bisdioxopiperazines, although the formation of a salt-stable complex in the presence of vanadate has not been examined previously. Given the similarities of the kinetic effects of bisdioxopiperazines and vanadate, we assessed the effect of vanadate on the ATP hydrolysis activity of the hTOP2α(Tyr50 → Phe) mutant. Vanadate was a potent inhibitor of the ATP hydrolysis of human wild-type topoisomerase IIα, reducing the ATP hydrolysis activity by 50% at 5 μM (Fig. 6A). However, no change in the ATP hydrolysis activity of the hTOP2α(Tyr50 → Phe) mutant was observed with the addition of up to 40 μM vanadate (Fig. 6B). The ATP hydrolysis activity of yeast wild-type topoisomerase IIα was also inhibited by vanadate (Fig. 6C), as has been reported previously (35); although similar to what we observed with ICRF-187, the yeast enzyme was more resistant, requiring 20 μM vanadate for a 50% reduction in ATP hydrolysis activity. Similar to what we observed with the hTOP2α(Tyr50 → Phe) mutant, the ATP hydrolysis activity of the yTop2(Tyr28 → Phe) mutant was unaffected by the addition of up to 40 μM vanadate.

We analyzed the effects of vanadate on the relaxation activity of the wild-type and mutant enzymes (Fig. 7). The relaxation of supercoiled pUC18 catalyzed by 1 unit of human wild-type topoisomerase IIα was inhibited by a vanadate concentration of 25 μM (Fig. 7A). Under the same conditions, the relaxation activity of the hTOP2α(Tyr50 → Phe) mutant was unaffected by the presence of up to 100 μM vanadate (Fig. 7B). Inhibition of the relaxation activity by 1 unit of yeast wild-type topoisomerase II required higher concentrations of vanadate compare with the human wild-type enzyme (50 μM vanadate). Nonetheless, under the same conditions, the yTop2(Tyr28 → Phe) mutant showed no reduction in relaxation activity with the addition of 100 μM vanadate. These results demonstrate that, similar to bisdioxopiperazines, vanadate inhibits both the ATP hydrolysis and relaxation activities of yeast and human topoisomerase II. The hTOP2α(Tyr50 → Phe) and yTop2(Tyr28 → Phe) mutants have catalytic activities resistant to both bisdioxopiperazines and vanadate.

\( hTOP2α(Tyr50 → Phe) \) Does Not Form Salt-stable Complexes in the Presence of Vanadate or ADP, but Does Form a Stable Closed Clamp with ADPPNP—We demonstrated that the hTOP2α(Tyr50 → Phe) and yTop2(Tyr28 → Phe) mutants display bisdioxopiperazine- and vanadate-resistant catalytic activities. Because of the parallels between bisdioxopiperazine and vanadate inhibition of topoisomerase II, we investigated the ability of vanadate to stabilize the closed clamp form of topoisomerase II using a filter binding assay (Fig. 8). In this assay, topoisomerase II was incubated with supercoiled plasmid (pUC18) in the presence of a nucleotide cofactor. In the absence of any inhibitor, DNA did not bind to the glass fiber filter and passed through the filter in the flow-through and low salt washes. When topoisomerase II was incubated with ATP and an inhibitor such as bisdioxopiperazine or a non-hydrolyzable ATP analog, the enzyme trapped the DNA in a salt-stable closed clamp, retaining the DNA through both low and high salt washes. The DNA was eluted from the filter by denaturing the enzyme with an SDS wash. When human wild-type topoisomerase IIα was incubated in the presence of ATP and 300 μM vanadate, DNA eluted both with the addition of a high salt.
buffer and with the addition of SDS (Fig. 8). By contrast, when the reaction was carried out in the presence of ADPPNP, a non-hydrolyzable ATP analog, DNA was detected in the SDS wash, but not in the high salt wash. This suggests that the closed clamp form has reduced salt stability compared with the bisdioxopiperazine-stabilized form of the enzyme.

When hTOP2α(Tyr50→Phe) was incubated with only ATP, all of the DNA eluted with the addition of a low salt buffer, similar to what was seen with the wild-type enzyme. In the presence of ADPPNP, hTOP2α(Tyr50→Phe) formed a salt-stable complex with DNA as shown by the presence of DNA in the SDS wash. However, no bands were observed to elute with either a high salt or SDS wash when the hTOP2α(Tyr50→Phe) mutant was incubated with ATP and 300 μM vanadate, indicating that vanadate does not lead to the production of salt-stable complexes.

In addition to ADPPNP, ADP has been demonstrated previously to stabilize the closed clamp form of human topoisomerase II (22). We investigated the ability of ADP to stabilize the closed clamp form of hTOP2α(Tyr50→Phe) (Fig. 9). As in the experiment shown in Fig. 8, ATP alone did not lead to retention of DNA on the glass fiber filters as a salt-stable complex with topoisomerase II, whereas DNA was detected in the SDS wash when the enzyme was incubated with ADPPNP. In the presence of ADP, DNA eluted in both the high salt and SDS washes with human wild-type topoisomerase IIα, whereas no DNA was detected in the SDS wash when the hTOP2α(Tyr50→Phe) mutant was incubated with ADP. This result demonstrates that hTOP2α(Tyr50→Phe) cannot be trapped by ADP in a salt-stable complex and is therefore resistant to this "inhibitor."

We also tested the ability of vanadate and ICRF-187 to stabilize the closed clamp form of yeast topoisomerase II and the yTop2(Tyr28→Phe) mutant (Fig. 10). In the presence of the enzyme alone or the enzyme and ATP, no DNA was detected in the SDS wash. For simplicity, in the experiment shown in Fig. 10, the low and high salt washes were combined and analyzed together. The addition of ADPPNP to either the yeast wild-type enzyme or the yTop2(Tyr28→Phe) mutant resulted in DNA eluting with the addition of SDS. Incubation of yeast wild-type topoisomerase II with vanadate and ATP or ICRF-187 and ATP also resulted in formation of salt-stable complexes as indicated by the presence of DNA eluting with the addition of SDS. In contrast, incubation of the yTop2(Tyr28→Phe) mutant with either vanadate and ATP or ICRF-187 and ATP resulted in all of the DNA eluting in the salt washes.

Incubation of wild-type topoisomerase II with bisdioxopiperazines and ATP, vanadate and ATP, or ADP produced topologically trapped complexes that were eluted only when the protein was denatured by SDS. This was similar to what was observed when the enzyme was incubated with ADPPNP. The hTOP2α(Tyr50→Phe) and yTop2(Tyr28→Phe) mutants were unable to form salt-stable complexes in the presence of vanadate and ATP or ADP, but were stabilized by ADPPNP. The resistance of the hTOP2α(Tyr50→Phe) and yTop2(Tyr28→Phe) mutants to bisdioxopiperazines, vanadate, and ADP, all of which stabilize the closed clamp form of the wild-type enzymes, suggests a difference in the stability of the closed clamp formed by the mutants. This difference in stability is most likely responsible for the resistance of the catalytic activities of

![Fig. 8. Vanadate stabilizes the closed clamp form of wild-type hTOP2α, but not hTOP2α(Tyr50→Phe). Salt-stable protein-DNA complexes formed by wild-type hTOP2α or hTOP2α(Tyr50→Phe) were assessed using a filter binding assay under the reaction conditions described under "Experimental Procedures." Wild-type hTOP2α was incubated with DNA in the presence of ATP alone, 300 μM vanadate and ATP, or ADPPNP. Bands corresponding to the formation of salt-stable topological complexes were observed when wild-type hTOP2α was incubated either with vanadate plus ATP or with ADPPNP. hTOP2α(Tyr50→Phe) was incubated with DNA in the presence of ATP alone, vanadate and ATP, or ADPPNP. The formation of salt-stable topological complexes was detected only in the presence of ADPPNP. F/L, flow-through and low salt washes; H, high salt wash; SDS, SDS wash.](http://www.jbc.org/)

![Fig. 9. ADP stabilizes the closed clamp of wild-type hTOP2α, but not hTOP2α(Tyr50→Phe). Salt-stable protein-DNA complexes formed by wild-type hTOP2α or hTOP2α(Tyr50→Phe) were assessed using a filter binding assay under the reaction conditions described under "Experimental Procedures." Wild-type hTOP2α (A) or hTOP2α(Tyr50→Phe) mutant (B) was incubated with 2 mM ATP, 4 mM ADPPNP, or 3 mM ADP as indicated. Closed clamp formation as determined by the appearance of DNA bands in the SDS wash was observed with wild-type hTOP2α incubated in the presence of ADP or ADPPNP, whereas the hTOP2α(Tyr50→Phe) mutant formed salt-stable topological complexes only in the presence of ADPPNP. F/L, flow-through and low salt washes; H, high salt wash; SDS, SDS wash.](http://www.jbc.org/)
Bisdioxopiperazines exert two biochemical effects on eukaryotic topoisomerase II. These drugs partially inhibit ATP hydrolysis by the enzyme and trap the enzyme in a salt-stable closed clamp conformation. Since we had characterized several mutant Top2 enzymes that confer bisdioxopiperazine resistance in both yeast and mammalian cells and that display bisdioxopiperazine resistance in vivo (24, 25), we examined whether bisdioxopiperazine-resistant mutants have altered ATPase activity. Although our initial analyses of mutant enzymes used mammalian TOP2 activity. As described above, the mutations affect the stability of the closed clamp conformation and also have the unexpected property of severely reducing DNA-stimulated ATPase activity. It has been suggested previously that DNA-stimulated ATPase activity is not essential for enzyme activity. Mutations have been identified in DNA gyrase with substantially reduced DNA-stimulated ATPase activity (38). A hTOP2α heterodimer with a single ATP-binding site was found to be able to carry out multiple rounds of relaxation, although with a dramatic reduction in activity compared with the wild-type protein (39). The ATPase activity of the heterodimeric protein lacks DNA stimulation, indicating that both subunits of the holoenzyme must be competent to bind ATP for DNA stimulation to occur.

How might the stability of the closed clamp form influence DNA stimulation of the ATPase activity of topoisomerase II? We suggest that, upon dimerization of the N-terminal gate, DNA cleavage and strand passage lead to hydrolysis of one ATP molecule, followed by release of ADP and P_i. This is in accord with the observation of Lindley and co-workers (40) that the ATP hydrolysis by the two subunits of the enzyme is sequential rather than simultaneous. The hydrolysis of the first ATP molecule does not lead to the dissociation of the N-terminal dimerization, but requires hydrolysis and release of the second bound ATP molecule. We suggest that, after release of the first ADP molecule, another molecule of ATP can bind,
maintaining the closed clamp in a stable form. The enzyme may carry out multiple rounds of ATP binding, hydrolysis, and release prior to clamp opening provided that the other subunit maintains a bound nucleotide. Upon ADP release when the second subunit does not contain a bound nucleotide, dissociation of the N-terminal dimerization proceeds rapidly, and the clamp opens. We suggest that the stability of the clamp with a bound G-segment is greater than that of the clamp formed in the absence of DNA. In the absence of DNA, hydrolysis of the first ATP molecule would be rapidly followed by hydrolysis of the second ATP molecule and clamp re-opening. By contrast, a bound G-segment results in a clamp of greater stability, allowing re-binding of ATP and additional rounds of ATP hydrolysis. We suggest that these additional cycles of ATP hydrolysis constitute the DNA stimulation of the ATPase activity.

The difference in the stability of the clamps formed in the presence versus the absence of DNA may explain why the enzyme carries out multiple rounds of ATP hydrolysis per catalytic cycle. If the enzyme forms a closed clamp in the absence of DNA, slow re-opening of the clamp would prevent the enzyme from associating with DNA. Thus, a biological rationale for our model may be to enhance the ability of the enzyme to effectively interact with DNA in an environment of high ATP concentration.

This model is consistent with several observations reported here. In the presence of vanadate, the ADP bound to the wild-type enzyme can be trapped prior to dissociation. This traps the enzyme as a closed clamp and inhibits further ATP hydrolysis. The reduced stability of the closed clamp with the mutant enzyme leads to a more rapid release of ADP, preventing effective vanadate binding and trapping of the ADP prior to release. Similarly, ADP would be unable to effectively trap the enzyme as a closed clamp because the reduced clamp stability would promote dissociation of ADP. Alternatively, reduced affinity for ADP would destabilize protein-protein interactions at the dimer interface, as suggested above.

This model is also consistent with several previous observations relating ATP hydrolysis to enzyme efficiency (13). Lindley and Wang (13) found that, at low ATP concentrations, Top2 carries out very efficient strand passage, with one DNA strand passage event per two ATP molecules hydrolyzed (one ATP molecule hydrolyzed per subunit). At high concentrations, ATP utilization was substantially greater, up to 20 ATP molecules hydrolyzed per subunit per strand passage event. In our model, at low ATP concentrations following hydrolysis of the first ATP molecule and release of ADP, there would be a very low probability of a second ATP molecule binding prior to hydrolysis of the second bound ATP molecule. This would lead to high efficiency of strand passage per ATP hydrolyzed. In the presence of high ATP concentrations, multiple rounds of ATP hydrolysis per strand passage event would occur, leading to lower catalytic efficiency.

Further support for this model comes from observations by Andersen and co-workers (39), who studied heterodimeric Top2 proteins with only a single ATP-binding site. These heterodimeric proteins are enzymatically active, albeit with reduced activity. Importantly, the heterodimers can carry out multiple rounds of strand passage, indicating that a single ATP-binding site is sufficient for enzyme turnover. Interestingly, Andersen and co-workers also found that DNA fails to stimulate the ATPase activity of heterodimeric Top2. When only a single ATP can bind to the protein, hydrolysis of the ATP and ADP release would rapidly lead to destabilization of the N-terminal dimerization and clamp opening. There would be no opportunity for multiple cycles of ATP hydrolysis per strand passage event. Notably, Andersen and co-workers have reported an insertion mutant at position 351 of human topoisomerase IIα that specifically prevents strand passage and also has reduced DNA stimulation. Similarly, mutations of the active-site tyrosine of yeast or human topoisomerase II that result in enzymes that are unable to cleave DNA also substantially reduce (but do not eliminate) DNA stimulation of the ATPase activity (41). These results suggest that the multiple cycles of ATP hydrolysis occur concurrent with or subsequent to DNA strand passage and that an inability to carry out strand passage substantially prevents DNA stimulation of ATP hydrolysis.

Of the two mutants analyzed in detail here, the yeast enzyme has an almost complete lack of DNA stimulation of its ATP hydrolysis activity, even though it retains substantially wild-type DNA relaxation activity. This mutant therefore differs from the active-site tyrosine mutant (41) and the mutants in the transducer domain described by Andersen and co-workers (42) in having wild-type strand passage activity. Although strand passage appears to be a prerequisite for full DNA stimulation of topoisomerase II ATPase activity, the DNA-stimulated ATPase activity is not required for normal strand passage. Several other bisdioxopiperazine-resistant mutants that have reduced closed clamp formation in the presence of bisdioxopiperazines have been described previously (24–26). It will be of considerable interest to determine whether these mutants also have alterations in DNA-stimulated ATPase as well as resistance to vanadate. A tight connection between insensitivity to agents that stabilize the closed clamp conformation of the enzyme and loss of DNA-stimulated ATPase in these mutants will provide additional support for our model.

Alternative models for our observation would require that the mutants we have described have altered DNA recognition. This could occur by the N-terminal domain interacting with one of the two strands of the Top2 reaction. However, no obvious DNA-binding domain is seen in the structures described by Berger and co-workers (10). Furthermore, in previous work (43), we did not observe differences in the DNA binding of the Tyr50→Phe mutant compared with the wild-type enzyme. These considerations suggest that the DNA-stimulated ATPase arises indirectly through steps occurring during the catalytic cycle and that the steps leading to DNA stimulation are not essential for strand passage or for the biological function of topoisomerase II.

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Stability of the Topoisomerase II Closed Clamp Conformation May Influence DNA-stimulated ATP Hydrolysis
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