Interaction of Human DNA Topoisomerase II α with DNA: Quantification by Surface Plasmon Resonance†

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ABSTRACT: DNA topoisomerase II is an ATP-operated clamp that effects topological changes by capturing a double-stranded DNA segment and transporting it through another duplex. Surface plasmon resonance (SPR) was used to characterize interactions of human topoisomerase II α with different topological forms of DNA. Using a linear fragment of pUC18 DNA, the equilibrium binding constant of topoisomerase II α was determined to be 0.16 nM. The affinity was not affected by the absence of ATP or the presence of the bisdioxopiperazine catalytic inhibitor ICRF-187. Besides, similar affinities were found for several bisdioxopiperazine-resistant mutant enzymes. These results suggest that the mechanism of topoisomerase II α inhibition by ICRF-187 and its resistance does not directly involve the interaction of DNA with the enzyme. SPR was also adapted to measure levels of the closed clamp form of topoisomerase II present on DNA. As expected, a stable closed clamp form of the enzyme was detectable on circular DNA but not on linear DNA. Detection of the closed clamp required the presence of ATP and a bisdioxopiperazine, or a non-hydrolyzable analogue of ATP. In the presence of ATP and ICRF-187, several bisdioxopiperazine-resistant mutant enzymes failed to form detectable levels of stable closed clamp. Interestingly, a mutant of human topoisomerase II α with an altered active site tyrosine showed lower levels of closed clamp formation. In conclusion, SPR is able to (1) determine the kinetics of topoisomerase II with its DNA substrate and (2) quantify the enzyme's closed clamp formation under varying circumstances.

DNA topoisomerases are nuclear enzymes that change the topological conformations of DNA (*1*, *2*). Eukaryotic topoisomerase II is a homodimer that passes one DNA duplex through a transient enzyme-bridged double-strand break in a second DNA segment, changing the linking number of DNA in steps of two. Mammalian topoisomerase II exists in two isozymes: a 170 kDa α isoform that is preferentially expressed in proliferating cells, and a 180 kDa *β* isoform found in both proliferating and nonproliferating cells.

In addition to the essential roles of topoisomerases in DNA metabolism, these enzymes are also the targets for several anticancer and antibacterial agents. There are two general classes of topoisomerase II inhibitors that interfere with different aspects of the enzyme cycle (*3*). Topoisomerase II poisons, such as doxorubicin, etoposide, and amsacrine, stabilize an enzyme−DNA cleavable complex that leads to irreversible DNA breaks and cell death (*4*). Topoisomerase II poisons can lead to elevated levels of covalent complexes either by inhibiting enzyme-mediated religation or by increasing the rate of cleavage by the enzyme. A wide body of evidence strongly suggests that the levels of topoisomerase II covalent complexes formed in the presence of topoisomerase II poisons, rather than inhibition of enzyme activity, is the major determinant of the cytotoxic potential of these agents.

The second major class of drugs is catalytic inhibitors of topoisomerase II (*5*). As these agents do not block the enzyme at a point where the DNA strands are broken, it is believed that their mechanism of cytotoxicity is due to the reduction of the essential activity provided by the enzyme. Catalytic inhibitors include agents that competitively inhibit ATP binding such as novobiocin and coumermycin A1 (*6*), agents that block DNA binding such as strong intercalating agents (*7*, *8*), and compounds such as merbarone that do not block DNA binding but inhibit DNA cleavage (*9*, *10*). A novel class of catalytic inhibitors are the bisdioxopiperazines, such as ICRF-187 and ICRF-193. They lock topoisomerase II as a closed clamp around DNA by stabilizing N-terminal dimer interaction formed upon ATP binding (*11*). Thus, the bisdioxopiperazines trap eukaryotic topoisomerase II at a specific point in their catalytic cycle and have also been suggested to have a unique mechanism of cytotoxicity (*12*). It has been hypothesized that the closed clamp form of topoisomerase II interferes with DNA metabolism when stabilized by bisdioxopiperazines. This hypothesis suggests that levels of closed clamps may be an important determinant of bisdioxopiperazine action.

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Table 1: Primers Used in Oligonucleotide-Directed Mutagenesis

primers	sequences[a]
Y50F-SN	CCG CCC AGA CAC CTT CAT TGG TTC TGT GG
Y50F-ASN	CCA CAG AAC CAA TGA AGG TGT CTG GGC GC
Y165S-SN	GGT CGA AAT GGC TCT GGA GCC AAA TTG TG
Y165S-ASN	CAC AAT TTG GCT CCA GAG CCA TTT CGA CC
L169F-SN	GGC TAT GGA GCC AAA TTT TGT AAC ATA TTC AGT
L169F-ASN	GGT ACT GAA TAT GTT ACA AAA TTT GGC TCC ATA GCC

[a] The primers are presented in the 5′ to 3′ direction. The single nucleotide change responsible for introduction of the mutation in the htopoIIα is underlined.

Several approaches have been described to measure topoisomerase II DNA closed clamps, including filter binding (*11*), equilibrium ultracentrifugation (*13*, *14*), and binding of topoisomerase II to beads carrying covalently closed DNA (*15*). Recently, new approaches have been described for quantitating macromolecular interactions. One such technology, based on surface plasmon resonance (SPR),[1] can sensitively detect changes in mass that occur when a macromolecule binds to a ligand. In this study, we have applied SPR as implemented by the Biacore© system to analyze the interaction of human topoisomerase II α (htopoIIα) with DNA. We first determined the apparent kinetic and equilibrium binding constants both for wt htopoIIα and for several mutants selected for resistance to bisdioxopiperazines. We also describe conditions for quantitating the binding of topoisomerase II to circular DNA and for measuring levels of the closed clamp form of topoisomerase II. We find that several bisdioxopiperazine-resistant mutants of htopoIIα, as well as an active site mutant, form substantially reduced levels of stable closed clamps in the presence of bisdioxopiperazines. These results may indicate that closed clamp formation plays a role in bisdioxopiperazine-mediated toxicity.

EXPERIMENTAL PROCEDURES

Instrumentation and Reagents. A BIACORE 3000 was used to perform all binding studies. Sensor Chip SA and HBS-EP buffer were from Biacore AB (Uppsala, Sweden). ICRF-187 was obtained from Chiron Corp. (Amsterdam, The Netherlands), dissolved in sterile distilled water and stored at − 80 °C.

Induction and Expression of WT and Mutant htopoIIα in Yeast Cells. For expression and purification of htopoIIα, we used a modification of the protocol in (*16*). The htopoIIα cDNA was in the expression vector YEpWOB6 controlled by a galactose-inducible GAL promoter (*17*). Both wt and mutant YEpWOB6 were transformed to the protease-deficient yeast strain Jel1Δtop1 (*trp1*, *leu2*, *ura-52*, *pbr1*−*1122*, *pep4*−*3*, Δ*his3::PGAL10-GAL4*, *top1::LEU2*).

Construction of Mutations in HtopoIIα. The mutations Y50F, Y165S, and L169F were reconstructed in the plasmid YEpWOB6 (*17*) by oligonucleotide-directed mutagenesis using a Quick change site-directed mutagenesis kit (Stratagene) as previously described (*18*) using the mutagenic primers depicted in Table 1.

[1] Abbreviations: AMPPNP, adenosine 5′-(*β*, *γ*-imidotriphosphate); bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; htopoIIα, human topoisomerase II α; PCR, polymerase chain reaction; RU, resonance unit; SPR, surface plasmon resonance; wt, wild type.

Table 2: Oligonucleotide Primers Used for Construction of the PUC18 Fragment

fragments	sequences[a]
85 bp	CCC GAA GAA CGT TTT CCA ATG ATG AGC
110 bp	CTG GAT CTC AAC AGC GGT AAG ATC C
166 bp	GAT GCT GAA GAT CAG TTG GGT GC
323 bp	GTA TCC GCT CAT GAG ACA ATA ACC CTG

[a] The primers are presented in the 5′ to 3′ direction.

DNA Preparation. Biotinylated DNA suitable for immobilization to the sensor surface via a streptavidin linkage was used. The linear DNAs were 85, 110, 166, or 323 bp fragments from plasmid pUC18. Each fragment was amplified by PCR from the pUC18 plasmid using the same 5′-biotinylated primer 5′-gctcttgcccggcgtcaatacggg-3′. Table 2 lists the second primer used for each fragment. The pUC18 fragments were purified from a 4% agarose gel by centrifuge tube filters (0.22 μm cellulose acetate, Corning Inc.), followed by ethanol precipitation (*19*). The circular DNA was a Biotin-Labeled pGeneGrip blank plasmid synthesized by Gene Therapy Systems Inc. (San Diego). pGeneGrip is a 5 kb plasmid which is efficiently and irreversibly labeled with Biotin at one known position on the DNA molecule without changing its supercoiled structure and transcriptional activity.

Immobilization of Biotinylated DNA on the Sensor Surface. The streptavidin matrix-coated Sensor Chip SA (Biacore Inc.) was preconditioned with three consecutive 10 μL injections of 1 M NaCl in 50 mM NaOH and equilibrated with HBS-EP buffer (10 mM HEPES, pH 7.4, 100 mM potassium acetate, 10 mM magnesium acetate, and 0.005% P20) at a flow rate of 10 μL/min. The biotinylated circular and linear DNAs in HBS-EP buffer containing 0.5 M NaCl were then injected at a flow rate of 5 μL/min for 5 min onto the corresponding flow cell (20 °C). The final change in resonance units (RU) following immobilization was approximately 300 RU for the circular DNA and 100−125 RU for the linear DNA. Flow cell 1, and in some cases flow cell 3 as well, was left unmodified to be used as a reference surface.

Establishment of Conditions for SPR Measurements with htopoIIα. To identify the optimal conditions for SPR measurements, we carried out a series of control experiments. To determine the effects of the transport of the enzyme to the DNA, control binding experiments were carried out at flow rates from 5 to 75 μL/min, with 30 μL/min showing acceptable minimization of mass transport effects. Only the small linear DNAs were used for the kinetic measurements. To further minimize mass transport limitations, the amount of the linear DNA immobilized to the chip was held to less than approximately 125 RU. Control experiments were also

carried out under a range of different temperatures and buffer conditions. Buffer compositions that were tested were conditions where the relaxation activity of the enzyme (in the presence of ATP) was within 50% of previously determined values. With buffer conditions that allowed normal enzyme activity, nonspecific binding (binding to a chip that had no DNA immobilized) was less than 15% only at temperatures up to 20 °C (data not shown). A range of enzyme concentrations was also examined, and nonspecific binding did not exceed 15% as long as the enzyme concentration did not exceed 20 nM.

Binding Studies. The Biacore flow rate was maintained at 30 μL/min at a temperature of 20 °C. The running buffer was 10 mM Tris-HCl, pH 7.6, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, and 30 μg/mL BSA. The purified protein was injected using the Biacore program "Kinject" in 100 μL running buffer at the beginning of the run. Buffers also included 1 mM ATP, 1 mM AMPPNP, and/or ICRF-187 where indicated. The association phase with protein solutions was monitored for 2 min; then running buffer was applied, and the dissociation phase was followed for 2−3 min. To identify the formation of the closed clamp intermediate of the protein, the protein−DNA complex was washed with 30 μL of 1 M KCl. Following the completion of each protein binding analysis, the surface of the chip was regenerated by injecting several times 30 μL of 0.5% SDS, which releases all bound protein without affecting the binding capacity of the immobilized DNA.

Data Analysis. Data are presented in RU, and all reported values were corrected by subtraction of the change of RU observed on the reference surface (flow cell 1 or 3) to correct for nonspecific binding and refractive index changes. For the kinetic measurements, a homogeneous 1:1 interaction on the sensor chip surface has been used and is described by the equation A + B ↔ AB, where A is htopoIIα and B is the surface-bound DNA. Assuming pseudo-first-order interaction kinetics, the rate of complex formation during sample injection is given by $d[AB]/dt = k_a[A][B] - k_d[AB]$, which is expressed in terms of the SPR signal as $dR/dt = k_aCR_{max} - (k_aC + k_d)R$, where dR/dt is the rate of change of the SPR signal, C is the htopoIIα concentration, R_{max} is the maximum protein binding capacity in RU, and R is the SPR signal in RU at time t. For the pseudo-first-order model, the dissociation phase is described by $dR/dt = -k_dR$. The kinetic parameters k_a and k_d were calculated by nonlinear fitting of the sensorgrams at various htopoIIα concentration using the BIAevaluation software version 3.1 (Biacore AB). This software fits the experimental curves to rate equations derived from models of the interaction mechanism. A 1:1 Langmuir interaction with mass transfer model was used. The goodness of fit of the experimental data is assessed by Chi squared analysis, and the values were considered satisfactory if <15. The equilibrium parameter K_D was calculated using the relationship $K_D = k_d/k_a$. The amount of closed clamp formation (percentage closed clamp) was determined by comparison of the amount of protein bound to the DNA at the end of the association and after one wash with 1 M of KCl.

RESULTS

Characterization of the WT and Mutant Topoisomerase IIα (htopoIIα). We used a modification of the protocol of (*16*) to express htopoIIα in yeast and to purify the wt and mutant proteins (*20*, *21*). To study the action of ICRF-187, we have used three previously described htopoIIα mutants known to confer resistance to this compound, Y50F (*18*), Y165S (*22*), and L169F (*23*). We also constructed a mutation changing the tyrosine involved in transesterification (Y805) to Phe to generate a mutant enzyme that was unable to cleave DNA. The enzymes were purified to >95% homogeneity (examples are shown in Figure 1). Their activities (except for the Y805F mutant) were similar to wt enzyme (data not shown), indicating that the native activity of htopoIIα is not significantly altered by these mutations.

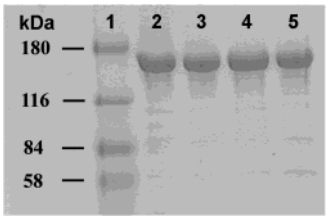

FIGURE 1: SDS−polyacrylamide gel electrophoresis analysis of highly purified htopoIIα proteins. The same amounts of each protein (10 μg) were denatured in a loading buffer containing SDS and subjected to electrophoresis on a 7% SDS−polyacrylamide gel. The protein was detected by Coomassie blue staining. Size markers (lane 1) have molecular masses of the band indicated in the left margin. Y165S mutant (lane 2 and 3) and wt (lane 4 and 5) htopoIIα obtained by two different purifications are seen as a 170 kDa band.

Binding of htopoIIα to pUC18 Fragment. We first examined the interaction of htopoIIα with a 166 bp fragment of pUC18 plasmid. The binding studies all utilized chips to which 5′ biotinylated DNA was immobilized on a streptavidin-coated surface. In the association phase of the experiment, a solution containing htopoIIα was introduced at a constant concentration and in the presence or absence of 1 mM ATP into the running buffer flow, and the progress of complex formation at the sensor surface was continuously monitored. After 2 min, the htopoIIα solution was replaced with running buffer lacking enzyme and the dissociation of the enzyme from the DNA recorded for 2−3 min. At the end of the dissociation phase, the sensor surface was regenerated to remove the remaining enzyme.

The association and dissociation of the enzyme was first examined as a function of htopoIIα concentration. A typical experimental run is illustrated in Figure 2. The data from these curves were used to calculate the rate constants (k_a and k_d), and the equilibrium binding constant (K_D) of the interaction of topoIIα and DNA. From analyses similar to Figure 2, we calculated a k_a of $9.10 \pm 4.50 \times 10^6$ M^{-1} s^{-1}, a k_d of $1.50 \pm 0.65 \times 10^{-3}$ s^{-1}, and a K_D of $1.65 \pm 0.65 \times 10^{-10}$ M.

Binding of htopoIIα to DNA Substrates of Differing Lengths. Similar sensorgrams to those shown in Figure 2 were obtained when pUC18 fragments of sizes ranging from 85 to 323 bp were used as immobilized substrates (data not shown). Kinetic parameters and the affinities of the interaction between htopoIIα and these other DNA fragments were similar, with no more than a 2-fold variation between DNA fragments of differing sizes (data not shown). The SPR response is related to the change in surface mass concentration of analyte and therefore depends on the molecular weight of the analyte in relation to the number of ligand sites on the surface. The stoichiometry of topoisomerase II binding

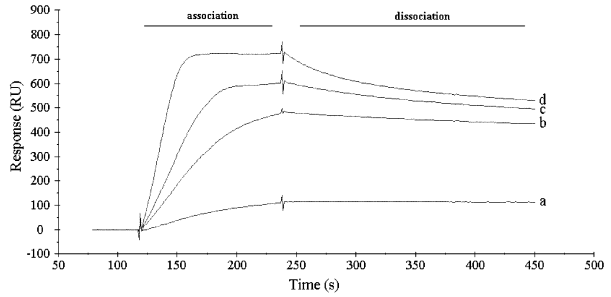

FIGURE 2: Example of a sensorgram used to obtain the kinetic parameters k_a and k_d and the equilibrium parameter K_D for the interaction of htopoIIα with linear DNA. The immobilization of a 166 bp pUC18 fragment to a SA sensor chip and the binding studies were performed as described in "Experimental Procedures". The wt htopoIIα was injected at concentrations of 1 (curve a), 5 (curve b), 10 (curve c), and 20 nM (curve d) dimer in the presence of 1 mM ATP. In each case, the signal is proportional to the enzyme concentration, i.e., the largest signal corresponds to the highest enzyme concentration and vice versa.

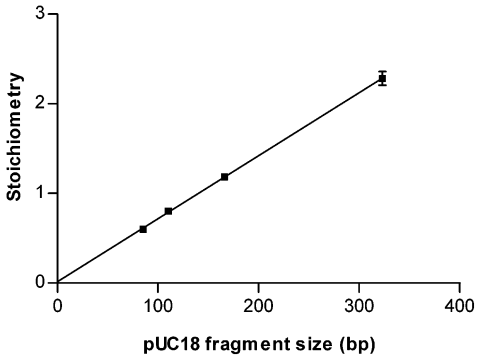

FIGURE 3: Numbers of htopoIIα bound to the DNA as a function of immobilized DNA fragment size. Immobilization and binding studies were performed as described in "Experimental Procedures". The wt htopoIIα was injected at concentrations of 1, 5, and 10 nM dimer in the presence of 1 mM ATP onto the surface, on which 100−125 RU of the pUC18 fragment had been immobilized. The R_{max} values used for the determination of stoichiometry were obtained from the BIAevaluation software. Each data point represents the average of two to five determinations.

to DNA can be calculated by the following equation (*24*): stoichiometry $S = R_{max}/RU_{DNA} \times$ DNA MW/htopoIIα MW $\times$ 0.8, where S is the number of analyte molecules which can bind to one ligand molecule, R_{max} is the maximum binding capacity of the surface ligand for the analyte in RU, RU_{DNA} is the increase in RU value upon binding of the DNA to the streptavidin surface, and MWs are the molecular weights of the DNA and the protein. The BIAevalution software was used to calculate the R_{max} for the different sized DNA fragments, and Figure 3 shows the stoichiometry calculated for each pUC18 fragment. The average number of htopoIIα holoenzymes that can bind to one ligand molecule was proportional to the pUC18 fragment size and ranged from 0.60 ± 0.01 for an 85 bp fragment to 2.28 ± 0.15 for a 323 bp fragment. The linearity over the entire range, with the calculated regression line going through zero, suggests that the binding of the oligonucleotide to the chip surface does not have a major influence on the calculated stoichiometry per base pair. This determination suggests that a DNA length of approximately 140 bp is required for optimal binding by topoisomerase II.

We also examined the association and dissociation of the enzyme with a 5 kbp circular DNA as the function of the htopoIIα concentration in order to determine the kinetic parameters, the affinity and the stoichiometry of the interaction (data not shown). However, when using this plasmid DNA, the goodness of fit observed was very poor being around 10 000 in repeated experiments. Therefore, this DNA could not be used in kinetic measurements. The reason for the large variation using this plasmid DNA may be that a large population of htopoIIα molecules interacts with the DNA at any given time, which could perturb the kinetic measurements.

Effect of ICRF-187 on the DNA Binding of htopoIIα. We next examined whether the topoisomerase II catalytic inhibitor, ICRF-187 affected the interaction of htopoIIα with linear DNA under our experimental conditions. We determined the kinetic and affinity constants of the binding of the wt enzyme with pUC18 fragments in the presence or absence of 1 mM ATP and in the presence or absence of 500 μM ICRF-187. No significant differences in kinetic or equilibrium binding constants were observed (data not shown). We also determined the kinetic and affinity constants of three mutants of htopoIIα that had been demonstrated to have ICRF-187-resistant catalytic activity (Y50F, Y165S, and L169F). For all three mutant proteins, we found constants similar to those observed with the wt enzyme (data not shown).

Quantitation of the Closed Clamp Form of htopoIIα in the Presence of ICRF-187. Bisdioxopiperazines stabilize topoisomerase II as a salt-stable clamp around DNA, preventing reopening of the enzyme clamp (*11*). We measured the level of the complex present between the enzyme and the DNA after association, followed by washing with 1 M KCl. Salt-stable complexes were assessed using the 166 bp linear pUC18 fragment and a covalently closed circular DNA. Figure 4A shows the sensorgram obtained with the linear DNA and Figure 4B shows the sensorgram with circular DNA. Following the KCl wash with linear DNA (Figure 4A), there is no difference in the level of bound topoisomerase II in the presence of ICRF-187 compared to the level seen in its absence. By contrast, a substantial level of topoisomerase II remains bound to the circular DNA substrate following the KCl wash in the presence, but not in the absence of ICRF-187 (Figure 4B). Setting the RU value at the end of the association phase as 100% binding, we calculated the percent enzyme bound after the KCl wash. For 500 μM ICRF-187, 75.5% ± 2.5% of htopoIIα remains bound to circular DNA, while 2.5% ± 2.5% of the enzyme remains bound to the linear DNA. Thus, a salt-stable closed clamp is formed in the presence of bisdioxopiperazines around circular, but not linear DNA.

We next examined the level of stable closed clamp formed as a function of enzyme or drug concentration. Figure 5 shows the level of wt htopoIIα bound to circular DNA as a salt-stable complex proportional to the ICRF-187 concentration. Increasing the drug concentration from 50 to 1000 μM increases the amount of closed clamp form. Binding saturates at approximately 500−1000 μM ICRF-187, with approximately 75−90% of the enzyme forming a salt-stable complex. The same percentages of salt-stable complexes were observed at differing enzyme concentrations (data not shown). These results suggest that nearly all of the bound htopoIIα can be trapped in a closed clamp form in the presence of a high concentration of ICRF-187.

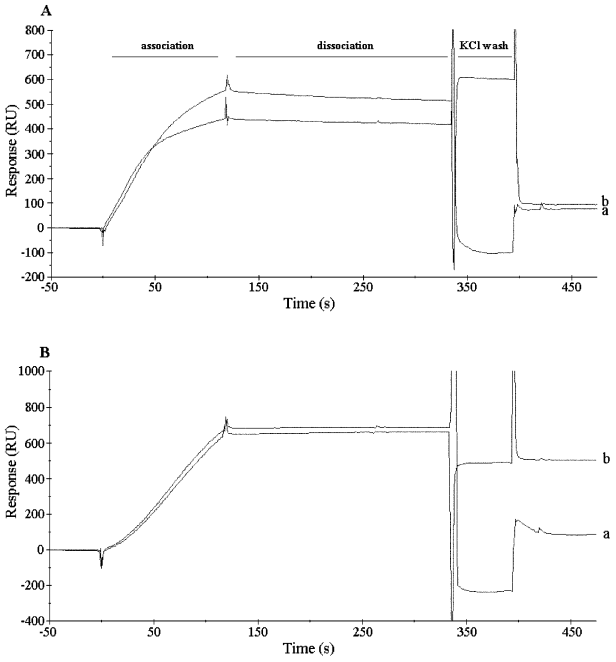

FIGURE 4: ICRF-187 effect on the binding of htopoIIα with linear DNA (A) and circular DNA (B) in the presence of 1 mM ATP. Immobilization and binding studies were performed as described in "Experimental Procedures". 125 RU of 166 bp pUC18 fragment (A) and 300 RU of 5kb-plasmid DNA (B) were immobilized on the sensor chip. The wt htopoIIα (10 nM) was injected over the DNA in the presence of 1 mM ATP (curve a) or 1 mM ATP and 500 μM ICRF-187 (curve b). Then, after association and dissociation, a 1 min injection of 1 M KCl was performed to investigate the stability of the complex between the enzyme and the DNA.

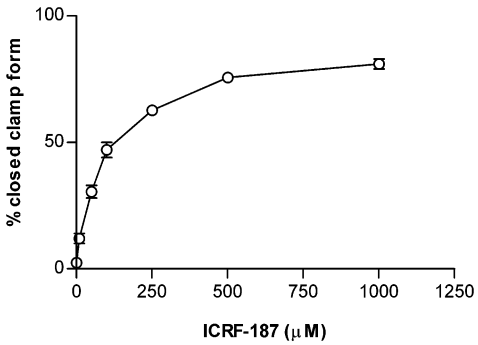

FIGURE 5: Determination of the amount of closed clamp intermediate formed with the wt htopoIIα in the presence of ICRF-187. Immobilization and binding studies were performed as described in "Experimental Procedures". The wt htopoIIα (10 nM) was injected over 300 RU of 5kb-plasmid DNA in the presence of 1 mM ATP and increasing concentrations of ICRF-187. Then, after association and dissociation, a 1 min injection of 1 M KCl was performed, and the amount of closed clamp intermediate was calculated in percentage. This value corresponds to the amount in RU of protein left after the KCl wash compared to the amount in RU of protein bound at the end of the association. Each data point represents the average of at least three determinations.

We next examined whether ICRF-187 was able to trap the different htopoIIα mutants known to be resistant to ICRF-187. Table 3 shows the levels of closed clamps formed with three mutant proteins, Y50F, Y165S, and L169F. In each case, detectable salt-stable closed clamps around the circular DNA were not observed in the presence of 1 mM ATP and 500 μM ICRF-187.

The formation of a salt-stable closed clamp by topoisomerase II in the presence of bisdioxopiperazines requires ATP (*11*). As described above, ATP binding does not affect the binding kinetics observed with linear DNA. To verify that the salt-stable complex detected using SPR showed the same behavior as the closed clamp intermediate detected using other approaches, we examined the stability of complexes formed by htopoIIα around the circular DNA in the presence of ICRF-187 and in the presence (Figure 6A) or absence (Figure 6B) of ATP. Figure 6A shows the presence of a salt-stable clamp, while htopoIIα was unable to form a stable closed clamp around the DNA when ATP was omitted (Figure 6B). From these results, we conclude that the salt-stable complex of topoisomerase II and circular DNA detected by SPR is indeed a closed clamp.

Table 3: Effect of AMPPNP, ATP, and ICRF-187 on the Closed Clamp Form of WT and Mutant HtopoIIα[a]

htopoIIα allele	% closed clamp		
	1 mM ATP	1 mM AMPPNP	1 mM ATP and 500 μM ICRF-187
WT	2.5 ± 2.5 ($n = 8$)	40 ± 2 ($n = 5$)	75.5 ± 2.5 ($n = 12$)
Y805F	10.5 ± 3.0 ($n = 3$)	45.0 ± 3.5 ($n = 4$)	27.5 ± 1.5 ($n = 3$)
Y50F	5 ($n = 1$)	23.5 ± 1.0 ($n = 3$)	<7
Y165S	5 ± 4 ($n = 4$)	16 ± 1 ($n = 3$)	<7
L169F	5 ± 4 ($n = 2$)	22.5 ± 4.0 ($n = 3$)	<7

[a] Immobilization and binding studies were performed as described in "Experimental Procedures". The htopoIIα enzyme (10 nM) was injected over 300 RU of 5kb-plasmid DNA in the presence of 1 mM ATP, 1 mM AMPPNP, or 1 mM ATP and 500 μM ICRF-187. Then, after association and dissociation, a 1 min injection of 1 M KCl was performed to investigate the stability of the complex between the enzyme and the DNA.

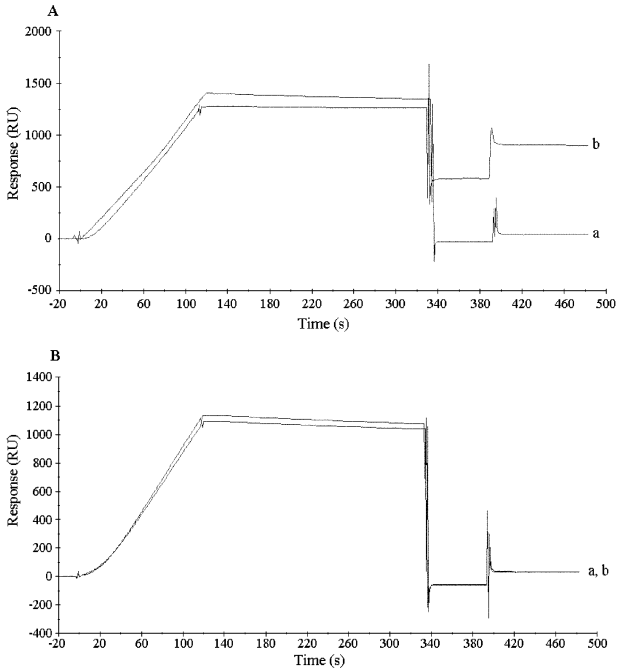

FIGURE 6: ICRF-187 effect on the binding of htopoIIα with circular DNA in the presence (A) or absence (B) of 1 mM ATP. Immobilization and binding studies were performed as described in "Experimental Procedures". The wt htopoIIα (10 nM) was injected over 300 RU of 5kb-plasmid DNA in the absence (curve a) or presence of 500 μM ICRF-187 (curve b) and in the presence of 1 mM ATP (A) or absence of ATP (B). Then, after association and dissociation, a 1 min injection of 1 M KCl was performed to investigate the stability of the complex between the enzyme and the DNA.

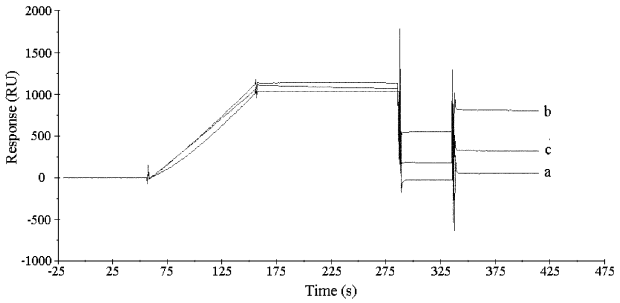

FIGURE 7: AMPPNP effect on the binding of htopoIIα with circular DNA. Immobilization and binding studies were performed as described in "Experimental Procedures". The wt htopoIIα (10 nM) was injected over 300 RU of 5kb-plasmid DNA in the presence of 1 mM ATP (curve a), 1 mM ATP and 500 μM ICRF-187 (curve b), or 1 mM AMPPNP (curve c). Then, after association and dissociation, a 1 min injection of 1 M KCl was performed to investigate the stability of the complex between the enzyme and the DNA.

Quantitation of the Closed Clamp Form of htopoIIα in the Presence of AMPPNP. In addition to bisdioxopiperazines, non-hydrolyzable ATP analogues such as AMPPNP are also able to trap eukaryotic topoisomerase II as a closed clamp (*25*, *26*). We next assessed the level of salt-stable closed clamps in the presence of different nucleotide cofactors. The salt-stable binding of wt htopoIIα with circular DNA was measured in the presence of 1 mM ATP (Figure 7, curve a), 1 mM ATP, and 500 μM ICRF-187 (Figure 7, curve b) or 1 mM AMPPNP (Figure 7, curve c). The percent closed clamp is summarized in Table 3. Efficient trapping of the wt enzyme is seen in the presence of 1 mM AMPPNP. Interestingly, the amount of salt-stable closed clamps (40±1%) is lower in the presence of 1 mM AMPPNP than the level obtained with 500 μM ICRF-187 in the presence of 1 mM ATP (75.5±2.5%). The quantity of closed clamp observed using 2.5 mM AMPPNP was similar to that observed with 1 mM AMPPNP, indicating that 1 mM AMPPNP probably results in maximal closed clamp formation (data not shown).

Table 3 also indicates the levels of salt-stable closed clamps obtained with bisdioxopiperazine-resistant htopoIIα mutants in the presence of 1 mM AMPPNP. For all three mutant proteins, Y50F, Y165S, and L169F, a reduced level of closed clamps was observed in the presence of AMPPNP, compared to the level seen with wt htopoIIα.

Mutating the Active-Site Tyrosine of htopoIIα Reduced Bisdioxopiperazine-Dependent Closed Clamp Formation. We have previously reported that expression of an active site mutation of htopoIIα did not result in bisdioxopiperazine sensitivity in yeast, whereas expression of the wt protein resulted in dominant bisdioxopiperazine sensitivity (*12*). We also found no qualitative difference in stable closed clamp formation between wt yeast topoisomerase II and an active site tyrosine mutant of the yeast enzyme. It was of interest therefore to assess the level of stable closed clamps formed by an active site mutation of htopoIIα. The quantitative SPR assay shows that mutating the active site tyrosine dramatically alters stable closed clamp formation (Table 3). Interestingly, in the presence of 1 mM ATP, the level of stable closed clamps formed by the Y805F mutant protein was significantly higher than that seen with the wt protein. The level of stable closed clamps was not significantly altered in the presence of AMPPNP, in agreement with results obtained by Lindsley and colleagues (*27*). The level of closed clamp formation was reduced in the presence of 1 mM ATP and 500 μM ICRF-187.

It is also important to note that no significant differences in kinetic or equilibrium binding constants were observed for the Y805F mutant compared to the wt enzyme (data not shown), as was also previously observed by Lindsley and colleagues using yeast topoisomerase II (*27*).

DISCUSSION

In this paper, we use surface plasmon resonance to measure the interactions of htopoIIα with DNA. One of our major goals is to understand how bisdioxopiperazines are able to trap topoisomerase II as a closed clamp around DNA, similar to what occurs when the enzyme binds to a non-hydrolyzable ATP analogue. We have previously described mutants in htopoIIα that are resistant to bisdioxopiperazines and wanted to develop an assay that would allow quantitation of the levels of closed clamps formed by the wt and mutant enzymes in the presence or absence of topoisomerase inhibitors. In this work, we demonstrate that SPR is a useful tool for examining the protein:DNA interactions of eukaryotic type II topoisomerases.

Before we could use SPR to examine the interactions of topoisomerase II with circular DNA, we needed to examine the interactions of the enzyme with linear DNA, both in the presence and in absence of topoisomerase II inhibitors. We were able to determine association and dissociation rate constants, which have not been previously reported for the interaction of topoisomerase II with DNA. From these constants, we also measured the equilibrium binding constant K_D to be 0.16 nM. This result shows that htopoIIα binds DNA with a very high affinity, which is coherent with its essential function in DNA metabolism. A K_D previously measured for yeast topoisomerase II by a filter binding approach was 100 fold lower than the one we found in this study (*27*). However, these two K_D have been measured by two different methods, which do not use the same equations to calculate the constant. The SPR method uses kinetic parameters with the equation $K_D = k_d/k_a$, while the filter binding method uses the DNA concentration with the equation $[DNA]_{bound}/[DNA]_{total} = [enzyme]/K_D + [enzyme]$ (*27*). In the simplest situation, the latter, also referred to as K_m, can be equal to the K_D identified by SPR, also known as K_s. However, care must be taken in the interpretation of the significance of K_m relative to K_s. Only when the complete reaction mechanism is known can the relationship between K_s and K_m be fully appreciated (*28*). The true affinity of the enzyme for its substrate is defined by K_s, and until now, only the SPR technique has been able to measure the real time kinetic parameters of the interaction of Toposiomerase II with DNA.

A further advantage of the Biacore system is its ability to easily examine several different DNA substrates. We applied this to determine the apparent stoichiometry of the binding of topoisomerase II to DNA molecules of different lengths (Figure 3). We calculated a 1:1 protein:nucleotide stoichiometry with a double-stranded DNA molecule of about 140 bp. This value is higher than that based on previous DNase I footprinting analysis, which suggested that *Drosophila* topoisomerase II protects a DNA segment of approximately

25 nucleotides (*29*). However, there are two interesting comparisons between our observed stoichiometry and other results with topoisomerase II. Wang and colleagues found that DNA gyrase wrapped a DNA segment of approximately 145 bp (*30*). The wrapping of DNA by DNA gyrase forms a positive writhe that is relaxed by a sign inversion mechanism resulting in negative supercoiling (*31*). It is important to note that there is no evidence that eukaryotic topoisomerase II wraps DNA in the same fashion that occurs with DNA gyrase. In addition, Maxwell and colleagues observed maximal ATPase stimulation of htopoIIα with pBR322 at a DNA concentration equivalent to approximately 150 bp per enzyme dimer (*32*). They proposed that the enzyme must bind to two DNA segments for maximal ATPase activity, one DNA fragment that will be cleaved, and a second DNA that binds in the vicinity of the ATPase domain. This second DNA strand could correspond to the segment to be translocated through the DNA break (*2*). Therefore, it is plausible that topoisomerase II interacts with more than one DNA segment and that the stoichiometry we observed reflects the need for the enzyme to interact with two DNA segments. DNA that is substantially shorter than 140 bp would require extreme bending to interact with topoisomerase II as both a strand to be cleaved and a strand to be passed through the break.

We found that ICRF-187 had little effect on the interaction of htopoIIα with linear pUC18 fragments because the drug did not modify the binding, as previously described by (*11*). Further, the kinetic and affinity constants were also unchanged. In addition, none of the htopoIIα mutants resistant to bisdioxopiperazines that we examined had any alteration in DNA binding compared to the wt protein. Thus, these experiments indicate that the inhibition of htopoIIα by ICRF-187 and the mechanism by which the mutants become resistant to ICRF-187 do not directly involve the interaction of DNA with the enzyme. With these results, we were then able to proceed to an examination of the closed clamp form of topoisomerase II using the Biacore system.

ICRF-187 has a unique mechanism of topoisomerase II inhibition, as it binds to the post strand passage closed clamp form of the enzyme, preventing its conversion to the open-clamp form. We used similar control experiments to those described by Roca and colleagues to show that the Biacore was detecting a closed clamp form of topoisomerase II rather than some other DNA complex. Thus, we detected a salt-stable complex in the presence of 1 mM ATP and ICRF-187 when the substrate immobilized to the Biacore chip was a circular DNA molecule. No salt-stable complex was detectable with a linear DNA substrate (Figure 4). The detection of a salt-stable complex with circular DNA and htopoIIα required the presence of ATP (Figure 6), as was previously observed by Roca and colleagues (*11*). The salt-stable complex was also seen in the absence of bisdioxopiperazines when the nonhydrolyzable ATP analogue, AMPPNP, was substituted for ATP (Figure 7). In the presence of AMPPNP, a salt-stable complex was only seen if the DNA was circular but not if the DNA was linear, as was previously reported using other techniques to detect closed clamps (*14*, *25*). Taken together, our results clearly indicate that the salt-stable complex detected using Biacore is the closed clamp form of the enzyme. Interestingly, the level of closed clamps formed with wt htopoIIα in the presence of AMPPNP (40%) was lower than the level seen with 500 μM ICRF-187 (75.5%) (Table 3). This difference may be due to a lower affinity for AMPPNP compared to ATP. It may also reflect that AMPPNP is less efficient in forming a closed clamp than ATP in the presence of bisdioxopiperazine and/or that the closed clamp formed is less stable in the presence of 1 M KCl. Further, the extreme salt concentration used to identify the formation of a closed clamp may explain that a higher concentration of ICRF-187 is needed to trap nearly all of the bound htopoIIα around the DNA compared to the enzymatic inhibition effect of the drug (*18*, *22*, *23*). Thus, it may also suggest that the two properties of bisdioxopiperazines (enzymatic inhibition and closed clamp inhibition) are not directly related.

When looking at the amount of salt-stable closed clamp complex formed in the presence of ATP alone, it is evident that the active site tyrosine Y805F mutant, which is incapable of performing strand passage, displays a four times higher level than seen with wt enzyme (Table 3). Detailed kinetic studies of yeast topoisomerase II have shown that topoisomerase II hydrolyzes its two bond ATP molecules in a sequential fashion (*33*, *34*). It was further found that hydrolysis of the first ATP actually drives strand passage (*35*). Finally, using pre-steady-state analysis, it was found that an active site tyrosine Y782F mutant incapable of strand passage hydrolyzes the first ATP 3−4 times more slowly that the corresponding wt enzyme (*27*). Interestingly, this reduction in ATP hydrolysis was insufficient to explain the observed decrease in the steady state ATP hydrolysis seen with the Y782F enzyme, indicating that (a) subsequent step(s) in the reaction cycle occurs more slowly with the mutant enzyme. The authors proposed that this slower step could be the hydrolysis of the second ATP, which is believed to trigger re-opening of the N-terminal clamp. This may explain the increased closed clamp formation seen with the Y805F mutant in the present study (the Y782 of yeast topoisomerase II is homologous to Y805 of htopoIIα). Here, the decreased ATP hydrolysis rates could shift the topoisomerase II catalytic cycle equilibrium toward the closed clamp form, which may well be more salt-stable than a more "open" configuration. This is, to our knowledge, the first example of topoisomerase II closed clamp quantitation done in the presence of ATP alone without the use of non-hydrolyzable cofactors. This was possible due to the high sensitivity of the SPR assay.

In the presence of the non-hydrolyzable ATP analogue AMPPNP, topoisomerase II is trapped as a salt-stable complex on DNA before the first reaction cycle is terminated. Therefore, the ability of topoisomerase II to cleave DNA and carry out strand passage is not expected to influence the formation of closed clamp complexes in the presence of this analogue. In addition, as the Y805F mutant protein has an intact ATP binding domain, it is expected to bind AMPPNP with an affinity similar to the wt enzyme. Therefore, our observation that wt and Y805F mutant enzymes are equally efficient in forming a closed clamp in the presence of AMPPNP (Table 3) is not surprising and is in agreement with results obtained by Lindsley and colleagues with the Y782F mutant of yeast topoisomerase II (*27*).

A detailed kinetic study of yeast topoisomerase II trapped as an ICRF-193 stabilized closed clamp on DNA showed

that topoisomerase II is capable of carrying out at least one strand passage event in the presence of saturating concentrations of ICRF-193 (*15*). It was further shown that hydrolysis of the first ATP, which is associated with DNA strand passage, was not inhibited by this drug. Finally, in the same study, isolated ICRF-193 stabilized closed clamp complexes were shown to carry out ATP hydrolysis at a reduced rate. From these observations, it was concluded that ICRF-193 most likely stabilizes a post strand passage closed clamp complex where the ATPase site of one monomer is blocked, while the ATPase site at the other monomer is still capable of hydrolyzing ATP. In our present study, the Y805F mutant enzyme is incapable of carrying out strand passage and concordantly is incapable of attaining the conformation suggested to be stabilized by bisdioxopiperazines in (*15*). This may explain the reduction in ICRF-187 stabilized closed clamp formation seen by the Y805F mutant protein (Table 3). It is possible that the asymmetrical conformation of the N-terminal clamp obtained after hydrolysis of the first ATP following DNA strand passage binds ICRF-187 more tightly than the symmetrical pre-strand passage complex with two ATP molecules bound. The finding that ICRF-187 is capable of stimulating some closed clamp formation with an active site mutant supports earlier data (*12*) and shows that the pre-strand passage closed clamp complex can be stabilized by the bisdioxopiperazine compounds to some extent.

Each of the bisdioxopiperazine-resistant mutants examined here, Y50F, Y165S, and L169F, failed to form significant levels of closed clamps in the presence of ATP and ICRF-187, as the amount of salt-stable closed clamp complex formed is equal to that seen in the absence of the drug (Table 3). These mutant enzymes have lost their ability to interact with ICRF-187, which strongly suggests that the corresponding mutations are directly involved in the binding of the bisdioxopiperazine with the enzyme. This result is further consistent with the observation that all of the mutants confer high levels of bisdioxopiperazine resistance both in mammalian cells and in yeast cells expressing the mutant htopoIIα proteins (*18*, *22*, *23*). The finding that all three mutants also showed reduced closed clamp formation in the presence of AMPPMP (Table 3) is expected given the fact that they all display mutations, located at or near the Walker A consensus ATP binding site of htopoIIα, impairing the interaction with ATP (*22*, *23*). Thus, it is likely that AMPPNP binds less strongly to these mutant enzymes than to wt, explaining the reduced closed clamp formation.

In conclusion, this work is the first demonstration that the SPR technology can provide a large amount of information on the DNA binding activity of topoisomerase II α. This method allowed us to compare the affinities of wt and mutant enzymes to several different DNA substrates. SPR was also adapted to quantify levels of the closed clamp form of topoisomerase II present on DNA. Taken together, the present results enable a more precise understanding of the biochemistry of topoisomerase II.

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