Kinetic Study of DNA Topoisomerases by Supercoiling-Dependent Fluorescence Quenching

Yunke Wang  
_Biomolecular Sciences Institute, Department of Chemistry & Biochemistry, and Environmental and Occupational Health, Robert Stempel College of Public Health & Social Work, Florida International University_, yunkwang@fiu.edu

Samantha Rakela  
_Biomolecular Sciences Institute, Department of Chemistry & Biochemistry, and Environmental and Occupational Health, Robert Stempel College of Public Health & Social Work, Florida International University_

Jeremy W. Chambers  
_Biomolecular Sciences Institute, Department of Chemistry & Biochemistry, and Environmental and Occupational Health, Robert Stempel College of Public Health & Social Work, Florida International University_

Zi-Chun Hua  
_School of Life Sciences, Nanjing University_

Mark T. Muller  
_Topogen, Inc._

Follow this and additional works at: https://digitalcommons.fiu.edu/biomolecular_fac

See next page for additional authors

**Recommended Citation**


This work is brought to you for free and open access by the College of Arts, Sciences & Education at FIU Digital Commons. It has been accepted for inclusion in Biomolecular Sciences Institute: Faculty Publications by an authorized administrator of FIU Digital Commons. For more information, please contact dcc@fiu.edu.
Kinetic Study of DNA Topoisomerases by Supercoiling-Dependent Fluorescence Quenching

Yunque Wang, Samantha Rakela, Jeremy W. Chambers, Zi-Chun Hua, Mark T. Muller, John L. Nitiss, Yuk-Ching Tse-Dinh, and Fenfei Leng

ABSTRACT: DNA topoisomerases are essential enzymes for all living organisms and important targets for anticancer drugs and antibiotics. Although DNA topoisomerases have been studied extensively, steady-state kinetics has not been systematically investigated because of the lack of an appropriate assay. Previously, we demonstrated that newly synthesized, fluorescently labeled plasmids pAB1_FL905 and pAB1_FL924 can be used to study DNA topoisomerase-catalyzed reactions by fluorescence resonance energy transfer (FRET) or supercoiling-dependent fluorescence quenching (SDFQ). With the FRET or SDFQ method, we performed steady-state kinetic studies for six different DNA topoisomerases including two type IA enzymes (Escherichia coli and Mycobacterium smegmatis DNA topoisomerase I), two type IB enzymes (human and variola DNA topoisomerase I), and two type IIA enzymes (E. coli DNA gyrase and human DNA topoisomerase IIα). Our results show that all DNA topoisomerases follow the classical Michaelis–Menten kinetics and have unique steady-state kinetic parameters, $K_M$, $V_{max}$, and $k_{cat}$. We found that $k_{cat}$ for all topoisomerases are rather low and that such low values may stem from the tight binding of topoisomerases to DNA. Additionally, we confirmed that novobiocin is a competitive inhibitor for adenosine S′-triphosphate binding to E. coli DNA gyrase, demonstrating the utility of our assay for studying topoisomerase inhibitors.

INTRODUCTION

DNA topoisomerases are enzymes that catalyze DNA structural alterations that include relaxation of positively and negatively supercoiled DNA and resolution of DNA knots and catenanes. These enzymes create transient DNA breaks to catalyze these changes in DNA topology. Because topoisomerase mechanisms are intrinsic to double-stranded DNA, topoisomerases are critical for DNA replication, transcription, recombination, and maintenance of the chromosome structure. DNA topoisomerases are important targets for anti-bacterial agents as well as anti-cancer drugs. Human topoisomerases I and II are targets of clinically important anti-cancer drugs, such as topotecan and doxorubicin. Bacterial DNA gyrase and Topo IV (another type II enzyme) are the targets of fluoroquinolones, such as ciprofloxacin, which are critically important antibiotics. Bacterial topoisomerases continue to be useful targets for the discovery of novel antibacterial drugs that could avoid cross resistance with current antibiotics to counter the serious global health problem of multidrug-resistant bacterial pathogens.

DNA topoisomerases are classified into type I and II families according to whether they make a transient single-stranded nick or double-stranded break during catalysis. Each family is further divided into different subfamilies depending on catalytic mechanisms. Type I enzymes cleave only one strand of the DNA templates, while type II enzymes transiently cleave both strands. During catalysis, type IA DNA topoisomerases (bacterial DNA topoisomerases I and III) link to the S′-phosphate. In contrast, type IB topoisomerases (human topoisomerase I and poxvirus DNA topoisomerase I) link to the 3′-phosphate of DNA. Although most DNA topoisomerases can relax (−) and/or (+) DNA supercoiling, bacterial DNA gyrase is the only enzyme that can actively introduce (−) DNA supercoiling to its DNA substrates.

One major assay for DNA topoisomerase activity is gel electrophoresis. Although gel electrophoresis is quick and convenient for resolving topoisomers of closed circular plasmid DNA molecules, it is less suitable for probing kinetics because gel electrophoresis cannot readily provide quantitative analysis of DNA supercoiling in real time. More recently,
single-molecule techniques, such as magnetic tweezers, have been used to analyze mechanisms of DNA topoisomerasescatalyzed supercoiling/relaxation reactions.\(^\text{16−21}\) Indeed, these elegant techniques provided mechanistic insights into various DNA topoisomerase-catalyzed supercoiling/relaxation reactions;\(^\text{22−29}\) however, DNA molecules must be physically linked onto solid surfaces.\(^\text{18,30}\) It is also not straightforward to derive constraint onto magnetic beads could influence diffusion or other aspects of radial DNA access of a topoisomerase before, during, or after the breaking/rescaling reaction.

Recently, we synthesized a type of uniquely fluorescently labeled DNA molecules that can be used to study DNA topoisomerases by fluorescence resonance energy transfer (FRET) or supercoiling-dependent fluorescence quenching (SFQ).\(^\text{31}\) This SFQ method stems from the fact that alternating adenine−thymine sequences (AT)\(_n\) in the closed circular plasmids undergo very rapid cruciform formation/deformation depending on the supercoiling density of the plasmids.\(^\text{32,33}\) A pair of fluorophore−quencher were inserted in the (AT)\(_n\) sequence, so that the distance between the fluorophore and quencher is dramatically changed when the plasmids adopt supercoiled (sc) or relaxed (rx) status.\(^\text{33}\) As a result, the fluorescence intensity of the plasmids is also greatly changed upon supercoiling transition.\(^\text{31}\) We demonstrated that these DNA molecules are excellent tools to examine relaxation/supercoiling kinetics of DNA topoisomerases and can be configured into rapid and efficient high-throughput screening assays to identify topoisomerase inhibitors.\(^\text{31}\) In this work, we report an improved procedure to rapidly and efficiently synthesize and purify fluorescently labeled plasmid DNA molecules. Using the SFQ assays, we find that all 6 DNA topoisomerases examined show characteristics of classical Michaelis−Menten kinetics. We also demonstrate that novobiocin is a competitive inhibitor for adenosine 5′-triphosphate (ATP) binding to Escherichia coli DNA gyrase.

## MATERIALS AND METHODS

### Materials.

Restriction enzyme Nt.BbvCI, T4 DNA polymerase, T5 exonuclease, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). E. coli DNA topoisomerase I, E. coli DNA gyrase, *Mycobacterium smegmatis* DNA topoisomerase I, varioila DNA topoisomerase I, human topoisomerase I, and human topoisomerase II\(_{\alpha}\) were purified as described previously.\(^\text{34−37}\) Ethidium bromide, buffer-saturated phenol, and isopropanol were purchased from Thermo Fisher Scientific, Inc. The synthetic oligonucleotides FL905 and FL924 were purchased from MWG-Biotec, Inc. (Huntsville, AL) and were described previously.\(^\text{31}\) Plasmid pAB1 was described previously\(^\text{31}\) and purified using commercial plasmid purification kits obtained from QIAGEN, Inc. (Valencia, CA).

### Synthesis of rx and sc pAB1_FL905 and pAB1_FL924.

The preparation of rx and sc pAB1_FL905 and pAB1_FL924 was described previously\(^\text{31}\) with some modifications. Briefly, 1 mg of pAB1 (~570 pmol) was digested by 2500 units of Nt.BbvCI in 20 mL of 1× CutSmart buffer for 1 h at 37 °C. After the digestion, 8000 pmol of phosphorylated FL905 or FL924 was added into the reaction mixture. The reaction mixture was incubated at 90 °C in a 4 L water bath for 2 min and then cooled down to room temperature in the water bath (~4~5 h; usually this step was carried out overnight). To generate rx pAB1_FL905 or pAB1_FL924, 25 000 units of T4 DNA ligase were added in the presence of 10 mM of dithiothreitol (DTT) and 2 mM of ATP (final concentrations). The reaction mixtures were incubated at 37 °C for 60 min to seal the nicks, thereby yielding the rx pAB1_FL905 or pAB1_FL924. T4 DNA polymerase (750 units) and dNTPs (100 μM) were added to the ligation mixture to increase the yield of the rx pAB1_FL905 or pAB1_FL924. After the ligation step, 5000 units of T5 exonuclease were added into the reaction mixture at 37 °C for 60 min to digest the nicked pAB1 and oligomer FL905 or FL924. The rx pAB1_FL905 or pAB1_FL924 sample was extracted with 20 mL of phenol, precipitated with isopropanol, washed once with 70% ethanol, and dialyzed against a large excess of 1× TE buffer solution (10 mM Tris- HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). To generate sc pAB1_FL905 or pAB1_FL924, the ligation reaction was carried out in the presence of 25 μM of ethidium bromide (EB). The other steps are the same as the synthesis of rx pAB1_FL905 or pAB1_FL924. The sc pAB1_FL905 or pAB1_FL924 sample was extracted with 20 mL of phenol, precipitated with isopropanol, washed once with 70% ethanol, and dialyzed against a large excess of 1× TE buffer solution.

### Fluorescence Spectroscopy.

Fluorescence measurements were performed using a Horiba FluorMax-3 spectrofluorimeter with an excitation wavelength of 494 nm or a Biotek Synergy H1 Hybrid Plate Reader with an excitation wavelength of 482 nm where plasmid pAB1_FL905 was used. Alternatively, fluorescence measurements were performed using a homemade spectrofluorimeter with a 532 nm laser for the excitation light source where pAB1_FL924 was used due to the fact that the fluorophore TAMRA of pAB1_FL924 was stable under this condition.

### DNA Supercoiling Density Determination.

sc pAB1_FL924 (4.6 μg) was relaxed by varioila DNA topoisomerase I in the presence of various concentrations of EB at 37 °C in 1× CutSmart buffer for 1 h. Subsequently, the relaxation reaction was stopped by addition of an equal volume of phenol. The reaction mixtures were extracted one more time with phenol (a total of 2 times of phenol extraction) and dialyzed against 1 L of 10 mM Tris- HCl, pH 8.0 twice overnight. The topological status of each DNA sample was analyzed by electrophoresis in a 1% agarose gel in 1× TAE buffer [40 mM Tris-acetate (pH 7.8) and 1 mM EDTA] containing different concentrations of chloroquine. After electrophoresis, agarose gels were stained with EB, destained, and photographed under UV light. The DNA linking number change (ΔLk) was determined by analyzing the distributions of the topoisomers, and the supercoiling density (σ) was calculated as follows

\[
\sigma = \frac{\Delta Lk}{Lk^0} = \frac{Lk - Lk^0}{Lk^0}
\]

where Lk^0 and Lk represent the DNA linking number for the relaxed and the supercoiled DNA, respectively.

The fluorescence intensity of these DNA samples was measured by using a microplate reader with λem = 550 nm and λex = 580 nm. DNA concentration was determined by UV absorbance at 260 nm using a Cary 50 spectrophotometer.

### Steady-State Kinetic Measurements.

All steady-state kinetic measurements were performed in 60 μL of a buffer solution containing either sc pAB1_FL905 (pAB1_FL924) or rx pAB1_FL905 (pAB1_FL924). CutSmart buffer [1× is 50
mM KAc, 20 mM Tris-Ac, 10 mM Mg(CH3COO)2, 100 μg/mL bovine serum albumin (BSA), pH 7.9] was used for E. coli DNA topoisomerase I, M. smegmatis DNA topoisomerase I, and variola DNA topoisomerase I. Human Top1 buffer (1x is 10 mM Tris-Cl, pH 7.9, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol) was used for human DNA topoisomerase I. Human Top2 buffer (1x is 50 mM Tris-Cl, pH 8, 100 mM KCl, 1 mM EDTA, 8 mM MgCl2, 35 mM β-mercaptoethanol, 0.5 mg/mL BSA, and 2 mM ATP or indicated in the assay) was used for human DNA topoisomerase II. DNA gyrase buffer (1x is 35 mM Tris-Cl, pH 7.5, 24 mM KCl, 4 mM MgCl2, 2 mM DTT, 5 mM spermidine, 0.1 mg/mL BSA, 6.5% glycerol, and 1.75 mM ATP or indicated in the assay) was used for E. coli DNA gyrase. Kinetic reaction mixtures were assembled on ice (without enzyme) and equilibrated to 37 °C usually for 5 min (in a cuvette inside the spectrofluorimeter). Then, one of DNA topoisomerases was added directly to the cuvette and mixed with other components of reaction mixtures by pipetting three times. The fluorescence intensities of the reaction mixture at 521 nm (for pAB1_FL905) or at 582 nm (for pAB1_FL924) were recorded every 5 s. The initial velocity of the reactions was calculated from linear-fitting of the first 5–10 data points. The steady-state kinetic parameters KM, Vmax, and kcat were obtained by fitting the Michaelis–Menten equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} \]  

(2)

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \]  

(3)

where \( V_0 \), \([S]\), \( K_M \), \( V_{\text{max}} \), \([E]\), and \( k_{\text{cat}} \) represent the initial velocity, substrate concentration, Michaelis constant, maximum velocity, enzyme concentration, and turn-over number, respectively.

**RESULTS AND DISCUSSION**

A rapid and efficient procedure to synthesize and purify fluorescently labeled plasmid DNA molecules. Recently, we reported the synthesis of a type of unique fluorescently labeled plasmid DNA molecules that can be used to study DNA topology and topoisomerases by FRET or SDFQ.31 Although the synthesis yield of the fluorescently labeled plasmid pAB1_FL905 is 35 mM Tris-HCl, pH 7.5, 24 mM KCl, 4 mM MgCl2, 2 mM DTT, 5 mM spermidine, 0.1 mg/mL BSA, 6.5% glycerol, and 1.75 mM ATP or indicated in the assay) was used for E. coli DNA gyrase. Kinetic reaction mixtures were assembled on ice (without enzyme) and equilibrated to 37 °C usually for 5 min (in a cuvette inside the spectrofluorimeter). Then, one of DNA topoisomerases was added directly to the cuvette and mixed with other components of reaction mixtures by pipetting three times. The fluorescence intensities of the reaction mixture at 521 nm (for pAB1_FL905) or at 582 nm (for pAB1_FL924) were recorded every 5 s. The initial velocity of the reactions was calculated from linear-fitting of the first 5–10 data points. The steady-state kinetic parameters KM, Vmax, and kcat were obtained by fitting the Michaelis–Menten equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} \]  

(2)

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \]  

(3)

where \( V_0 \), \([S]\), \( K_M \), \( V_{\text{max}} \), \([E]\), and \( k_{\text{cat}} \) represent the initial velocity, substrate concentration, Michaelis constant, maximum velocity, enzyme concentration, and turn-over number, respectively.

**RESULTS AND DISCUSSION**

A rapid and efficient procedure to synthesize and purify fluorescently labeled plasmid DNA molecules. Recently, we reported the synthesis of a type of unique fluorescently labeled plasmid DNA molecules that can be used to study DNA topology and topoisomerases by FRET or SDFQ.31 Although the synthesis yield of the fluorescently labeled plasmid pAB1_FL905 is 35 mM Tris-HCl, pH 7.5, 24 mM KCl, 4 mM MgCl2, 2 mM DTT, 5 mM spermidine, 0.1 mg/mL BSA, 6.5% glycerol, and 1.75 mM ATP or indicated in the assay) was used for E. coli DNA gyrase. Kinetic reaction mixtures were assembled on ice (without enzyme) and equilibrated to 37 °C usually for 5 min (in a cuvette inside the spectrofluorimeter). Then, one of DNA topoisomerases was added directly to the cuvette and mixed with other components of reaction mixtures by pipetting three times. The fluorescence intensities of the reaction mixture at 521 nm (for pAB1_FL905) or at 582 nm (for pAB1_FL924) were recorded every 5 s. The initial velocity of the reactions was calculated from linear-fitting of the first 5–10 data points. The steady-state kinetic parameters KM, Vmax, and kcat were obtained by fitting the Michaelis–Menten equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} \]  

(2)

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \]  

(3)

where \( V_0 \), \([S]\), \( K_M \), \( V_{\text{max}} \), \([E]\), and \( k_{\text{cat}} \) represent the initial velocity, substrate concentration, Michaelis constant, maximum velocity, enzyme concentration, and turn-over number, respectively.

**RESULTS AND DISCUSSION**

A rapid and efficient procedure to synthesize and purify fluorescently labeled plasmid DNA molecules. Recently, we reported the synthesis of a type of unique fluorescently labeled plasmid DNA molecules that can be used to study DNA topology and topoisomerases by FRET or SDFQ.31 Although the synthesis yield of the fluorescently labeled plasmid pAB1_FL905 is 35 mM Tris-HCl, pH 7.5, 24 mM KCl, 4 mM MgCl2, 2 mM DTT, 5 mM spermidine, 0.1 mg/mL BSA, 6.5% glycerol, and 1.75 mM ATP or indicated in the assay) was used for E. coli DNA gyrase. Kinetic reaction mixtures were assembled on ice (without enzyme) and equilibrated to 37 °C usually for 5 min (in a cuvette inside the spectrofluorimeter). Then, one of DNA topoisomerases was added directly to the cuvette and mixed with other components of reaction mixtures by pipetting three times. The fluorescence intensities of the reaction mixture at 521 nm (for pAB1_FL905) or at 582 nm (for pAB1_FL924) were recorded every 5 s. The initial velocity of the reactions was calculated from linear-fitting of the first 5–10 data points. The steady-state kinetic parameters KM, Vmax, and kcat were obtained by fitting the Michaelis–Menten equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} \]  

(2)

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \]  

(3)

where \( V_0 \), \([S]\), \( K_M \), \( V_{\text{max}} \), \([E]\), and \( k_{\text{cat}} \) represent the initial velocity, substrate concentration, Michaelis constant, maximum velocity, enzyme concentration, and turn-over number, respectively.
that reached plateau within 200–300 s (Figure 3B). Initial velocities were calculated from these time courses and successfully fitted to the Michaelis–Menten equation to yield a \( K_M = 1.2 \pm 0.3 \) nM, \( V_{max} = 50 \pm 4 \) pm/s, and \( k_{cat} = 3.0 \times 10^{-3} \) s\(^{-1}\). These values are lower than those that we obtained from agarose gel electrophoresis published previously.\(^{34}\) Possible reasons for this disparity include the following: (1) Gel electrophoresis is not as quantitative for analyzing the kinetics of DNA topoisomerases. For example, the disappearance of the "most sc species" used in the agarose-gel based assays may not represent the real initial velocity of \( E. \ coli \) DNA topoisomerase I because of the presence of supercoiled forms in the agarose-gel based results.\(^{34,41}\) (2) DNA molecules other than pAB1_FL905 may be present in the reaction mixture. (3) DNA molecules other than pAB1_FL905 may be present in the reaction mixture.
enzyme is released from relaxed DNA products. We also carried out a time course of the relaxation reaction of *E. coli* DNA topoisomerase I using agarose gel electrophoresis (Figure 3D). At 300 s, all pAB1_FL905 is fully relaxed, which is consistent with our fluorescence results.

Previous studies showed that high salt concentrations inhibited the relaxation activities of *E. coli* DNA topoisomerase I and also changed the final topological status of the relaxed topoisomers. Here, we performed kinetic studies of *E. coli* topoisomerase I using plasmid pAB1_FL924 in two different solution conditions, 1× CutSmart buffer and 1× CutSmart buffer plus 100 mM NaCl. Figure S2 shows the results. Indeed, high salt concentration decreased the fluorescence intensity of the final DNA topoisomers, presumably because of the topological status difference of the final relaxed topoisomers (Figure S2A), and also inhibited the relaxation activities of *E. coli* DNA topoisomerase I (Figure S2B). After fitting our kinetic data to the Michaelis–Menten equation, we obtained the following kinetic parameters: *K*~cat~ of 1.2 ± 0.2 nM, *V*~max~ of 51 ± 4 pM/s, and *K*~M~ of 3.0 × 10^−3 s^−1^ for 1× CutSmart buffer and *K*~cat~ of 1.5 ± 0.2 nM, *V*~max~ of 40 ± 3 pM/s, and *K*~M~ of 2.3 × 10^−3 s^−1^ for 1× CutSmart buffer plus 100 mM NaCl. The *K*~M~ value does not change significantly, while *V*~max~ decreases in the high salt buffer condition, which is similar to the effect of a noncompetitive inhibitor of the enzyme.

*M. smegmatis* DNA topoisomerase I, a 110 kDa monomeric type IA topoisomerase, was also examined. It is the only type IA DNA topoisomerase of *M. smegmatis*, essential for this bacterium, and a validated target for antibiotic discovery. In DNA relaxation time courses, we measured *K*~cat~ of 4.5 ± 3.0 nM, *V*~max~ of 130 ± 5 pM/s, and *K*~M~ of 9.8 × 10^−3 s^−1^ for 0.2 nM, 3 s and 3 s, respectively.

Steady-State Kinetics of Type IIA DNA Topoisomerases. Next, we studied the steady-state kinetics of the type IIA DNA topoisomerases, *E. coli* DNA gyrase, and human DNA topoisomerase IIb. Both enzymes require ATP for catalytic activity. *E. coli* DNA gyrase is a tetrameric protein and contains two different kinds of subunits, gyrA and gyrB that form an active A,B complex. DNA gyrase is the only known DNA topoisomerase that actively introduces (−) supercoils in DNA substrates under normal reaction conditions and therefore requires hydrolysis of ATP. DNA gyrase only exists in bacteria, is an essential enzyme, and could be used to identify inhibitors without significantly affecting host human enzymes. Figures 6 and S4 show our kinetic results. Addition of DNA gyrase to solutions containing rx pAB1_FL905 resulted in a significant decrease of the fluorescence intensity of the solution at 521 nm that reached the plateau within 300–400 s (Figure 6B) and a complete (−) supercoiling of the rx pAB1_FL905 (Figure 6C). Because *E. coli* gyrase has two substrates, rx DNA pAB1_FL905 and ATP, we determined the pseudo first-order kinetics for both substrates by fitting the initial velocity results to the Michaelis–Menten equation (Figure 6D,E). For rx DNA pAB1_FL905, we kept the ATP concentration at 1.75 mM and...
Figure 5. Steady-state kinetics of human DNA topoisomerase I. (A) Proposed reaction scheme for the relaxation reaction catalyzed by human DNA topoisomerase I. (B) Time courses of human DNA topoisomerase I-catalyzed DNA relaxation reactions monitored by fluorescence intensity change. For the relaxation reaction, 60 μL of 1× human Top1 buffer (10 mM Tris-Cl, pH 7.9, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol) containing different concentrations of sc pAB1_FL905 was prepared and equilibrated to 37 °C, and 25 nM of human DNA topoisomerase I was used to relax the sc pAB1_FL905. The fluorescence intensity at λem = 521 nm was monitored using a Biotek Synergy H1 Hybrid Plate Reader with an excitation wavelength of 482 nm. (C) Initial velocities of relaxation reaction were calculated from (B), plotted against the substrate concentration to fit into the classical Michaelis–Menten equation to determine K_M, V_max, and k_cat.

Figure 6. Steady-state kinetics of E. coli DNA gyrase. (A) Proposed reaction scheme for the supercoiling reaction catalyzed by E. coli DNA gyrase. The reaction includes two substrates: ATP and DNA (pAB1_FL905). (B) Time courses of E. coli DNA gyrase-catalyzed DNA supercoiling reactions monitored by fluorescence intensity change. For the supercoiling reaction, 60 μL of 1× DNA gyrase buffer (35 mM Tris-HCl, 24 mM KCl, 4 mM MgCl₂, 2 mM DTT, 1.75 mM ATP, 5 mM spermidine, 0.1 mg/mL BSA, 6.5% glycerol, pH 7.5) containing different concentrations of rx pAB1_FL905 was prepared and equilibrated to 37 °C, and 44.6 nM of E. coli DNA gyrase was used to supercoil the rx pAB1_FL905. The fluorescence intensity at λem = 521 nm was monitored with λex = 494 nm using a Horiba FluoroMax-3 spectrofluorimeter. (C) Time courses of E. coli DNA gyrase-catalyzed DNA supercoiling reactions monitored by 1% agarose gel electrophoresis. 384 μL of 1× DNA gyrase buffer containing 7.2 nM of rx pAB1_FL905 was prepared and equilibrated to 37 °C, and 44.6 nM of E. coli DNA gyrase was used to supercoil the rx pAB1_FL905. The reactions were stopped by adding 20 mM EDTA and 0.1% SDS into the reaction mixtures. Lanes 1–7 represent DNA samples from 0, 30, 60, 120, 300, 450, and 600 s of the supercoiling assay, respectively. rx and sc represent relaxed and supercoiled DNA molecules, respectively. (D) Initial velocities of supercoiling reaction were calculated from (B), plotted against the substrate concentration (sc pAB1_FL905) to fit into the classical Michaelis–Menten equation to determine K_M, V_max, and k_cat for the DNA substrate (rx pAB1_FL905). (E) Initial velocities of supercoiling reaction were plotted against ATP concentration and fitted into the classical Michaelis–Menten equation to determine K_M and V_max for ATP.

varied DNA concentration from 0.75 to 7.2 nM to yield a K_M of 2.7 ± 0.2 nM, 50 ± 2 pM/s, and 1.1 × 10⁻³ s⁻¹, respectively. For ATP, we kept the concentration of rx pAB1_FL905 at 7.2 nM and varied the ATP concentration from 0.1 mM to 2 mM to generate a K_M and V_max of 0.33 ± 0.14 mM and 50 ± 7 pM/s, respectively. The K_M values of DNA gyrase are consistent with previously published results using ATPase assays and single-molecule techniques.

Previously, it was demonstrated that novobiocin is a competitive inhibitor of DNA gyrase to prevent ATP binding to gyrase subunit B. In this study, we also performed kinetic studies of E. coli DNA gyrase in the absence and presence of 60 nM of novobiocin. Figure 7 shows our results. As expected, novobiocin significantly inhibited the supercoiling reaction of E. coli DNA gyrase (Figure 7A). Fitting of these kinetics to the Michaelis–Menten equation produced the following kinetic parameters: K_M of 0.4 ± 0.1 mM and V_max of 50 ± 3 pM/s in the absence of novobiocin and K_M of 2.4 ± 0.1 mM and V_max of 50 ± 2 pM/s in the presence of 60 nM novobiocin. V_max is identical while K_M is significantly higher in the presence of novobiocin. This result demonstrates that novobiocin is a competitive inhibitor of ATP for E. coli DNA gyrase. In the Lineweaver–Burk or double-reciprocal plot, the intercept on the y-axis of the plot of 1/V_o versus 1/[ATP] is the same in the presence or absence of novobiocin showing that novobiocin competes with ATP for its binding sites on E. coli DNA gyrase. The K_i value was calculated to be 11.6 nM which is consistent with the previous determination.

Human DNA topoisomerase IIα is a homodimer with 1531 residues and 174 385 Da per monomer. It can relax both (−) and (+) supercoiled DNA in the presence of ATP and plays an essential role during mitosis and meiosis as a decatenuate. This enzyme is also a target of several important anticancer drugs, including doxorubicin and etoposide. Similar to DNA gyrase, human DNA topoisomerase IIα has two substrates, sc plasmid DNA and ATP. Therefore, we determined the pseudo first order steady-state kinetic parameters for both substrates (Figure S5). For sc DNA pAB1 FL924, we kept the ATP concentration at 2 mM and varied DNA concentration from 1.3 to 8 nM to yield K_M of 5.7 ± 1.5 nM, V_max of 187 ± 25 pM/s, and 6.7 × 10⁻³ s⁻¹, respectively. For ATP, we kept the concentration of sc pAB1 FL924 at 4 mM and varied the ATP concentration from 0.025 mM to 2 mM to generate a K_M of 0.22 ± 0.06 mM and 99 ± 8 pM/s, respectively. The K_M value of human DNA topoisomerase IIα is consistent with previously published results using ATPase assays and single-molecule techniques for human and other eukaryotic DNA topoisomerase IIα.
Figure 7. Novobiocin is a competitive inhibitor of *E. coli* DNA gyrase to prevent ATP binding. (A) Inhibition of *E. coli* DNA gyrase catalyzed supercoiling reaction as a function of ATP. DNA supercoiling reactions were performed as described in “Materials and Methods” and also in the legend of Figure 6. The kinetic results were fitted to the classical Michaelis–Menten equation to yield $K_M$, $K_p$, and $V_{max}$. (B) Lineweaver–Burk plot or double-reciprocal plot of *E. coli* DNA gyrase in the absence or presence of novobiocin. Closed circles and squares represent supercoiling reactions in the absence and presence of 60 nM novobiocin, respectively.

## SUMMARY

Table 1 summarizes the steady-state kinetic parameters for 6 DNA topoisomerases that we examined in this study. The Michaelis constants of these topoisomerases are all in the nanomolar range. Because $K_M$ can be used to estimate the strength of the enzyme substrate (ES) complex (Figure 8A), low $K_M$ values indicate strong binding of topoisomerases to DNA, which is consistent with previously published results.3,6 Intriguingly, the $K_{cat}$ values of these 6 DNA topoisomerases are all low (Table 1). To explain this, we are considering a kinetic pathway for topoisomerases (Figure 8B). The enzyme and substrate form an ES complex and become an enzyme-product complex through an activation ES* state. After relaxation or supercoiling steps, the topoisomerases may need to dissociate from the DNA product for the next round of catalysis. DNA topoisomerases may need to dissociate from the DNA product before the next round of catalysis. If topoisomerases still tightly bind to their product, $k_{cat}$ should be low, which yields a low $k_{cat}$. Indeed, the $k_{cat}$ value of *E. coli* DNA topoisomerase I was determined previously and shown to be quite low69 (0.017–0.043 s⁻¹). In other words, the low values of $K_M$ of *E. coli* DNA topoisomerase I stem from its tight binding to DNA. Recently, it was reported that *Streptomyces coelicolor* DNA topoisomerase I is a highly processive enzyme and can relax ~150 (−) supercoils in a single burst in the single-molecule assay.70 Steady-state kinetic parameters were also determined using a gel-based assay and pUC1819 to yield a $K_M$ of 5.1 nM and a $V_{max}$ of 108 pM/s (6.5 nM/min).71 Because 5 nM of DNA topoisomerase I was used, this produces a $k_{cat}$ of 2.2 × 10⁻² s⁻¹, comparable to $k_{cat}$ values determined in this article (Table 1). Although the SDFQ method provides quantitative measurements for kinetic studies of DNA topoisomerases, we noticed some limitations for this method. This assay cannot be used to measure relaxation of (+) sc DNA. Additionally, this method only works in a relative narrow range of $\sigma$, that is, $−0.02 \leq \sigma \leq 0.04$ and cannot be used to study the transition of hypernegatively sc DNA to (−) sc DNA with $\sigma \leq −0.04$.

In conclusion, we developed a rapid and efficient method to generate rx and sc fluorescein labeled plasmid DNA molecules for DNA topoisomerases. Utilizing the fluorescein labeled rx and sc pAB1_FL905 and pAB1_FL924, we found that all 6 DNA topoisomerases including type IA, IB, and IIA topoisomerases follow the classical Michaelis–Menten kinetics.

<table>
<thead>
<tr>
<th>enzyme*</th>
<th>subfamily type</th>
<th>$K_M$ (nM)</th>
<th>$V_{max}$ (pM/s)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcTopI</td>
<td>IA</td>
<td>1.2 ± 0.3b</td>
<td>50 ± 4b</td>
<td>3.0 × 10⁻³b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 ± 0.2c</td>
<td>40 ± 3c</td>
<td>2.3 × 10⁻³c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5 ± 2.2 (ref 34)d</td>
<td>8.0 ± 1.0 (ref 34)d</td>
<td>12 ± 0.5 (ref 34)d</td>
</tr>
<tr>
<td>MsTopI</td>
<td>IA</td>
<td>4.3 ± 3.0</td>
<td>130 ± 5</td>
<td>9.8 × 10⁻³</td>
</tr>
<tr>
<td>vTopI</td>
<td>IB</td>
<td>2.4 ± 1.0</td>
<td>120 ± 23</td>
<td>6.0 × 10⁻³</td>
</tr>
<tr>
<td>hTopI</td>
<td>IB</td>
<td>2.7 ± 0.2</td>
<td>31 ± 5</td>
<td>1.2 × 10⁻³</td>
</tr>
<tr>
<td>EcGyrase</td>
<td>IIA</td>
<td>2.7 ± 0.2</td>
<td>50 ± 2</td>
<td>1.1 × 10⁻³</td>
</tr>
<tr>
<td>EcGyrase* (ATP, mM)</td>
<td>IIA</td>
<td>0.33 ± 0.14</td>
<td>51 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 (refs 58 and 61)d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16 (ref 60)d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hTopIIα</td>
<td>IIA</td>
<td>5.7 ± 1.5</td>
<td>187 ± 25</td>
<td>6.7 × 10⁻³</td>
</tr>
<tr>
<td>hTopIIα* (ATP, mM)</td>
<td>IIA</td>
<td>0.2 ± 0.1</td>
<td>99 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.56 ± 0.17 (ref 65)d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 ± 0.3 (ref 68)d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*EcTopI, *E. coli* DNA topoisomerase I; MsTopI, *M. smegmatis* DNA topoisomerase I; vTopI, variola DNA topoisomerase I; hTopI, human topoisomerase I; EcGyrase, *E. coli* DNA gyrase; hTopIIα, human DNA topoisomerase IIα. These kinetic parameters were determined in 1× CutSmart buffer. These kinetic parameters were determined in 1× CutSmart buffer plus 100 mM NaCl. These values were determined in the cited references. The concentration unit for $K_M$ of ATP is mM.
We determined the steady-state kinetic parameters, that is, $K_{M}$, $V_{max}$ and $k_{cat}$, for these DNA topoisomerases. Using this kinetic study, we also confirmed that novobiocin is a competitive inhibitor of DNA gyrase to prevent ATP binding.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02676.

Effects of EB on the supercoiling status of pAB1_FL905; effects of NaCl on the kinetics of *E. coli* topoisomerase I; steady-state kinetics of *M. smegmatis* DNA topoisomerase I; time courses of *E. coli* DNA gyrase-catalyzed DNA supercoiling reactions monitored by fluorescence intensity change in the presence of 1 or 2 mM of ATP; and steady-state kinetics of human DNA topoisomerase IIα (PDF)

### Accession Codes

The following are accession codes from UniProt for the proteins used in this article: *E. coli* DNA topoisomerase I, P06612; *E. coli* DNA gyrase subunit A, P0AES4; *E. coli* DNA gyrase subunit B, P0AES6; *M. smegmatis* DNA topoisomerase I, A0R5D9; Variola DNA topoisomerase I, P32989; human DNA topoisomerase I, P11387; human DNA topoisomerase IIα, P11388.

### AUTHOR INFORMATION

**Corresponding Author**

*E-mail: lengf@fiu.edu.* Phone: 305-348-3277. Fax: 305-348-3772.

**ORCID**

John L. Nitiss: 0000-0002-1013-4972

Fenfei Leng: 0000-0002-9024-1216

**Author Contributions**

F.L. designed research; Y.W., S.R., and F.L. performed research; F.L., J.W.C., Z.-C.H., J.L.N., and Y.-C.T.-D. analyzed data; M.T.M. and J.L.N. provided critical reagents; F.L. wrote the paper.

**Notes**

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

This work was supported by grants 1R15GM109254-01A1 and 1R21AI125973-01A1 from the National Institutes of Health (to F.L.). We thank Dr. H Peter Lu at Bowling Green State University, and Drs. Geoff Wilson and Gregory J. Lohman at New England Biolabs, Inc. for helpful discussion. Additional support was from NIH Grant R43GM113286 (to M.T.M.), a State of Colorado Advanced Industry Grant from the Office of Economic Development and International Trade (grant number CTGG1-2016-1825 to M.T.M.), and BZ2018017 from the Jiangsu Department of Science and Technology of China (to Z.C.H.).

### ABBREVIATIONS

rx, relaxed; sc, supercoiled; nk, nicked; FRET, fluorescence resonance energy transfer; SDFQ, supercoiling dependent fluorescence quenching

### REFERENCES


