INSIGHTS INTO THE CATALYTIC MECHANISM OF EUKARYOTIC AND
BACTERIAL TYPE II TOPOISOMERASES AND THE ACTIONS OF
TOPOISOMERASE II POISONS

By

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Robert Hunter Lindsey, Jr. 1971-2013

placed in the medical school biochemistry course instead of the interdisciplinary course taken by uncommitted graduate students. Historically, the few graduate students who landed in the medical school class were outcasts. However, typical of Hunter, he fit in immediately. During Hunter's second year in his graduate program, he became one of my best tutors for the medical students!

Hunter had never done research before, so it took him a little time to get used to life in the laboratory. However, he persevered and learned quickly. Before he was finished in 2004, he published first author papers in *Biochemistry*, *Chemical Research in Toxicology*, and *Chemico-Biological Interactions*, and a second author paper in *Biochemistry*. This was a trememdous publication record for a Master's student. I have seen students receive their Doctoral degrees with less.

During Hunter's first period in my laboratory, he worked with benzoquinone, a metabolite of benzene that is a causative agent of leukemia. The metabolite is believed to trigger leukemia, at least in part, by acting as a topoisomerase II poison. Hunter's work was well received by the field. Furthermore, it opened the door to a class of compounds that we now refer to as "covalent topoisomerase II poisons." It was groundbreaking research that my group is still following up today. In fact, in Chapter II of this dissertation, Hunter made seminal findings that have helped us to understand how covalent topoisomerase II poisons increase levels of enzyme-mediated DNA cleavage.

Hunter spent the next three years as an Instructor in the Department of Chemistry at West Point. By all accounts, he did extremely well and the departmental administration made it clear that they would love to have Hunter return in the future. With this as a backdrop, Hunter decided that he would like to finish his Army career as a teacher at the

Military Academy. However, in the Army system, he could not simply finish his Ph.D. and return to the Academy. The Army required Hunter to take a new deployment for several years before he could apply to finish his Ph.D. and report back to the Military Academy as an Assistant Professor. Finally, in 2011, Hunter (now a Lieutenant Colonel working in the Defense Threat Reduction Agency) got the green light to report back to my laboratory for his Ph.D. When Hunter arrived, it was as though no time had passed. He got to work and, once again, fit in with a new group of students.
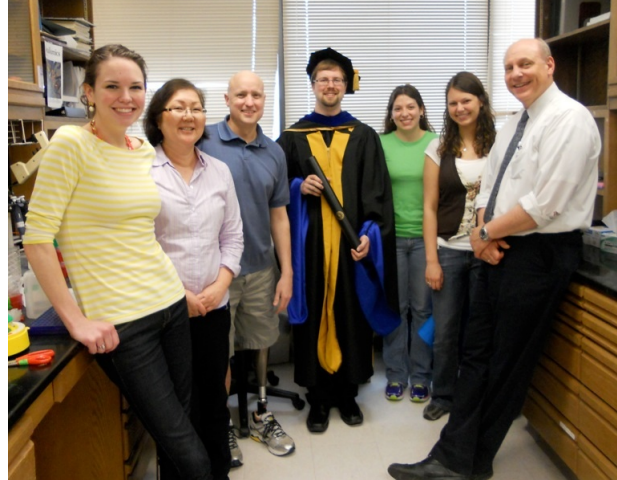
Hunter was a distance runner. One day in 2012, something "popped" in his left leg. The problem was treated as a typical runner's injury. Unfortunately, it did not seem to get better. As the tests became more drawn out and more serious, it became apparent that the injury was not typical. Ultimately, Hunter was diagnosed with a synovial sarcoma, a rare and virulent soft tissue cancer. Over the next several months, Hunter lost his left leg and went through several rounds of chemotherapy and radiation. Throughout all of his treatments, knowing that he had a poor prognosis, Hunter remained steadfastly upbeat. He retained his smile and sense of humor (admittedly, it had become a bit darker), even when those who knew and cared for him had trouble keeping up their spirits.

While Hunter was in the hospital, he and I had several very serious conversations about how he wanted to spend his remaining time. Over and over he re-iterated his commitment to the Ph.D. program.

The following spring, after having endured more than most individuals could have tolerated, Hunter returned to my laboratory with a smile on his face. During the next six weeks, he generated several key pieces of data that are included in Chapter II and resulted in a first author paper in *Biochemistry*. Unfortunately, by this time, Hunter's cancer had

metastasized to his lungs, which debilitated him. Once he left the laboratory, he was never able to return. He succumbed to his disease before he had the opportunity to complete his work.

This dissertation is based on Hunter's research. I had the privilege of assembling the dissertation and submitting it on behalf of Hunter posthumously.

The world was a better place with Hunter in it. He was a model of perseverance and courage. He was a genuinely good person who liked everyone and was liked by everyone in return. Over the years, I watched Hunter develop from an eager student with no experience into an excellent scientist and an outstanding teacher. I am extremely proud of all of Hunter's scientific achievements. I am more proud of his achievements as a human being. I am a better person for having known Hunter and I still miss him badly.

I am grateful to all of the laboratory members who worked with Hunter and helped to train him. I am especially grateful to Jo Ann Byl, who did so many things for Hunter during both of his "deployments" at Vanderbilt. Special recognition also has to go to MaryJean Pendleton and Rachel Ashley, who filled in some of the data and worked with me to put together Hunter's paper. Both put their hearts and souls into the work.

Additional thanks go to Jo Ann and MaryJean for keeping in such close contact with Hunter and his family. Your friendship and caring meant the world to them.

Throughout all of Hunter's trials with his cancer, his wife Jenn was by his side. I still do not know how she managed to keep it together as well as she did. Jenn is the

strongest woman I know. Thank you for taking such good care of Hunter and for loving him so much.

I wish to thank Dr. Joe Deweese for the catalytic core of topoisomerase IIα that Hunter used for many of the experiments in Chapter II. I also would like to thank Dr. Chuck Turnbough and Sylvia McPherson for providing some of the *Bacillus anthracis* gyrase used in Chapter III, working out the purification scheme for the enzyme subunits, and training Hunter how to express and purify them.

I am extremely grateful to the members of Hunter's continuing education committee: Dr. Martin Egli, Dr. Dan Liebler, Dr. Nicholas Reiter, and Dr. Eric Skaar. All of them contributed to Hunter's development as a scientist before his cancer diagnosis. Following his diagnosis, they displayed tremendous personal support for him and for my assembling this dissertation.

I also wish to thank the Chair of the Department of Biochemistry, Dr. John York, and the Director of Graduate Studies, Dr. Chuck Sanders, for all of the help and support that they showed Hunter and for their support for my efforts to obtain his degree for him. Chuck was the first faculty member to obtain a posthumous Ph.D. for a student at Vanderbilt University. Without his trailblazing efforts, Hunter's Ph.D. might never have been awarded. Finally, many thanks to Lindsay Meyers, who is the Program Manager, Biomedical Research Education and Training, for the Department of Biochemistry. She took care of much of the paperwork and the coordination with the Graduate School that were necessary for this Hunter to receive his degree.

Neil Osheroff, Ph.D.
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John G. Coniglio Chair in Biochemistry

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LIST OF ABBREVIATIONS

3'-(AM)P	3'-(aminomethyl)pyrrolidinyl
A	alanine
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
D	aspartic acid
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
FQ	fluoroquinolone
Glu	glutamic acid
His	histidine
Ile	isoleucine
K	lysine
kDa	kilodalton
L	leucine
Leu	leucine
Lys	lysine
M	methionine

Met	methionine
PCR	polymerase chain reaction
R	arginine
(–)SC	negatively supercoiled
(+)SC	positively supercoiled
S	serine
SDS	sodium dodecyl sulfate
Ser	serine
Tyr	tyrosine
V	valine
Val	valine
WT	wild-type
W	tryptophan
Y	tyrosine

CHAPTER I

INTRODUCTION

Type II topoisomerases are ubiquitous enzymes that regulate levels of DNA under- and overwinding and remove knots and tangles from the genetic material (*1-7*). These enzymes are essential for cell survival and play vital roles in virtually every nucleic acid process, including DNA replication, transcription, and recombination. They also are required for proper chromosome organization and segregation (*1-7*).

Beyond their critical physiological functions, type II topoisomerases are the targets for a number of drugs that significantly impact human health (*2, 8-12*). The human type II enzymes (topoisomerase IIα and topoisomerase IIβ) are the targets for some of the most widely prescribed anticancer drugs in clinical use (*2, 8-12*). Furthermore, the bacterial type II enzymes (gyrase and topoisomerase IV) are the targets for quinolones, which are the most efficacious and broad-spectrum class of oral antibacterial agents used worldwide (*13-20*).

Given the importance of type II topoisomerases to eukaryotic and bacterial cells and to human health, it is critical to understand how these enzymes interact with their DNA substrates and with drugs. These are the issues that form the focus for this dissertation.

Type II Topoisomerases

Catalytic Cycle

Type II topoisomerases regulate superhelical density and remove tangles and knots by the double-stranded DNA passage reaction depicted in Figure 1 (*1, 3, 5, 6, 8, 21-23*). These enzymes require a divalent metal ion (Mg^{2+} appears to be the physiological ion) and ATP in order to carry out their complete catalytic cycle.

Type II enzymes bind two segments of DNA (Step 1). The first segment bound by the enzyme is the double helix that will be cleaved and is referred to as the "Gate-" or "G-segment." The second segment is the double helix that will be transported through the transient DNA gate and is referred to as the "Transport-" or "T-segment." DNA binding requires no cofactors. In the presence of the active site Mg^{2+} ions, type II topoisomerases sample the DNA for malleability (Step 2) (*24*). Sequences that can be cleaved are bent to an angle of ~150° (depending on the enzyme) (*25*). Conversely, sequences that cannot be bent are not cleaved (*24, 26*). DNA bending induces significant strain in the G-segment, which is maximal at the scissile bonds on either strand of the double helix. A double-stranded break is generated in the G-segment (Step 3) using a noncanonical two-metal-ion mechanism (*22, 27, 28*). The type II enzymes contain two active site tyrosyl residues (located on different subunits), each of which makes a single-stranded DNA break. The scissile bonds on the two strands of the double helix are staggered, and cleavage generates 5'-termini with four-base single-stranded cohesive ends. During the scission event, type II topoisomerases covalently attach to the 5'-termini of the cleaved DNA. These covalent enzyme-cleaved DNA complexes are known as "cleavage complexes."

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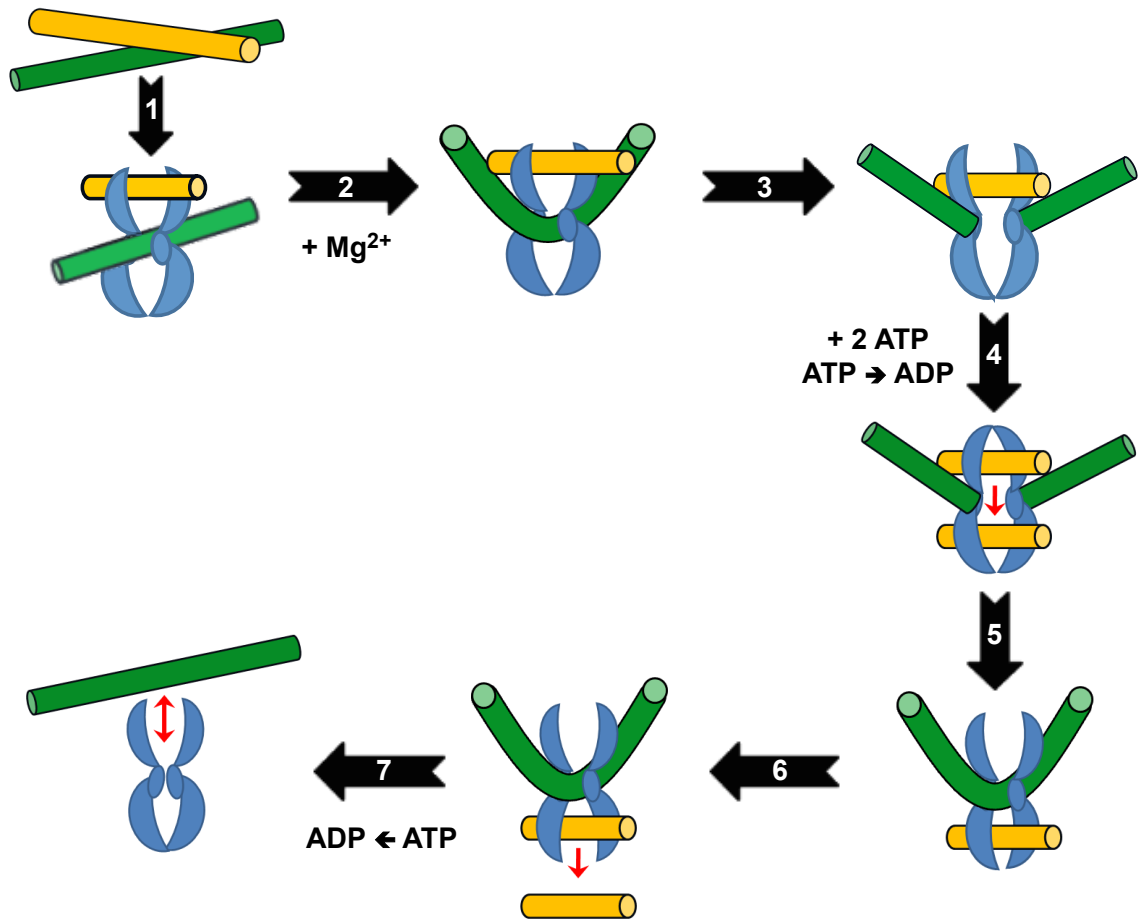
Figure 1. Catalytic cycle of type II topoisomerases. The homodimeric enzyme is shown in blue, the DNA double helix that is cleaved and acts as the DNA gate (G-segment) is shown in green, and the double helix that is transported through the DNA gate (T-segment) is shown in yellow. Details of the individual reaction steps are given in the text.

Two molecules of ATP are bound by the enzyme, which triggers the closing of the N-terminal protein gate, the opening of the DNA gate, and the translocation of the T-segment through the gate (Step 4). Although hydrolysis of the cofactor is not a prerequisite for DNA translocation, it appears that this step proceeds more rapidly if it is preceded by hydrolysis of one of the bound ATP molecules. The cleaved DNA is rejoined (Step 5), the T-segment is released through the C-terminal protein gate (Step 6), and, upon hydrolysis of the second ATP molecule, type II enzymes regain the ability to initiate a new round of catalysis (Step 7).

Bacterial Type II Topoisomerases

Bacteria contain two distinct type II topoisomerases, gyrase and topoisomerase IV. Gyrase was discovered in 1976 (*29*). It was the first type II topoisomerase to be described and is the only enzyme in this class to retain its historical name. Gyrase is comprised of two distinct subunits, GyrA and GyrB (~96 kDa and ~88 kDa, respectively) (Figure 2) and functions as an A_2B_2 tetramer (*1, 3, 6, 7, 21*). GyrA contains the active site tyrosyl residue that forms the covalent bond with DNA during scission, and GyrB contains consensus sequences for ATP binding.

Of the known type II topoisomerases, gyrase is the only enzyme that is capable of generating negative supercoils in the double helix without the assistance of additional DNA binding proteins or intercalators (*1, 3, 6, 7, 21, 23*). Gyrase accomplishes this feat by using a sign inversion mechanism (*3, 7, 23, 30*). The enzyme wraps the double helix around itself in a right-handed fashion such that it generates a constrained positive supercoil and a compensatory unconstrained negative supercoil. The C-terminal domain

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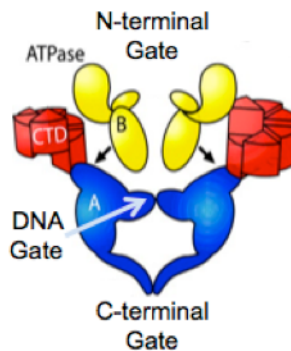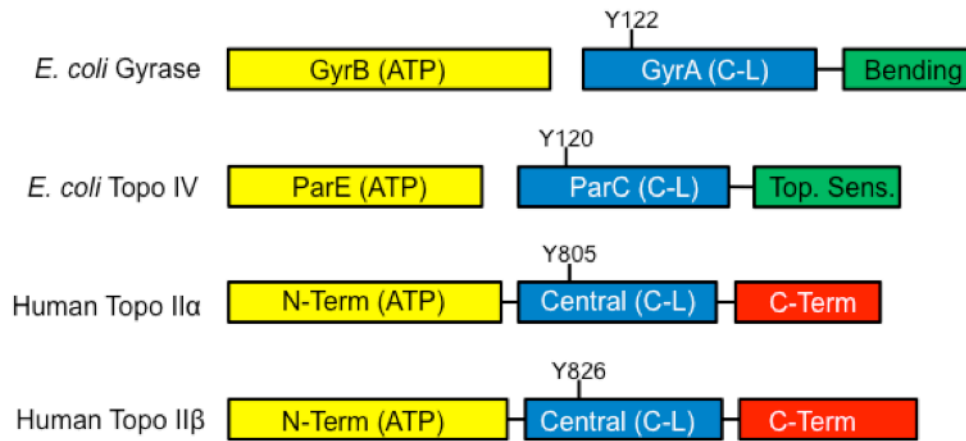
Figure 2. Domain structures of type II topoisomerases. Bacterial (*E. coli* gyrase and topoisomerase IV) and eukaryotic (human topoisomerase IIα and IIβ) type IIA enzymes are shown. Regions of homology among the enzymes are indicated by colors. The N-terminal (*i.e.*, GyrB/ParE) homology domains (yellow) contain the regions responsible for ATP binding and hydrolysis. The central (*i.e.*, GyrA/ParC) homology domains (blue) contain the active site tyrosyl residue that performs cleavage and ligation (C-L) and forms the covalent bond with DNA during scission. The variable C-terminal domains are shown in green for the bacterial enzymes and in red for the eukaryotic enzymes. These domains are involved in DNA topology sensing during the strand passage reaction. Subunits and domains are drawn proportionally to their length. The active site tyrosyl residue is indicated for each enzyme.

of the GyrA subunit, which adopts a unique DNA bending β-pinwheel conformation (*31*), is required for this chiral DNA wrapping event. As a result of the double-stranded DNA passage event, gyrase converts the positive supercoil to a negative supercoil.

Because of the intramolecular DNA wrapping mechanism used by gyrase, its ability to generate negative supercoils in the double helix is much more efficient than its ability to untangle or unknot the genetic material (which often requires an intermolecular interaction between separate DNA molecules) (*1, 3, 6, 7, 21, 23*). Consequently, the major physiological roles of DNA gyrase stem directly from its ability to underwind the double helix. DNA gyrase plays a critical role in opening DNA replication origins and removing positive supercoils that accumulate in front of replication forks and transcription complexes. In addition, because prokaryotes lack the highly organized chromatin structure that helps regulate supercoiling in eukaryotes, gyrase works in conjunction with the ω protein (a type IA topoisomerase that removes negative supercoils from the double helix) to set and maintain the global superhelical density in bacterial cells.

The second bacterial type II topoisomerase, topoisomerase IV, was discovered in 1990 (*1, 3, 6, 7, 21, 23*). Like gyrase, this enzyme is an A_2B_2 tetramer (Figure 2). The two subunits of topoisomerase IV were first identified in Gram-negative species as proteins required for chromosome partitioning and were designated ParC (~88 kDa) and ParE (~70 kDa). Based on sequence analysis, it was determined that the ParC and ParE proteins were homologous to the A and B subunits of gyrase, respectively. Subsequent functional studies led to the discovery that the ParC/ParE complex constituted a novel

type II topoisomerase. In Gram-positive species, the subunits of topoisomerase IV are designated as gyrase-like proteins, GrlA and GrlB, respectively.

Despite the homology between topoisomerase IV and gyrase, the two enzymes display distinct properties that affect their physiological functions (*1, 3, 6, 7, 21, 23*). First, there are critical differences between the C-terminal domains of ParC/GrlA and GyrA. Instead of maintaining the "closed" β-pinwheel conformation seen in gyrase (*31*), this region of topoisomerase IV adopts an "open" conformation (*32*). This "broken" pinwheel cannot perform the chiral wrapping function that is necessary for the gyrase supercoiling reaction. Consequently, topoisomerase IV can remove positive and negative supercoils from DNA but cannot actively underwind the double helix. Rather, the C-terminal domain of topoisomerase IV functions as a topology sensor that allows the enzyme to distinguish the handedness of DNA supercoils. As a result, topoisomerase IV is able to remove positive DNA supercoils (like those found ahead of replication forks) much more efficiently than it does negative supercoils (*33, 34*). This ability has led to speculation that topoisomerase IV may act ahead of DNA tracking systems to help gyrase alleviate overwinding of the double helix. However, the precise role of topoisomerase IV in this process has yet to be defined.

Second, because topoisomerase IV does not utilize the intramolecular chiral DNA wrapping mechanism of gyrase, it is much more efficient at catalyzing intermolecular reactions (*i.e.*, those that require actions on two separate DNA molecules) (*1, 3, 6, 7, 21, 23*). Thus, the ability of topoisomerase IV to resolve DNA tangles and knots is much more efficient than that of gyrase. As a result, the most important cellular functions of topoisomerase IV are the unlinking of daughter chromosomes following DNA replication

and the removal of DNA knots that are formed during recombination and other physiological processes.

Eukaryotic Type II Topoisomerases

The eukaryotic type II enzyme, topoisomerase II, was discovered in 1980 (*1, 3, 6, 7, 21, 23*). Like bacterial topoisomerase IV, topoisomerase II can remove supercoils from the double helix and can resolve DNA tangles and knots. Eukaryotes encode no "gyrase-equivalent" topoisomerase that is able to actively underwind DNA. The supercoiling activity of gyrase has been obviated by the evolution of histones. Because nucleosomes wrap the genetic material in a left-handed superhelix (which underwinds the DNA), removal of the resulting compensatory positive supercoils by a non-gyrase topoisomerase leads to a net negative supercoiling of the eukaryotic genome (*30*).

Eukaryotic species such as yeast and *Drosophila* encode only a single type II topoisomerase (*i.e.*, topoisomerase II). However, vertebrates express two isoforms, topoisomerase IIα and topoisomerase IIβ (*1, 3, 4, 6, 8, 21, 22, 35*). These two isoforms share extensive amino acid sequence identity (~70%) but are encoded by separate genes (located at chromosomal bands 17q21-22 and 3p24 in humans, respectively). Topoisomerase IIα and topoisomerase IIβ also can be distinguished by their protomer molecular masses (~170 kDa and ~180 kDa, respectively).

Eukaryotic type II topoisomerases are homologous to the bacterial type II enzymes (Figure 2) (*1, 3, 4, 6, 8, 21, 22, 35*). However, the two subunits have fused into a single polypeptide, and the eukaryotic enzymes function as homodimers (as opposed to an A_2B_2 tetramer). On the basis of amino acid sequence comparisons with bacterial

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gyrase, each topoisomerase II protomer can be divided into three distinct domains (Figure 2). The N-terminal domain of the enzyme is homologous to GyrB and contains the binding site for ATP. The central domain is homologous to much of GyrA and contains the active site tyrosyl residue. The C-terminal domain of topoisomerase II, which occupies the same location on the protein as the C-terminal domain of GyrA/ParC/GrlA, is highly variable. This region shares little to no sequence similarity to the equivalent region in DNA gyrase or topoisomerase IV and differs considerably between type II topoisomerases, even across eukaryotic species. The C-terminal domain of eukaryotic topoisomerase II contains nuclear localization sequences as well as amino acid residues that interact with cellular components or are phosphorylated *in vivo*.

It is not obvious why vertebrates encode two distinct topoisomerase II isoforms. In contrast to gyrase and topoisomerase IV, enzymological differences between topoisomerase IIα and topoisomerase IIβ are subtle (*1, 3, 4, 6, 8, 21, 22*). In this regard, the only major characteristic that distinguishes topoisomerase IIα and topoisomerase IIβ is the ability to recognize the handedness of DNA supercoils (*36*). While the α isoform removes positive DNA supercoils ~10–fold faster than it does negative, the β isoform removes both at similar rates. As with topoisomerase IV, this topology sensing function of topoisomerase IIα is embodied in the C-terminal domain of the protein.

Topoisomerase II plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves movement or organization of the genetic material (*1, 3, 4, 6, 8, 21, 22, 35*). The enzyme unlinks tangled daughter chromosomes following replication and resolves DNA knots that are formed during recombination. It also helps to alleviate the torsional stress that accumulates ahead of

replication forks and transcription complexes. Topoisomerase II is required for proper chromosome condensation, cohesion, and segregation and appears to play roles in centromere function and chromatin remodeling. Finally, the enzyme is important for the maintenance of proper chromosome organization and structure and is the major non-histone protein of the mitotic chromosome scaffold and the interphase nuclear matrix.

Topoisomerase IIα and topoisomerase IIβ have distinct patterns of expression and separate nuclear functions (*1, 3, 4, 6, 8*). Topoisomerase IIα is essential for the survival of proliferating cells and is regulated over cell and growth cycles. Enzyme levels increase throughout S-phase of the cell cycle and peak at the G_2/M boundary. Although topoisomerase IIα is nearly non-existent in quiescent or differentiated tissues, rapidly proliferating cells contain as many as ~500,000 copies of the enzyme. Topoisomerase IIα is associated with replication forks, and its ability to preferentially relax positive supercoils (*36*) has led to speculation that it helps remove torsional stress ahead of the replication machinery. Furthermore, the enzyme remains tightly bound to chromosomes during mitosis. In light of the enzymological characteristics, regulation, and cell biology described above, it is believed that topoisomerase IIα is the isoform that functions in growth-related cellular processes (*1, 3, 4, 6, 8*).

Topoisomerase IIβ is dispensable at the cellular level, and its presence cannot compensate for the loss of topoisomerase IIα in mammalian cells (*1, 3, 4, 6, 8, 37*). However, the β isoform is required for proper neural development in mice (*38*). In contrast to topoisomerase IIα, the concentration of topoisomerase IIβ is independent of the cell cycle, and high levels of this isoform are found in most cell types regardless of proliferation status (*1, 3, 4, 6, 8, 37*). Topoisomerase IIβ dissociates from chromosomes

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during mitosis. The sequences that govern the association/dissociation of topoisomerase II with mitotic chromosomes reside in the C-terminal domain (*39*). Ultimately, the physiological functions of the β isoform have yet to be fully defined. However, recent evidence suggests that topoisomerase IIβ plays an important role in the transcription of hormonally- or developmentally-regulated genes (*40*).

<u>Type II Topoisomerases as Cellular Toxins</u>

Because type II topoisomerases must generate double-stranded DNA breaks prior to strand passage, they are inherently dangerous proteins. Thus, while necessary for cell viability, these enzymes also have the capacity to fragment the genome (*2, 5-11*). As a result of this "Jekyll-Hyde" persona, levels of cleavage complexes must be maintained in a critical balance (Figure 3).

Cleavage complexes are requisite intermediates in the strand passage reaction catalyzed by type II topoisomerases. Thus, a decrease in their concentration generally reflects a decrease in overall catalytic activity. Consequently, if cleavage complexes drop below threshold levels, topoisomerase IIα and gyrase are unable to maintain necessary rates of DNA replication and topoisomerase IIα and topoisomerase IV are unable to completely disentangle daughter chromosomes following replication. Consequently, cells die as a result of mitotic failure.

Conversely, if levels of cleavage complexes generated by gyrase or topoisomerase IV in bacteria or topoisomerase IIα or topoisomerase IIβ in eukaryotes increase, cells also suffer catastrophic physiological effects, but for different reasons (*2, 3, 8-11, 41*). When replication forks, transcription complexes, or other DNA tracking systems attempt to

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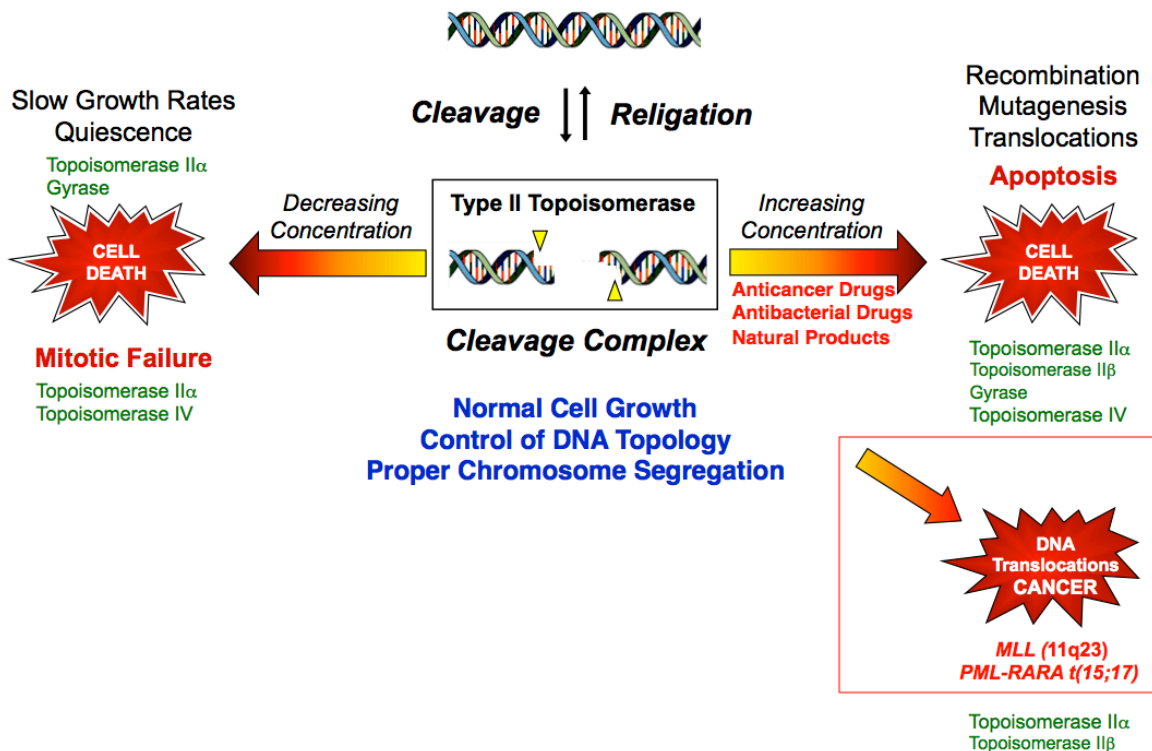
Figure 3. Type II topoisomerases are essential but genotoxic enzymes. The balance between enzyme-mediated DNA cleavage (which is required for their physiological functions) and ligation is critical for the survival of bacterial and eukaryotic cells. If the level of enzyme–mediated DNA cleavage decreases below threshold levels, cells are not able to untangle daughter chromosomes and ultimately die of mitotic failure (left). If the level of cleavage becomes too high (right), the actions of DNA tracking systems can convert transient cleavage complexes to permanent double-stranded breaks. The resulting DNA breaks, as well as the inhibition of essential DNA processes, initiate recombination/ repair pathways and can generate chromosome translocations and other DNA aberrations. If the strand breaks overwhelm the cell, they can trigger cell death. This is the basis for the actions of several widely prescribed anticancer and antibacterial drugs. If cell death does not occur, mutations or chromosomal aberrations may be present in surviving populations. Exposure of human cells to topoisomerase II poisons is associated with the formation of specific types of t-AMLs and infant leukemias that involve the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 and t-APLs that feature t(15:17) chromosomal translocations between the *PML* (promyelocytic leukemia) and *RARA* (retinoic acid receptor α) genes (lower right arrow). The specific type II topoisomerases involved in the individual cellular processes described above are indicated in green.

traverse the covalent topoisomerase-DNA "roadblock," accumulated cleavage intermediates are converted to strand breaks that are no longer tethered by protein-linked bridges. The ensuing damage induces recombination/repair pathways that can trigger mutations, chromosomal translocations, or other aberrations. If the DNA breaks overwhelm the repair process, their presence can initiate cell death pathways. However, if cells recover sufficiently, they may survive but contain damaged chromosomes. In some cases, chromosome aberrations may initiate a leukemogenic transformation in humans (*2, 8, 42-47*).

<div align="center">Topoisomerase II Poisons</div>

Chemicals that increase levels of topoisomerase II-DNA cleavage complexes convert the enzyme to a potent cellular toxin that generates the chromosomal damage described above. These compounds are called topoisomerase II poisons to distinguish them from catalytic inhibitors of the enzyme (*2, 8-11*). Topoisomerase II poisons kill cells by a gain of function, inducing the enzyme to generate DNA strand breaks, as opposed to robbing the cell of the essential functions of the enzyme.

This section will focus on poisons that affect the human type II topoisomerases, topoisomerase IIα and topoisomerase IIβ. Quinolones, which poison the bacterial type II enzymes, gyrase and topoisomerase IV, are discussed in the following section.

Based on their mechanism of action, topoisomerase II poisons can be categorized into two distinct classes, "interfacial poisons" and "covalent poisons" (*2, 8, 10, 11, 48*). Selected topoisomerase II poisons are shown in Figure 4. The characteristics and
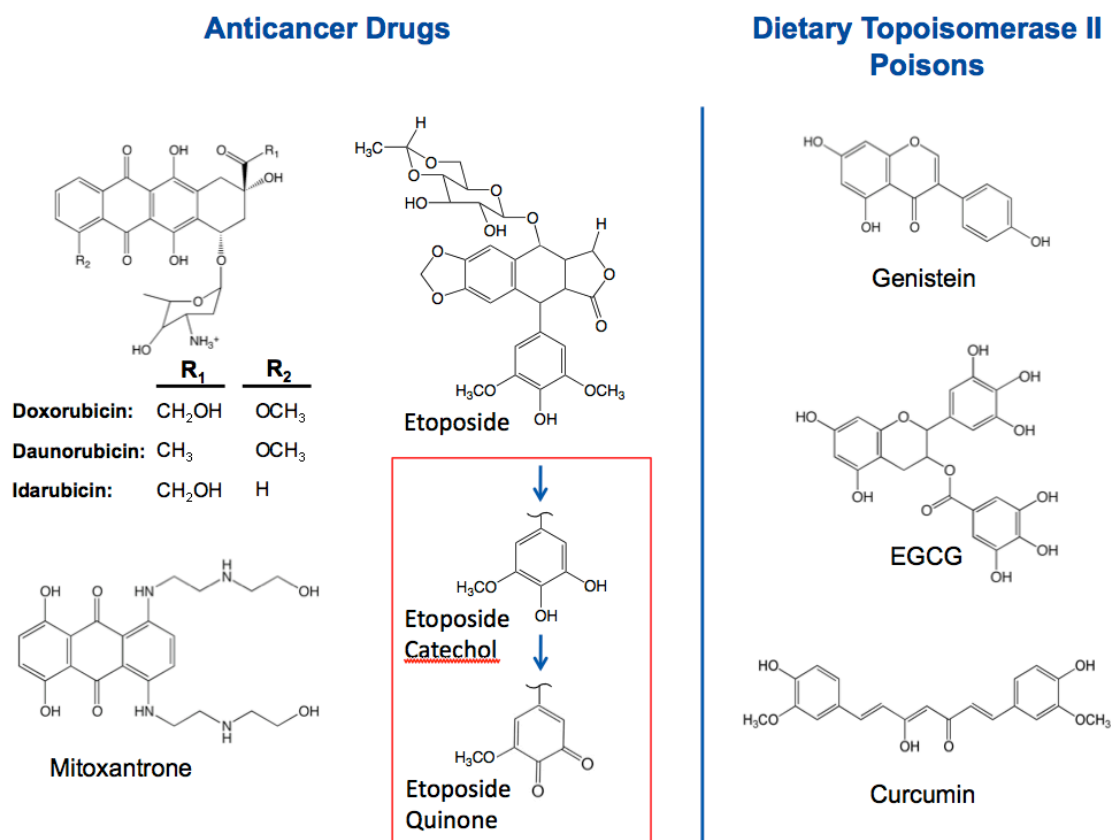
Figure 4. Structures of selected topoisomerase II poisons that target the eukaryotic type II enzymes. Clinically used anticancer drugs that target topoisomerase II are shown on the left. Dietary topoisomerase II poisons are shown on the right. The catechol and quinone metabolites of etoposide (generated by CYP3A4 and cellular oxidases or redox cycling, respectively) are highlighted in the red box. Epigallocatechin gallate is abbreviated as EGCG.

distinguishing features of the two classes of topoisomerase II poisons are described in Figure 5.

Interfacial poisons bind non-covalently to the cleavage complex at the protein-DNA interface. They intercalate into the double helix at the cleaved scissile bond and impede the ability of topoisomerase II to rejoin the DNA ends (*8, 10, 11, 49*). In essence, interfacial poisons act as "molecular doorstops" and prevent the DNA gate from being closed. Examples, including etoposide, doxorubicin, mitoxantrone, and bioflavonoids such as genistein, are shown in Figure 4.

Covalent poisons function distal to the active site of topoisomerase II (*2, 8, 48*). They contain reactive groups such as quinones or maleiamides and covalently adduct to cysteine (and potentially other amino acid) residues (*48, 50-53*). It is believed that covalent poisons increase levels of enzyme-mediated DNA cleavage by altering the conformation of the topoisomerase II N-terminal protein gate. Examples, including epigallocatechin gallate (EGCG) (*54*), which is prevalent in green tea, and curcumin (*55*), which is the major flavor and aromatic component in turmeric, are shown in Figure 4.

Covalent topoisomerase II poisons are found in a number of fruits, vegetables, and other plants that are common components in the human diet (*48, 50, 53*). Many of these foods, such as green tea and soy, are believed to have chemopreventative properties (*48, 56*). Covalent topoisomerase II poisons are also prevalent in medicinal herbs, such as turmeric and black seed (*55, 57*).

Topoisomerase II poisons represent some of the most important and widely prescribed anticancer drugs worldwide (*2, 8-11, 48*). At the present time, six of these agents are approved for use in the United States. Topoisomerase II-targeted drugs

Interfacial Poisons

- Acts non-covalently at the cleavage/ligation active site at the interface between topoisomerase II and the cleaved DNA

- Activity is unaffected by reducing agents

- Enhances enzyme-mediated DNA cleavage when added to the enzyme-DNA complex

- Enhances DNA cleavage when incubated with the enzyme prior to the addition of DNA

- Inhibits DNA ligation by intercalating into the cleaved scissile bond

- Examples: etoposide, amsacrine, doxorubicin

Covalent Poisons

- Covalently adducts topoisomerase II at amino acid residues distal to the active site

- Activity is blocked by reducing agents

- Enhances enzyme-mediated DNA cleavage only when added to the enzyme-DNA complex

- Inactivates topoisomerase II when incubated with the enzyme prior to the addition of DNA

- Inhibits DNA ligation by a mechanism that may be related to the ability to close the N-terminal gate of the protein

- Examples: benzoquinone, EGCG, curcumin

Figure 5. Properties of interfacial and covalent topoisomerase II poisons.

encompass a diverse group of natural and synthetic compounds and are used to treat a variety of human malignancies (*2, 8-11, 48*). For example, etoposide and doxorubicin (and its derivatives) are front-line therapies for a myriad of systemic cancers and solid tumors, including leukemias, lymphomas, sarcomas, breast cancers, lung cancers, neuroblastoma, and germ-cell malignancies. Furthermore, mitoxantrone is used to treat breast cancer, acute myeloid leukemia, and non-Hodgkin lymphoma. In addition, it is used as a single agent to treat multiple sclerosis.

All clinically relevant topoisomerase II-targeted anticancer drugs act as interfacial poisons. Furthermore, they all affect the activities of both enzyme isoforms. However, the degree to which topoisomerase IIα and IIβ are targeted by any given drug and the relative contributions of the two isoforms to drug efficacy are not well understood (*2, 8-11, 48*). Although some drugs "prefer" one isoform over the other, no truly topoisomerase IIα- or topoisomerase IIβ-specific drugs are available for clinical use at the present time (*12*).

Quinolone Antibacterials

Bacterial DNA gyrase and topoisomerase IV are the targets for quinolone-based antibacterial agents (*13-20*). Structures of selected quinolones are shown in Figure 6.

Quinolones are an extremely successful drug family and are widely prescribed for the prophylaxis and treatment of infections in humans. Members of this drug class include the most active and most broad-spectrum oral antibacterials currently in clinical use. These agents are effective against a variety of pathogens, including Gram-negative and Gram-positive species (*13-20*).
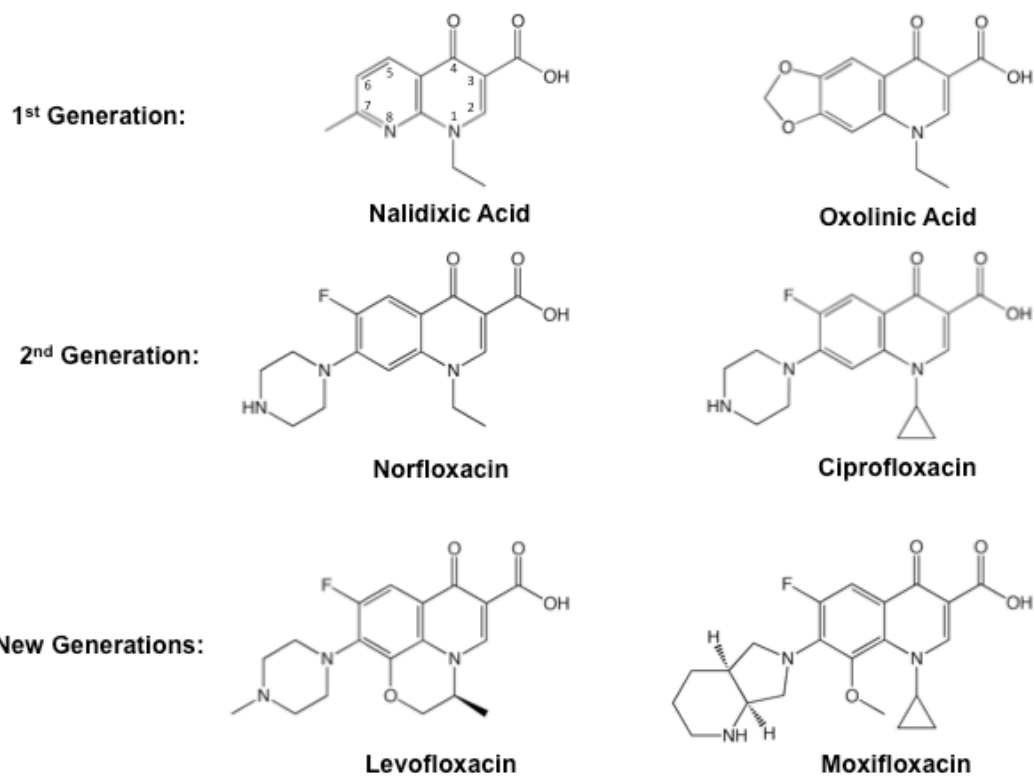
Figure 6. Structures of selected quinolone antibacterial agents that target gyrase and topoisomerase IV. The figure shows the first generation quinolones nalidixic acid and oxolinic acid, the second-generation fluoroquinolones norfloxacin and ciprofloxacin, and the newer generation fluoroquinolones levofloxacin and moxifloxacin. Quinolone numbering is given on the structure of nalidixic acid.

In parallel to the effects of anticancer drugs on human topoisomerases, quinolones increase levels of gyrase- and topoisomerase IV-DNA cleavage complexes (in large part) by inhibiting enzyme-mediated ligation of cleaved DNA (*19, 58-60*). Recently generated crystal structures provide strong evidence that these drugs contact both the protein and the DNA in the ternary complex and block ligation by intercalating into the double helix at the cleaved scissile bond (Figure 7) (*61*).

The founding quinolone, nalidixic acid (Figure 6), was first synthesized in the early 1960s (*14, 18*). This compound, along with the other early generation quinolones (such as oxolinic acid), was introduced into clinics throughout that decade for the treatment of urinary tract infections. Ultimately, these first-generation quinolones were dropped from clinical use due to their limited efficacy.

By the early 1980s, advancements in quinolone chemistry resulted in the development of second-generation compounds with considerably improved activity and pharmacokinetics (*13-20*). The most critical change was the introduction of a fluorine atom at the C-6 position (Figure 6). This alteration dramatically enhanced potency against gyrase and promoted drug uptake by the bacterial cell. The first clinically important "fluoroquinolone" was norfloxacin (Figure 6). It displayed much greater activity against Gram-negative bacteria than did earlier quinolones and also exhibited modest activity against some Gram-positive species. Due to poor tissue distribution and low serum levels, norfloxacin was restricted to the treatment of urinary tract infections and sexually transmitted diseases. Subsequent fluoroquinolones (Figure 6), such as ciprofloxacin, were the first family members to display clinical activity outside of the urinary tract. Ciprofloxacin is used to treat a variety of Gram-negative (and, to a lesser

Figure 7. Crystal structure of a moxifloxacin-stabilized *Acinetobacter baumannii* topoisomerase IV-DNA cleavage complex. The catalytic core of the enzyme is shown. Moxifloxacin is shown in red, the topoisomerase IV A and B subunits are shown in blue and green, respectively, and DNA is shown in yellow. Top: A top view of the cleavage complex showing two quinolone molecules intercalating four base pairs apart at the sites of DNA cleavage. Bottom: A front view (rotated by 90° from the top view) of the cleavage complex. Protein Data Bank accession 2XKK was visualized using Discovery Studio 3.5 Visualizer (Accelrys Software Inc.). Adapted from Wohlkonig *et al.* (*61*).

extent, Gram-positive) pathogens and has come to worldwide attention as the drug of choice for the treatment of anthrax.

Recently, newer generations of quinolones have emerged that display markedly higher activity against Gram-positive bacteria (*13-20*). Drugs such as levofloxacin and moxifloxacin (Figure 6) also display excellent activity against Gram-positive respiratory tract infections and have greatly extended the clinical range of this drug class.

Quinolone Targeting

Gyrase was first identified as the toxic target for quinolones in *Escherichia coli* in 1977 (*62, 63*). The discovery that the ParC/ParE subunits constituted topoisomerase IV raised the question of whether this topoisomerase also was a target for quinolones (*64*). Most subsequent studies concur that both enzymes are targets for quinolones in bacterial cells (*17, 65*). However, the relative contributions of gyrase and topoisomerase IV to drug efficacy are not well understood. Early studies suggested that gyrase rather than topoisomerase IV was the primary cytotoxic target for quinolones in Gram-negative bacteria, but that the opposite was true in Gram-positive species (*14*). However, recent studies indicate that this paradigm does not hold in all cases and that the issue of quinolone targeting needs to be evaluated on a strain-by-strain and drug-by-drug basis (*17, 19*).

Quinolone Resistance and Enzyme Interactions

Resistance to antibacterial agents has become an increasingly important clinical issue (*17-19, 65*). Unfortunately (due in part to overuse), quinolone resistance is

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becoming more prevalent. Although increased levels of multidrug efflux pumps often play a role in cases of high resistance, initial resistance to quinolones is usually associated with point mutations in the DNA cleavage-ligation subunits of gyrase (GyrA) and/or topoisomerase IV (ParC/GrlA).

The two most common mutations associated with quinolone resistance occur at Ser83 and Glu87 of GyrA (sequence numbering is based on *E. coli* GyrA) or the homologous residues of ParC/GrlA (*17-19, 65*). The enzyme in which a mutation occurs first is defined as the primary target for quinolones in a given bacterial species. This event generally confers <10–fold resistance. Subsequent mutation of the second enzyme often confers an additional order of magnitude of quinolone resistance.

Quinolone resistance is associated with a decreased affinity of gyrase or topoisomerase IV for the drug as well as a decreased ability of the drug to induce stable cleavage complexes and inhibit DNA ligation (*18, 19, 66*). Recent structural and biochemical evidence indicates that the basis for Ser83/Glu87-mediated resistance is related to the ability of quinolones to bind divalent metal ions (Figure 8) (*19, 60, 61, 66-68*). The primary interaction of clinically relevant quinolones with gyrase and topoisomerase IV is coordinated through this metal ion *via* four water molecules, two of which are anchored by Ser83 and Glu87. Thus far, the presence of the water-metal ion bridge and its role in mediating interactions with clinically relevant quinolones has been demonstrated in topoisomerase IV. Although mutagenesis studies in gyrase are consistent with a critical role for the water-metal ion bridge, the existence of the bridge has yet to be demonstrated in gyrase.

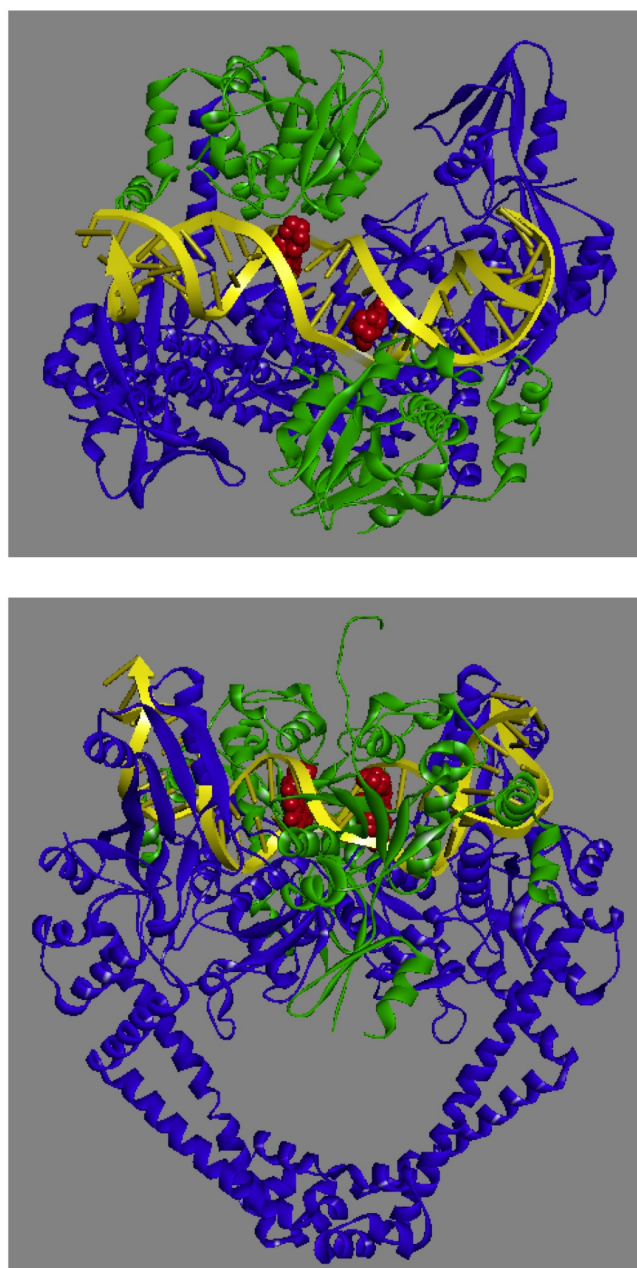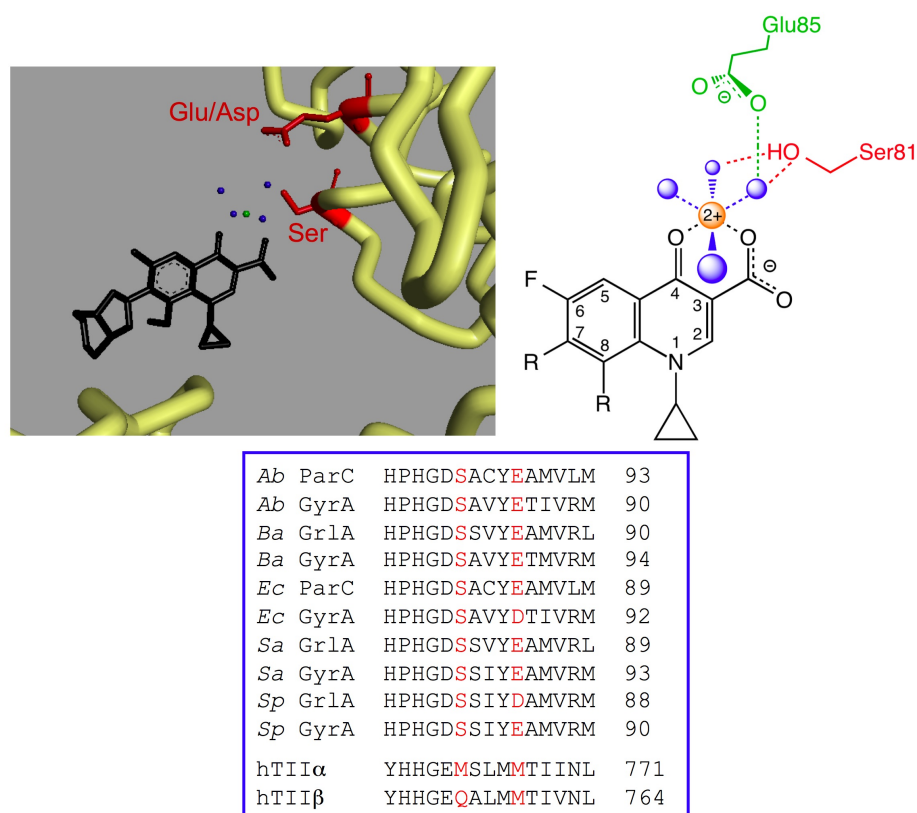Figure 8. Quinolone-topoisomerase IV binding is mediated by a water-metal ion bridge. Left: Crystal structure of a moxifloxacin-stabilized *Acinetobacter baumannii* topoisomerase IV-DNA cleavage complex. Moxifloxacin is shown in black and the non-catalytic Mg^{2+} ion that is chelated by the C3/C4 keto acid of the quinolone and participates in the bridge is shown in green. The four water molecules that fill out the coordination sphere of the Mg^{2+} ion are shown in blue. The backbone of selected portions of the protein amino acid chain is shown in yellow. The side chains of the serine and acidic residues that form hydrogen bonds with the water molecules in the water-metal ion bridge are shown in red. For clarity, DNA has been omitted from the picture. Protein Data Bank accession 2XKK was visualized using Discovery Studio 3.5 Visualizer (Accelrys Software Inc.). Adapted from Wohlkonig *et al.* (*61*) Right: Simplified diagram of the water-metal ion bridge adapted from Aldred *et al.* (*19*). A generic quinolone is in black, the non-catalytic Mg^{2+} is orange, water molecules are blue, and the coordinating serine and acidic residues (*B. anthracis* topoisomerase IV numbering) are red and green, respectively. Blue dashed lines indicate the octahedral coordination sphere of the divalent metal ion. The red or green dashed lines represent hydrogen bonds between the serine side chain hydroxyl group or the acidic residue side chain carboxyl group and the water molecules. Bottom: Sequence alignment of the A subunits showing the serine and acidic residues (red) that coordinate the water-metal ion bridge. Sequences of *A. baumannii* (*Ab*), *Bacillus anthracis* (*Ba*), *Escherichia coli* (*Ec*), *Staphylococcus aureus* (*Sa*), and *Streptococcus pneumoniae* (*Sp*) gyrase (GyrA) and topoisomerase IV (ParC/GrlA) are shown. The homologous regions of human topoisomerase IIα (hTIIα) and IIβ (hTIIβ), which lack the residues necessary to coordinate the water-metal ion bridge interaction, are shown for comparison.

Because of the importance of type II topoisomerases to nucleic acid functions in human and bacterial cells and because of the wide use of anticancer and antibacterial agents that target these enzymes, it is important to understand how type II topoisomerases interact with it DNA substrate and with drugs. Therefore, the goals of this dissertation are to further our understanding of how human and bacterial type II topoisomerases interact with DNA and discern the handedness of DNA supercoils, how human topoisomerase IIα interacts with covalent poisons, and how bacterial gyrase interacts with quinolone antibacterials.

An introduction to bacterial and human type II topoisomerases, topoisomerase II poisons, and quinolone antibacterials is presented in Chapter I.

Chapter II describes the characterization of the catalytic core of human topoisomerase IIα. Results further define the distinct contributions of the N-terminal gate and the catalytic core to topoisomerase II function. The work demonstrates that the catalytic core senses the handedness of DNA supercoils during cleavage, while the N-terminal gate is critical for capturing the transport-segment and for the activity of covalent poisons. These findings have been published (*69*).

Chapter III describes the ability of *Bacillus anthracis* gyrase to discern the handedness of DNA supercoils during DNA cleavage. Results also lay the groundwork for future studies on assessing the role of the water-metal ion bridge in mediating interactions between gyrase and clinically relevant quinolones.

Concluding remarks on the research presented in this dissertation are provided in Chapter IV.

CHAPTER II

THE CATALYTIC CORE OF HUMAN TOPOISOMERASE IIα: INSIGHTS INTO ENZYME–DNA INTERACTIONS AND DRUG MECHANISM

Introduction

As discussed in the previous chapter, eukaryotic type II topoisomerases function as homodimeric proteins. On the basis of homology with DNA gyrase, these enzymes can be divided into three domains: the N-terminal domain, the catalytic core, and the C-terminal domain (*4-8, 70*). The N-terminal domain contains the site of ATP binding and hydrolysis. ATP binding triggers dimerization of the N-terminal domain, which helps capture the T-segment and closes the N-terminal protein gate (*71*). This action induces the transport of the T-segment passage through the open gate in the G-segment (*4-8, 70, 71*). The catalytic core of topoisomerase II contains the active site tyrosine that cleaves and covalently attaches to the DNA. It also forms a second protein gate that allows the T-segment to exit the enzyme following strand passage. The C-terminal domain is the least understood portion of topoisomerase II. It is highly variable and contains nuclear localization sequences and sites of phosphorylation (*2, 7, 8*). Although it is not necessary for catalytic activity, the C-terminal domain is involved in the recognition of DNA geometry during strand passage and provides different type II topoisomerases with unique capabilities. In human topoisomerase IIα, the C-terminal domain allows the enzyme to relax positively supercoiled (*i.e.*, overwound) DNA that accumulates ahead of replication forks ten times faster than it does negatively supercoiled (*i.e.*, underwound) molecules

(*36, 72*). In contrast, topoisomerase IIβ relaxes positive and negative DNA supercoils at the same rate (*36, 72*).

Although only topoisomerase IIα is able to recognize the handedness of DNA supercoils during relaxation, both isoforms are able to distinguish between positive and negative supercoils during DNA cleavage (*73*). Topoisomerase IIα and topoisomerase IIβ maintain higher levels of cleavage complexes with underwound as compared to overwound molecules. In spite of the important role played by the C-terminal domain in distinguishing DNA geometry during relaxation, this portion of the enzyme is not involved in recognizing supercoil handedness during DNA cleavage (*72*). It is not obvious which domain of topoisomerase II is responsible for this recognition. Given the role of the N-terminal gate in capturing the T-segment and that of the catalytic core in cleaving the G-segment, both are likely candidates.

In addition, despite the suggested role of the N-terminal gate in the actions of covalent poisons (*74, 75*), the portion of topoisomerase II that mediates the effects of these compounds has not been established. Further complicating this issue, covalent poisons have been shown to adduct cysteine residues in both the N-terminal gate and the catalytic core of topoisomerase IIα (*51, 53*).

To address the above issues, the DNA cleavage activity of the catalytic core of human topoisomerase IIα was characterized. Results indicate that the catalytic core is sufficient for the enzyme to recognize DNA supercoil handedness during the cleavage reaction. However, the catalytic core alone displayed little ability to cleave DNA substrates that did not intrinsically provide the enzyme with a transport segment (*i.e.*,

substrates that did not contain crossovers). Finally, the N-terminal gate is necessary for cleavage enhancement by covalent poisons.

Experimental Procedures

Enzymes

The truncated hTop2$\alpha\Delta$1175 (containing amino acids 1-1175) was constructed as described previously (*76*). Human topoisomerase IIα and hTop2$\alpha\Delta$1175 were expressed in *S. cerevisiae* JEL-1Δtop1 and purified as described by Kingma *et al*. (*77*). The catalytic core of human topoisomerase IIα (containing residues 431-1193) (*78*) was expressed in yeast cells and purified using a Ni^{2+}-nitriloacetic acid agarose column (Qiagen) as described previously (*79, 80*). The enzyme was stored at -80 °C as a 1.5 mg/mL stock in 50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 750 mM KCl, 5% glycerol. For all of the enzymes examined, the concentration of dithiothreitol carried over from purification protocols was <2 μM in final reaction mixtures.

DNA Substrates

Negatively supercoiled pBR322 plasmid DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Positively supercoiled pBR322 DNA was prepared by treating negatively supercoiled molecules with recombinant *Archaeoglobus fulgidus* reverse gyrase (*36, 81*). The number of positive supercoils was comparable to the number of negative supercoils in the original pBR322 preparations (*36*). For experiments comparing positively and negatively supercoiled DNA, the negatively supercoiled plasmid was processed identically to the positively supercoiled

molecules except that reverse gyrase was omitted from reaction mixtures. Relaxed pBR322 plasmid DNA was generated by treating negatively supercoiled pBR322 with topoisomerase I and purified as described previously (*67*).

Drugs

Etoposide, benzoquinone, and thymoquinone were purchased from Sigma-Aldrich. Etoposide was stored at room temperature as a 20 mM solution in 100% DMSO. Benzoquinone was stored at -20 °C as a 20 mM solution in water. Thymoquinone was stored at 4 °C as a 40 mM solution in 100% DMSO. The quinolone CP-115,953 was the gift of Thomas D. Gootz and Paul R. McGuirk (Pfizer). It was stored at -20 °C as a 40 mM solution in 0.1 N NaOH and was diluted five-fold with 10 mM Tris–HCl (pH 7.9) immediately prior to use. Etoposide quinone was synthesized as described previously (*82-84*) and was stored at 4 °C as a 20 mM solution in 100% DMSO.

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*85*). Reaction mixtures contained 10 nM pBR322 and 150 nM wild-type topoisomerase IIα, 80 nM hTop2αΔl175, or 430 nM catalytic core in a total of 20 μL of cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. Reaction mixtures were incubated at 37 °C for 6 min, and enzyme–DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the

enzyme. Samples were mixed with 2 μL of agarose loading dye [60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 2 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized by UV light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

Note that lower levels of hTop2$\alpha\Delta$1175 were used in reaction mixtures because in the presence of Mg^{2+}, the protein displays ~2–fold higher levels of DNA cleavage than does wild-type topoisomerase IIα (*76*). Conversely, higher levels of the catalytic core were used because it displays lower levels of baseline DNA cleavage than does the wild-type enzyme in reactions containing Mg^{2+} (see Figure 10).

DNA cleavage reactions were carried out in the presence of 0–250 μM etoposide, 0–20 μM CP–115,953, or 0–100 μM benzoquinone, thymoquinone, or etoposide quinone. Alternatively, $MgCl_2$ was omitted from the cleavage buffer and reaction mixtures contained 0–5 mM $CaCl_2$.

DNA Cleavage Site Utilization

DNA cleavage sites were mapped using a modification (*86*) of the procedure of O'Reilly and Kreuzer (*87*). A unique derivative of pUC19 (pMP-bcr6) was used as substrate for DNA cleavage site utilization experiments. The substrate was generated by modifying pUC19 to include a region of *PML* intron 6 that contains an established breakpoint associated with therapy-related acute promyelocytic leukemia. The genomic

DNA of human CEM cells was prepared using standard protocols according to the manufacturer's instructions for the DNeasy Blood and Tissue Kit (Qiagen). 5'-GGGGGGATCCTTCTGCAAAGGCCACCTACC-3' and 5'-AGGGGAAGCTTCACT-GTCCCCATTCTCAGC-3' primers were synthesized for amplifying a 319-bp region of the PML gene (44157-44475 on GenBank accession number NG029036) with the CEM genomic DNA as template. Purified clones containing the insert were transformed into XL1-Blue cells and sequenced with M13 primers by Vantage (Vanderbilt Technologies for Advanced Sequencing.) pMP-bcr6 was linearized by treatment with *Acc*651. Terminal 5'-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA was treated with *Eco*RI, and the 2968-bp singly end-labeled fragment was purified from the small *Eco*RI–*Acc*651 fragment by being passed through a CHROMA SPIN+TE-100 column (Clontech).

Reaction mixtures contained 1 nM labeled pMP-bcr6 and 60 nM wild-type human topoisomerase IIα or 115 nM catalytic core in 50 μL of DNA cleavage buffer (containing 5 mM Ca^{2+}) in the absence or presence of compounds. Reaction mixtures were incubated at 37 °C for 6 min, and enzyme–DNA cleavage complexes were trapped by the addition of 5 μL of 5% SDS followed by 4 μL of 250 mM EDTA (pH 8.0). Proteinase K (5 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. DNA products were precipitated with ethanol and resuspended in 5 μL of polyacrylamide gel loading buffer [10% agarose gel loading buffer, 80% formamide, 100 mM Tris-borate (pH 8.3), and 2 mM EDTA]. Samples were subjected to electrophoresis in denaturing 6% polyacrylamide sequencing gels. Gels were dried *in*

vacuo, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

<div align="center">Results and Discussion</div>

Recognition of Supercoil Geometry by Human Topoisomerase IIα

Human type II topoisomerases can distinguish the handedness of DNA supercoils during scission and maintain levels of cleavage complexes with negatively supercoiled DNA that are ~2-4–fold higher than those seen with positively supercoiled molecules (Figure 9, left) (*73*). Several lines of evidence indicate that the C-terminal domain of topoisomerase II is not involved in this recognition (*72, 73*). Most notably, deletion of the C-terminal domain of human topoisomerase IIα does not affect the ability of the enzyme to preferentially cleave underwound molecules (*72*).

It is not clear which portion of topoisomerase IIα is responsible for the recognition of DNA geometry during cleavage. Although DNA cleavage is mediated by the catalytic core of the enzyme, rates of cleavage can be modulated by changes in the N-terminal gate (*24, 88*) Therefore, the ability of the catalytic core of topoisomerase IIα to cleave negatively and positively supercoiled DNA was assessed. As shown in Figure 9 (right), the catalytic core retained the ability to recognize supercoil handedness and preferentially cleaved negatively supercoiled plasmid.

The above experiments substituted Ca^{2+} for Mg^{2+} as the required divalent metal ion in order to generate levels of DNA cleavage that were high enough to reliably quantify enzyme-mediated DNA scission under conditions that did not include anticancer

Figure 9. The catalytic core of human topoisomerase IIα preferentially cleaves negatively supercoiled DNA in the presence of Ca^{2+}. The ability of wild-type topoisomerase IIα (WT, left panel) and the catalytic core (CC, right panel) to cleave negatively [(-)SC, filled circles] or positively [(+)SC, open circles] supercoiled plasmid DNA is shown. Error bars represent the standard deviation of at least three independent experiments.

drugs (*22*). Previous work demonstrated that Ca^{2+} does not affect DNA cleavage site selection by topoisomerase IIα or the ability of type II enzymes to recognize DNA supercoil geometry (*73, 89*). Although the catalytic core displayed a preference for negatively supercoiled DNA that was similar to that of wild-type topoisomerase IIα (Figure 9), it exhibited unexpectedly high levels of DNA cleavage in the presence of Ca^{2+}. The underlying basis for this high level of Ca^{2+}-supported DNA cleavage is not known.

To further confirm the ability of the catalytic core to distinguish supercoil geometry, DNA cleavage was examined in the presence of Mg^{2+} and topoisomerase II poisons (Figure 10). Etoposide and the quinolone CP-115,953 are well-characterized interfacial poisons that do not intercalate into DNA (which would change the apparent topology of the plasmid substrate) (*90, 91*). Similar to results seen with Ca^{2+}, the catalytic core maintained higher levels of cleavage complexes with negatively as compared to positively supercoiled DNA in the presence of Mg^{2+} and interfacial topoisomerase II poisons.

These results provide strong evidence that the ability to distinguish the geometry of DNA supercoils during cleavage is embedded in the catalytic core of human topoisomerase IIα.

Role of the N-Terminal Gate of Topoisomerase IIα in Mediating Interactions with the T-Segment of DNA

Topoisomerase II binds negatively supercoiled DNA at sites of helix-helix crossovers, and it has been proposed that this ability to bind DNA crossovers allows topoisomerase II to distinguish between relaxed and supercoiled molecules (*89, 92*).
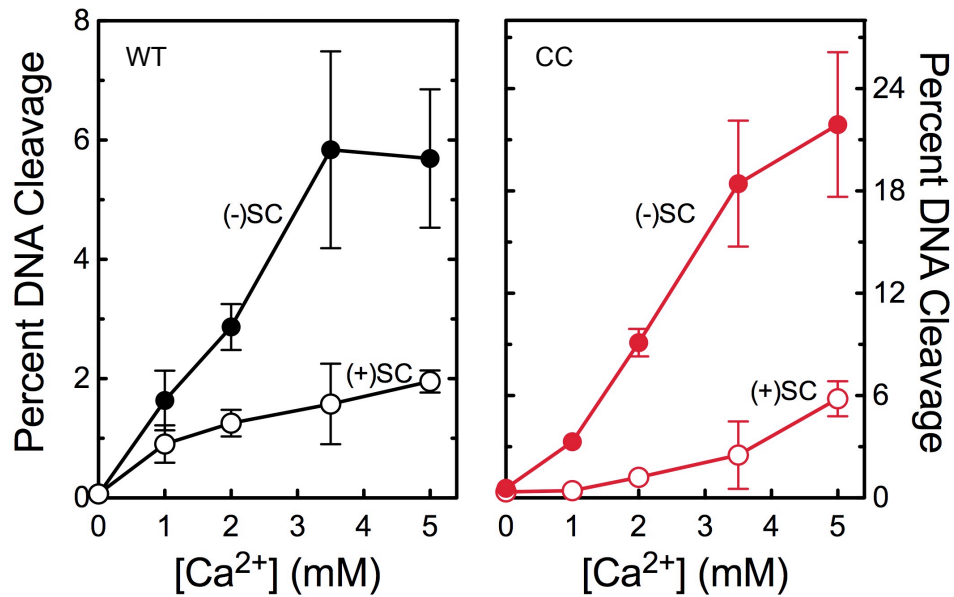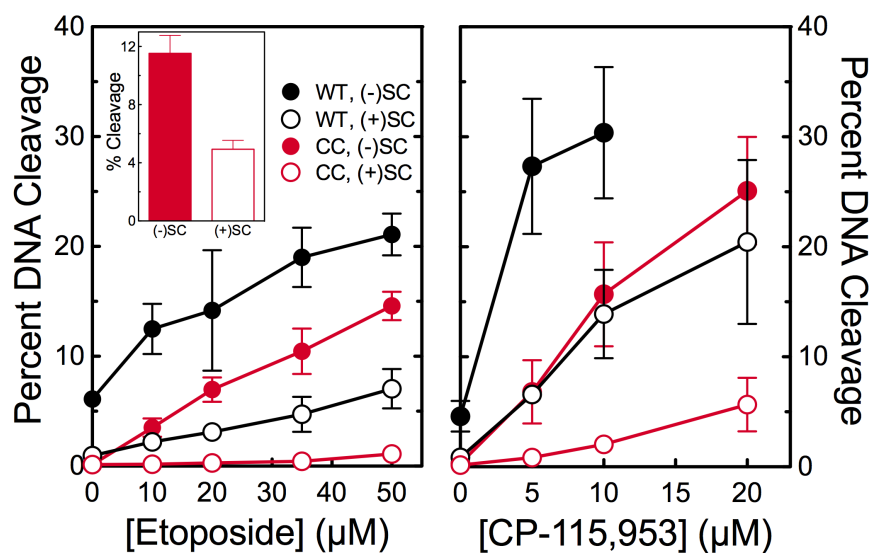
Figure 10. The catalytic core of human topoisomerase IIα preferentially cleaves negatively supercoiled DNA in the presence of etoposide and CP-115,953. The ability of wild-type topoisomerase IIα (WT, black) and the catalytic core (CC, red) to cleave negatively [(-)SC, filled circles] or positively [(+)SC, open circles] supercoiled plasmid DNA in the presence of etoposide (left panel) or CP-115,953 (right panel) is shown. Results for CP-115,953 are not shown at concentrations above 10 μM with the wild-type enzyme because the drug induced multiple cleavage events per plasmid. The inset shows cleavage induced by the catalytic core in the presence of 250 μM etoposide. Error bars represent the standard deviation of at least three independent experiments.

Although it has not been rigorously demonstrated, it is believed that the two DNA helices at the crossover become the G- and T-segments.

Studies with oligonucleotides indicate that binding of the T-segment greatly stimulates topoisomerase II-mediated cleavage of the G-segment (*24, 93*). These findings suggest that the T-segment plays an important role in the ability of topoisomerase II to identify and relax DNA supercoils. Although the N-terminal gate of the protein plays a critical role in capturing the T-segment and passing it through the DNA gate, it is not known whether the initial interaction with the T-segment is mediated by this portion of topoisomerase II or by the catalytic core of the enzyme. Therefore, we examined the ability of the catalytic core of topoisomerase IIα to cleave DNA that does not contain intrinsic crossovers.

In the first experiment, a linearized plasmid was used as the DNA substrate. Etoposide was included in experiments to increase levels of DNA scission. As seen in Figure 11, wild-type human topoisomerase IIα was able to cleave the DNA in the absence or presence of drug. Cleavage was enhanced when ATP was added to reaction mixtures. One interpretation of this finding is that in the presence of ATP, the N-terminal gate is able to capture the T-segment, thereby stimulating DNA scission. In contrast, the catalytic core of the enzyme was unable to cleave the linearized plasmid under any of the above conditions. Furthermore, no DNA cleavage was seen when the concentration of the catalytic core was increased 5-fold, or when Mg^{2+} was used in place of Ca^{2+} in reaction mixtures (not shown). These findings suggest that the N-terminal gate plays a critical role in mediating interactions with the T-segment.
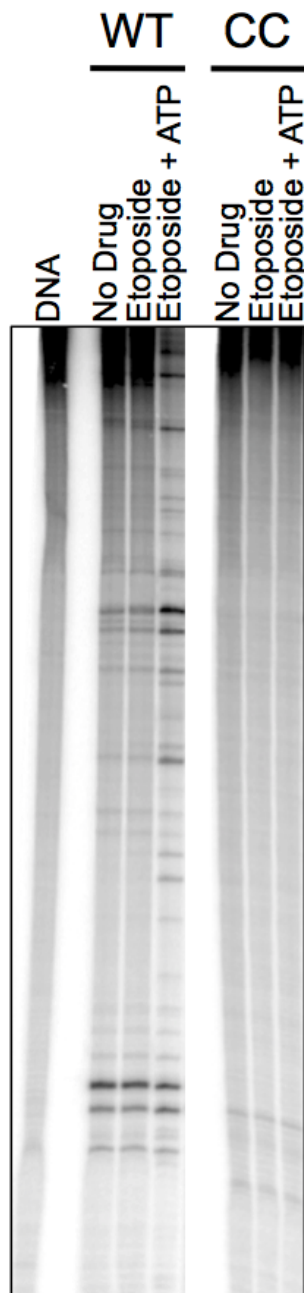
Figure 11. Effects of etoposide on sites of DNA cleavage mediated by wild-type human topoisomerase IIα and the catalytic core. An autoradiogram of a polyacrylamide gel depicting DNA sites cleaved by wild-type topoisomerase IIα (WT) and the catalytic core (CC) is shown. Reaction mixtures contained no enzyme (DNA), enzyme in the absence of drug (No Drug), or enzyme in the presence of 20 μM etoposide with or without 1 mM ATP. Lanes shown were taken from different portions of the same gel. The autoradiogram is representative of three independent experiments.

To further explore this conclusion, the ability of wild-type topoisomerase IIα and the catalytic core to cleave relaxed DNA was examined (Figure 12). In contrast to negatively supercoiled plasmid, which contains numerous inherent DNA crossovers, relaxed molecules contain few, if any intrinsic sites of helix-helix juxtaposition. For this reason, topoisomerase II preferentially cleaves negatively supercoiled over relaxed molecules (compare scission in Figures 10 and 12) (*89*).

Wild-type topoisomerase IIα was able to cleave relaxed plasmid (Figure 12, left), suggesting that the full-length enzyme can capture a transport-helix even when there are few intrinsic crossovers in the DNA substrate. When ATP was added to reaction mixtures, levels of cleavage increased ~9–fold (left), consistent with the conclusion that ATP induces closing of the N-terminal gate, stabilizing the capture of the T-segment. Although ATP also stimulates the ability of the intact enzyme to cleave negatively supercoiled plasmid (Figure 12, right), this enhancement is much smaller (~2–fold). The presence of high levels of helix-helix crossovers in the negatively supercoiled plasmid facilitates interactions between the intact enzyme and helix-helix crossovers, partially obviating the need for T-segment capture by the addition of ATP.

In contrast to topoisomerase IIα that contained its N-terminal gate, the catalytic core was unable to cleave relaxed plasmid in the absence or presence of ATP (Figure 12, left). Moreover, the addition of ATP did not enhance cleavage of negatively supercoiled plasmid (right).

These experiments lead to the conclusion that the catalytic core of human topoisomerase IIα cannot efficiently capture the T-segment. More importantly, they also lead to the conclusion that the N-terminal gate of the enzyme plays a critical role in

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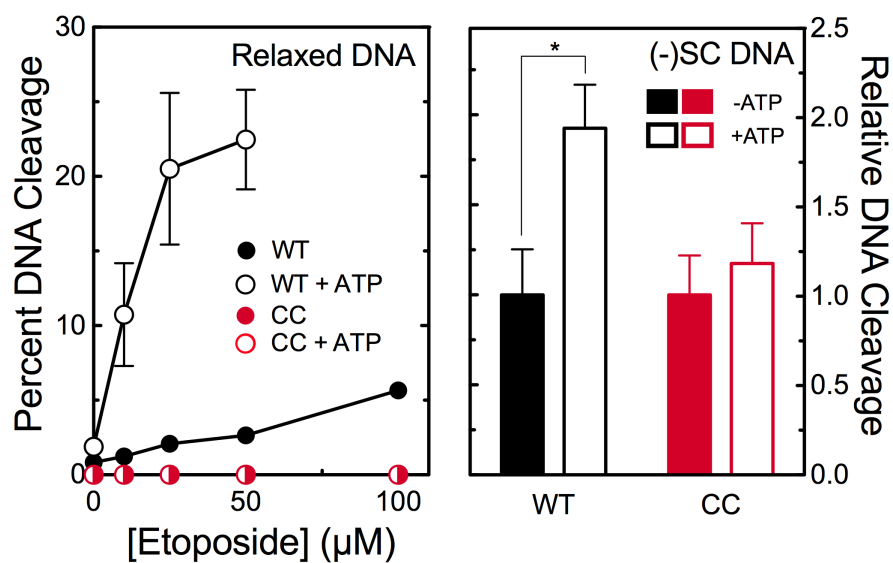
Figure 12. Effects of ATP on cleavage of relaxed and negatively supercoiled DNA by wild-type human topoisomerase IIα and the catalytic core. The ability of wild-type topoisomerase IIα (WT, black) and the catalytic core (CC, red) to cleave relaxed DNA (left panel) or negatively supercoiled DNA (right panel) in the presence of etoposide is shown. Experiments were carried out in the absence (filled symbols) or presence (open symbols) of 500 μM ATP. Experiments with negatively supercoiled DNA contained 50 μM etoposide. Error bars represent the standard deviation of at least three independent experiments. Statistically significant difference is noted by an asterisk (*$p < 0.01$).

mediating the initial interaction with the T-segment. In the absence of this protein domain, the catalytic core is able to cleave supercoiled plasmid primarily because the substrate carries intrinsic DNA crossovers. Thus, the substrate is able to present the T-segment to the enzyme, even in the absence of the protein domain that normally mediates the interaction with the second DNA double helix.

Role of the N-Terminal Gate of Human Topoisomerase IIα in Mediating the Actions of Covalent Poisons

A number of environmental, dietary, and medicinal compounds act as covalent topoisomerase II poisons (*48, 50, 53-55, 57, 74, 75, 94-96*). Compared to interfacial poisons, the mechanistic basis for the actions of covalent poisons is less well understood. These compounds form adducts with the enzyme (*48, 50-53*). At the present time, only cysteine adducts have been characterized. It has been proposed that the ability of covalent poisons to close the N-terminal gate plays an important role in mediating their ability to increase levels of topoisomerase II-DNA cleavage complexes (*74, 75*). However, modified residues have been identified in both the N-terminal gate and the catalytic core (*51, 53*).

In order to explore the role of the N-terminal gate in the actions of covalent poisons, the effects of benzoquinone (*94*) and thymoquinone (*57*) on DNA cleavage mediated by wild-type topoisomerase IIα and the catalytic core were examined. Effects on cleavage mediated by a truncated human enzyme lacking the C-terminal domain (hTop2αΔl175) also were determined as a control. Benzoquinone and thymoquinone displayed similar abilities to increase cleavage complexes formed with full-length topoisomerase IIα or hTop2αΔl175 and negatively supercoiled plasmid (Figure 13). This
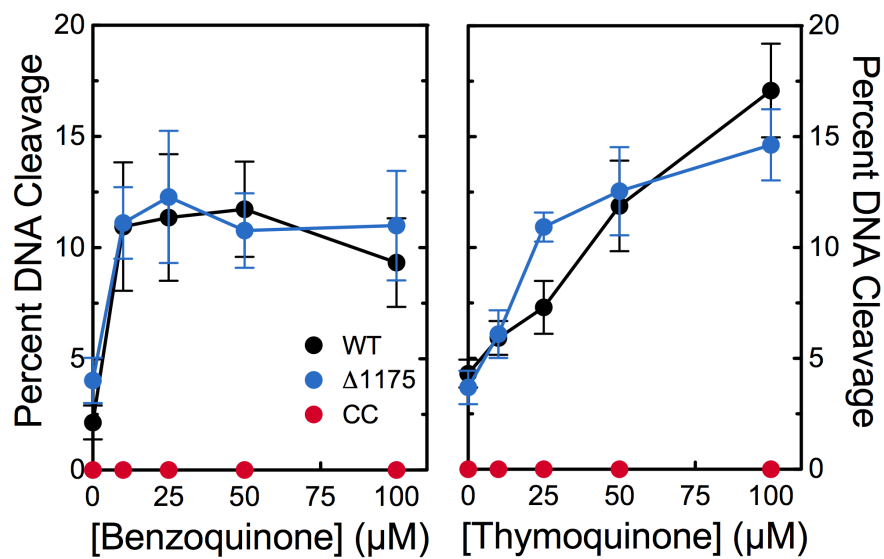
Figure 13. Covalent poisons do not enhance DNA cleavage mediated by the catalytic core of human topoisomerase IIα. Effects of benzoquinone (left) and thymoquinone (right) on DNA cleavage mediated by wild-type human topoisomerase IIα (black), the catalytic core (red), and hTop2αΔ1175 (blue) are shown. Error bars represent the standard deviation of at least three independent experiments.

finding demonstrates that the C-terminal domain plays no significant role in mediating the actions of covalent topoisomerase II poisons. In marked contrast, neither compound displayed any ability to enhance DNA cleavage mediated by the catalytic core. This result indicates that the N-terminal gate of topoisomerase IIα is critical for the actions of covalent poisons. Furthermore, it provides yet another distinction between interfacial poisons (which do not require the N-terminal gate to stimulate topoisomerase II-mediated DNA cleavage; see Figure 10) and covalent poisons.

In further contrast to interfacial poisons, covalent topoisomerase II poisons display the hallmark characteristic of inactivating the enzyme when the two are incubated prior to the addition of DNA (*50, 94*). Even though the inactivation can be explained by the ability of covalent poisons to close the N-terminal protein gate (thus preventing DNA from entering the active site of topoisomerase II) (*51, 75*), this proposed mechanism is controversial. Indeed, treatment of human topoisomerase IIα with benzoquinone or PCB quinones blocks the ability of the enzyme to cleave oligonucleotides that are able to bind to the protein and diffuse into the active site without entering through the protein gate (*51*). This finding implies that mechanisms besides the proposed closing of the N-terminal gate may contribute to enzyme inactivation by covalent poisons.

To address this controversy, benzoquinone and thymoquinone were incubated with wild-type topoisomerase IIα, hTop2$\alpha\Delta$1175, or the catalytic core prior to the addition of negatively supercoiled plasmid and the effects on DNA cleavage were assessed. Assays with the catalytic core were carried out in the presence of Ca^{2+} in order to raise baseline levels of DNA cleavage (see Figure 9). As seen in Figure 14, benzoquinone and thymoquinone inactivated all three enzymes. Thus, while covalent

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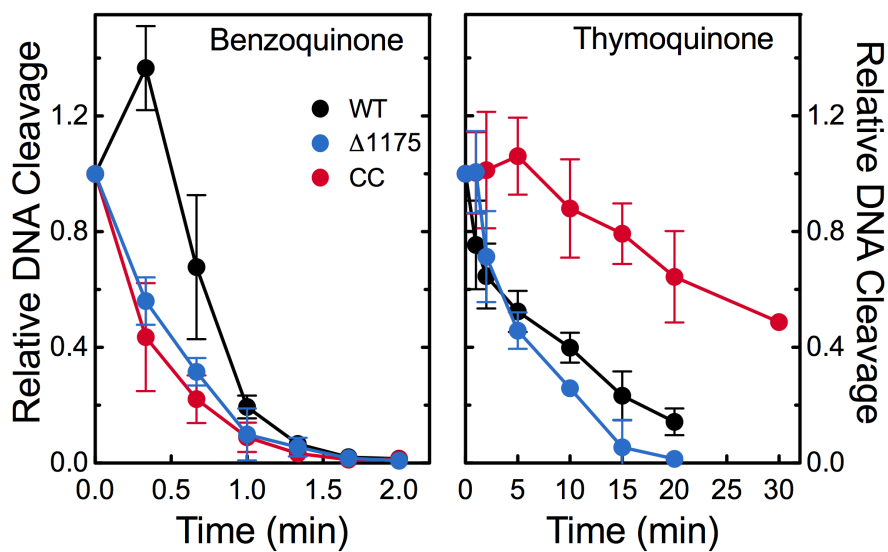
Figure 14. Covalent poisons inactivate human topoisomerase IIα enzymes when incubated with the protein prior to the addition of DNA. The DNA cleavage activities of wild-type human topoisomerase IIα (black), the catalytic core (red), and hTop2αΔ1175 (blue) were monitored in the presence of 50 μM benzoquinone (left) or 50 μM thymoquinone (right). DNA cleavage levels were calculated relative to cleavage induced when the drug and the enzyme were not incubated prior to DNA addition. Error bars represent the standard deviation of at least three independent experiments.

poisons require the N-terminal gate in order to stimulate DNA cleavage mediated by topoisomerase II, they do not require this portion of the protein to inactivate the enzyme. Although the closing of the N-terminal gate may contribute to topoisomerase II inactivation, clearly other mechanisms can produce a similar effect.

Mechanism of Action of Etoposide Quinone

Etoposide has been linked to the generation of treatment-related acute myeloid leukemia (*12, 42, 45, 46, 97*), and etoposide quinone, a metabolite of etoposide (*83, 98*), has been implicated in this process (*99*). These leukemias feature rearrangements of the *MLL* gene at chromosomal band 11q23 and are believed to be triggered by drug-induced DNA cleavage events mediated by human type II topoisomerases (*12, 42, 45, 46, 97*). Although etoposide is an interfacial topoisomerase II poison, several studies indicate that etoposide quinone acts primarily as a covalent poison (*84, 100, 101*). However, it is not known whether the covalent interaction of the quinone with topoisomerase II masks the fact that the metabolite also can act as an interfacial poison.

Previous studies indicate that the pendant E-ring of etoposide is critical to its actions as an interfacial poison (Figure 15) (*102-104*). Substitution of either the 3' or 5' methoxy groups with a hydroxyl moiety has little effect on drug activity (*100, 105*). Thus, the catechol metabolite of etoposide displays an activity (and mechanism) similar to that of the parent drug. Removal of the 4' hydroxyl moiety or substitution by a methoxy group greatly compromises the activity of etoposide (*103, 104*). However, it is not known whether substitution by a carbonyl group affects the ability of etoposide to function as an interfacial poison.
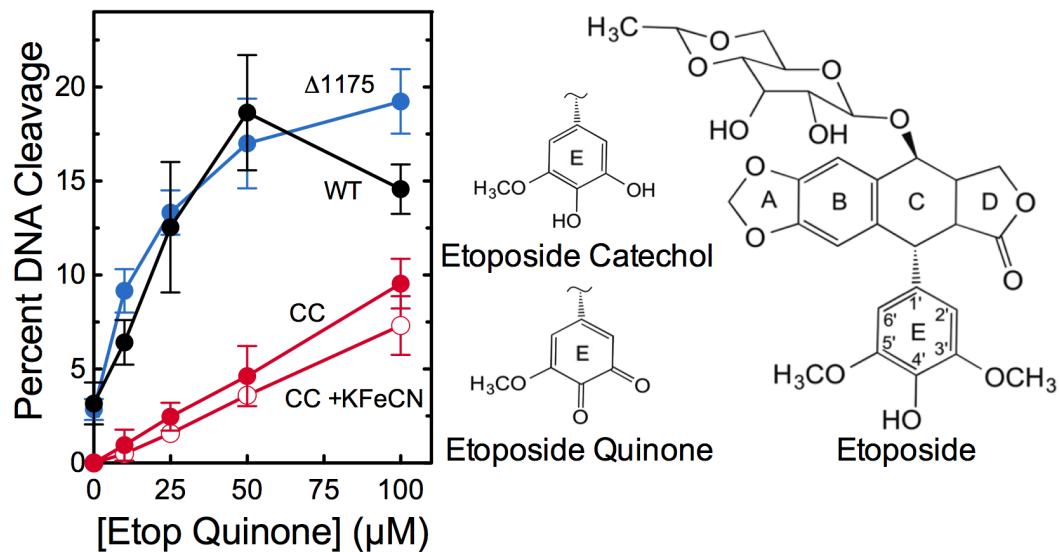
Figure 15. Etoposide quinone enhances DNA cleavage mediated by the catalytic core of human topoisomerase IIα. The effects of etoposide quinone on DNA cleavage mediated by wild-type human topoisomerase IIα (black), the catalytic core (red), and hTop2αΔ1175 (blue) are shown. Cleavage reactions with etoposide quinone and the catalytic core that included 10 μM K$_3$[Fe(CN)$_6$] (open circles) also are shown. The structure of etoposide and the E-rings of etoposide catechol and etoposide quinone are depicted at right. Error bars represent the standard deviation of at least three independent experiments.

The experiments shown in Figure 13 provide a method to determine whether etoposide quinone can function as an interfacial poison in addition to acting as a covalent poison. If etoposide quinone functions purely as a covalent poison, it should have no effect on DNA cleavage mediated by the catalytic core. However, if it retains the ability to act as an interfacial poison (despite the fact that it can also act as a covalent poison), it should display at least some activity against the catalytic core. As seen in Figure 15, etoposide quinone retains partial activity against the catalytic core of human topoisomerase IIα. It is possible that this activity reflects the fact that a portion of the etoposide quinone preparation has been reduced over time to the catechol, which is an interfacial topoisomerase II poison. To address this possibility, the effect of 10 μM $K_3[Fe(CN)_6]$ on the ability of etoposide quinone to enhance DNA cleavage mediated by the catalytic core was assessed. The oxidant, which converts the catechol to the quinone (*100*), had little effect on the actions of etoposide quinone against the catalytic core. Although etoposide quinone functions primarily as a covalent topoisomerase II poison, these findings indicate that it still retains a modest ability to act as an interfacial poison of human topoisomerase IIα.

Conclusions

The N-terminal gate and the catalytic core of type II topoisomerases work coordinately to capture, cleave, and transport DNA during the DNA strand passage reaction. Although this coordination is essential for proper enzyme function, it has obscured the individual contributions of these two domains to important aspects of enzyme–DNA interactions and drug mechanism. Previous studies have shown that the C-

terminal domain of human topoisomerase IIα is responsible for DNA geometry recognition during relaxation; however, the present results indicate that the catalytic core is the portion of the enzyme that senses the handedness of DNA supercoils during the cleavage reaction (Figure 16). Conversely, the N-terminal gate plays critical roles in the capture of the T-segment.

The use of different topoisomerase IIα constructs also provided considerable insight into the actions of covalent topoisomerase II poisons (Figure 16). Whereas the N-terminal gate is necessary for the enhancement of DNA cleavage by these compounds, residues within the catalytic core may be responsible for the inhibition of catalytic function that follows the incubation of covalent poisons with topoisomerase II prior to the addition of DNA. Finally, the ability of interfacial poisons, but not covalent poisons, to enhance DNA cleavage mediated by the catalytic core allowed us to further characterize the mechanism of action of etoposide quinone. Although this important drug metabolite functions primarily as a covalent poison, it still retains the ability to act in an interfacial manner.
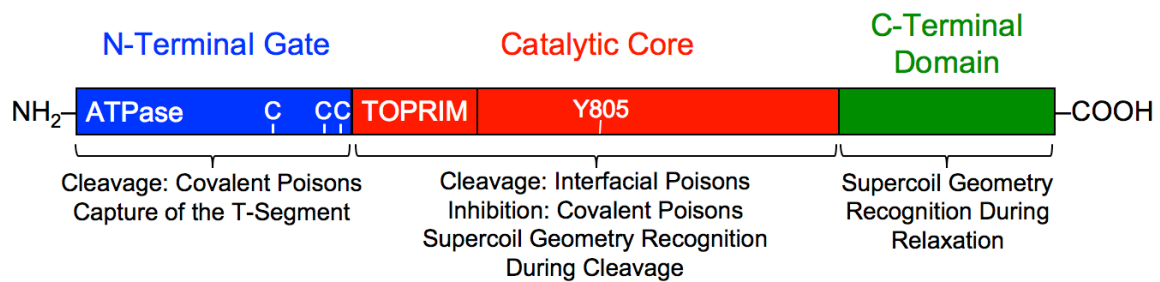
Figure 16. Domains of human topoisomerase IIα and their involvement in DNA geometry recognition and drug activity. The enzyme is divided into three domains: the N-terminal gate (blue, amino acid residues 1-430), which contains the ATPase active site; the catalytic core (red, residues 431-1193), which contains the TOPRIM domain (the portion that binds the catalytic divalent metal ions) and the DNA cleavage/ligation active site tyrosine residue (Y805); and the C-terminal domain (green, residues 1193-1531) (*1, 3, 4, 6, 8, 21, 22, 35*). Functions associated with each domain are indicated. Three cysteine residues (C) are indicated in the N-terminal gate (C300, C392, and C405 from left to right). Cys300 has been identified as a site of attachment of isothiocyanate-based covalent topoisomerase II poisons (*53*). However, it has not been established whether attachment at this residue plays a role in the actions of these agents (*53*). Cys392 and Cys405 have been identified as sites of attachment of quinone-based covalent poisons (*51*), and substitution of alanine residues at these positions results in an ~2–fold resistance to a variety of covalent poisons (*51, 55, 57*).

CHAPTER III

PRELIMINARY CHARACTERIZATION OF DNA GEOMETRY RECOGNITION BY
BACILLUS ANTHRACIS GYRASE AND DRUG INTERACTIONS WITH THE
BACTERIAL ENZYME

Introduction

Globally, the bacterial chromosome is negatively supercoiled (it is ~6% underwound) (*30*). However, when helicases convert double-stranded to single-stranded DNA, they do so without unwinding the two plectonemically coiled strands of the double helix. Thus, in contrast to bulk DNA, the DNA immediately ahead of replication forks, transcription complexes, and other tracking systems is overwound (*i.e.*, positively supercoiled) (*30, 106*). Because collisions with DNA tracking systems are critical for the conversion of transient topoisomerase-DNA cleavage complexes to permanent strand breaks, the cleavage complexes most likely to produce permanent strand breaks should be formed on overwound DNA (*8, 107*). Consequently, the ability of type II topoisomerases to cleave positively supercoiled DNA significantly impacts the potential response of cells to antibacterial drugs.

Previous studies indicate that human topoisomerase IIα and topoisomerase IIβ both are able to discern the geometry of DNA supercoils and maintain lower levels (~2– to 4–fold) of cleavage complexes with positively as compared to negatively supercoiled plasmids (*73*). Conversely, studies with *E. coli* topoisomerase IV suggest that this bacterial type II enzyme maintains higher levels of cleavage complexes with positively supercoiled molecules (*33*).

Gyrase is believed to be the enzyme primarily responsible for removing positive supercoils ahead of DNA tracking systems in bacteria (*1, 7, 30*). However, at the present time, nothing is known about the effects of supercoil geometry on DNA cleavage mediated by gyrase or on the sensitivity of the enzyme to quinolone antibacterials.

As described in Chapter I, interactions of quinolones with topoisomerase IV are mediated through a water-metal ion bridge that is anchored by a highly conserved serine and an acidic residue located four amino acids away (Ser81 and Asp85 in *B. anthracis* gyrase, respectively) (*19, 60, 61, 66-68*). However, the role of the bridge appears to vary between species. Although *B. anthracis* topoisomerase IV uses the water-metal ion bridge as the primary mechanism to bind quinolones (*19, 60, 66, 68*), *E. coli* topoisomerase IV uses the bridge to properly align clinically relevant quinolones (*67*).

Even though gyrase is the primary cellular target for quinolones in many bacterial species, including *B. anthracis*, the role of the water-metal ion bridge in mediating drug interactions has not yet been assessed. Alterations in the above conserved serine and acidic amino acid residues are the most common resistance mutations observed in gyrase from a multitude of species (*19*). Thus, it is highly likely that the water-metal ion bridge plays a critical role in coordinating quinolones and gyrase. However, the use of the bridge by gyrase has not yet been demonstrated and the role that the bridge plays in coordinating quinolone interactions remains an open question.

In order to address the two issues described above, the ability of *B. anthracis* gyrase to discern supercoil geometry during DNA cleavage was assessed. Results indicate that like the human type II enzymes, both the wild-type bacterial enzyme and a common quinolone resistant mutant enzyme maintain lower levels of cleavage complexes

with positively supercoiled substrates. Finally, as a first step toward characterizing the role of the water-metal ion bridge in mediating quinolone interactions with gyrase, the sensitivity of wild-type and GyrA^{S85L} gyrase to quinolones and quinazolinediones [a quinolone-like compound that does not require the bridge to interact with bacterial type II enzymes (*60, 66, 68*)] was assessed. The GyrA^{S85L} mutant enzyme was utilized for these experiments because it is the most common mutation observed when selecting for quinolone resistant strains of *B. anthracis* in laboratory settings (*108*). Preliminary findings support the hypothesis that gyrase uses the bridge to coordinate quinolone interactions and lay the groundwork for future studies on this important mechanistic issue.

<u>Experimental Procedures</u>

Enzymes

  B. anthracis gyrase enzymes were purified using the procedure of Dong *et al*. (*109*) with modifications. Genes encoding wild-type *B. anthracis* GyrA and GyrB were individually PCR-amplified from *B. anthracis* Sterne 34F2 chromosomal DNA and cloned into the pET15b (Novagen) expression vector, which provided an N-terminus 6×His tag. Mutant quinolone-resistant GyrA^{S85L} gyrase was generated by QuikChange (Stratagene) site-directed mutagenesis of the wild-type expression vector. Recombinant subunits were individually expressed in an *E. coli* BL21(DE3) ΔslyD strain. Cells were lysed by resuspension in CelLytic B (Sigma) containing protease inhibitors (Roche Complete Protease Inhibitor Cocktail, EDTA-free) and one passage through a French

press at 20,000 psi. The cell lysate was centrifuged at $20,000 \times g$ for 30 min at 4°C to remove debris. The cleared lysate was incubated with 2 mL Ni-NTA agarose beads (Qiagen) for 1 h at 4°C with end-over-end rotation. Following batch binding, the beads were pelleted by centrifugation at $100 \times g$ at 4°C and were then washed with a buffer containing 1 M NaCl, 60 mM imidazole, and 20 mM Tris-HCl (pH 7.9) for 30 min at 4°C with end-over-end rotation. Next, the beads underwent a series of four 1-min washes with a buffer containing 500 mM NaCl, 60 mM imidazole, and 20 mM Tris-HCl (pH 7.9). Finally, the beads were loaded into a column, and the proteins were eluted at 4°C with 12 mL of a buffer containing 500 mM NaCl, 1 M imidazole, and 20 mM Tris-HCl (pH 7.9). Twelve 1-mL fractions were collected, and fractions 2-11 were combined, injected into a 20kDa MWCO Slide-a-Lyzer dialysis cassette (Thermo), and dialyzed against 200 mM NaCl and 50 mM Tris-HCl (pH 7.5) at 4°C for 4 hours. The proteins were then dialyzed overnight at 4°C into 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 20% glycerol and were subsequently stored at -80°C. Protein concentrations were determined by A_{280} measurements, and the quality and purity of the subunits was determined by Coomassie staining following separation on a 7.5% SDS-PAGE gel. In all assays, gyrase was used as a 1:1 mixture of GyrA:GyrB.

DNA Substrates

Negatively and positively supercoiled pBR322 plasmid DNA was prepared as described in Chapter II.

Drugs

Ciprofloxacin was obtained from LKT Laboratories and CP-115,953 was the gift of Thomas D. Gootz and Paul R. McGuirk (Pfizer). Both quinolones were stored at -20°C as a 40 mM stock solution in 0.1 N NaOH, and diluted five-fold with 10 mM Tris-HCl (pH 7.9) immediately prior to use. Levofloxacin was obtained from Sigma-Aldrich and 8-methyl-3'-(AM)P-quinazoline-2,4-dione was the gift of Dr. Robert Kerns (University of Iowa). These latter two compounds were stored at 4°C as 20 mM stock solutions in DMSO.

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*85*). Reactions contained 250 nM wild-type or $GyrA^{S85L}$ mutant gyrase and 10 nM negatively or positively supercoiled pBR322 in a total of 20 μL of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM potassium glutamate, 50 μg/mL bovine serum albumin, and 5 mM DTT. Reaction mixtures were incubated at 37°C for 30 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45°C for 45 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45°C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized and quantified as described above. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

Results and Discussion

Recognition of Supercoil Geometry by Wild-Type B. anthracis *Gyrase*

As a first step toward analyzing the recognition of DNA geometry by gyrase, the effects of supercoil handedness on the level of DNA cleavage mediated by the wild-type *B. anthracis* enzyme were assessed in the presence of ciprofloxacin. The data shown in Figure 17 provide strong evidence that gyrase is able to discern the geometry of DNA supercoils. As was found with the human type II enzymes (*73*), gyrase maintained ~2–fold lower levels of cleavage complexes when positively supercoiled (as opposed to negatively supercoiled) DNA was used as a substrate. Assays were carried out in the presence of ciprofloxacin in order to increase levels of DNA scission. However, as reflected in the zero ciprofloxacin data point, the enzyme is able to discern DNA geometry even in the absence of drug (a similar result can be seen in Figure 19). This conclusion recently has been confirmed by a more extensive study carried out by Rachel Ashley in the Osheroff laboratory.

The fact that gyrase maintains lower levels of cleavage complexes with positively supercoiled substrates contributes to the ability of the enzyme to alleviate torsional stress ahead of replication forks in a safe manner. However, it also renders gyrase an intrinsically less sensitive physiological target for quinolone antibacterial drugs.

Characterization of GyrA^{S85L} B. anthracis Gyrase

The most commonly observed quinolone resistance mutations occur in amino acid residues that are proposed to anchor the water-metal ion bridge. Consequently, the
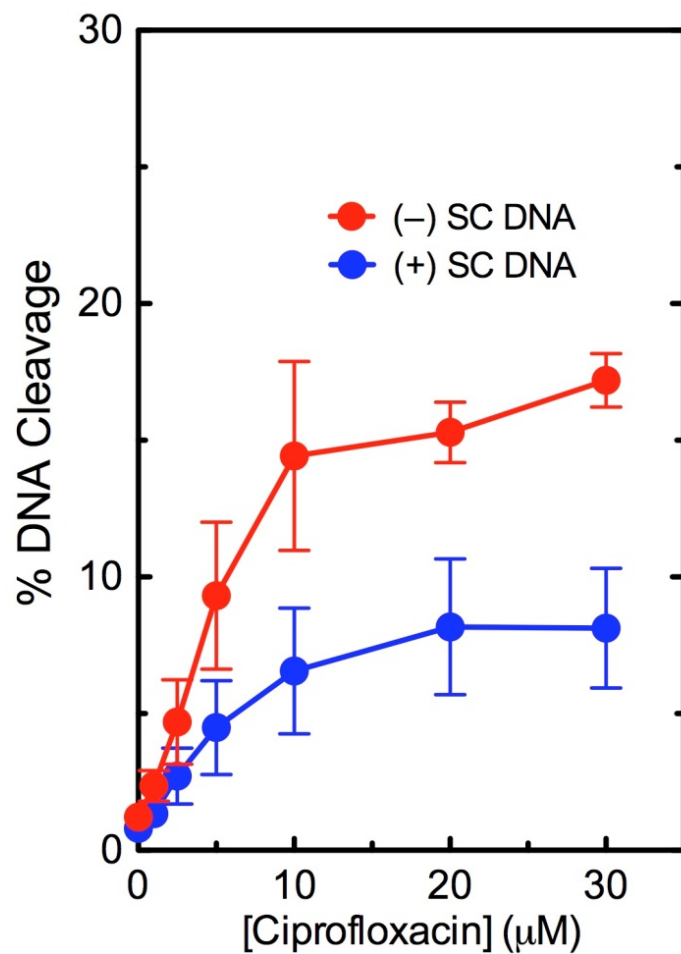
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Figure 17. Recognition of DNA geometry by *B*. *anthracis* gyrase during DNA cleavage in the presence of ciprofloxacin. DNA cleavage with negatively [(–SC), red circles] and positively [(+SC), blue circles] supercoiled DNA is shown. Error bars represent the standard deviation of three or more independent experiments.

characterization of these enzymes and their drug interactions has played an instrumental role in furthering our understanding of the role that the bridge plays in coordinating quinolone-based drugs. It also has provided tremendous insight into the basis of quinolone resistance and potential mechanisms for overcoming this drug resistance.

Although a series of structural and biochemical studies have provided a reasonable understanding of how the water-metal ion bridge affects quinolone interactions with topoisomerase IV (*19, 60, 61, 66-68*), parallel studies have yet to be carried out with gyrase. Therefore, a preliminary characterization of GyrA^{S85L} *B. anthracis* gyrase was undertaken. GyrA^{S85L} gyrase was used because it contains the most common quinolone resistance mutation observed in laboratory studies with *B. anthracis* (*108*). Note that Ser85 is homologous to the serine residue that has been shown to anchor the water-metal ion bridge in topoisomerase IV (*61*).

Because quinolones increase levels of enzyme-generated DNA cleavage complexes, these drugs depend on enzyme activity to support their function. Thus, if a mutation results in a loss of enzyme activity, it could cause quinolone resistance, even if it did not directly affect drug-enzyme interactions. Therefore, before characterizing the quinolone sensitivity of GyrA^{S85L} gyrase, its intrinsic DNA cleavage activity was compared to that of the wild-type *B. anthracis* enzyme (Figure 18). The DNA cleavage activity of the mutant enzyme in the absence of drugs was actually somewhat higher than that seen with wild-type gyrase. A similar increase in DNA scission activity has been seen in resistance mutations at the equivalent residue in *B. anthracis* topoisomerase IV (*66*). Thus, quinolone resistance observed in cells carrying GyrA^{S85L} gyrase is not due to a loss of enzyme activity.
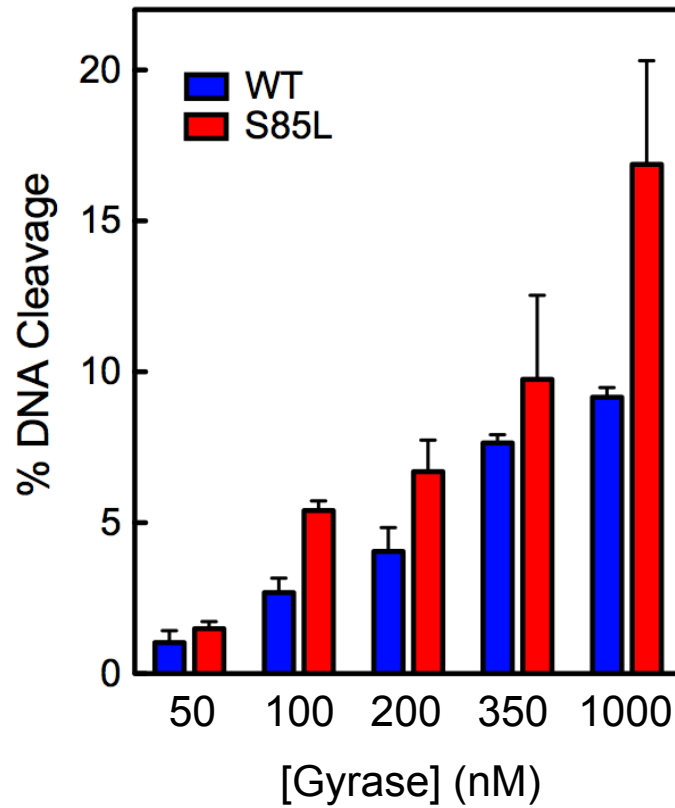
Figure 18. DNA cleavage activity of wild-type and GyrA^{S85L} *B. anthracis* gyrase. The activities of the wild-type (WT, blue bars) and GyrA^{S85L} (S85L, red bars) mutant enzymes are shown. Error bars represent the standard deviation of three or more independent experiments.

In order to explore the mechanism of quinolone action and resistance, the next experiments examined the effects of drugs on DNA cleavage mediated by wild-type and GyrA^{S85L} gyrase. Ciprofloxacin and levofloxacin, two clinically used quinolones, as well as CP-115,953, an experimental quinolone that displays high activity against bacterial and eukaryotic type II enzymes (*91*), were used for these studies. In addition, 8-methyl-3'-(AM)P-quinazoline-2,4-dione, a quinolone-like drug that lacks the C3,4-keto acid required to chelate divalent metal ions, was used. This latter compound occupies the same interaction domain on topoisomerase IV as clinically relevant quinolones (*110*), but does not utilize the water-metal ion bridge (*66*). Rather, it interacts with the enzyme through novel contacts with its 3'-(AM)P moiety (*68*).

Although some differences in drug potency were noted, all of the compounds fundamentally had the same effect on wild-type *B*. *anthracis* gyrase and enhanced DNA cleavage >20–fold (Figure 19). In contrast, the quinolones displayed much lower activity against GyrA^{S85L} gyrase. The two clinically used quinolones showed nearly no ability to enhance DNA cleavage and the potency of CP-115,953 was reduced considerably compared to its effects on wild-type gyrase (Figure 19). Despite the significant drop in activity seen with the quinolones, the quinazolinedione, which does not require a divalent metal ion, maintained the majority of its activity against GyrA^{S85L} gyrase (Figure 19). These findings, although preliminary, strongly suggest that *B*. *anthracis* gyrase interacts with clinically relevant quinolones through the water-metal ion bridge.
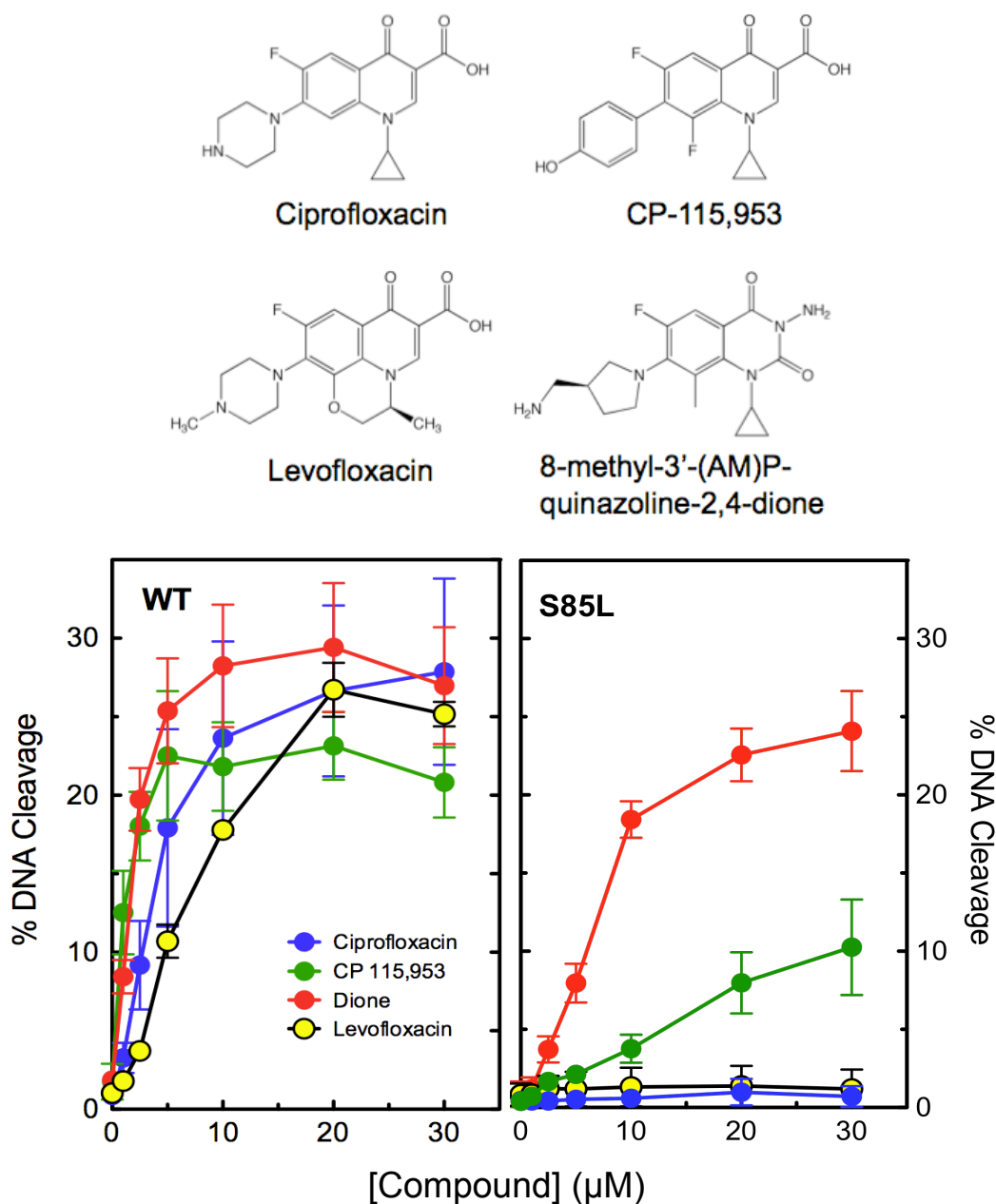
Figure 19. Effects of quinolones and 8-methyl-quinazoline-2,4-dione on the DNA cleavage activities of wild-type and GyrA[S85L] *B. anthracis* gyrase. DNA cleavage mediated by wild-type (WT) GyrA[S85L] gyrase in the presence of drugs are shown in the left and right panels, respectively. Results with ciprofloxacin (blue circles), CP-115,953 (green circles), levofloxacin (yellow circles), and 8-methyl-3'-(AM)P-quinazoline-2,4-dione (Dione, red circles) are shown for the mutant enzymes. Error bars represent the standard deviation of three or more independent experiments. Structures of the drugs are shown above the graphs.

Recognition of DNA Geometry by GyrA^{S85L} B. anthracis Gyrase

The finding that GyrA^{S85L} gyrase is still sensitive to 8-methyl-3'-(AM)P-quinazoline-2,4-dione provided an opportunity to determine whether this common quinolone resistance mutation alters the ability of the enzyme to discern supercoil geometry during cleavage. As seen in Figure 20, the mutant enzyme retained its ability to recognize supercoil geometry and maintained lower levels of cleavage complexes with the positively supercoiled substrate.

Conclusions

Although the ability of topoisomerase IV to discern supercoil geometry and utilize the water-metal ion bridge to coordinate quinolones has been examined (*33*), we know very little about these two critical issues for gyrase. The present chapter describes preliminary studies designed to address this deficiency.

Results indicate that *B. anthracis* gyrase can discern supercoil geometry and (like the eukaryotic type II enzymes) maintains lower levels of DNA cleavage complexes with positively supercoiled substrates. As discussed earlier, this makes gyrase a "safer" enzyme to act ahead of replication forks. However, it may decrease the ability of quinolones to enhance gyrase-mediated DNA cleavage in bacterial cells. Thus, it may have a negative impact on drug sensitivity.

Finally, preliminary studies with GyrA^{S85L} gyrase strongly suggest that the *B. anthracis* enzyme interacts with quinolones through the water-metal ion bridge. This work sets the stage for more detailed future studies on gyrase.
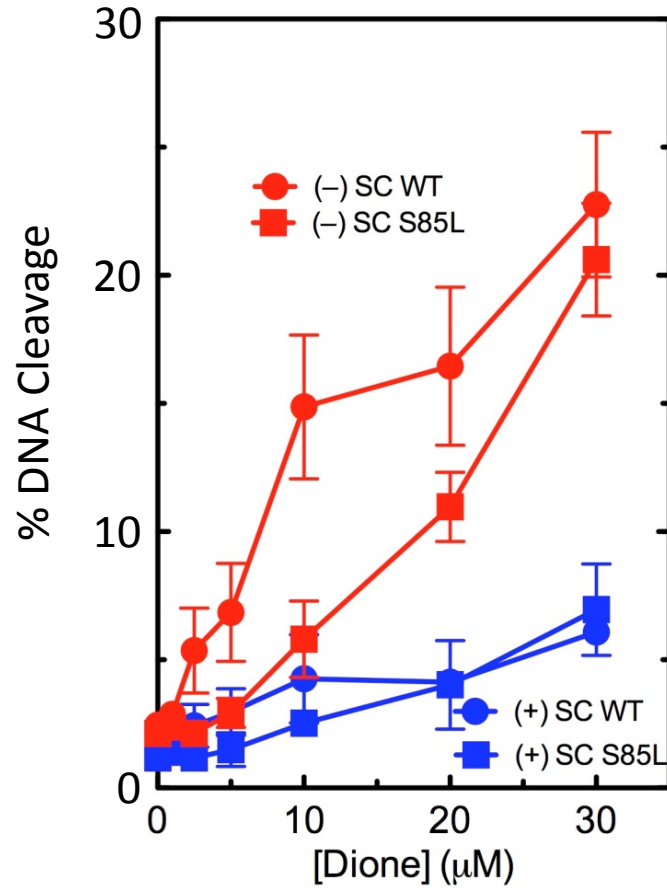
Figure 20. Recognition of DNA geometry by wild-type and GyrA[S85L] mutant *B*. *anthracis* gyrase during DNA cleavage in the presence of 8-methyl-3'-(AM)P-quinazoline-2,4-dione. DNA cleavage by wild-type (WT, circles) and GyrA[S85L] (S85L, squares) mutant gyrase with negatively [(–SC), red] and positively [(+SC), blue] supercoiled DNA is shown. Error bars represent the standard deviation of three or more independent experiments.

CHAPTER IV

CONCLUSIONS

The work described in this dissertation has addressed a number of fundamental issues regarding the basis by which eukaryotic and bacterial type II topoisomerases recognize and interact with their DNA substrates and topoisomerase II poisons. Studies presented in Chapter II demonstrate that the ability of human topoisomerase IIα to discern supercoil geometry during DNA cleavage resides in the catalytic core of the enzyme. They also define new roles for the N-terminal domain in capturing the T-segment of DNA during the strand passage reaction.

Chapter II demonstrates that covalent poisons require the N-terminal domain of topoisomerase IIα in order to enhance DNA cleavage. This finding is consistent with previous models for how covalent poisons function (*74, 75*). An intriguing result from the work in this chapter is the finding that covalent poisons do not require the N-terminal domain in order to inhibit enzyme activity when incubated with topoisomerase IIα prior to the addition of DNA. This finding strongly suggests that the ability of covalent poisons to increase or abrogate enzyme-mediated DNA cleavage (depending on the incubation conditions) results from different mechanisms.

Finally, the work presented in Chapter III, although preliminary in nature, adds substantially to our understanding of how gyrase recognizes and interacts with DNA and quinolones. This study lays the groundwork for detailed mechanistic studies.

<u>Future Directions</u>

The studies described above have opened several new vistas for the Osheroff laboratory. First, the finding that covalent poisons require the N-terminal domain of topoisomerase IIα to enhance enzyme-mediated DNA cleavage, but only require the catalytic core to inhibit enzyme function, provides strong evidence that our earlier "unified" theory for the actions of covalent poisons is incorrect. Therefore, the Osheroff laboratory plans to incubate the catalytic core of the enzyme with a variety of covalent poisons and determine the residues that may be responsible for the inhibitory activities of these compounds. One intriguing possibility is that covalent poisons adduct one or more active site amino acid residues when DNA is not present.

The work on the recognition of supercoil geometry by *B. anthracis* gyrase currently is being extended to DNA supercoiling reactions. Preliminary results indicate that the enzyme removes positive supercoils >10–fold faster than it generates negative supercoils in relaxed DNA. These findings suggest that gyrase may be an enzyme that was designed to function primarily on overwound molecules. It also raises the question of whether the conversion of positively supercoiled DNA to relaxed molecules by gyrase utilizes a different mechanism than the conversion of relaxed substrates to negatively supercoiled molecules.

Finally, given the importance of gyrase as a drug target, it is imperative to determine the basis by which quinolones interact with the enzyme. The preliminary studies described in Chapter III have set the stage for a detailed analysis of the role played by the water-metal ion bridge in mediating interactions of quinolone antibacterials with *B. anthracis* gyrase. These studies currently are underway.

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