DNA topoisomerases I from Pseudomonas aeruginosa and vaccinia virus and their use as drug targets

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DNA TOPOISOMERASES I FROM *PSEUDOMONAS AERUGINOSA* AND VACCINIA VIRUS AND THEIR USE AS DRUG TARGETS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by

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May, 2009
Dedication

To my family.
Acknowledgements

I thank Dr. Anne Grove for giving me the opportunity to work on this project. I will always remember her gentle guidance and patience during the challenging times. I also thank my committee members Dr. Patrick DiMario, Dr. Craig Hart, and Dr. Marcia Newcomer for their support and guidance. My lab members deserve a special acknowledgement for being extremely friendly and helpful. Inoka thank you for teaching me many of the computer skills. I thank all of the undergraduate students in the lab especially Brian, Weedad, Cara, and Kevin for giving me helping hands when I had to leave work and go teaching. The former Grove lab members Steven, Nick, Edwin, and Anirban gave me a lot of moral support.

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Abstract

Pseudomonas aeruginosa encodes a putative topoisomerase with sequence similarity to the type IB topoisomerase enzyme from vaccinia virus. Residues in the active site are conserved, notably Tyr292 which would be predicted to form the transient covalent bond to DNA. The gene encoding the P. aeruginosa topoisomerase I (PAT) was cloned and expressed in E. coli. The enzyme relaxes supercoiled DNA, while a mutant of P. aeruginosa containing a Tyr292 to Phe substitution at the active site was found to be catalytically inert. This is consistent with the role of Tyr in forming the covalent intermediate. Like vaccinia topoisomerase (VT), PAT relaxes DNA in the absence of ATP. Unlike VT, PAT does not relax supercoiled DNA without MgCl₂ (or MnCl₂) present. In addition, high concentration of NaCl is not able to substitute for MgCl₂ as seen for VT. A truncated derivative of the topoisomerase lacking residues 1-98 relaxes DNA in the absence of the N-terminal domain, with both full length and truncated enzyme exhibiting equivalent requirements for divalent cations. Data shows that P. aeruginosa encodes a functional topoisomerase with significant similarity to the type IB enzyme encoded by poxviruses.

Fluoroquinolones are antibacterial agents in clinical use with activity against DNA gyrase and DNA topoisomerase IV. P. aeruginosa is an opportunistic pathogen causing life-threatening diseases. Sparfloxacin, enrofloxacin and norfloxacin fluoroquinolones, are able to inhibit the PAT at high concentrations but other drugs belonging to this family are unable to do so.

VT is ≈32 KDa and one-third the size of the human topoisomerase ≈ 100 KDa. It shares sequence and biochemical similarities with the human topoisomerase. The VT binds duplex DNA with stringent specificity for transesterification at 5’-(C/T) CCTT↓ site, where the 3’ phosphate of the incised strand is linked to the Tyr274 of the enzyme to form a covalent cleavage complex. The fluoroquinolone enrofloxacin inhibits relaxation of supercoiled DNA by VT in a
Mg$^{2+}$-dependent fashion. Further results indicate that the mechanism by which enrofloxacin inhibits VT is by preventing formation of the covalent complex which suggest that fluoroquinolones may be structurally optimized to target type IB topoisomerases.
1 Introduction

1.1 Supercoiling

Supercoiling is a very important property of the DNA structure. It shows a high degree of structural organization. DNA is folded in an extremely compact state and is at the same time accessible to other factors/proteins required to accomplish various processes in the cell. A supercoil is defined as the twisting (coiled) of helical molecule on itself and supercoiled DNA as DNA that twists upon itself because it is under- or overwound relative to the B-form DNA. Compared to supercoiled DNA, relaxed DNA shows no bending of the DNA axis upon itself. There are a numbers of parameters for measuring supercoiling. Supercoiling provides a lot of insight into the structure of the DNA. The branch of mathematics called topology, studies the properties of an object that do not change under continuous deformations. There are right handed coils defined by negative numbers and referred to as negative supercoiling. Likewise, there are left handed supercoils defined by positive numbers and defined as positive supercoils. During the process of replication as the transcription bubble moves forward there are unpaired bases behind and compaction in front of the replication fork. This leads to positive supercoiling or overwinding in front of the replication fork and negative supercoiling or underwinding behind the replication fork. DNA compaction require a special kind of supercoiling. Plectonomic supercoiling is the right handed supercoiling in a negatively supercoiled DNA molecule, and it tends to be extended and narrower rather than compacted, often with multiple branches (1). Solenoidal supercoiling is adopted by negatively supercoiled DNA and involves tight left handed turns (1). Toroidal supercoiling is yet another form of supercoiling found in E. coli in which the DNA is wrapped around proteins and is restrained, although transient in bacteria it is stable in
the form of nucleosomes in eukaryotes (1). Two forms of circular DNA that differ only in their topology are called topoisomers. The enzymes that manage the topology of DNA in the cell are called topoisomerases.

1.2 DNA Topoisomerases

DNA topoisomerases are enzymes that manage the topology of DNA in the cell and therefore perform vital functions in nearly all cellular processes like replication, transcription, recombination, chromosome segregation, chromosome condensation and decondensation, chromosome organization, genome stability, repair, transcription regulation, cell cycle control, post transcriptional modifications and chromatin remodeling (2). Topology is the mathematics dealing with structural properties. Molecules which differ in their linking number are called topological isomers. Therefore, linking number is defined as a topological property of the DNA molecule which is equal to the number of times that a strand of DNA winds in right handed direction around the helix axis when the axis is constrained to lie in a plane. Another mathematical definition of linking number (Lk) is the sum of twist (Tw) and writhe (Wr). Twist is defined as helical winding of DNA strands around each other. Writhe is the measure of the coiling of the axis of the double helix (3).

1.3 Classification of Topoisomerases

DNA topoisomerase are basically of two kinds. Those enzymes that cleave one strand of the DNA are defined as type I, while those that cleave both strands of DNA are type II. Type I topoisomerases are further classified as type IA subfamily or type IB subfamily. In the type IA subfamily members the protein is linked to the 5’ phosphate and in the type IB subfamily the protein is linked to the 3’ phosphate (2). Further divisions of the topoisomerases are based on structure (2). A few examples are given in Table 1-1.
The interconversion of topoisomers of DNA is catalyzed by enzymes called Topoisomerases. Topoisomerases can also be defined as DNA modifying enzymes. Their nature of relaxing DNA is very similar to a nuclease. The only difference being that it behaves as a reversible nuclease. The topoisomerase first breaks the phosphodiester bond in the DNA backbone to form a DNA phosphotyrosine bond. The energy in the phosphodiester bond is stored as a phosphotyrosine bond. DNA cleavage is followed by relaxation activity and finally the enzyme is released and the phosphodiester bond forms again and no additional energy is required.

The type I topoisomerases attack the DNA to cause a nick in the single stranded DNA forming a covalent bond with the DNA backbone and the opposite strand rotates around the nicked strand either/or any tension ahead or before the nick will cause a rotation to release the stress. No energy is required for this reaction (Fig. 1-1). The type II topoisomerases attack the DNA to cause double stranded breaks to form a covalent bond with both strands of the DNA helix. The other double helix passes through this double stranded break in the DNA to release the tortional stress. In the final step the enzyme is released and the DNA backbone is restored (Fig. 1-2). The type II are used to solve DNA tangling problems.

These relaxation reactions catalyzed by eukaryotic DNA topoisomerase I enzyme alter the linking number of DNA by catalyzing a three step process.

1. The cleavage of one or both strands of DNA. DNA cleavage is accompanied by the formation of a transient phosphodiester bond between a tyrosine residue in the protein and one of the broken strands.
2. The passage of one segment of the DNA through this break.
3. Then resealing this break of DNA.
Figure 1-1. Topoisomerase Mechanisms.
Adapted from Genomes second edition:- On binding to DNA, topoisomerase I cleaves one strand of DNA/ topoisomerase II cleaves both strands of the DNA, through a tyrosine residue attacking a phosphate. When the strands have been cleaved, it rotates in a controlled manner around the other strand. The rotation is completed by religation of the cleaved strand.

Figure 1-2. Topoisomerase IB Mechanism
Adapted from The Molecular Biology of the Cell, third edition:- On binding to DNA, topoisomerase I cleaves one strand of DNA through a tyrosine residue attacking a phosphate. When the strands have been cleaved, it rotates in a controlled manner around the other strand. The rotation is completed by religation of the cleaved strand. This process results in partial or complete relaxation of supercoiled plasmid.
Table 1-1 Classification of topoisomerases.

<table>
<thead>
<tr>
<th>Topoisomerase</th>
<th>Subfamily Type</th>
<th>Subunit Structure</th>
<th>Size (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacterial DNA topoisomerase I (<em>E. coli</em>)</td>
<td>IA</td>
<td>Monomer</td>
<td>865</td>
</tr>
<tr>
<td>Eubacterial DNA topoisomerase III (<em>E. coli</em>)</td>
<td>IA</td>
<td>Monomer</td>
<td>653</td>
</tr>
<tr>
<td>Yeast DNA topoisomerase III (<em>S. cerevisiae</em>)</td>
<td>IA</td>
<td>Monomer</td>
<td>656</td>
</tr>
<tr>
<td>Mammalian DNA topoisomerase IIIα (human)</td>
<td>IA</td>
<td>Monomer</td>
<td>1001</td>
</tr>
<tr>
<td>Eubacterial reverse gyrase (<em>Methanopyrus Kandleri</em>)</td>
<td>IA</td>
<td>Heterodimer</td>
<td>A,358 B, 1221</td>
</tr>
<tr>
<td>Mammalian DNA topoisomerase IIIβ (human)</td>
<td>IA</td>
<td>Monomer</td>
<td>862</td>
</tr>
<tr>
<td>Eukaryotic DNA topoisomerase I (human)</td>
<td>IB</td>
<td>Monomer</td>
<td>765</td>
</tr>
<tr>
<td>Poxvirus DNA topoisomerase (vaccinia)</td>
<td>IB</td>
<td>Monomer</td>
<td>314</td>
</tr>
<tr>
<td>Eubacterial DNA gyrase (<em>E. coli</em>)</td>
<td>IIA</td>
<td>A₂B₂ heterotetramer</td>
<td>GyrA, 875 GyrB, 804</td>
</tr>
<tr>
<td>Eubacterial DNA topoisomerase IV (<em>E. coli</em>)</td>
<td>IIA</td>
<td>C₂E₂ heterotetramer</td>
<td>ParC, 752 ParE, 630</td>
</tr>
<tr>
<td>Mammalian DNA topoisomerase II α,β</td>
<td>IIA</td>
<td>Homodimer</td>
<td>α 1531, β 1626</td>
</tr>
<tr>
<td>Yeast DNA topoisomerase II (<em>S. cerevisiae</em>)</td>
<td>IIA</td>
<td>Homodimer</td>
<td>1428</td>
</tr>
<tr>
<td>Archael DNA topoisomerase VI (<em>Solfolobus shibatae</em>)</td>
<td>IIB</td>
<td>A₂B₂ heterotetramer</td>
<td>A, 389 B, 530</td>
</tr>
</tbody>
</table>
1.4 Cellular Roles of Topoisomerases

1.4.1 The Type 1A Topoisomerases Share the Following Properties

All type IA topoisomerases are monomeric with the exception of Methanopyrus kandleri reverse gyrase. Reverse gyrase is a topoisomerase which induces positive supercoiling in hyperthermophiles (4). In the type IA topoisomerases cleavage of a DNA strand is accompanied with covalent attachment of one of the DNA ends to the enzyme through a 5` phosphodiester bond to the tyrosine at the active site. All the type IA topoisomerases require Mg$^{2+}$ for DNA relaxation. Plasmids containing negative, but not positive supercoils are substrates for the DNA relaxation reaction. The relaxation of negative supercoils in this family does not go to completion (2).

1.4.2 Type IB Topoisomerases

The type IB topoisomerases belong to three classes- firstly, the eukaryotic topoisomerase for example the human topoisomerase I, secondly, the poxvirus topoisomerase for example the vaccinia virus topoisomerase and the mimivirus topoisomerase and thirdly, the prokaryotic topoisomerase V from Methanopyrus kandleri. Deinococcus radiodurans topoisomerase IB has recently been shown to posses sequence, structural and mechanistic similarity to Human and Vaccinia virus topoisomerase (5, 6). It has been shown that the topoisomerase IB shows structural, functional and catalytic properties similar to tyrosine recombinases exemplified by bacteriophage P1, Cre, E.coli, XerD recombinases, and certain phage integrases (7-11)The topoisomerase type IB share the following properties (2). They are monomeric, they all cleave the DNA strand forming a covalent complex with the DNA, in which the enzyme is linked through a 3` phosphodiester bond to the tyrosine at the active site. They may or may not require cations for activity, they can relax both positively and negatively supercoiled DNA, relaxation of
DNA goes to completion and there is no requirement that the substrate DNA is single stranded (2).

1.4.3 Type II Topoisomerasess Share the Following Properties

The type II topoisomerases are dimeric enzymes that bind duplex DNA and cleave both strands of the DNA. Cleavage involves covalent attachment of each subunit of the dimer to the 5’ end of the DNA through a phosphotyrosine bond. In the type II enzymes, a conformational change pulls the two ends of the duplex DNA apart to create the G segment. A second DNA duplex [T segment] from the same molecule or a different molecule can pass through the G segment and thus relax the DNA. The reaction requires Mg\(^{2+}\) and ATP hydrolysis. They are further classified as Type IIA and Type IIB. An example of a Type IIA enzyme is DNA Gyrase and of Type IIB is Topoisomerase VI found in the archaeon Sulfolobus shibatae (2).

1.4.4 Topoisomerasess in Different Species

1.4.4.1 E. coli

E. coli encodes four kinds of topoisomerases two of which are type IA (DNA Topoisomerase I and III) and two are type IIA enzymes (DNA Gyrase and DNA topoisomerase IV). DNA gyrase is a type IIA topoisomerase that introduces negative supercoils in the DNA at the expense of ATP hydrolysis. The DNA gyrase together with the Muk protein in E. coli help to condense DNA leading to proper chromosome partitioning at cell division. DNA topoisomerase IV is known to play a role in decatenation of DNA. It has been shown recently that topoisomerase I together with IV play a role to prevent excessive negative supercoiling by DNA gyrase (2).

Replication and transcription leads to the generation of positive supercoils ahead of the replication fork and negative supercoils behind the translocating RNA polymerase in
transcription and these are resolved by DNA gyrase and DNA topoisomerase I respectively (2). In *E. coli*, type IV topoisomerases are primarily responsible for decatenating the DNA, and type III topoisomerase supports the movement of the replication fork in spite of the fact that it cannot relax positively supercoiled DNA. The preferred DNA substrate for DNA topoisomerase III is single stranded DNA and it therefore acts behind the replication fork where the DNA template is discontinuously synthesized and is therefore single stranded (2, 12). The preferred substrate for DNA topoisomerase IV is double stranded DNA and it therefore acts ahead of the replication fork. Also under normal conditions topoisomerase I relaxes negatively supercoiled DNA and topoisomerase III is acting to prevent excessive negative supercoiling and requires a hypernegatively supercoiled substrate (2).

1.4.4.2 Other Eubacteria

Genome analysis of 17 mesophilic eubacterial organisms revealed that several bacteria including *Haemophilus influenzae*, *Bacillus subtilis*, and *Xylella fastidiosa* share topoisomerases similar to *E. coli* and they probably serve similar roles in these bacteria as they do in *E. coli* (2). A number of mesophilic bacteria lack topoisomerase III but this is obvious due to the redundant role of the enzyme. But they have other topoisomerases. This group includes *Borrelia burgdorferi*, *Chlamyphila pneumoniae*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Neisseria meningitidis*, *Rickettsia prowazekii*, *Synechocystis PCC6803*, and *Ureaplasma urealyticum*. In these bacteria, topoisomerase IV is responsible for unlinking precatenates as well as daughter molecule catenates (2).

A few of the mesophiles like *Campylobacter jejuni*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, and *Treponema pallidum* possess only two topoisomerase genes. This is probably the minimal topoisomerase makeup. One is topoisomerase I *topA* gene and the
second gene is a homolog of the *E. coli* DNA gyrase. In these species, topoisomerase I relaxes negative supercoils and is associated with transcription and prevents excessive negative supercoiling by DNA gyrase. On the other hand DNA gyrase would be associated with negative supercoiling of DNA, relaxing positive supercoils and decatenating replicated DNA (2).

The hyperthermophilic bacteria like *Aquifex aeolicus* and *Thermotoga maritime* encode for example reverse gyrase which derives energy from ATP hydrolysis to introduce positive supercoils into chromosomal DNA. They also have DNA gyrase and a homologue of the topoisomerase type IA enzyme. The type IA plays a role to relax negative supercoils associated with transcription and replication and type II, DNA gyrase plays a important role for decatenating activity during replication. Apart from this they may also play a role to oppose reverse gyrase and prevent excessive positive supercoiling (2). Topoisomerase V has been identified in the hyperthermophile *M. kandleri*.

The vaccinia virus encodes a type IB topoisomerase which is ≈32 KDa and 314 amino acids (13-15). It is one third the size of the human topoisomerase of ≈100 KDa and 765 amino acids (16). It has all the residues required for catalytic activity. It is used as a model to study the human enzyme because it is easy to purify and work with. The *Deinococcus radiodurans* also encodes a type IB topoisomerase with sequence similarity to the pox virus encoded enzyme (17).

### 1.4.4.3 Archaea

Hyperthermophiles *Aeropyrum pernix*, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Pyrococcus horikoshii*, and *Pyrococcus abyssi* are examples of archaea that have the complete genome sequenced. These hyperthermophiles possess reverse gyrase, a type IA topoisomerase and a topoisomerase VI. In addition to these enzymes, *Archaeoglobus fulgidus* also appears to possess a atypical DNA gyrase. The reverse gyrase from this organism helps to
introduce positive supercoils, the type IA relaxes negative supercoils and the type VI can relax both positive and negative supercoiling, thus preventing the excessive supercoiling generated due to transcription and other processes (2).

1.4.4.4 Yeast

Saccharomyces cerevisiae and Schizosaccharomyces pombe code for three topoisomerases (2). They are:-topoisomerase IB, which is dispensable for growth, relaxes both positively and negatively supercoiled DNA, the topoisomerase IIA subfamily is essential for decatenating linked chromosomes and segregating chromosomes for mitosis in the cell cycle, and topoisomerase III is dispensable for growth but in its absence the cells grow slowly.

1.4.4.5 Higher Eukaryotes

They contain topoisomerase I, topoisomerase type IIA, topoisomerase III and its two isoforms. Topoisomerase I plays a major role in the movement of the replication fork and relaxation of transcription related supercoils (2). They contain the two isoforms of IIA that is type IIα which is essential and found in all cells and is responsible for unlinking intertwined daughter duplexes during DNA replication and likely contributes to DNA relaxation during transcription. Type IIβ is responsible for suppressing recombination or supporting transcription in early neurons. The topoisomerase IIIα is required for early embryogenesis and topoisomerase IIIβ is responsible for cell viability and development.

1.4.4.6 Vaccinia Virus DNA Topoisomerase IB

The vaccinia topoisomerase IB belongs to the eukaryotic-like sub-family and is biochemically similar to human topo yet is only one-third the size (7). Due to the large size of the human topoisomerase (765 amino acids), the vaccinia virus DNA topoisomerase I (314 amino acids) is studied. It is used as a model system to study the eukaryotic type IB
topoisomerases. The mimivirus also encodes a topoisomerase IB with structural and functional homology to the vaccinia virus topoisomerase IB (18). The residues involved in transesterification are conserved in pox and the Mimi virus even though the Mimi virus shares primary structure with the bacterial enzyme (18). The Mimi virus IB like the vaccinia topoisomerase IB incises the duplex DNA to form the covalent complex with stringent specificity at the 5' -CCCTT pentameric sequence (18). The Deinococcus radiodurans bacteria also encodes a functional type IB topoisomerase (5).

1.5 Topoisomerases as Drug Targets

The topoisomerases have become an important drug target because of three primary reasons, firstly, they act as molecular machines that manage the topology of the DNA (7); secondly, numerous topoisomerase target drugs have been identified and these drugs trap the enzyme in the covalent complex with the DNA. These include antimicrobials and anticancer chemotherapeutic drugs (16, 19-24). Finally, a lot of structures of topoisomerases have been solved (6, 8, 11, 16) and they complement the biochemical data and provide more insight on the molecular machine and therefore making the studies on the topoisomerase more interesting.

1.5.1.1 Quinolones

The quinolones are a group of broad spectrum antibiotics (25). The parent compound is nalidixic acid. The majority of the quinolones belong to a subset of fluoroquinolones, which have a fluoro group attached to the central ring system, typically at position 6. (Fig. 1-3) The Quinolone antimicrobial agents exert their antibacterial action via inhibition of type II topoisomerase, DNA gyrase, DNA topoisomerase IV and recently discovered type IB topoisomeras (21, 26-29). Fluoroquinolones (Table. 1-1) are thought to stabilize the enzyme DNA complex together shortly after the duplex strand breakage, and this results in the release of
Figure 1-3. Structure of the quinolone molecule using the accepted numbering scheme for positions on the molecule.

An R indicates possible sites for structural modifications. Molecules at positions marked by a thick lined boxes can also be changed; however the most commonly used structure is here.
Table 1-2 Some of the drugs used against topoisomerase.

<table>
<thead>
<tr>
<th>Parent compound of the fluoroquinolone Nalidixic acid</th>
<th>Fluoroquinolone: Moxifloxacin</th>
<th>Fluoroquinolone: Lomefloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluoroquinolone</strong>: Sparfloxacin</td>
<td><strong>Fluoroquinolone</strong>: Ciprofloxacin</td>
<td><strong>Coumarin drug</strong>: Coumermycin</td>
</tr>
<tr>
<td><strong>Fluoroquinolone Enrofloxacin</strong></td>
<td><strong>Fluoroquinolone</strong>: Norfloxacin</td>
<td>The sole target of the human topoisomerase: Camptothecin</td>
</tr>
</tbody>
</table>
free DNA, chromosomal disruption and cell death (26). They also have the ability to stimulate the forward reaction rate of topoisomerase II-mediated DNA scission; after binding the cleavable complex, the fluoroquinolone induces conformational change in the enzyme that prevents religation (30-32). Sparfloxacin is a synthetic fluoroquinolone, broad-spectrum antimicrobial agent in the same class as ofloxacin and norfloxacin. Sparfloxacin has in vitro activity against a wide range of gram positive and gram negative microorganisms. Sparfloxacin exerts its antimicrobial activity by inhibiting DNA gyrase and topoisomerase IV (33). Quinolones may, therefore, be active against bacteria resistant to (beta)-lactum antibiotics (34). Although cross resistance has been observed between sparfloxacin and other fluoroquinolones, some organisms resistant to other fluoroquinolones may be susceptible sparfloxacin (35). Most quinolone antimicrobials appear to have a preferential affinity for topoisomerase IV in gram positive bacteria, sparfloxacin is reported first to interact with GyrA (34). Studies have reported facts which suggest to us that structural modifications have profound impact on drug target interactions (35).

Besides mutations other pathways for development of resistance to quinolones exist in *P. aeruginosa*. Notably, there are no known enzymes that degrade fluoroquinolones (36). *P. aeruginosa* probably has developed a mutation at the so called Quinolone resistance determining region [QRDR] or there could be a limitation of intracellular penetration by efflux of agents through “pumps” and transposable *qnr* gene (25, 37). Structural modifications have profound impact on the drug target interactions.

### 1.5.2 Fluoroquinolone DNA Topoisomerase Interactions

In order to study the interaction of the vaccinia virus topoisomerase with the fluoroquinolones a 18/24 mer suicide substrate is used. This suicide substrate has previously
been used to study single turnover and steady-state kinetics of the vaccinia topoisomerase-DNA complex (21, 38, 39). Vaccinia virus topoisomerase binds the DNA at a specific site (40, 41). It then cleaves the DNA at 5´-(C/T)CCTT↓ sites (40, 41). Suicide substrates are designed after careful consideration and these substrates contain the CCCTT↓ cleavage site and are such that when the vaccinia topoisomerase cleaves the DNA it releases a 6 nt product which is unable to religate. The suicide substrate used in our studies is 5´-CGTGTCGCCCTTATTCCG-3 (18 mer top strand) and 5´-CACTATCGGAATAAGGCGACACG-3` (24 mer bottom strand).

Four steps take place for the topoisomerase IB to relax DNA completely. First, noncovalent binding of the topoisomerase to DNA; second, scission of one strand of the DNA; thirdly, formation of the covalent bond between the topoisomerase and the DNA; finally, release of the topoisomerase and religation of DNA using energy stored in the phosphodiester bond (7).

It has previously been shown that the vaccinia topoisomerase IB is inhibited by coumarin drugs novobiocin and coumermycin (23) at concentrations 200 µM and 500 µM respectively. Both the coumarin drugs are inhibitors of the topoisomerase type II. They bind to the GyrB subunit near the ATP binding site therefore preventing the binding of ATP and blocking DNA relaxation (42, 43). The eukaryotic type II topoisomerases are also inhibited by novobiocin and coumermycin (44-47). The plant alkaloid camptothecin inhibits the human topoisomerase by stabilizing the covalent complex between the topoisomerase and the DNA (16). The vaccinia virus topoisomerase is resistant to camptothecin (13). Fluoroquinolones have recently been shown to inhibit relaxation by vaccinia topoisomerase at 1-1.5 mM concentration (21).

### 1.6 Vaccinia Topoisomerase Structure

The vaccinia virus topoisomerase I amino acid sequence similarity with the eukaryotic-like cellular topoisomerases is at best marginal [similarity=43%, identity=19.7%] (48, 49). The
Vaccinia virus topoisomerase as shown in Figure 1-4 has a tripartite structure in which three protease resistant structures are separated by two protease sensitive [Bridge and Hinge] interdomain segments. The amino terminal domain is 80 amino acids, 9 KDa long. The N-terminal domain promotes DNA binding and increases enzyme processivity (14). The amino terminal domain binds to the major groove and is responsible for site specificity (14). Also residues implicated in noncovalent DNA binding are situated within the bridge and in the N-terminal domain just proximal to the bridge (14). The Tyr-70 and Tyr-72 residues contact the +3C and +4C bases of the CCCCCTT element in the major groove (8). Vaccinia virus topoisomerase binds and cleaves the DNA at a specific sequence 5` [T/C]CCTT↓ (40, 50-53).

Residues Arg-80, Lys-83, Arg-84 are the trypsin accessible sites; Arg-67, Tyr-70, Tyr-72, Arg-80 contribute to target site specificity (14).

The NH2-terminal domain consists of a five stranded antiparallel β sheets and two helices and several loops that join the β strands (14). There are a network of hydrogen-bonding interactions between the protein main chain atoms, side chain atoms and water molecules (14). All these interactions, in the form of deep hydrophobic core, salt bridges, side chain stacking interactions, and hydrogen bonds serve to form a very compact and stable protein fragment (14). Loops are responsible for connectivity (14). The salt bridges serve to stabilize the solvent exposed side chains of acid and basic residues (14). The catalytic domain of the vaccinia virus topoisomerase I is 234 amino acids long Topo-[81-314] and 20 kDa (15). This domain includes the active site nucleophile Tyr-274 and four other residues which are important for the DNA cleavage and religation steps that is Gly-132, Lys-167, Arg-223 and His-265 (8, 15). It is proposed that these residues catalyze the attack of Tyr-274 on the scissile phosphate through transition state stabilization and general acid catalysis of the expulsion of the 5`OH leaving
**Figure 1-4.** Domain structure of vaccinia topoisomerase IB.
The tripartite structure of the 314 amino acid wild type vaccinia topoisomerase.

**Figure 1-5.** Important residues in the vaccinia virus topoisomerase catalytic domain.
The catalytic domain of the vaccinia virus topoisomerase is shown in gray, ribbon and the conserved residues in color. The active site tyrosine Tyr274 is shown in red.
group (8, 38). Three other residues Gly-132, Tyr-136, Ser-204, which are important for strand cleavage but not religation, are implicated in a proposed precleavage conformation step (8, 38) (Fig. 1-5).

The Topo-[81-234] crystallized structure consists of 10 α helices and three stranded β sheets (8). The N-terminal lobe[81-210] and the C-terminal lobe [211-310] have an overall temperature factor of 57 Å² and 37 Å². This could be due to the fact that the N-terminal lobe does not make crystal contacts, and this contributes to the high temperature factor (8). The recombinases are enzymes that catalyze the exchange of short pieces of DNA between two long DNA strands, particularly the exchange of homologous regions between the paired maternal and paternal chromosomes (2, 7-11, 16, 54-56). They display topoisomerase I activity in vitro (2, 7-11, 16, 54-56). They are mechanistically related to the eukaryotic type IB topoisomerases (2, 7-11, 16, 54-56). They also cut one strand of DNA to yield a free 5´OH strand and a covalent DNA-[3´-phosphotyrosyl]-enzyme intermediate (2, 7-11, 16, 54-56). As the functional mechanism of both enzymes is similar it is thought that residues important for transesterification are also conserved in the HP1 integrase and Cre recombinase. For example Vaccinia virus Lys 167 is located in the loop connecting the second and third antiparallel β sheets; a lysine is present at the same position in HP1 integrase and λ integrases, Cre recombinases, and Xer D. A homologous Lysine mutation in the human topoisomerase also results in loss of activity (8).

In the vaccinia topoisomerase, the tyrosine side chain at the active site is actually oriented away from the putative binding pocket (8). A similar case of the Tyr pointing away from the binding pocket is seen in XerD where the Tyr is buried in the protein with the hydroxyl moiety pointing away from the putative phosphate binding site (11). In contrast, the Tyr nucleophile in HP1 integrase and Cre recombinase is pointing towards the putative phosphate
binding site formed by the catalytic side chains (54). The Tyr in the Vaccinia virus will have to be reoriented when the topoisomerase binds to the target DNA (Fig. 1-6). The vaccinia virus topoisomerase does not have a preassembled active site conformation but, must undergo a conformational change on DNA binding.

Figure 1-6. A model of vaccinia Topoisomerase bound to the drug target site. The topoisomerase catalytic domain was manually docked on to the 24 bp B form DNA (backbone colored red and purple). The sulfates on the scissile phosphate of the CCCTT strand are colored green. The CCCTT sequence is shown in yellow. In blue is shown the Nterminal domain of vaccinia topoisomerase and in dark green is shown the catalytic domain. Important residues include Ser 268 in purple, lys 271 in gray, Lys 220 in red, Arg 223 in pink, His 265 in orange, ser 270 in yellow and tyrosine 274 in wheat. Tyr 70 and 72 are shown in yellow.
1.7 Deinococcus radiodurans Topoisomerase IB [DraTopIB] Crystal Structure

DraTop IB consists of an N terminal domain (aa 1-90, β-sheets) and a predominantly α-helical C-terminal domain (aa 91-346) that closely resembles the corresponding domains of Vaccinia virus topoisomerase IB (5, 6). The five amino acids of DraTopIB that comprise the catalytic pentad are Arg-137, Lys-174, Arg-239, Asn280 and Tyr-289 and they are preassembled at the active site in the absence of DNA in a manner similar to the human topoisomerase I bound to DNA and unlike the vaccinia virus topoisomerase I (5, 6). There is one major difference between the Virus topoisomerases and the DraTopIB, the only bacterial type IB topoisomerase so far characterized. Besides the conformation of the active site, it is known that the virus topoisomerase IB is sequence specific but the DraTop IB is likely not. Also the cleavage complex for the DraTopIB has not been captured either reflecting the absence of a preferred cleavage site or a religation event that is very fast. The Human topoisomerase I is a much larger protein when compared to both the bacterial and the viral topoisomerase.

1.8 Human Topoisomerase

The topoisomerase IB is exemplified by the Vaccinia virus topoisomerase IB, D. radiodurans bacterium topoisomerase IB and the Human Topoisomerase I. The Human topoisomerase I is a monomeric protein of 765 amino acids. The Human topoisomerase I consists of four domains the NH2-terminal domain (210 residues), core (435 residues), linker (77 residues) and COOH-terminal domain (52 residues) (16). The Human topoisomerase studied is a reconstituted enzyme. It means that the 58 kDa core domain which is expressed in baculovirus insect cell system and the 6.3 kDa COOH-terminal domain expressed in E. coli are mixed to constitute a tightly associated 1:1 complex (16). The full-length human topoisomerase has never been purified and so its properties are only predicted but not tested.
The protein completely wraps around the DNA duplex, contacting 4 bp upstream and 6 bp downstream of the cleavage site (16). The positively charged nose-cone helices on the cap of the enzyme are positioned above the major groove of the DNA, but do not directly contact the DNA (16). There is close contact of the h-Topo (human topoisomerase) and the DNA substrate. The h-topo wraps around the DNA and buries a total of 4700Å surface accessible area upon complex formation. All the three domains and the COOH domain interact with the DNA (16). Sub domain I and III interact extensively with the DNA compared with Sub domain II and the COOH domain (16). The subdomain I attacks the major groove of the DNA (16). Sub domain I and sub domain II forms a clamp around the DNA before cleavage and covalently attachment (16). The protein-DNA contacts made in the noncovalent complex are as important as the contacts made in the covalent complex. The “nose cone” helices offer a highly positively charged interior for the sugar phosphate backbone yet the DNA does not interact directly or indirectly with these positively charged residues in the covalent and non-covalent complex (16).

Residues near the active site make several DNA contacts, especially directly across the minor groove from the cleavage site. The protein contacts only the central 10 bp of the DNA [i.e. positions -4 to +6], and the vast majority of the contacts involve the DNA phosphate groups (16). The active site Tyr723 was mutated to Phe [Y723F] to obtain this structure. The Y723F is facing the active site which shows a preassembled active site conformation.

1.9 Structural Comparison between the Human Topoisomerase, the Vaccinia and the Deinococcus Topoisomerase

To summarize the difference in structures,

1. The human and the DraTop have a preassembled cleavage site as apposed to the vaccinia virus topoisomerase. The structure of the topoisomerase suggests that a pre-cleavage
conformational change in the catalytic domain would be necessary to orient Tyr-274 to attack the scissile phosphate in the vaccinia enzyme. It is predicted that the PAT also has a preassembled cleavage site. The prediction is based on the purification schemes and the behavior of the protein in various experiments.

2. The amino terminal of the human topoisomerase contributes to but is not required for the topoisomerase activity and is missing in all the human topoisomerases purified and studied to date. The human topoisomerase is thought to have high affinity for a sequence from the ribosomal DNA of *Tetrahymena thermophilus*. The amino terminal of the vaccinia virus topoisomerase is important for sequence specificity. I predict that the amino terminal of the human topoisomerase could add sequence specificity to the enzyme.

3. The linker region (residues 129-137) in the vaccinia virus topoisomerase is not defined. This linker region is required to orient the active site tyrosine to the catalytic site for cleavage and relegation steps/ transesterification chemistry. So the role of this region of the topoisomerase is not known, yet is indispensable for activity.

1.10 Perspectives to the *Pseudomonas aeruginosa* Topoisomerase

*Pseudomonas aeruginosa* is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients (57-60). The bacterium’s virulence depends on a large number of cell-associated (flagellum, pilus, nonpilus adhesions, alginate/ biofilm, lipopolysaccharide (LPS)), extracellular factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin,) and efflux pumps (57-61) (Figure 1-7). The specific hypothesis behind the proposed research is that *Pseudomonas aeruginosa* topoisomerase (PAT) can act as an essential target of the therapeutic agents.
The hypothesis is based on the following observations:

1. The topoisomerases are essential enzymes responsible for modulation of DNA topology in the cell. They have been identified in all cell types studied to date (2). The eukaryotic-like sub-family includes vaccinia virus DNA topoisomerase I (7, 8). The PAT shares sequence similarity with that. The PAT can thus be used to study the eukaryotic type I topoisomerases. PAT can act as a model organism to study in detail the biochemical and mechanistic characteristics of the eukaryotic topoisomerases and their interactions with chemotherapeutic agents.

2. *P. aeruginosa* is one of the most prevalent opportunistic human pathogens found in nosocomical infections (pneumonia, UTI), surgical wound infections, blood stream infections, immunocompromised patients such as neutropenic cancer and bone marrow transplant patients, AIDS, and cystic fibrosis (57). DNA topoisomerases are essential for vital functions of DNA during normal growth so by inhibiting the enzyme the organism could be inhibited.

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**Figure 1-7.** *Pseudomonas aeruginosa* virulence factors.
3. Working with the yeast mutants, Dr. James Wang demonstrated a unique aspect of the DNA topoisomerase as a target of the therapeutic agents (22). He discovered that a nonessential yeast DNA topoisomerase I can be the sole target of a cytotoxic drug. Therefore, it is more likely that bacterial topoisomerases, whether essential or not, could serve as targets of antibiotics and antiviral agents. In addition there is a plethora of evidence suggesting the use of topoisomerases as drug targets (16, 20-24, 62-64).

4. Experimental framework from the vaccinia virus topoisomerase-DNA complex reveals why the human cancer drug camptothecin, which kills cells by targeting their topoisomerase, does not affect the viral enzyme (13, 19). The vaccinia virus being 314 amino acids (≈ 32 KDa) is considerably smaller than the human counterpart (765 amino acids, ≈100 KDa) and therefore it is predicted that the vaccinia topoisomerase lacks a DNA binding domain required for the drug to bind. This suggests that structure based drug design can be used to develop homologs of camptothecin, or other drugs, which can target the PAT. DNA topoisomerases are essential for vital functions of DNA, for example normal growth, but under various physiological and non physiological stresses because of their delicate act of breaking and rejoining DNA, they are highly vulnerable and can convert to DNA breaking nucleases, resulting in death and genomic instability (63).

1.10.1 *Pseudomonas aeruginosa* as a Human Pathogen

*P. aeruginosa* is a prevalent opportunistic pathogen. It is the most commonly found gram negative bacteria in nosocomial infections. It has been found that *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases (65), 12% of the hospital acquired urinary tract infections (66), 8% of the surgical wounds infections (36), 10% of the bloodstream infections (67), and immunocompromised patients such as neutropenic cancer, and bone marrow transplant
patients are particularly susceptible to *Pseudomonas* infection (57). Besides this, the pathogen is also responsible for pneumonia and septicemia with the death toll reaching almost 30% (68, 69). *P. aeruginosa* is also a common cause of ventilator–associated pneumonia in intubated patients, which accounts for 38% of the deaths (70). Burn patients are also affected by *P. aeruginosa* (57). But the bacteremia has declined due to better hygiene during treatment of wounds (36). Death rates associated with burns are around 60% and AIDS 50% (71, 72). People suffering from cystic fibrosis are susceptible to *P. aeruginosa* infection (73).

There are two reasons, which make the *P. aeruginosa* infection extremely difficult to resolve and the study of PAT extremely important. First, the intrinsic multidrug resistance in *P. aeruginosa* (59). *P. aeruginosa* shows a significant degree of intrinsic resistance to a wide variety of antimicrobial agents, including most β-lactams, tetracyclines, chloramphenicol, and fluoroquinolones (22). The outer membrane of this species shows a very low nonspecific permeability to small, hydrophilic molecules. In addition to the low permeability, removal of the incoming antibiotic molecules via mechanisms such as degradation or modification produces a significant level of resistance. Further, active efflux of tetracycline, chloramphenicol, and norfloxacin by efflux pumps of *P. aeruginosa* makes a major contribution to the general resistance of *P. aeruginosa* (71). The presence of R plasmids against many antimicrobial agents adds to the resistance (57).

Secondly, the sessile biofilm lifestyle of the bacterium makes it resistant to antimicrobial agents (58). Many persistent infections are caused by biofilms (61). One particular example is the devastating lung infections caused by *P. aeruginosa* biofilm in people with the genetic disease cystic fibrosis. Once *P. aeruginosa* colonizes the cystic fibrosis lung it cannot be eradicated by even the most aggressive antibiotic therapy (60, 61, 74, 75). Bacteria growing in
the biofilms possess characteristics distinct from free floating or swimming [planktonic] counterparts. Biofilm bacteria are resistant to antimicrobial treatments and to the immune defense systems of the hosts (61, 76). Biofilm bacteria form structured communities of cells embedded in the extracellular polymeric (EPS) matrix (61, 77, 78). Biofilm formation by *P. aeruginosa* occurs in discrete steps - surface attachment and multiplication followed by microcolony formation and finally differentiation into mature, structured, antibiotic resistant communities (61, 79).

The topoisomerases have recently attracted attention not because they play a critical role in managing the topology of the DNA in the cell but because, firstly, it has been discovered that a large number of topoisomerases can act as drug targets; for example the drug camptothecin and its derivatives act as inhibitors of the human topoisomerase IB by capturing the enzyme in a covalent complex with DNA (16). Fluoroquinolones act against the DNA topoisomerase II, vaccinia virus topoisomerase IB, the *Pseudomonas aeruginosa* topoisomerase I, DNA gyrase, DNA topoisomerase IV and *Candida albicans* topoisomerase I (21, 28, 62, 80, 81). These drugs include antibacterial agents, anticancer chemotherapeutics and bacteriostatic agents. Secondly, the crystal structures of a large number of topoisomerases are available, such as human topoisomerase, the vaccinia topoisomerase IB and the *Deinococcus radiodurans* topoisomerase IB and these provide valuable insight into the mechanism of the molecular machine and complement the biochemical literature already available (5, 8, 16).

In my thesis I have shown that *P. aeruginosa* encodes a functional topoisomerase I with sequence similarity to the *Vaccinia* and *D. radiodurans* topoisomerase IB. The studies also indicate structural similarities between the *P. aeruginosa* topoisomerase and the human topoisomerase IB. PAT requires Mg²⁺ in the reaction, but not ATP and high concentration of
NaCl cannot substitute for Mg$^{2+}$ in the reaction, unlike what has been reported for the Vaccinia topoisomerase IB. In addition, a comparable domain organization and constellation of catalytic residues are observed for the PAT and the Vaccinia topoisomerase IB. The fluoroquinolone drugs activity against the PAT was explored and PAT showed inhibition by selected fluoroquinolones. The PAT and the Vaccinia virus topoisomerase IB behave similarly to some drugs. The Vaccinia-encoded homolog suggested that fluoroquinolones require Mg$^{2+}$ for inhibitory activity and they function by preventing covalent complex formation.
A Functional Type I Topoisomerase from *Pseudomonas aeruginosa*

2.1 Introduction

DNA topoisomerases are enzymes that solve topological problems associated with important processes such as DNA replication, transcription, recombination and chromatin remodeling by introducing transient single or double stranded breaks in the DNA and releasing accumulated strain (2). DNA cleavage leads to a transient phosphodiester bond between the tyrosine at the active site of the protein and one of the broken DNA strands. The final step in the reaction is resealing of the DNA break, the end result being an altered DNA linking number (2). DNA topoisomerases are of two kinds; those enzymes that cleave only one strand of DNA are type I topoisomerases and those enzymes that cleave both strands of DNA are referred to as type II. The type I topoisomerases are further classified as type IA or IB. In the type IA subfamily the protein is linked to the 5` phosphate and in the type IB subfamily the protein is linked to the 3` phosphate of the DNA substrate (2).

The topoisomerases have attracted attention precisely because of their diverse roles in controlling DNA topology and therefore in regulating DNA-dependent processes. They can act as potential targets for anticancer drugs; for example, the plant alkaloid camptothecin blocks DNA and RNA synthesis in the treated cells and acts as an anticancer drug for human cancer by targeting the human topoisomerase I (16). Coumermycin, which targets the Vaccinia topoisomerase, prevents DNA binding. Both the DNA topoisomerases and the site-specific DNA recombinases perform cleavage and ligation reactions (2, 7-11, 16, 54-56). Therefore, the topoisomerases can also provide insight to the function of their close cousins, the tyrosine recombinases, which could also act as drug targets.
Besides the linkage of the protein to either the 3’- or 5’- end of the DNA, there is another difference in catalytic mechanism between the type IA and type IB topoisomerases. In reactions catalysed by the type IA enzymes, the breaking of the duplex DNA and the rejoining event occurring after the stress relief occurs in a single stranded region of the DNA template (7, 12, 82). This is in contrast with the type IB topoisomerases, which generate a nick in a double stranded region of the DNA template (2, 7, 13, 40). The type IB topoisomerase might be able to attack a single stranded region in a double stranded DNA template but when such a reaction occurs the enzyme might detach from the 5’ end and yield a linear intermediate (2, 83).

In contrast to the type I topoisomerases, the type II topoisomerases catalyze the breakage of both strands of DNA and also catalyze the catenation and decatenation of double stranded DNA circles (84-86). The type II enzymes use the energy released from ATP hydrolysis to create breaks in the double stranded DNA, to cause the nearby double helix to pass through this beak, to reseal the break and dissociate the DNA (7). By contrast, the type I enzymes do not require ATP.

The eukaryotic type IB DNA topoisomerase family includes the topoisomerase I encoded by the vaccinia virus (49, 87). It is the smallest topoisomerase known. It is a 314 amino acid protein that binds duplex DNA with stringent specificity for transesterification at 5’-(C/T)CCTT sites (40, 87). Indeed, all poxviruses studied encode a topoisomerase IB homolog (88-92). The vaccinia virus DNA topoisomerase I is biochemically similar to the eukaryotic type I topoisomerase and yet is only one-third the size. Proteins with homology to the vaccinia topoisomerase IB are also encoded by a few eubacteria; for example, the *Pseudomonas aeruginosa* topoisomerase I (PAT) shares significant sequence similarity with vaccinia virus topoisomerase IB. The sizes of the bacterial enzymes are similar to that of poxvirus enzymes, and the tyrosine nucleophile as well as other residues implicated in catalysis are well conserved.
Therefore, the *P. aeruginosa* topoisomerase I can act as a model to study the mechanism of topoisomerases as well as provide important insight into the physiology of *P. aeruginosa*. A functional eubacterial topoisomerase IB has so far only been described in *Deinococcus radiodurans* (5, 93).

*P. aeruginosa* is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients. The bacterium’s virulence depends on a large number of cell-associated and extracellular factors. Considering that topoisomerases serve vital cellular functions, the topoisomerase from this organism may be a potential drug target as well (57). In the present study, we show that *P. aeruginosa* encodes a functional topoisomerase I with sequence similarity to the vaccinia virus and *D. radiodurans* topoisomerases IB. PAT requires Mg$^{2+}$ for relaxation, but not ATP, and high concentration of NaCl cannot substitute for Mg$^{2+}$ in the reaction, unlike what has been reported for the vaccinia-encoded homolog. A comparable domain organization and constellation of catalytic residues is suggested by the observation that substitution of the tyrosine nucleophile in the active site leads to a mutant that binds DNA but is unable to relax DNA, and that the catalytic fragment is able to relax supercoiled DNA in the absence of the N-terminal domain.

2.2 Experimental

2.2.1 Cloning and Expression of *P. aeruginosa* Topoisomerase I

The PAT gene was PCR amplified from the genomic DNA UCBPP-PA14 using forward primer 5’-CGGAACCCACATATGAGCGCAGC-3’ and reverse primer 5’-GCACGGCATGGATCCGCACC-3’. The NdeI and BamHI restriction sites were incorporated into the oligonucleotide primers, and the fragment containing the *P. aeruginosa* topoisomerase I
coding region was cloned into the *E. coli* expression vector pET5a. The identity of the cloned fragment was confirmed by DNA sequencing, and the recombinant plasmid was used to transform *E. coli* strain BL21(DE3)pLysS. The transformants were plated on MDAG media which is an enriched, fully defined non inducing media; the MDAG media was prepared as described in (94), except the 1000X metals was made according to (95).

### 2.2.2 Mutagenesis

The point mutation on *P. aeruginosa* topoisomerase I to replace the tyrosine with phenylalanine at the active site was created using whole plasmid PCR. The sequence of the forward primer is 5’-CGGCAATGCTTCATCCACCCCGG-3’ and the reverse primer is 5’-GCAGATCGCCACGCTGTTGCC-3’. The template was removed by DpnI digestion and the PCR product was used to transform *E. coli* TOP10 (Invitrogen). The catalytic fragment was created by amplification of the C-terminal region using forward primer 5’-GCGCGAAGCATATGGATGCC-3’ and reverse primer 5’-CCTTTCGTCTTCAAGAATTCGGATCC-3’. NdeI and BamHI restriction sites were introduced at either end of the PCR product which was cloned into pET5a. Constructs were confirmed by sequencing.

Plasmids harboring the gene encoding the full length enzyme (PAT), catalytic mutant (PATCAT) or the Y292F mutant were transformed into BL21(DE3)pLysS, cells were grown in LB media at 37°C, 250 rpm, until OD≈0.2 and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. After one hour, the cells were harvested by pelleting and the over-expression was confirmed by SDS-PAGE followed by Coomassie Brilliant Blue staining. For PAT, Y292F and PATCAT the cells were harvested by centrifugation and the pellets were stored at -80°C.
2.2.3 Protein Purification

Six 1500 ml cultures of *E. coli* BL21(DE3)pLysS containing plasmid carrying the PAT gene were induced as noted above, cells were pelleted, and the pellet was redissolved in HA buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol and 0.25 M NaCl, 7.2 mM β-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride (PMSF)) and lysed by sonication; about 20% *P. aeruginosa* topoisomerase I was in the pellet at this time. After removing the insoluble material in the crude lysate by centrifugation at 5,000-6,000 rpm for 15 minutes, the soluble fractions were subjected to 60% ammonium sulfate precipitation. The supernatant remaining after the final precipitation was found to contain 80% *P. aeruginosa* topoisomerase I. The supernatant was dialyzed overnight against HA buffer. The dialyzed supernatant was applied to a phosphocellulose column previously equilibrated to pH 8.0. The elution was performed by applying a step gradient from 0.4-1.5 M NaCl. The glycerol concentration in the eluate was increased to 30% and the protein was stored at -20°C.

The pellet from the overexpressed Y292F mutant was dissolved in HA buffer and subjected to 30% ammonium sulfate precipitation. The pellet from the precipitation was redissolved in HA buffer, lysed by sonication and loaded on a heparin agarose column in HA buffer. Protein was eluted by a gradient from 0.4-1.5 M NaCl in HA. The overexpressed PATCAT mutant was similarly subjected to 30% ammonium sulfate precipitation. The supernatant from the precipitation was dialyzed against HA buffer overnight and loaded onto the phosphocellulose column pre-equilibrated in the same buffer. Protein was eluted by step gradient from 0.4-1.5 M NaCl. Protein concentrations were determined by Coomassie-stained SDS-PAGE gels using BSA standards.
2.2.4 Oligonucleotide Labeling

Oligonucleotides were purchased from Operon. The top strand oligonucleotides were labeled at the 5’ end with T4 polynucleotide Kinase (New England Biolabs) and $[\gamma^{32}\text{P}]$ ATP and annealed to the bottom strand. The sequences of the oligonucleotides are given in Table 1.

2.2.5 Relaxation Assay

Relaxation assay (per 20 μl) was performed with negatively supercoiled pUC18 DNA (≈110ng) unless otherwise stated, and the indicated amount of PAT in topoisomerase buffer (50 mM Tris (pH 8.0), 2.5 mM MgCl₂, 0.1 mM EDTA) for two hours at 37 °C. The reaction also contained a final concentration of 0.24 M NaCl and≈19% glycerol from the protein storage buffer. Reactions were stopped with 5 μl 1(10% SDS):1(30% glycerol) stop buffer. The reactions were loaded on a 1% agarose gel in 0.5X TBE buffer (45 mM Tris-borate (pH 8.3), 1 mM EDTA). The gels were run at 2 V/cm for 16 hours. The gels were stained with ethidium bromide (EtBr) and visualized using an Alpha Innotech digital imaging System.

The positively supercoiled DNA was prepared by first nicking pUC18 with N.BstNBI for an hour, followed by phenol extraction and ethanol precipitation. Nicked pUC18 was then incubated with netropsin and T4 DNA ligase and again phenol extracted and ethanol precipitated to remove the drug, resulting in the production of positively supercoiled DNA. The production of positively supercoiled DNA was confirmed by two dimensional agarose gel electrophoresis, as described (96). To confirm production of covalently closed DNA on incubation with PAT, reactions were also electrophoresed on a gel containing ethidium bromide.

2.2.6 Electrophoretic Mobility Shift Assay

The reaction mixture per 20 μl contained fifty fmol of the labeled DNA substrate which was incubated with the indicated amount of *P. aeruginosa* topoisomerase I or mutant protein in
topoisomerase buffer (50 mM Tris (pH 8.0), 2.5 mM MgCl₂, 0.1 mM EDTA) for one hour at 37°C. The reaction mixture contained 0.67 M NaCl final concentration. The glycerol concentration in each sample was different as noted in the figure legend; this was due to the instability of the protein which precluded its concentration following elution. Samples were loaded on 8% (w/v) PAGE (37:1 acrylamide/bisacrylamide) gels in 0.5XTBE and run for 90 minutes. Gels were visualized on a Molecular Dynamics Storm Phosphoimager using software supplied by the manufacturer.

2.3 Results

2.3.1 Sequence Alignment

Screening of the NCBI database with the BLAST search engine and PAT as the query yielded several homologs including vaccinia virus topoisomerase IB. A group of conserved amino acid side chains in the active site coordinate the attack of the tyrosine nucleophile on the scissile phosphodiester. This catalytic pentad, Arg-130, Lys-167, Lys-220, Arg-223 and His-265 as well as Tyr-274 for the vaccinia virus topoisomerase I, is conserved among the poxvirus enzymes, while the closely related tyrosine recombinases typically contain a histidine in place of the lysine in position 220 of vaccinia topoisomerase. The two arginines and the histidine interact directly with the scissile phosphodiester and may serve to stabilize a negatively charged transition state (38, 56, 97-99). The homologous residues in *P. aeruginosa* topoisomerase I are Arg-148, Lys-185, Lys 247, Arg 250, Asn-283 and Tyr-292 (indicated with an asterisk in Fig. 2-1) and in the *D. radiodurans* encoded homolog, Arg-137, Lys-174, Lys-235, Arg-237, Asn-280 and Tyr-289. The His-265 is replaced with Asn-283 in *P. aeruginosa* topoisomerase and Asn-280 in *D. radiodurans* topoisomerase (5). The *P. aeruginosa* topoisomerase I showed 13% sequence identity with Entomopox virus, 20% with Bovine Papular Stomatitis virus, 23% with
Orf virus, 14% with Vaccinia virus IB, 14% with Fowl Pox virus and the *D. radiodurans* topoisomerase aligned particularly well at 36% identity. The C-terminal catalytic domain is structurally conserved, and the catalytic pentad occupies equivalent positions in the structures of the topoisomerases IB and tyrosine recombinases. In contrast, the N-terminal domain is not conserved between topoisomerases and recombinases.

The 333 amino acid *P. aeruginosa* topoisomerase I is predicted to consist of three protease-resistant structural domains demarcated by two protease-sensitive segments referred to as the bridge and the hinge (Fig. 2-2A) (100). Residues 1 through 98 are implicated as the N-terminal domain and residues 99 through 333 are predicted to form the catalytic C-terminal domain. The C-terminal domain which includes the bridge and the hinge also includes the active site nucleophile (Tyr-292) which is implicated in transesterification. While the N-terminal domains of poxvirus topoisomerases and tyrosine recombinases are divergent, this domain is conserved between poxvirus and bacterial topoisomerases. To establish whether the *P. aeruginosa*–encoded homolog is functional, we cloned the gene and characterized the purified protein (Fig. 2-2B). Based on sequence features, we also created two mutants of PAT; one is the truncated C-terminal domain consisting of residues 99-333 (PATCAT) (Fig. 2-2C) and the other is the active site mutant where the active site tyrosine nucleophile is changed to phenylalanine, referred to here as Y292F (Fig. 2-2D). Initial expression analyses of PAT revealed that the protein is highly unstable and loses activity upon storage. For this reason, we performed a relaxation assay each time before using the PAT for other experiments. Under typical growth conditions, the protein was also toxic, and showed poor expression when expressed using BL21(DE3) pLysS; therefore, the transformants were grown on non-inducing media containing low concentrations of glucose and amino acids. Glucose and amino acids prevent induction by
Figure 2-1. Sequence alignment of *P. aeruginosa* topoisomerase I with other homologous topoisomerases IB.

The amino acid sequences from Entomopoxivirus (MSV130), Bovine Papular Stomatitis virus (BPSVgORF062), Orf virus (ORFVgORF062), Vaccinia virus (H6R, Vaccinia topoisomerase type IB), Fowl Pox virus (FPV143) and *D. radiodurans* topoisomerase IB (DR_0690) are aligned with the *P. aeruginosa* topoisomerase I (PA2G_01347). Sequence gaps are indicated by dashes. The residues in blue and red correspond to identical and conserved residues, respectively. The asterisks in black identify the conserved catalytic residues. The N- and C-terminal domains are demarcated by arrows.
Figure 2-2. **Domain structure of P. aeruginosa topoisomerase I.**

(A) Schematic representation of the tripartite structure of the 333 amino acid predicted for *P. aeruginosa* topoisomerase I is illustrated using the vaccinia topoisomerase IB as a template. The mutant protein lacking residues 1-98 that is the N-terminal domain is the catalytic fragment (PATCAT, residues 99-333). For the active site tyrosine mutant the active site tyrosine is changed to phenylalanine (referred to as the Y292F mutant protein). (B) *P. aeruginosa* topoisomerase I wild type purified. The enzyme fraction was analyzed by SDS-PAGE. In lane 1 is the molecular weight marker, lane 2 shows the PAT. (C) *P. aeruginosa* topoisomerase I catalytic fragment purified, lane 1 shows the molecular marker and lane 2 shows the PATCAT. (D) *P. aeruginosa* topoisomerase tyrosine mutant purified, lane 1 shows the molecular weight marker and lane 2 shows the Y292F mutant protein.

Lactose during log phase growth, and metabolic balancing of pH allowed development of reliable non-inducing media. Expression strains grown on non-inducing media retain plasmids and remain fully viable for months in the refrigerator. While comparable DNA binding sites for the
PAT and PATCAT may be suggested by the observation that both proteins interact strongly with phosphocellulose, the Y292F mutant does not bind to the phosphocellulose and was instead purified using heparin agarose chromatography. These mutants and the purified wild type protein are shown in Fig. 2-2B, 2-2C, 2-2D.

### 2.3.2 DNA Relaxation Activity of *P. aeruginosa* Topoisomerase I

The DNA relaxation activity of the *P. aeruginosa* topoisomerase I enzyme was assayed by incubation with supercoiled plasmid pUC18. Reaction mixtures containing freshly prepared *P. aeruginosa* topoisomerase I and supercoiled pUC18 were incubated for different times and the reactions were terminated with SDS. The full-length enzyme (9.2 ng) starts to relax 110 ng supercoiled pUC18 after 0.5 minutes and all the DNA is relaxed in 15 minutes; in comparison, the less efficient PATCAT (808.2 ng) starts to relax 110 ng of supercoiled pUC18 after 0.5 min and the DNA is completely relaxed in 15 minutes (Fig. 2-3A and 2-3B). For vaccinia topoisomerase, 2.7 ng enzyme relaxes 300 ng supercoiled pUC18 in 0.25 min in the presence of Mg\(^{2+}\) and in the absence of Mg\(^{2+}\) it relaxes DNA in 2 minutes (15). The *D. radiodurans* topoisomerase IB is also not as efficient as the Vaccinia homolog, about 350 ng enzyme relaxes 300 ng of supercoiled pUC18 in 5 minutes (5). Consistent with its role in the transesterification reaction, the Y292F mutant is unable to relax supercoiled DNA (Fig. 2-3C). To determine if the product of PAT mediated DNA relaxation is covalently closed, reactions were electrophoresed also on gels containing ethidium bromide, under which conditions only covalently closed DNA will become supercoiled. As seen in Fig. 2-4, PAT does produce covalently closed product (compare lanes 5). We also note that the fraction of nicked DNA appears to increase as the protein preparation ages, reflecting instability of the enzyme.
Figure 2-3.  (A) Kinetics of DNA relaxation by PAT.

Lane 1 shows the relaxed DNA. The supercoiled pUC18 (110 ng) was relaxed with 9 ng PAT in the topoisomerase buffer. Lanes 2 through 10 contain 110 ng of DNA and 9 ng PAT in the topoisomerase buffer. Reactions were stopped after 0.5, 1, 2, 3, 4, 5, 10, 15 and 20 minutes. The relaxed DNA (R) and supercoiled DNA (S) are identified on the left. (B) Relaxation of supercoiled pUC18 in the presence of PATCAT. Lane 1 shows the relaxed DNA. The supercoiled pUC18 (110 ng) was relaxed with 9 ng PAT in the topoisomerase buffer. Lanes 2 through 11 contain 110 ng DNA and 808.2 ng PATCAT in the topoisomerase reaction buffer. Reactions were stopped after 0.5, 1, 2, 3, 4, 5, 10, 15, 20, and 25 minutes in lanes 2 to 11 respectively. The relaxed and supercoiled DNA are identified by (R) and (S) on the left. (C) The Y292F mutant is not able to relax supercoiled pUC18. The relaxed and supercoiled DNA are identified by (R) and (S) on the left. In lane 1, is shown the negative control containing supercoiled pUC18 in the topoisomerase buffer and lane 2 is showing the relaxed DNA (supercoiled pUC18 (110 ng) relaxed with PAT (9 ng)) in the topoisomerase buffer. Lanes 3 through 7 contain 110 ng DNA and 241 ng Y292F in the topoisomerase reaction buffer. Reactions were stopped after 5, 10, 15, 20 and 25 minutes, respectively.
Figure 2-4. PAT produces covalently closed, relaxed DNA.

(A) Lane 1 contains the 1 Kb ladder, lane 2 contains 120 ng of supercoiled DNA, lane 3 contains 130 ng nicked DNA, lane 4 contains 140 ng linear DNA and lane 5 contains 140 ng supercoiled pUC18 and 63 ng protein in topoisomerase buffer with MgCl₂. The reaction mixtures are run on 1% agarose gel.

(B) Lane 1 contains the 1 Kb ladder, lane 2 contains 120 ng supercoiled DNA, lane 3 contains 140 ng linear DNA, lane 4 contains 130 ng nicked DNA, lane 5 contains 140 ng supercoiled pUC18 and 63 ng protein in topoisomerase buffer with MgCl₂. The reaction mixtures are run on an 1% agarose gel with 2 μg/ml of Ethidium Bromide. Reactions represent a single sample, of which one-half is loaded on each gel.
Surprisingly, PAT is unable to relax DNA in the absence of MgCl₂ in the topoisomerase buffer (Fig. 2-5). DNA relaxation was performed by titrating increasing MgCl₂ from 0 to 2.5 mM and *P. aeruginosa* topoisomerase I (202 fmole). The *P. aeruginosa* topoisomerase I relaxed supercoiled pUC18 DNA when the salt concentration is 2.5 mM. This is in contrast to vaccinia topoisomerase I which was reported not to require Mg²⁺ for activity although its activity is stimulated in its presence (13). DNA relaxation assay by *P. aeruginosa* topoisomerase I with different salts in the reaction mixtures showed that the *P. aeruginosa* topoisomerase I requires divalent cations for relaxation and that high concentration of NaCl cannot substitute for MgCl₂ in the reaction mixture. A more detailed examination of the cation specificity is shown in Fig. 2-6A, Fig. 2-6B. MgCl₂, MnCl₂, CuCl₂ and ZnCl₂ had a stimulatory effect on the relaxation activity. For Vaccinia topoisomerase I, higher concentration of monovalent salt can substitute for MgCl₂. By contrast, high concentration of NaCl cannot substitute for MgCl₂ in the reaction catalyzed by PAT (Fig. 2-6C). Calcium and cobalt cannot substitute for MgCl₂ at relaxation of supercoiled DNA when compared with zinc, copper, magnesium and manganese. The *P. aeruginosa* topoisomerase I differs from vaccinia virus topoisomerase IB in the sense that vaccinia virus topoisomerase IB does not relax DNA in the presence of ZnCl₂ and for *P. aeruginosa* topoisomerase I, ZnCl₂ is able to substitute for MgCl₂ in the relaxation reaction (Fig. 2-6A, 2-6B) (13). The PATCAT shares similar requirements for divalent cations as the PAT (data not shown). Vaccinia virus topoisomerase IB requires a monovalent salt for relaxation activity and in the presence of EDTA the activity was optimal between 100 and 250 mM NaCl (13). This was not true for *P. aeruginosa* topoisomerase I which fails to relax supercoiled DNA under comparable conditions (Fig. 2-6C). High [NaCl] (1 M), however, was inhibitory for enzyme activity (data not shown).
Figure 2-5. MgCl$_2$ is required for DNA relaxation.

Lane 1 shows relaxed DNA (R) containing 110 ng pUC18 DNA and 9 ng PAT in topoisomerase buffer with final concentration of 2.5 mM MgCl$_2$ and lane 2 shows supercoiled (S) DNA without PAT. Reaction mixtures containing 110 ng of supercoiled pUC18, 9 ng of PAT and increasing concentration of MgCl$_2$ from 0, 0.00025, 0.0025, 0.025, 0.25, 2.5 mM in lanes 3-8, respectively.
Figure 2-6. (A) Relaxation of supercoiled DNA in the presence of different cations.

Reactions contained 200 ng of supercoiled pUC18 and the indicated cation at 2.5 mM final concentration in topoisomerase buffer without MgCl$_2$ and 0.24 M NaCl. Lane 1 contains relaxed DNA (R). Relaxation of 200 ng supercoiled pUC18 was performed with 9 ng of PAT in topoisomerase buffer with MgCl$_2$ for 2 hours at 37°C. Lane 2 contains 200 ng supercoiled pUC18 in the topoisomerase buffer indicated by (S) on the left hand side. Lane 3 through 7 contain 9 ng of PAT in addition to (B) Reactions contain 75 ng of supercoiled pUC18, 9 ng PAT, indicated cation at 2.5 mM concentration. The relaxed DNA (R) and supercoiled pUC18 (S) are identified on the left and were obtained as described in (A). (C) Effect of NaCl on topoisomerase activity. Reaction mixtures contained 110 ng of supercoiled pUC18 DNA, 2.5 mM EDTA, 9 ng PAT, and increasing concentration of NaCl 265 mM, 290 mM, 315 mM, 340mM, 365 mM, 390 mM, 415 mM, 440 mM, 465 mM, 490 mM, 540 mM and 590 mM in lanes 3 through 14 respectively in topoisomerase buffer without MgCl$_2$. Lane 1 is the positive control containing relaxed DNA and lane 2 is the negative control containing supercoiled pUC 18 DNA. The relaxed DNA (R) and supercoiled pUC18 (S) are identified on the left and were obtained as described in (A).
Figure 2-7. PAT does not require ATP for relaxation of supercoiled DNA.

All reactions contain 110 ng of supercoiled pUC18 and 9 ng of PAT in the topoisomerase buffer. R indicates relaxed DNA and S indicates supercoiled pUC18 in lane 1. Lane 2 contains 0.1 mM ATP and 0 mM MgCl₂, lane 3 contains 1 mM ATP and 0 mM MgCl₂, lane 4 contains 0.1 mM ATP and 0.25 mM MgCl₂, lane 5 contains 1 mM ATP and 0.25 mM MgCl₂, lane 6 contains 0.1 mM ATP and 2.5 mM MgCl₂, lane 7 contains 1 mM ATP and 2.5 mM MgCl₂.

Figure 2-8. PAT relaxes positively supercoiled DNA as well as negatively supercoiled DNA.

In lane 1 is positively supercoiled pUC18 (S1), lane 2 contains negatively supercoiled pUC18 (S2), lane 3 contains positively supercoiled DNA relaxed, lane 4 contains negatively supercoiled DNA relaxed. Lanes 3 and 4 contain 110 ng of supercoiled DNA, 9 ng PAT in the topoisomerase buffer with 2.5 mM MgCl₂.
The type II topoisomerases utilize ATP for activity, while the Type IA and IB topoisomerases do not require ATP to relax DNA. Type I topoisomerases can thus be differentiated from type II by either the requirement of ATP or by the fact that the type I enzymes bring a change in ±1 in the linking number and type II change the linking number by ±2 (101). As shown in Fig. 2-7, PAT does not require ATP for relaxation, nor does ATP inhibit activity. Eukaryotic type I topoisomerases differ from the prokaryotic type I topoisomerase in the ability to relax positively supercoiled DNA (102). Indeed, Vaccinia topoisomerase IB was reported to relax positively supercoiled DNA more efficiently (7). Notably, PAT is able to relax positively supercoiled DNA (Fig. 2-8). We also noted that the observed activity is unlikely to derive from contaminating E. coli topoisomerase; type II enzymes would be sensitive to fluoroquinolone antibiotics, and the activity observed here is insensitive to ciprofloxacin (data not shown). While contaminating Type IA activity may not be readily addressed using inhibitors, such activity would be expected to result in a population of topoisomers with different linking numbers (e.g., (103)). As we consistently observe instead the generation of covalently closed and nicked, relaxed species, we conclude that the observed activity (which is absent in the preparations of Y292F and significantly reduced for PATCAT) is due to the PAT, and that this enzyme relaxes supercoiled DNA to completion following DNA strand incision.

2.3.3 Sequence Preference of PAT

Vaccinia topoisomerase I is specific for cleavage of the sequence (C/T)CCTT↓ in the scissile strand. To evaluate the sequence preference of PAT, we therefore first compared complex formation with 37 bp DNA containing the Vaccinia topoisomerase recognition sequence, a 37 bp DNA of average G+C content, or the equivalent 37 bp DNA in which two 4 nucleotide loops were introduced (Table 2-1). In addition, a previously published shorter duplex
used to monitor vaccinia topoisomerase cleavage kinetics was used; PAT does not yield a complex with this 18/24 duplex DNA used by the vaccinia virus topoisomerase IB (data not shown) perhaps because it is too short for stable complex formation. However, PAT does bind the 37 bp DNA containing the pentameric Vaccinia sequence and forms two complexes as seen in Fig. 2-9A. Some preference for the CCCTT-containing DNA is suggested by the observation that PAT does not form a discrete complex with the 37 bp perfect duplex (Fig. 2-9B), but does interact more stably with the corresponding looped DNA (Fig. 2-10A). The Y292F mutant also binds DNA (37 bp looped DNA Fig. 2-10B) but does not relax DNA. Regardless of DNA substrate, the presence of MgCl₂ in the reaction has no effect on complex formation (data not shown).

The PAT forms two distinct complexes with the 37 bp CCCTT-containing DNA. It might be that the enzyme molecule may bind to the DNA at different places, thus giving rise to more than one complex of distinct mobility. Maybe one PAT molecule binds to CCCTT, which in turn causes a second PAT to bind DNA, resulting in complexes that are stuck in the well. The preferred binding to 37 bp DNA containing the vaccinia topoisomerase site suggests that PAT recognizes certain sequence elements. While all poxvirus topoisomerases favor cleavage at (C/T)CCTT sequences (40), surrounding sequences also effects DNA cleavage (104); for example, only four of the eight CCCTT sequences in pUC19 are cleaved by MCV topoisomerase (92). However, the *D. radiodurans* encoded homolog was reported not to share the preference for cleavage at (C/T)CCTT sites as evidenced by the failure to detect covalent adduct with CCCTT-containing duplex DNA (5). Similarly, we have been unable to capture this covalent adduct using the 37 bp DNA (data not shown), reflecting either a lack of preference for cleavage at this site or a religation reaction that is too rapid to allow detection. For poxvirus-encoded
Table 2-1 The 37 bp DNA constructs.

(A) The sequence of the 37 bp containing the Vaccinia recognition pentameric sequence in bold. (B) The sequence of the loop-containing duplex: the sequence of the bottom strand is modified to generate tandem mismatches of identical opposing nucleotides with spacing of 9 bp. The sequence generating the loops is in boldface. (C) The sequence of the perfect duplex without loops.

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<th>5’-CGTGTCGATTCCGACGTCCTTGCAATTTATCAAATAT-3’</th>
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<td>A</td>
<td>bp VacSeq</td>
<td>3’-GCACAGCTAAGGCTGCAGGGAACGTAAATAGTTAATA-5’</td>
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<th>5’-CCTAGGCTACACCTACTCTTTGTAAGATTTAAGCTTC-3’</th>
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<tr>
<td>B</td>
<td>37bp Loops</td>
<td>3’-GGATCCGATGTGGATGAGAAACATTCTTAATCGAAG-5’</td>
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<th>5’-CCTAGGCTACACCTACTCTTTGTAAGATTTAAGCTTC-3’</th>
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<tr>
<td>C</td>
<td>37bp without loops</td>
<td>3’-GGATCCGATGTGGATGAGAAACATTCTTAATCGAAG-5’</td>
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Figure 2-9. Binding of PAT to 37 bp DNA with and without CCCTT-containing sequence.

(A) PAT 0, 20, 40, 68, 136, 200, 268, 336, 400, 468, 712 fmol respectively in lanes 1-11 in topoisomerase buffer with MgCl₂. The glycerol concentration in lanes 1 through 11 varies from 8.34% to 24.5%. Free DNA (F), complex (C) and aggregates (A) are indicated on the right.

(B) Binding of PAT to 37 bp perfect duplex DNA; PAT 0, 20, 40, 68, 136, 200, 268, 336, 400, 468, 712 fmol respectively in lanes 1-11 in topoisomerase buffer without MgCl₂. The glycerol concentration varies from 8.34% to 24.5% in lanes 1 through 11. Free DNA (F) and aggregates (A) are indicated on the right.
Figure 2-10. Binding of PAT and Y292F to 37 bp looped DNA.

(A) PAT 0, 20, 40, 68, 136, 200, 268, 336, 400, 468, 712 fmol respectively in lanes 1-11 in topoisomerase buffer with MgCl₂ and 37 bp looped DNA. The glycerol concentration in lanes 1 through 11 varies from 8.34% to 24.5%. Free DNA (F), complex (C) and aggregates (A) are indicated on the left. (B) Binding of Y292F to 37 bp looped DNA. Y292F 0, 0.53, 1.06, 1.8, 3.6, 5.3, 7.1, 8.9, 10.6, 12.4, 18.8 pmol respectively in lanes 1-11 in topoisomerase buffer with MgCl₂ and 37 bp looped DNA. The glycerol concentration in lanes 1 through 11 varies from 8.3% to 32.26%. Free DNA (F), complex (C) and aggregates (A) are indicated on the right.
enzymes, such covalent adducts have been readily detected; in contrast, for other topoisomerases, the covalent intermediate is quite short-lived and generally cannot be captured in absence of specific inhibitors or without the use of suicide substrates in which the non-covalently held DNA dissociates to prevent religation. That both bacterial topoisomerases fail to yield detectable covalent intermediate suggest a mechanistic difference from poxvirus-encoded homologs, either in terms of substrate recognition or religation kinetics.

2.4 Discussion

Few bacterial species encode a type IB topoisomerase with similarity to the poxvirus-encoded enzymes, and *P. aeruginosa* topoisomerase I is only the second bacterial homolog to be characterized and shown to be functional in vitro. The structural gene for the *P. aeruginosa* topoisomerase I when induced to express in *E. coli* results in the appearance of the expected Mr 38 KDa polypeptide which is responsible for the relaxation activity. While PAT relaxes both positively and negatively supercoiled DNA in absence of ATP and appears to retain the domain organization and constellation of catalytic residues characteristic of the poxvirus-encoded enzymes, PAT does exhibit a unique requirement for divalent cations for catalytic activity.

2.4.1 Effect of Salt on Relaxation Activity

Analysis of the purified enzyme showed that while PAT does not require any divalent cation for DNA binding, it does require divalent cations for relaxation of supercoiled DNA, which suggests that either the divalent cations are required for cleavage complex formation or for religation of DNA after removal of the torsional strain. The divalent cation could also play a role in site specific binding, including the enzyme’s ability to discern the topological state of the nucleic acid substrate. The poxvirus topoisomerases were reported not to require Mg$^{2+}$ for
binding and cleavage, however, the vaccinia enzyme activity is stimulated 10-to 20-fold by
divalent cations.

2.4.2 Domain Organization of PAT

The catalytic fragment (235 amino acids) of the _P. aeruginosa_ topoisomerase I lacking
the amino terminal domain is able to relax DNA in the absence of the N-terminal domain, albeit
less efficiently than the full length enzyme. Similarly, the vaccinia topoisomerase catalytic
domain is less efficient than the full length enzyme, a phenomenon ascribed to lower affinity
binding to the CCCTT target sequence and a reduced rate of DNA cleavage (15). The structure
of poxvirus topoisomerase IB in complex with its cognate DNA site confirms the contribution of
the N-terminal domain to site-specific binding (105). A \( \beta \)-strand from the N-terminal domain of
the major groove makes contacts to several bases within this sequence; these residues (Tyr-70,
Tyr-72 and Gln-69 of poxvirus topoisomerase) are all conserved in the _P. aeruginosa_-encoded
homolog. Thus, removal of the N-terminal domain of PAT may likewise attenuate any
sequence-preferences exhibited by the full-length enzyme. Failure to engage the cognate site
adequately may in turn result in suboptimal positioning of catalytic residues. Consistent with this
interpretation, mutation of Tyr-70, Tyr-72 and Gln-69 of poxvirus topoisomerase results in
defects in relaxation activity (105).

The human topoisomerase (Fig. 2-11A) and the _Deinococcus_ topoisomerase have a
preassembled cleavage site as opposed to the vaccinia virus topoisomerase in which the active
site is not preassembled prior to DNA binding (Fig. 2-11B) (8). Comparison of the structures
shows that three of the vaccinia topoisomerase catalytic residues, Arg-130, Lys-167 and Tyr-274
are not in the correct position for transesterification to occur (99, 106). A precleavage
conformational change in the catalytic domain would be necessary to orient Tyr-274 to attack the
scissile phosphate in the DNA backbone (6, 16, 101), a conformational change triggered by specific interaction with either strand of the CCCTT target sequence (105). Based on the sequence conservation between the bacterial enzymes and the properties of the Y292F mutant, we speculate that PAT may likewise have a preassembled cleavage site. While the PAT and PATCAT were both purified by chromatography on phosphocellulose, the Y292F mutant did not bind to phosphocellulose and was purified on the heparin column. If Y292F was far removed from the active site, its replacement with Phe would be unlikely to alter features of the active site. Therefore, we hypothesize that by changing the tyrosine at the active site we altered the cleavage site, resulting in attenuated interaction with the phosphocellulose column. Consistent with this interpretation, DNA-binding properties of wild type PAT and the Y292F mutant are comparable, including preferred binding to CCCTT-containing and looped DNA.

2.4.3 DNA Binding by PAT

PAT binds to the 37 bp CCCTT-containing sequence as opposed to little binding and no distinct complexes with 37 bp perfect duplex without this pentameric sequence. Both N-and C-terminal domains of the poxviral enzymes contribute to sequence-specific binding, and several residues involved in direct contacts are conserved, notably Tyr-70 and Tyr-72 that contact the central CCT of the CCCTT recognition sequence and Arg-80 and Lys-167 that along with Tyr-72 contact the TT at the scissile phosphate; Arg-80 is not conserved in the \textit{D. radiodurans}-encoded enzyme. However, Lys-133 which contacts the G opposite the less stringently conserved 5' - C and contributes to positioning Arg-130 appropriately at the active site is not conserved in the bacterial homologs, and neither is Asp-168 that makes a water-mediated contact to A at the scissile phosphate (105). It is therefore conceivable that the bacterial enzymes exhibit
Figure 2-11. The cleavage site assembly for the human topoisomerase (PDB IK4T) (panel A) and Vaccinia topoisomerase IB (PDB IA4I) (panel B).

The human topoisomerase IB has a preassembled precleavage site but in the vaccinia enzyme, the catalytic tyrosine is located away from the cleavage site and is reoriented after DNA binding.
only a modest preference for the sequence stringently preferred by the poxviral enzyme. PAT also binds 37 bp looped DNA; this could reflect a binding mode similar to that of eukaryotic topoisomerases which exhibit preferential binding to curved DNA \((17, 107)\). The reactions catalyzed by the purified PAT proceed via an intermediate in which there is a covalent linkage between the DNA and the enzyme. Such complexes can generally be captured on denaturing gels, however, this complex has not been captured for PAT.

There could be several reasons for this. PAT may exhibit a high degree of sequence preference in selection of the cleavage site, a sequence preference only partly emulated by the CCCTT sequence or its context. Secondly, PAT might be recognizing not only sequences but certain structures in the DNA perhaps structures promoted by DNA supercoiling. Thirdly, the reaction may be so fast that a suicide substrate needs to be defined in order to capture the cleavage complex. Based on the partial conservation of residues involved in sequence specific DNA interactions we propose that bacterial type IB topoisomerases exhibit a relaxed sequence preference, reflected in failure to capture a cleavage complex with CCCTT-containing DNA and a reduced relaxation activity compared to the poxviral enzymes.
3 Interaction of Fluoroquinolones with the Pseudomonas aeruginosa and Vaccinia Topoisomerases

3.1 Introduction

Quinolones are broad-spectrum antibiotics. The fluoroquinolones are a subset of quinolones in which a fluoro group is attached to the central ring (Fig. 3-1). Quinolones are antibiotics, targeting topoisomerase II (DNA gyrase and topoisomerase IV) in bacterial cells (26-28, 108). They inhibit DNA synthesis by trapping topoisomerase II in a covalent complex with DNA (29). Their target in gram negative bacteria is DNA gyrase and in gram positive bacteria it is type IV topoisomerase (108). The mechanism of action of the quinolones in inhibiting the DNA gyrase and the topoisomerase IV is similar. The gyrase is encoded by two proteins, gyrA and gyrB (109-112), and the topoisomerase IV is encoded by parC and parE (113-116), where the parC is homologous to gyrA and parE is homologous to gyrB (114). The mechanism of action of the quinolones is ambiguous as indicated by conflicting reports. Some reports indicate that the drug binds directly to DNA (117). Other reports suggest that drug binding is largely determined by the structure of the DNA, and that the quinolone drugs bind with higher affinity to single stranded DNA showing a higher affinity towards poly(G) and poly(dG) as compared to poly(dI) (117). It has been shown that drug binding to DNA increases binding of gyrase to DNA (118). In addition, the extent of DNA binding is controlled by the topology of the DNA (119). Conflicting data include that quinolone drugs bind to DNA gyrase (14) at their inhibitory concentration, bind poorly to relaxed double stranded DNA and preferentially to single stranded DNA, but in a nonspecific and noncooperative manner (120). Reports confirm that binding of the fluoroquinolone norfloxacin stabilizes the binding of topoisomerase IV to DNA, using a topoisomerase mutant with substitutions at the active site (where the active site tyrosine has been
changed to phenylalanine); this mutant is incapable of DNA cleavage but still able to bind norfloxacin, suggesting that DNA cleavage is not required for drug binding and that these are mutually exclusive events (121, 122). The drugs, however, cause structural perturbations in the DNA upon binding (122). It is therefore predicted that the quinolone forms a ternary complex with the DNA and the gyrase/topoisomerase IV after strand cleavage, targeting the staggered single strands and therefore preventing religation (28, 117, 121, 123-125). The magnesium concentration is also a key to the quinolone interaction with DNA and the enzyme. Experimental data suggests absence of magnesium as well as excess of magnesium prevents norfloxacin from interacting with DNA, and that optimal binding occurs at a concentration of 1-2 mM (126). It is proposed that Mg$^{2+}$ acts as a bridge between the phosphates in the DNA backbone and the carbonyl and carboxyl moieties of norfloxacin (126).

![Structure of the Fluoroquinolone](image)

**Figure 3-1. Structure of the Fluoroquinolone.**

The molecule is shown with the hydrogen-bonding domain, hypothetical domain for the interaction of the drug with the type II enzyme and drug-drug self association domain indicated.
Topoisomerases are important enzymes that manage the topological state of DNA in the cell. They play important roles necessary for efficient replication, transcription, recombination and chromatin remodeling. Topoisomerases IB perform the relaxation reaction in three steps, first cleaving the backbone of the DNA, followed by covalent attachment of the topoisomerase to the backbone of DNA, disrupting a phosphodiester bond to form a phosphotyrosine bond. Energy is stored in the form of this phosphotyrosine bond. The other strand of the DNA now rotates relative to the covalently linked strand to release the tortional stress. This release could be in a controlled fashion as predicted for the human topoisomerase or a free rotation as predicted for the Vaccinia topoisomerase IB (39, 127). Third, the topoisomerase IB dissociates and the energy stored in the phosphotyrosine bond is used to restore the DNA backbone forming the phosphodiester bond.

Topoisomerases IB are usually found in eukaryotes, but a few eubacteria encode homologs of the enzyme. *Pseudomonas aeruginosa* is among the few eubacteria that encode a topoisomerase with sequence and biochemical characteristics similar to those of Vaccinia virus topoisomerase IB. *P. aeruginosa* is an opportunistic pathogen responsible for a large number of nosocomial infections (100), such as hospital acquired urinary tract infections (128), surgical wound infections (100), bloodstream infections (67), etc. *P. aeruginosa* is also responsible for many respiratory tract infections, including ventilator-associated pneumonia in intubated patients (129), immunocompromised patients, for example neutropenic cancer patients and bone marrow transplant patients, burn patients (71), cystic fibrosis patients and AIDS patients.

The Vaccinia virus is a live virus used in smallpox vaccines and a major bioterrorism agent. Vaccinia virus topoisomerase is a well studied type IB DNA topoisomerase with sequence specificity for transesterification at 5´-(C/T)CCTT↓ sites. The tyrosine 274 at the active site is
linked to the 3’ phosphate of the incised strand of DNA to form a protein DNA covalent complex (8). Fluoroquinolones have been shown to have activity against Vaccinia virus DNA topoisomerase IB (VT) while the human topoisomerase is resistant (21). It has been reported that the Vaccinia topoisomerase IB is sensitive to the fluoroquinolone drug enrofloxacin [1 mM] (21) and coumarin drugs novobiocin [≈0.5 mM] and coumermycin [0.2 mM] (23). Ofloxacin, another fluoroquinolone derivative, has been shown to inhibit Vaccinia virus and to inhibit 30% of the topoisomerase I purified from Vaccinia cores at ≈ 2 mM concentration (130). The mechanism by which fluoroquinolones inhibit the type IB topoisomerases is unknown. We show here that the *P. aeruginosa* encoded enzyme is inhibited by select fluoroquinolones. Assays with the Vaccinia-encoded homolog suggests that fluoroquinolones require Mg$^{2+}$ for inhibitory activity, and that they function by preventing covalent complex formation.

3.2 Results

A number of fluoroquinolones have been shown to have activity against topoisomerases. Fig. 3-1 shows the common backbone shared by all fluoroquinolones. For the Vaccinia topoisomerase (VT), several but not all fluoroquinolones were seen to inhibit relaxation, suggesting specific structural requirements for activity. The Vaccinia enzyme also exhibits unique pharmacological properties in that it is resistant to the topoisomerase poison camptothecin, but inhibited by coumarin drugs. We therefore explored first the range of compounds with potential activity against the *P. aeruginosa*-encoded homolog.

3.2.1 Resistance of *P. aeruginosa* Topoisomerase to Camptothecin

Camptothecin is a cytotoxic plant alkaloid from the plant *Camptotheca acuminata* found in China (131). When incubated with HeLa cells or adenovirus infected HeLa cells in the G1 or S phase of the cell cycle, it results in the inhibition of nucleic acid synthesis and fragmentation of
RNA chains (81, 132-135). It is hypothesized that camptothecin inhibits at the single stranded replication fork, and that the drug does not affect non-replicating cells (81). Camptothecin inhibits the human topoisomerase IB by capturing it in a covalent complex with DNA (133, 136-139). The human topoisomerase I is the sole target of camptothecin and its derivatives, which act as anti-cancer drugs stabilizing the enzyme-DNA complex (16, 140). Like the VT, P. aeruginosa topoisomerase (PAT) is effectively resistant to camptothecin at concentrations up to 2 mM (Fig. 3-2), perhaps reflecting a closer structural relationship between VT and PAT compared to the human enzyme.

3.2.2 Resistance of P. aeruginosa Topoisomerase to Coumermycin

Vaccinia topoisomerase IB is sensitive to the coumarin drugs novobiocin and coumermycin (23). Coumarin drugs are also DNA gyrase inhibitors, binding to the ATPase unit and inhibiting the activity of DNA gyrase by preventing supercoiling (42, 43). The VT is inhibited at 500 µM by novobiocin and at 100 µM by coumermycin A1 (23). PAT also shows modest inhibition by Coumermycin, at 500 µM. As coumermycin is thought to bind at a site on VT that coincides with or overlaps the DNA binding site (23), this result suggests that VT and PAT may only have moderately conserved DNA-binding interfaces (Fig. 3-3).

3.2.3 Interaction of P. aeruginosa with Fluoroquinolones

Fluoroquinolones have been reported to interact nonspecifically with relaxed DNA (119) and binding is also influenced by the topological state of the DNA in the cell (119). It has been reported that fluoroquinolones bind various targets, such as DNA gyrase, topoisomerase IV and DNA topoisomerase IB (21, 30, 34, 117, 119, 120, 141, 142). A number of fluoroquinolones, some of which inhibit the Vaccinia virus topoisomerase, were tested against the PAT.
Figure 3-2. PAT is not inhibited by Camptothecin.

In lane 1 is supercoiled DNA. Lane 2 shows relaxed DNA. The supercoiled DNA (75 ng) was relaxed with 100 ng PAT. Lane 3 contains supercoiled DNA incubated with camptothecin (2 mM). Lanes 4 through 13 contain supercoiled pUC18 incubated with PAT (100 ng) and increasing concentration of camptothecin 2.5 μM, 25 μM, 250 μM, 500 μM, 750 μM, 1000 μM, 1250 μM, 1500 μM, 1750 μM, 2000 μM respectively.

Figure 3-3. PAT shows modest sensitivity to Coumermycin.

In lane 1 is supercoiled DNA. Lane 2 shows relaxed DNA. The supercoiled DNA was relaxed with 100 ng PAT. Lane 3 contains supercoiled DNA incubated with camptothecin (2 mM). Lanes 4 through 9 contain supercoiled pUC18 (75 ng) incubated with PAT (100 ng) and increasing concentration of coumermycin 2.5 μM, 25 μM, 250 μM, 500 μM, 750 μM, 1000 μM.
Figure 3-4. Sensitivity of PAT to different Fluoroquinolones.

(A) Moxifloxacin: Lane 1 shows supercoiled DNA, lane 2 shows relaxed DNA. In this lane DNA was relaxed in the presence of 100 ng of PAT. Lane 3 contains supercoiled pUC18 and 2 mM moxifloxacin. Lanes 4 through 13 contain supercoiled pUC18 in the presence of 100 ng PAT and increasing concentration of moxifloxacin 2.5 µM, 25 µM, 250 µM, 500 µM, 750 µM, 1000 µM, 1250 µM, 1500 µM, 1750 µM and 2000 µM. (B) Ciprofloxacin: Lane 1 shows supercoiled DNA, lane 2 shows relaxed DNA. In this lane DNA was relaxed in the presence of 100 ng of PAT. Lane 3 contains supercoiled pUC18 and 2 mM ciprofloxacin. Lanes 4 through 13 contain supercoiled pUC18 in the presence of 100 ng PAT and increasing concentration of ciprofloxacin 2.5 µM, 25 µM, 250 µM, 500 µM, 750 µM, 1000 µM, 1250 µM, 1500 µM, 1750 µM and 2000 µM. (C) Norfloxacin: Lane 1 contains supercoiled DNA, lane 2 contains supercoiled DNA in the presence of 2 mM norfloxacin. Lane 3 contains relaxed DNA. In this lane, DNA was relaxed in the presence of 100 ng of PAT. Lanes 4 through 11 contain supercoiled pUC18 in the presence of 100 ng PAT and increasing concentration of norfloxacin 25 µM, 250 µM, 500 µM, 750 µM, 1000 µM, 1250 µM, 1500 µM and 2000 µM.
Figure 3-5.  Sensitivity of PAT to Enrofloxacin:
Lane 1 shows supercoiled DNA, lane 2 shows relaxed DNA. In this lane DNA was relaxed in the presence of 100 ng of PAT. Lanes 3 through 12 contain supercoiled pUC18 in the presence of 100 ng PAT and increasing concentration of enrofloxacin 2.5 µM, 25 µM, 250 µM, 500µM, 750 µM, 1000 µM, 1250 µM, 1500 µM,1750 µM and 2000 µM.

Figure 3-6.  Sensitivity of PAT to Sparfloxacin.
Lane 1 shows supercoiled DNA, lane 2 shows relaxed DNA. The DNA was relaxed in the presence of 100 ng of PAT. Lane 3 contains supercoiled pUC18 and 2 mM sparfloxacin. Lanes 4 through 13 contain supercoiled pUC18 in the presence of 100 ng PAT and increasing concentration of sparfloxacin 2.5 µM, 25 µM, 250 µM, 500 µM, 750 µM, 1000 µM, 1250 µM, 1500 µM,1750 µM and 2000 µM.
Moxifloxacin does not inhibit either the VT or PAT (Fig. 3-4A) while norfloxacin inhibits both VT and PAT (Fig. 3-4C) Ciprofloxacin inhibits PAT but not VT (Fig. 3-4B) and ciprofloxacin in contrast inhibits VT but not PAT (Fig. 3-4B) (21). Norfloxacin inhibits VT at 62.5 µM and PAT at 500 µM. Since enrofloxacin was reported to inhibit relaxation by VT completely at 1 mM (21), we investigated whether PAT would likewise be sensitive to inhibition by enrofloxacin. The DNA relaxation activity of PAT when assayed by incubation of the PAT with supercoiled DNA and increasing amounts of enrofloxacin. Enrofloxacin resulted in inhibition of PAT at 1 mM (Fig. 3-5). Sparfloxacin is a potent antibacterial agent that also belongs to the quinolone family, showing inhibitory activity against DNA gyrase and type IV topoisomerases from *Escherichia coli* (28, 114, 143-145). Reports have shown that sparfloxacin also shows activity against DNA gyrase from *Streptococcus pneumoniae* (34). It inhibits the PAT at 1 mM (Fig. 3-6). That both VT and PAT are inhibited only by one set of fluoroquinolones suggests a structure specific interaction with the enzyme.

### 3.2.4 Fluoroquinolones Prevent Cleavage Complex Formation by Vaccinia Topoisomerase

Fluoroquinolones have been reported to bind DNA in a magnesium ion-dependent manner with binding being inhibited by both lack and excess of magnesium ions (126). The optimal concentration of Mg$^{2+}$ for drug binding is at 1-2 mM (126). To investigate the role of Mg$^{2+}$ in fluoroquinolone mediated inhibition of DNA relaxation, we used Vaccinia topoisomerase IB as this enzyme does not require Mg$^{2+}$ for activity, while PAT does. In this assay, we performed DNA relaxation by VT in the presence of the antibiotic norfloxacin with and without Mg$^{2+}$ in the topoisomerase reaction buffer. Our data shows that the fluoroquinolone-
dependent inhibition of DNA relaxation by Vaccinia topoisomerase IB occurs only in the presence of MgCl₂ (Fig. 3-7).

### 3.2.5 Mode of Inhibition of VT

The mechanism of inhibition of VT by enrofloxacin was investigated to determine the step at which Vaccinia topoisomerase is inhibited. DNA relaxation assays conducted in the presence and absence of enrofloxacin in the topoisomerase buffer with Mg²⁺ confirmed that the antibiotic inhibited covalent complex formation between the DNA and VT. Suicide substrates were used for these DNA binding reactions (Fig. 3-8A). These suicide substrates are short duplex DNAs containing a single CCCTT↓ cleavage site in the top strand; when the VT binds, it releases a 6 nt DNA, leaving the VT in a trapped covalent complex with DNA. Such suicide substrates have been used extensively in the past to analyze cleavage and religation kinetics (38, 39, 50, 146). To monitor cleavage complex formation for VT in the presence and absence of the antibiotic, VT was incubated with the suicide substrate and reactions stopped by adding equal volumes of 10% SDS. SDS causes the disruption of all non-covalent interactions between the DNA and protein allowing detection of covalent complexes only. These reactions were run on a 10% SDS page. As shown in Fig. 3-8C, enrofloxacin prevents cleavage complex formation by Vaccinia virus topoisomerase IB while covalent complex accumulates in the absence of the drug (Fig. 3-8B) (26-28, 30-32, 117, 126, 147, 148).

### 3.3 Discussion

The fluoroquinolones are widely in clinical use, and inappropriate use of these agents leads to antibiotic resistance. It is therefore important to understand their interaction with their various targets, inducing DNA gyrase, DNA topoisomerase IV, and DNA topoisomerase IB. The
Figure 3-7. Fluoroquinolones Require MgCl₂ for Inhibition of Vaccinia Topoisomerase IB.

Lane 1 contains supercoiled pUC18 DNA in topoisomerase reaction buffer with 2.5 mM MgCl₂ and 100 mM NaCl, lane 2 contains relaxed DNA without antibiotic, lanes 3-8 contain pUC18 and 6 ng VT in topoisomerase buffer with 2.5 mM MgCl₂ and 100 mM NaCl and increasing concentration of norfloxacin 5, 10, 50, 100, 500, 1000 µM, respectively; lane 9 contains relaxed DNA without antibiotic, lanes 10-15 contain pUC18 and 6 ng VT in topoisomerase buffer without MgCl₂ and containing 100 mM NaCl and increasing concentration of norfloxacin 5, 10, 50, 100, 500, 1000 µM, respectively (Fig. 3-8).
structures of some fluoroquinolones are given in Table 3-1. All these fluoroquinolones have the same DNA backbone except for the side chain at position R7 (Fig. 3-1). The PAT is resistant to many fluoroquinolones, such as moxifloxacin and ciprofloxacin, but it is sensitive to enrofloxacin, sparfloxacin and norfloxacin at 1000 µM. This suggests that there are some specific interactions occurring at the active site. Further experimental data also suggests that sparfloxacin does not inhibit PAT binding to DNA, but it has yet to be determined at what step the PAT is inhibited. However, the cleavage complex for PAT has yet to be captured, hampering a detailed mechanistic analysis. Based on structural similarity to VT, a reasonable prediction is that it may be inhibited by a mechanism similar to that seen for VT (Fig. 3-8). PAT is able to
bind DNA in the presence of the antibiotic, indicating interference with downstream steps for both enzymes. Like VT, PAT is inhibited by coumarin drugs and resistant to camptothecin, indicating that VT and PAT share conserved sites of interaction by coumarin drugs, although the higher concentration of drug required for inhibition of PAT points to dissimilarities in the respective binding pockets. In addition, although the fluoroquinolones target the DNA-enzyme cleavage complex in the case of topoisomerase IV and DNA gyrase, the fluoroquinolones prevent the formation of the cleavable complex for VT. This mode of inhibition has not been seen before. The interaction of norfloxacin with DNA has been investigated; some data suggest that Mg$^{2+}$ is required as a bridge between the negatively charged DNA backbone and norfloxacin, and it has also been reported that norfloxacin does bind DNA in absence of Mg$^{2+}$(126, 147, 148). Our data indicates that the ability of norfloxacin to inhibit DNA relaxation by Vaccinia topoisomerase depends on Mg$^{2+}$. Since the inhibitory activity depends on the structure of the fluoroquinolone, we find it less likely that DNA-binding by the drug results in the observed inhibition, but favor the interpretation that specific interactions between fluoroquinolone, Mg$^{2+}$ and enzyme must be involved. Consistent with this interpretation, PAT and VT do not exhibit the same sensitivity to the fluoroquinolones tested.

Vaccinia topoisomerase removes an average of five superhelical turns per cleavage and religation event in a process proposed to be a free rotation of the non-covalently held DNA (146). In contrast, the human enzyme has extensive ionic contacts between DNA and protein and is proposed to release superhelical tension in a “controlled rotation” event (16). Comparison of the sequence of PAT with sequence and structures of Vaccinia topoisomerase in complex with DNA reveal lack of conservation of several residues seen to contact DNA in the poxviral enzymes. It is therefore possible that PAT releases superhelical turns in an unhindered rotation
Table 3-1 Some drugs used against topoisomerases.

<table>
<thead>
<tr>
<th>Parent compound of the Fluoroquinolone:</th>
<th>Fluoroquinolone:</th>
<th>Fluoroquinolone:</th>
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<tbody>
<tr>
<td>Fluoroquinolone: Nalidixic acid</td>
<td>Moxifloxacin</td>
<td>Lomefloxacin</td>
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<tr>
<td>Fluoroquinolone:</td>
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<td>Fluoroquinolone:</td>
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<td>Fluoroquinolone: Sparfloxacin</td>
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<tr>
<td>Fluoroquinolone:</td>
<td></td>
<td>Coumarin drug:</td>
</tr>
<tr>
<td>Fluoroquinolone:</td>
<td></td>
<td>Coumermycin</td>
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<tr>
<td>Fluoroquinolone:</td>
<td></td>
<td></td>
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<tr>
<td>Fluoroquinolone: Enrofloxacin</td>
<td></td>
<td>The sole target of the human topoisomerase: Camptothecin</td>
</tr>
</tbody>
</table>

68
event that leads to complete relaxation upon DNA cleavage, as reflected in the formation of a population of topoisomers on inhibition of VT, while inhibition of PAT largely leads to retention of completely supercoiled DNA. This could also imply that PAT might explore a mechanism of relaxation different from Vaccinia and the human DNA topoisomerases.

*Bacillus anthracis* spores, small pox, cholera, plague, tuberculosis, and tularemia are a few weapons of biological warfare. Fluoroquinolones have an important role in post exposure prophylaxis and chemotherapy but there is always a danger of developing resistance against these fluoroquinolones. In addition there is evidence which proves that knocking out the topoisomerase gene in Vaccinia virus reduces transcription and therefore the infectivity of the virus can be controlled (149). *P. aeruginosa* is a gram negative aerobic rod responsible for a number of nosocomial infections and a potent human pathogen (57). By studying the mechanism of action of drug inhibition of VT and PAT, these enzymes could be explored as targets for drugs.

### 3.4 Materials and Methods

#### 3.4.1 Oligonucleotide Labeling

Oligonucleotides were purchased from Operon. The top strand oligonucleotides were labeled at the 5′ end with T4 polynucleotide kinase (New England Biolabs) and [γ-32P] ATP and annealed to the bottom strand. The sequence of the top strand is 5′-CGTGTCGCCCTTATTCCG-3′ and the bottom strand is 5′-CACTATCGGAATAAGGGCGACACG-3′.

#### 3.4.2 DNA Relaxation Assay

PAT was purified and characterized as described in chapter 2. For protein purification the phosphocellulose column was used and various DNA binding assays were performed for the
characterization. The VT was purchased from Epicentre Technologies. Topoisomerase inhibitors were purchased from Sigma. Relaxation assays (per 20 µl) were performed with negatively supercoiled pUC18 DNA (≈75 ng) unless otherwise stated, and the indicated amount of PAT or VT in topoisomerase buffer (50 mM Tris (pH 8.0), 2.5 mM MgCl₂, 0.1 mM EDTA) containing the indicated antibiotic at the mentioned concentration for two hours at 37°C. For PAT, the reaction also contained a final concentration of 0.24 M NaCl and ≈19% glycerol from the protein storage buffer. Reactions with VT contained 0.1 M NaCl. Reactions were stopped with 5 µl (1(10% SDS):1(30% glycerol)) stop buffer. The reactions were loaded on a 1% agarose gel in 0.5X TBE buffer (45 mM Tris-borate (pH 8.3), 1 mM EDTA). The gels were run at 2 V/cm for 16 hours. The gels were stained with ethidium bromide (EtBr) and visualized using an Alpha Innotech digital imaging system.

### 3.4.2.1 Vaccinia virus Topoisomerase Suicide Substrate Cleavage Assay

The reaction mixture (50 µl) contained topoisomerase reaction buffer (10 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 2.5 mM MgCl₂, 100 mM NaCl) containing 0.5 pmole DNA, with or without enrofloxacin at 1 mM. The reaction was initiated with the addition of 50 units of the VT (≈36 ng). Time points were taken at 0.5, 1, 5, 7, 10 minutes. For each time point, 10 µl of the reaction mixture was quenched by addition of 10 µl of 10% SDS. In addition 10 µl of Laemmli loading buffer was added. The samples were then run on a 10% SDS PAGE gel in Laemmli buffer. Covalent complexes were visualized by phosphoimaging.
4 Summary and Conclusions

DNA topoisomerases are enzymes that manage the topology of DNA in the cell and therefore perform vital functions in nearly all cellular processes, such as replication, transcription, recombination, chromosome segregation, chromosome condensation and decondensation, chromatin organization, genome stability, repair, transcriptional regulation, cell cycle control, post transcriptional modifications and chromatin remodeling (7). DNA supercoiling also has effects on DNA structure and its interactions with other molecules (7). The DNA topoisomerases are probably the most important enzymes that solve all the major problems that arise due to the DNA being a double helix and the need for the strands to separate, for example, in order for each strand to act as a template for the synthesis of the complementary strand.

The topoisomerases are a hot topic in recent years not because of their critical role in managing the topology of the DNA in the cell but because, firstly, it has been discovered that a large number of topoisomerases can act as drug targets; for example, the drug camptothecin and its derivatives act as inhibitors of the human topoisomerase IB by capturing the enzyme in a covalent complex with DNA (16). These drugs include antibacterial agents, anticancer chemotherapeutics and bacteriostatic agents. Secondly, the crystal structures of a large number of topoisomerases are available, such as the human topoisomerase and the vaccinia topoisomerase IB and these provide valuable insight into the mechanism of the molecular machine and complement the extensive biochemical literature already available (8, 16). The vaccinia virus topoisomerase is ≈32 KDa and the human topoisomerase is ≈100 KDa.

The vaccinia topoisomerase is one third the size of the human topoisomerase yet is maintaining the integrity of the enzyme by conserving important residues and sharing the
catalytic mechanism (8, 13, 14, 19). The PAT shares sequence similarity with the vaccinia topoisomerase, forming a covalent protein DNA complex where the active site tyrosine attacks a phosphodiester bond on one DNA strand. Residues in the active site include the conserved Tyrosine 292 forming the covalent bond to DNA. *Pseudomonas aeruginosa* is the most potent opportunistic pathogen and the topoisomerase from this organism could act as a target for drugs. Fluoroquinolones are a potent class of antibacterial and anticancer agents in use against DNA gyrases and DNA topoisomerase IV, and as recently discovered, they inhibit vaccinia topoisomerase IB.

It is shown here that the PAT is sensitive to sparfloxacin, enrofloxacin, and norfloxacin at high concentrations. In this dissertation I describe DNA relaxation by PAT, effect of salt on relaxation activity, DNA binding and sequence preference by PAT, inhibition of PAT by certain fluoroquinolones, resistance of PAT to camptothecin and the inability of VT to form the cleavage complex in the presence of enrofloxacin.

4.1 DNA Relaxation by PAT

PAT was cloned from *P. aeruginosa* genomic DNA, overexpressed in *E. coli* and purified. It was found to be active, but to exhibit features distinct from those of the poxviral enzymes. The DNA relaxation activity of PAT was assayed by incubation with supercoiled pUC18 in topoisomerase reaction buffer with Mg$^{2+}$. The PAT (9 ng) relaxes 110 ng of supercoiled pUC18 in 0.5 minutes and the PATCAT (808.2 ng) relaxes 110 ng of supercoiled pUC18 in 0.5 minutes. The VT relaxes 300 ng of supercoiled pUC18 in 0.25 min in the presence of Mg$^{2+}$ and in 2 minutes in the absence of Mg$^{2+}$. The *Deinococcus radiodurans* topoisomerase (300 ng) is also not as efficient as the Vaccinia homolog, relaxing 350 ng of the supercoiled pUC18 DNA in 5 minutes (15, 17). Evidently, the bacterial enzymes are not as efficient as the
vaccinia-encoded homolog, perhaps because an optimal recognition sequence is not present, resulting in a suboptimal disposition of catalytic residues.

While the larger human topoisomerase IB contacts DNA through numerous non-covalent interactions and is thought to relax DNA by a “controlled rotation” of non-covalently held DNA, VT relaxes an average of five superhelical turns per cleavage and religation event by a mechanism proposed to involve free rotation of DNA downstream of the cleavage site. Since PAT appears to relax DNA, essentially to completion, it is conceivable that it further limits contact to downstream DNA, resulting in DNA relaxation by a completely “uncontrolled rotation” mechanism.

4.2 Effect of Salt on Relaxation Activity of PAT

The experimental results showed that the PAT does not require divalent cation for DNA binding, but it does require divalent cation for relaxation activity, with 2.5 mM concentration optimal for the relaxation to go to completion. The vaccinia virus and the *D. radiodurans* topoisomerase IB do not require a divalent cation for relaxation activity (13, 17). The mechanistic basis for this Mg$^{2+}$-dependence remains to be determined.

4.3 DNA Binding by PAT

PAT binds the 37 bp CCCTT-containing sequence as opposed to little or no distinct binding to a 37 bp perfect duplex without this pentameric sequence. PAT also binds 37 bp looped DNA. The vaccinia topoisomerase IB binds 18/24 duplex DNA with stringent specificity for transesterification at 5′-(C/T)CCCT↓ sites (40, 50-53) whereas this DNA is not a substrate for PAT. The *D. radiodurans* topoisomerase IB does not show specificity for cleavage at the pentameric sequence as opposed to VT (17). It is possible that the bacterial enzymes only exhibit
a modest preference for the sequence strictly recognized by the vaccinia homolog due to lack of conservation of residues seen to contact DNA.

4.4 Inhibition of the PAT by Drugs

Sparfloxacin is an antibacterial agent belonging to the family of fluoroquinolones inhibiting DNA gyrase and DNA topoisomerase IV (28, 34, 114, 142-145). The PAT is inhibited by sparfloxacin at 1500 µM, by enrofloxacin at 1000 µM and by norfloxacin at 500 µM. In contrast, other fluoroquinolones are unable to inhibit activity, including for example ciprofloxacin which inhibits the vaccinia enzyme (22). The fact that both VT and PAT are inhibited by certain fluoroquinolones suggests that a structure specific interaction occurs between the enzyme, the drug and the DNA.

The human topoisomerase is inhibited by camptothecin at 100 µM (16, 150). Camptothecin stabilizes the covalent human topo I intermediate (16). The vaccinia virus topoisomerase IB is resistant to camptothecin and so is the PAT at concentrations up to 2000 µM (13). The fact that the PAT is resistant to camptothecin like the vaccinia topoisomerase and unlike the human topoisomerase, it not only suggests that the PAT is structurally closer to VT as compared with human topoisomerase but also that PAT could form the evolutionary link between the vaccinia and the human.

VT is inhibited by the coumermycin at 100 µM, but PAT only shows a modest inhibition even at 2000 µM. Coumermycin is thought to bind at a site on VT that coincides with the DNA binding site. The fact that PAT only shows a modest inhibition implies that the VT and PAT do not share similar DNA-binding interfaces. This is true, on comparison of the residues at the DNA-binding site.
4.5 Inability of the Vaccinia Virus Topoisomerase to Form the Cleavage Complex in the Presence of Enrofloxacin

The mechanism of enrofloxacin inhibition of VT was investigated using the suicide substrate and the VT in presence of 1 mM Enrofloxacin concentration, and it was determined that the enrofloxacin prevents cleavage complex formation. The cleavage complex of the PAT has yet not been captured and it is hypothesized that the mode of action of the fluoroquinolones would be the same in the PAT and VT. These data suggest that fluoroquinolones may be structurally optimized to target type IB topoisomerases.
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Vita

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