DNA gyrase: Mechanism of supercoiling and interaction with quinolones

by

Sotirios C. Kampranis

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Department of Biochemistry
University of Leicester

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To my mother, Eleni, and the memory of
my father, Charalampos
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Abbreviations

ADPNP  5'-adenylyl-β,γ-imidodiphosphate
CFX  ciprofloxacin
DTT  dithiothreitol
EtBr  ethidium bromide
GyrA  gyrase A protein
GyrA59  the 59-kDa N-terminal domain of the A protein
GyrA64  the 64-kDa N-terminal domain of the A protein
GyrB  gyrase B protein
k-DNA  kinetoplast DNA
Lk  DNA linking number
ΔLk  linking number difference
OXO  oxolinic acid
PAGE  polyacrylamide gel electrophoresis
SDS  sodium dodecyl sulphate
σ  superhelical density
topo  topoisomerase
ts  temperature sensitive
Abstract

DNA gyrase: Mechanism of supercoiling and interaction with quinolones

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DNA gyrase is unique among topoisomerases in its ability to introduce negative supercoils into closed-circular DNA. Deletion of the C-terminal DNA-binding domain of the A subunit of gyrase gives rise to an enzyme that behaves like a conventional type II topoisomerase, suggesting that the unique properties of DNA gyrase are attributable to the wrapping of DNA around the C-terminal DNA-binding domains of the A subunits. However, these results do not unveil the detailed mechanism by which the transported DNA segment is captured and directed through the DNA gate. This mechanism was addressed by probing the topology of the bound DNA segment at distinct steps of the catalytic cycle. A model is proposed in which gyrase captures a contiguous DNA segment with high probability, irrespective of the superhelical density of the DNA, while the efficiency of strand passage depends on the superhelical free-energy. This mechanism is concerted, in that capture of the transported segment induces opening of the DNA gate, which in turn, stimulates ATP hydrolysis.

Mutation of Glu42 to Ala in the B subunit of DNA gyrase abolishes ATP hydrolysis but not nucleotide binding. Gyrase complexes that contain one wild-type and one Ala42 mutant B protein were formed and the ability of such complexes to hydrolyse ATP was investigated. It was found that ATP hydrolysis was able to proceed only in the wild-type subunit, albeit at a lower rate. With only one ATP molecule hydrolysed at a time, gyrase could still perform supercoiling but the limit of this reaction was lower than that observed when both subunits can hydrolyse the nucleotide.

Limited proteolysis was used to identify conformational changes in DNA gyrase and the proteolytic signatures observed were interpreted in terms of four complexes of gyrase, each representing a particular conformational state. Quinolone binding to the gyrase-DNA complex induces a conformational change that results in the blocking of supercoiling. Under these conditions gyrase is still capable of ATP hydrolysis. The kinetics of this reaction have been studied and found to differ from those of the reaction of the drug-free enzyme. By observing the conversion of the ATPase rate to the quinolone-characteristic rate, the formation and dissociation of the gyrase-DNA-quinolone complex can be monitored. Comparison of the time dependence of the conversion of the gyrase ATPase with that of DNA cleavage reveals that formation of the gyrase-DNA-quinolone complex does not correspond to the formation of cleaved DNA. Quinolone binding and drug-induced DNA cleavage are separate processes constituting two sequential steps in the mechanism of action of quinolones on DNA gyrase.
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1 Introduction

1.1. The requirement for DNA topoisomerases

"Since the two chains in our model are intertwined, it is essential for them to untwist if they are to separate. .... Although it is difficult at the moment to see how these processes occur without everything getting tangled, we do not feel that this objection would be insuperable."

-J. D. Watson and F. H. C. Crick, 1953

From the moment of the discovery of the double-helical structure of DNA, it became apparent that any interaction requiring the unravelling of the two intertwined strands would create a topological problem. Initially, it was thought that such problems could be overcome by rotation of the double-stranded portion of the molecule around its axis. However, this idea was soon rendered unfavourable as it was discovered that an entire chromosome consisted of one DNA molecule containing millions of base pairs, and was organised in a compact form with multiple loops. As information accumulated for a number of intracellular processes involving DNA, the topological problems posed by the structure of the DNA became more specific. To give an insight into such problems the processes of transcription and replication will be discussed.

DNA transcription. As RNA polymerase tracks along the DNA template, transcribing a particular gene, it must follow the helical path of the DNA, a movement that would result in the rotation of the transcription complex around the DNA axis. The conventional view of this interaction was that the DNA was stationary and the transcribing polymerase was rotating
around the DNA axis. However, the transcription complex comprises the RNA polymerase, the nascent mRNA, and, in the case of prokaryotic cells, ribosomes translating the mRNA in a coupled manner. It has been suggested (Liu & Wang, 1987) that such a complex would be restricted from rotating around the DNA axis since, because of its size it would be subjected to significant frictional drag. Alternatively, it has been proposed that the transcription complex is anchored to an intracellular matrix (Cook, 1989). In either case, the DNA would have to be translocated past the stationary polymerase, rotating about its own axis as it does so. However, both in prokaryotes and eukaryotes, the DNA is organised into discrete topological domains as a result of its attachment to cellular structures such as membranes in prokaryotes or the nuclear matrix in eukaryotes. The existence of such points of anchorage would prevent the free rotation of the DNA, resulting in the overwinding of the DNA (positive supercoiling) ahead of the transcribing polymerase and the unwinding of the DNA (negative supercoiling) behind it (Fig. 1.1a-c).

Figure 1.1. The 'twin supercoiled domain' model for transcription. a-c, the transcription complex consisting of RNA polymerase, mRNA and the attached ribosomes is shown transcribing a topologically constrained domain of DNA. This process leads to the formation of positive supercoils ahead of the polymerase and negative supercoils behind it. d, a similar situation is shown in the case of a plasmid with two transcription complexes moving in opposite directions.
In the case of transcription on a circular plasmid, the positive and negative supercoiling would cancel out by diffusing around the circle. However, transcription of two genes on the same plasmid proceeding in opposite directions would generate a similar effect (Fig. 1.1d). Accumulation of positive supercoiling ahead of the polymerase would eventually block transcription, and survival of the cell requires the existence of a mechanism that would remove the supercoils generated during this process.

**DNA replication.** The elongation stage in DNA replication can be considered as a special case of the situation in transcription. Progress of the replication fork generates positive supercoils ahead of the replication machinery (Fig. 1.2a). Behind the fork the separated parental strands can be thought of as an extreme case of negative supercoiling (separation of the two strands reduces their linking number to zero) (Wang, 1996). At this stage, the requirement for the removal of the positive supercoils seems obvious since, otherwise, these would soon inhibit the progress of the replication machinery.

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**Figure 1.2. Topological problems encountered during DNA replication.**

- **a.** At the process of elongation, positive supercoiling builds ahead of the replication complex.
- **b-c.** At the termination of replication, if untwining of the daughter molecules has not finished before the completion of elongation, subsequent ligation would produce catenated progeny.
At the termination of replication, when two replication forks converge at the end of DNA synthesis, the unravelling of the parental DNA strands may not have been completed before the progeny strand synthesis has finished (Fig. 1.2b). In this case, after ligation of the newly formed strands, the residual intertwists between the parental strands would be converted to intertwines (catenanes) between the newly replicated progeny molecules (Fig. 1.2c). Clearly decatenation is required before cell division can successfully occur.

The necessity of disentangling DNA strands or duplexes also arises in most of the other cellular manipulations of DNA, including chromosome condensation and decondensation, and recombination (for a review, see (Wang, 1996)).

1.2. DNA topoisomerases

A family of enzymes has evolved to solve the topological problems of DNA. These are the DNA topoisomerases, a group of enzymes which are able to disentangle DNA strands and duplexes by passing the one through the other as if there were no physical boundaries. There are two types of topoisomerases which are classified according their ability to cleave one or both DNA strands. Type I topoisomerases break only one strand of the DNA and in so doing change the linking number of a DNA molecule in steps of one. Type II enzymes transiently break a pair of complementary strands and pass a double-stranded segment through the break, therefore altering the linking number of the DNA in steps of two.

The first topoisomerase activity reported was that of the \( \alpha \) protein of \( E. coli \), now known as topoisomerase I, which was found to relax supercoiled DNA (Wang, 1971). A eukaryotic type I topoisomerase was found the following year in mouse cell extracts (Champoux & Dulbecco, 1972). DNA gyrase was discovered in \( E. coli \) by Gellert and co-workers (Gellert et al., 1976) in an attempt to identify host factors required for the site-specific integration of bacteriophage \( \lambda \). Since then topoisomerases have been identified in every cell type studied so far. Many cells have more than one type of topoisomerase and where a genetic test is possible it has been shown that at least one is essential for cell growth (reviewed in (Wang, 1996)). The range of reactions found to be catalysed by the DNA topoisomerases \textit{in vitro} include: (1) the relaxation of both negatively and positively supercoiled DNA; (2) the introduction of negative and positive supercoils into DNA; (3) the catenation and decatenation of DNA circles and (4) the knotting and unknotting of DNA. Not all topoisomerases can carry out the full range of reactions listed above. For example, \( E. coli \) topoisomerase I (a type I enzyme) can only relax negatively supercoiled DNA, while the eukaryotic type I enzyme can relax both positively and negatively supercoiled DNA. DNA gyrase is the only type II topoisomerase able to introduce negative supercoiling into DNA using the free energy of ATP hydrolysis. In prokaryotes another type II topoisomerase exists, topoisomerase IV. This enzyme shares a high sequence similarity with DNA gyrase but is unable to perform supercoiling; rather it is more efficient in DNA relaxation and decatenation. Some characteristic members of the family together with their properties are shown in Table 1.1.
Table 1.1. Some characteristic members of the topoisomerase family and their properties.

1.3. The role of DNA topoisomerases

It is apparent that the different topoisomerases have different roles in the cell since mutations in each of them result in different phenotypes. Evolution has produced a whole range of topoisomerases, each one specialising in a different aspect of DNA manipulation. A number of genetic and biochemical studies has dealt with the particular role of each topoisomerase in vivo. Let's consider again the two examples given in paragraph 1.1 to see how topoisomerases in different organisms have evolved to deal with these topological problems.

In bacteria containing a plasmid with two genes in opposite directions (such as the tet and bla genes of pBR322), inactivation of topoisomerase I results in the isolation of plasmid DNA with high levels of negative supercoiling (Pruss & Drlica, 1986). Moreover, inhibition of DNA gyrase resulted in the isolation of positively supercoiled plasmid (Lockshon & Moris, 1983). In prokaryotes, topoisomerase I can only relax negatively supercoiled DNA while gyrase can only relax positively supercoiled DNA (Table 1.1). These results suggest that the activities of these two topoisomerases are directed to the oppositely supercoiled domains generated by transcription. In a plasmid containing two genes in opposite directions...
the transcriptional supercoiled domains will reinforce each other. Inhibition of gyrase should abolish the relaxation of positively supercoiled domains and therefore lead to positively supercoiled plasmid while inhibition of topoisomerase I should lead to enhanced negative supercoiling of the plasmid.

As it was described previously (paragraph 1.1) during the elongation step of replication, positive supercoiling is being built ahead of the replication fork. In bacteria, DNA gyrase is probably the major activity responsible for the removal of these supercoils since bacterial topoisomerase I can only relax negative supercoils. DNA synthesis in E. coli gyrase temperature-sensitive mutants was significantly reduced immediately after the cells were subjected to the non-permissive temperature (Filutowicz & Jonczyk, 1983, Kreuzer & Cozzarelli, 1979). In yeast, inactivation of DNA topoisomerase I or II alone could not inhibit DNA replication although inactivation of topoisomerase I resulted in a temporary delay in the extension of the nascent DNA chains (Kim & Wang, 1989). However, synthesis was blocked by inactivation of both topoisomerases I and II. These results suggested that in yeast, topoisomerase I probably serves as the major relaxation activity during replication elongation, but its role can be substituted by DNA topoisomerase II.

At the end of replication, the daughter chromosomes must be segregated. For this a type II topoisomerase is required to create a double-stranded break in order to unlink the two catenated molecules. In prokaryotes, although DNA gyrase can decatenate replication products, it has been shown that topoisomerase IV is the potent decatenase (Peng & Marians, 1993). In yeast and Drosophila, only one type II DNA topoisomerase is known. Using yeast temperature-sensitive topoisomerase II mutants, catenated pairs of plasmid DNA are formed at the non-permissive temperature (DiNardo et al., 1984). Moreover, when chromosomal pairs are separated during meiosis, inactivation of topoisomerase II results in a lethal phenotype (the in vivo role of yeast topoisomerase II is reviewed in (Wang, 1996)).

1.4. Scope of this thesis

This thesis will focus on a unique member of this family of enzymes, DNA gyrase. Gyrase not only is spectacular in its mechanism but also is an important drug target. From the enzymological standpoint, the mechanism by which these enzymes transport one DNA segment through another has always been an intriguing issue. More so gyrase as it is unique in its catalytic abilities. DNA gyrase acts as a molecular motor, transforming the free-energy of ATP hydrolysis to superhelical free energy. Also, gyrase is the target of a widespread group of antibacterial agents, the quinolones. A broad spectrum of activity and good pharmacokinetic properties make quinolones a frequent choice in clinical practice (Hooper & Wolfson, 1985, Wolfson & Hooper, 1985). Moreover, a number of structurally related compounds, which are active against eukaryotic cells, are used as potent anti-cancer agents. The purpose of this work was to improve our understanding of the mechanism and the energetics of supercoiling by gyrase and to combine this knowledge to the study of the inhibition of the enzyme by the quinolones.
1.5. References


Conversion of DNA gyrase into a conventional type II topoisomerase

Abstract
DNA gyrase is unique among topoisomerases in its ability to introduce negative supercoils into closed-circular DNA. We have demonstrated that deletion of the C-terminal DNA-binding domain of the A subunit of gyrase gives rise to an enzyme that cannot supercoil DNA but relaxes DNA in an ATP-dependent manner. Novobiocin, a competitive inhibitor of ATP binding by gyrase, inhibits this reaction. The truncated enzyme, unlike gyrase, does not introduce a right-handed wrap when bound to DNA and stabilises DNA crossovers; characteristics reminiscent of conventional type II topoisomerases. This new enzyme form can decatenate DNA circles with increased efficiency compared to intact gyrase and, as a result, can complement the temperature-sensitive phenotype of a parC<sup>*</sup> mutant. Taken together these results suggest that the unique properties of DNA gyrase are attributable to the wrapping of DNA around the C-terminal DNA-binding domains of the A subunits, and provide an insight into the mechanism of type II topoisomerases.

2.1. Introduction
DNA topoisomerases are a ubiquitous class of enzymes responsible for the alteration of the topological state of DNA (Wang, 1996). As they are essential to all cells, topoisomerases are potential targets of antibacterial and antineoplastic drugs (Froelich-Ammon & Osheroff, 1995, Liu, 1989). The topoisomerisation reaction is achieved by the cleavage of DNA and the formation of a transient DNA gate through which a part of the same or another DNA molecule is passed. Mechanistic differences in this reaction distinguish topoisomerases into two types. Type I enzymes introduce a single-stranded break in DNA and facilitate the passage of a second strand through this gate. Type II topoisomerases create a double-stranded break and allow the passage of a double-stranded DNA segment. Covalent linkage between type II enzymes and DNA occurs by a transesterification reaction between
two tyrosine residues and a pair of phosphates four base-pairs apart in the DNA (Sugino et al., 1980, Tse et al., 1980). This results in the attachment of the 5'-ends of each cleaved DNA strand to the enzyme and the formation of a double-stranded DNA gate. Another DNA segment is then passed through this gate and the break is resealed. Turnover of type II enzymes in topoisomerisation reactions generally requires the hydrolysis of ATP.

Type II topoisomerases are able to catalyse a number of reactions, including the relaxation of negative and positive supercoils, the catenation and decatenation of DNA circles, and the knotting and unknotting of DNA (Hsieh, 1990, Reece & Maxwell, 1991b). DNA gyrase is the only topoisomerase able to actively introduce negative supercoils into DNA molecules, in a reaction dependent upon ATP hydrolysis (Gellert et al., 1976). In the absence of ATP, gyrase can relax supercoiled DNA (Gellert et al., 1977, Sugino et al., 1977). Although gyrase can decatenate DNA (Liu et al., 1980), this reaction is not as efficient as with other type II enzymes (Hiasa et al., 1994, Hiasa & Marians, 1994, Peng & Marians, 1993a). In the presence of quinolone drugs, gyrase can introduce double-stranded breaks in DNA after treatment with SDS and protease (Gellert et al., 1977, Sugino et al., 1977). In addition to DNA gyrase, a second type II enzyme, topoisomerase IV (topo IV), exists in prokaryotes. Topo IV is unable to supercoil DNA but, unlike gyrase, can, through its decatenating activity, support the final stages of replication during in vitro oriC DNA replication (Hiasa & Marians, 1994); in vivo, topo IV mutants are deficient in chromosomal partitioning (Adams et al., 1992). Topo IV is also targeted by quinolones (Khodursky et al., 1995, Peng & Marians, 1993b). Eukaryotic cells contain at least one type II enzyme; topo II in lower eukaryotes (e.g. S. cerevisiae and D. melanogaster) and the two isoforms topo IIα and topo IIβ in human cells (Watt & Hickson, 1994). All type II enzymes share a degree of sequence similarity, but tend to differ at their C-termini.

DNA gyrase is composed of two subunits, GyrA, (97 kDa) and GyrB (90 kDa); the active form being an A₂B₂ heterotetramer (Reece & Maxwell, 1991b). The holoenzyme, with a total mass of 374 kDa, binds ~130 bp of DNA (Orphanides & Maxwell, 1994) in a positive-superhelical sense around its core (Liu & Wang, 1978). Topo IV is a heterotetramer like gyrase, with ParC and ParE being the equivalents of GyrA and GyrB (Kato et al., 1992). Eukaryotic topo IIs are homodimers where the monomer is equivalent to a fusion of the A and B subunits of gyrase such that the B protein corresponds approximately to the N-terminus of the eukaryotic proteins while the A subunit is equivalent to the C-terminus (Lynn et al., 1986). The latter enzymes bind DNA without wrapping it and protect only 25-35 bp from nuclease digestion (Lee et al., 1989, Peng & Marians, 1995).

When GyrA is treated with either trypsin or chymotrypsin, two large fragments are generated with approximate masses of 64 and 33 kDa (Reece & Maxwell, 1989). The 64-kDa fragment comprises the N-terminal 571 amino acids of the intact protein and contains both the active site tyrosine (122) and all the sites in GyrA to which quinolone resistance has been mapped (Maxwell, 1992). In vitro experiments in the presence of GyrB have shown that this fragment performs quinolone-induced DNA cleavage as efficiently as the intact protein.
Subsequent deletion experiments showed that the smallest fragment able to catalyse DNA cleavage was a 58-kDa protein (residues 7-523) (Reece & Maxwell, 1991c). The C-terminal 33-kDa fragment (residues 572-875) is unable to catalyse any of the reactions of gyrase, but has been shown to bind DNA in a Mg\(^{2+}\)-independent manner, in contrast to the binding of the holoenzyme or the A protein. When the 33-kDa domain is allowed to bind nicked DNA and the nick is resealed by *E. coli* ligase, the DNA becomes positively supercoiled (Reece & Maxwell, 1991a). Thus, it is likely that this domain provides a non-specific DNA-binding function responsible for the positive wrapping of DNA around the enzyme. Such a mode of binding could present the transport DNA segment to the DNA gate in an orientation such that subsequent strand passage will favour the introduction of negative supercoils. The fact that the C-terminus of gyrase bears little sequence similarity to that of the other type II enzymes, when compared with other parts of the protein (Caron & Wang, 1993), could account for the differences in mechanism between gyrase and other type II topoisomerases. In this case, removal of the C-terminal domain of GyrA may give rise to an enzyme that relaxes instead of supercoiling DNA and catalyses intermolecular strand passage more efficiently than intact gyrase, i.e. this new enzyme form may behave like a conventional type II topoisomerase. In this paper we have tested this proposal using a 59-kDa N-terminal fragment of GyrA.

### 2.2. Results

**The A59\(_2\)B\(_2\) complex can relax supercoiled DNA in the presence of ATP.** The N-terminal domain of GyrA has been identified as being responsible for the DNA breakage-reunion reaction of DNA gyrase (Reece & Maxwell, 1989). When expressed as a 64 kDa fragment [GyrA(1-572)] and complexed with GyrB (A64\(_2\)B\(_2\)), it was found to support low levels of DNA supercoiling and ATP-independent relaxation [~0.1% compared with A\(_2\)B\(_2\) (Reece & Maxwell, 1991c)]. When expressed as a 59 kDa fragment [GyrA(1-523)] and complexed with GyrB (A59\(_2\)B\(_2\)), it was again found to exhibit low levels of relaxation activity but undetectable supercoiling activity (Reece & Maxwell, 1991c). Subsequently the supercoiling activity of A64\(_2\)B\(_2\) has been re-evaluated and found to be undetectable (Critchlow & Maxwell, 1996). We assume that the low-level supercoiling activity previously found (Reece & Maxwell, 1991c) is attributable to contamination by GyrA. Both the A64\(_2\)B\(_2\) and A59\(_2\)B\(_2\) complexes show levels of quinolone-induced DNA cleavage comparable to that of intact gyrase (Critchlow & Maxwell, 1996, Reece & Maxwell, 1991c).

We have confirmed that the A59\(_2\)B\(_2\) complex is unable to supercoil relaxed DNA, even at high protein concentrations, and supports only low levels of ATP-independent relaxation (less than 10% compared with intact gyrase, data not shown); this again may be affected by contamination by GyrA. However, we have found that the A59\(_2\)B\(_2\) complex is able to efficiently relax negatively-supercoiled DNA in the presence of ATP (Fig. 2.1A). Neither the 59-kDa protein nor GyrB alone can support this reaction. The relaxation reaction supported by the A59\(_2\)B\(_2\) complex is distributive and not processive as is the case for supercoiling by DNA gyrase. The time dependence of relaxation clearly suggests a catalytic nature for this
interaction (Fig. 2.1A). To rule out the possibility of a slow stoichiometric reaction, time-courses of relaxation at low protein:DNA ratios were performed (Fig. 2.1A). Under these conditions each A592B2 complex increased the linking number of the DNA by at least 25. We also found that A642B2 is capable of ATP-dependent relaxation of DNA.

Novobiocin is a specific inhibitor of DNA gyrase that has recently been shown (Gormley et al., 1996, Lewis et al., 1996) to act by competitively inhibiting the binding of ATP. Addition of novobiocin completely inhibited DNA relaxation by the A592B2 complex (Fig. 2.1B). Moreover, the non-hydrolysable ATP analog, ADPNP, failed to support the reaction. These results demonstrate the ATP-dependent nature of the relaxation reaction. The A592B2 complex is also able to relax positively- and highly negatively-supercoiled DNA in ATP-dependent reactions. Gyrase, by contrast, converts positively-supercoiled DNA to the negatively-supercoiled form in the presence of ATP. The efficiency of the ATP-dependent relaxation reaction of A592B2 was compared to that of the supercoiling reaction of gyrase. The rate by which A592B2 removes supercoils is not constant throughout the reaction (Fig. 2.1A and Fig. 2.4C). Under the same conditions, in the first steps of the reaction the rate of relaxation by A592B2 is approximately 20% of the rate of supercoiling by gyrase during the same time period. At the later stages of the reaction this rate drops to less than 10% that of the initial steps of the gyrase supercoiling reaction (see Fig. 2.4 B,C). Control experiments have shown that this is not due to loss of enzyme activity and can be explained in terms of the decreasing concentration of DNA crossovers (see below).

To test whether the supercoiling ability of the A592B2 complex could be restored, we tried adding back the 33-kDa C-terminal DNA-binding domain of GyrA, which has been expressed as a separate gene product (Reece & Maxwell, 1991a). In the presence of high
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Concentrations of the 33-kDa domain only weak positive supercoiling was observed. This was probably caused by relaxation by the A592B2 complex of the negative writhe introduced into relaxed DNA upon the binding of the 33-kDa domains. This result is in contrast to the N-terminal 64-kDa domain of GyrA which shows relatively efficient supercoiling activity in the presence of the 33-kDa domain (Critchlow & Maxwell, 1996, Reece & Maxwell, 1991a). It is likely that the approximately 5 kDa missing between the C-terminus of the 59-kDa domain and the N-terminus of the 33-kDa protein is important for the stability of the complex.

The A592B2 complex is a potent decatenating enzyme. The ability of A592B2 to decatenate DNA circles was investigated by examining its ability to release minicircles from kinetoplast DNA (k-DNA) networks. These are networks of thousands of interlocked DNA minicircles in which each circle is linked to an average of three others (Chen et al., 1995). The size of the network is so large that when subjected to electrophoresis on an agarose gel only the released minicircles will enter the gel while the network remains in the wells. The decatenation of k-DNA was performed under ATP-dependent relaxation conditions and the ability of A592B2 to perform the reaction was compared to that of gyrase (Fig. 2.2).

Comparing the enzyme concentration required for the release of the same number of minicircles, it was estimated that the truncated enzyme is ~30 times more efficient in decatenation than gyrase. These levels of decatenation are approximately one order of magnitude lower than those exhibited by human topo IIa using k-DNA as substrate (T. R. Hammonds, personal communication). Control experiments have shown that the failure of previous studies to detect decatenation by the N-terminal domain of GyrA (Reece & Maxwell, 1991c) can largely been attributed to the different reaction conditions employed; the earlier studies were conducted at a lower temperature (25°C compared with 37°C) and lower salt concentration. Enzyme concentrations that, under the conditions used in this work, yielded full decatenation of k-DNA, produced only low levels of free minicircles under the conditions employed previously (Reece & Maxwell, 1991c). Differences could also be due to the different catenated substrates used: a pair of interlinked circles in the previous work.
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(Reece & Maxwell, 1991c) and k-DNA in the present experiments; k-DNA may present a better substrate due to the high local concentration of crossovers (see below).

DNA gyrase can catalyse the catenation of plasmid DNA under conditions of high spermidine and low Mg$^{2+}$ concentration (A.P. Tingey, unpublished observations). Under these conditions, we were unable to detect significant levels of catenation by the A592 B2 complex. An extensive investigation of alternative reaction conditions failed to detect any catenation activity with the truncated enzyme.

The interaction of A592 B2 with DNA is characterised by binding to crossovers. Using either supercoiled or relaxed radiolabelled pBR322, the amount of DNA retained on nitrocellulose filters by the A592 B2 complex was found to be less than 10% of that retained by intact gyrase (A2 B2). It has previously been shown that gyrase shows a preference for binding to relaxed as compared to negatively-supercoiled DNA (Higgins & Cozzarelli, 1982); we found that the truncated enzyme exhibits a preference for the supercoiled form.

When gyrase binds to nicked-circular DNA and the nick is resealed by E. coli ligase the DNA is found to be positively supercoiled (Liu & Wang, 1978). We performed this experiment with the A592 B2 complex and found that, unlike gyrase, the truncated enzyme does not wrap DNA (Fig. 2.3A); on the contrary, the A592 B2 complex reduces the linking number of DNA by less than -0.03 per enzyme molecule. This is likely to be a manifestation of the absence of the C-terminal DNA-binding domain which has been shown to wrap DNA in a positive sense even when incubated with DNA on its own (Reece & Maxwell, 1991a). This experiment was performed both in the absence and the presence of ciprofloxacin (CFX) and the results were found to be identical; CFX stabilises the relatively weak enzyme-DNA complex.

The ATPase activity of DNA gyrase is stimulated by the binding of DNA (Maxwell & Gellert, 1984). We found that linear pBR322 failed to stimulate the ATPase of the A592 B2 complex. Investigation of ATP hydrolysis in the presence of a large excess of negatively-

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**Fig. 2.3. A,** Determination of the extent of DNA wrapping. Open-circular pBR322 (400 ng) was incubated for 30 min at 25°C with the enzyme:DNA ratios indicated and then the nick was resealed with E. coli ligase. The star (*) indicates the starting open-circular material. Samples were analysed on an 1% agarose gel containing 0.4 μg/mL chloroquine. **B,** Stabilisation of crossovers by A592 B2. Negatively-supercoiled pBR322 (400 ng) was relaxed by topo I in the presence of the indicated ratios of enzyme:DNA, in the absence and presence of ciprofloxacin (CFX). The tracks labelled with the star (*) contain the starting supercoiled material while those labelled (0) contain no enzyme and are fully relaxed by topo I. Samples were analysed on an agarose gel containing 0.8 μg/mL chloroquine.
supercoiled DNA showed a rate which decreased during the course of the reaction (Fig. 2.4). Samples were removed from the reaction and analysed by electrophoresis to monitor the progress of relaxation. As the reaction proceeded the rate of hydrolysis gradually decreased (Fig. 2.4), whereas intact gyrase exhibits a stable ATPase rate during DNA supercoiling even when all the substrate is fully supercoiled [Fig. 2.4 and also (Bates et al., 1996)]. The rate of hydrolysis by A$_2$B$_2$ was stable at 1.04 s$^{-1}$ throughout the experiment while the rate of the A$_{592}$B$_2$-supported reaction dropped from 0.24 s$^{-1}$ in the first 5 min to 0.09 s$^{-1}$ in the last stages of the reaction. A similar trend can be observed also in the rate of supercoil relaxation and DNA supercoiling (Fig. 2.4 B,C and Fig. 2.1A).

The rate of ATP hydrolysis in the presence of linear DNA remains constant throughout the course of this experiment indicating that this phenomenon is not due to loss of enzyme activity. It is evident that the binding of the A$_{592}$B$_2$ complex to linear or relaxed DNA does not efficiently stimulate the ATPase. In the presence of the quinolone drug ciprofloxacin the ATPase activity of the A$_{592}$B$_2$ complex in the presence of linear and relaxed DNA was stimulated (data not shown); consistent with the stabilisation of the DNA-protein complex in the presence of the drugs (Higgins & Cozzarelli, 1982). These results can be interpreted as preferential binding to DNA crossovers stabilising the complex and increasing

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**Fig. 2.4. A, ATPase of A$_{592}$B$_2$ and A$_2$B$_2$.** The experiment was performed using an enzyme-linked assay (Ali et al., 1993) and the decrease in NADH concentration reflects the amount of ATP hydrolysed. Reactions contained 100 nM pBR322 DNA (supercoiled for the A$_{592}$B$_2$ reaction and relaxed for the A$_2$B$_2$ reaction) and were simultaneously monitored for topoisomerase activity by analysing samples on 1% agarose gels containing 1 μg/mL chloroquine. **B,** supercoiling by 10 nM A$_2$B$_2$, and **C,** relaxation by 50 nM A$_{592}$B$_2$. The rate of hydrolysis by A$_2$B$_2$ was stable at 1.04 s$^{-1}$ throughout the experiment while the rate of the A$_{592}$B$_2$-supported reaction dropped from 0.24 s$^{-1}$ in the first 5 min to 0.09 s$^{-1}$ in the last stages of the reaction.
the rate of hydrolysis, subsequent strand passage would remove the crossovers and thus
decrease the rate. Close investigation of a time-course of relaxation (Fig. 2.1A) reveals that the
rate of the reaction slows down with time; again consistent with preferential binding to
crossovers.

In order to investigate the binding of the A592B2 complex to DNA crossovers further,
supercoiled DNA was relaxed by chicken erythrocyte topo I in the presence of different
concentrations of the truncated enzyme. Topo I does not require ATP and thus allows the
experiment to be carried out in the absence of nucleotide (Wang, 1996). As the interaction
between A592B2 and DNA is relatively weak, this experiment was performed in parallel in the
presence of CFX in order to stabilise the A592B2-DNA complex and obtain a clearer result.
As seen previously with topoisomerase II (Roca et al., 1993), A592B2 inhibits full relaxation
by topo I (Fig. 2.3B). This is a clear indication of the truncated enzyme binding to DNA
crossovers, thus stabilising them and preventing them from being removed by topoisomerase
I. This interaction is consistent with the higher affinity of the truncated enzyme for
supercoiled DNA and the effects of different forms of DNA on the ATPase rate of the
A592B2 complex. Control experiments have shown that the slight unwinding of relaxed DNA
observed in the experiment containing CFX is probably due to the well documented
interaction between quinolones and DNA, (Shen & Pernet, 1985, Tornaletti & Pedrini, 1988),
and does not interfere with the results of this experiment.

**Fig. 2.5. Complementation of a parCts strain.** Histogram showing the relative
efficiency with which plasmids expressing different proteins complemented the parCts
phenotype. In the column labelled 'none' the cells were transformed with plasmid
pTTQ18* which is the vector used in the construction of the gyrase plasmids (Reece & Maxwell, 1991a)

Complementation of a parC temperature-sensitive mutant. So far the truncated
enzyme seems to behave *in vitro* like a typical type II topoisomerase, being able to support
all the reactions catalysed by topo IV, or its eukaryotic counterpart topo II. We tested
whether this enzyme can behave the same way *in vivo* and complement a topo IV-deficient
strain. For this purpose, a temperature-sensitive parCts E. coli mutant was used (gift of Dr.
K. Marians). The ts strain and its parent strain *E. coli* C600 were transformed with plasmids
encoding wild-type ParC (pET-3c-parC), the 59-kDa fragment (pRJR10.18), GyrA (pPH3),
GyrB (pAG111), and the vector pTTQ18* (Reece & Maxwell, 1991a). The various strains
were incubated at the permissive (30°C) and non-permissive (42°C) temperatures and the
results are shown in Fig. 2.5. The 59-kDa protein could clearly complement the ParC deficiency at 42°C in contrast to GyrA which failed to do so at any inducer concentration. The strains transformed with the wild-type ParC-expressing plasmid showed the highest levels of complementation, while the A59 protein was less effective (approximately 5%). On the other hand, the strains transformed with the GyrA- or the GyrB-expressing plasmids or the vector alone failed to survive at the non-permissive temperature (Fig. 2.5).

2.3. Discussion

DNA gyrase is an atypical type II topoisomerase. While the other type II enzymes (topo IV and topo II) carry out relaxation and decatenation reactions, gyrase is so far the only topoisomerase able also to negatively supercoil DNA. The type II enzymes exhibit a great degree of sequence similarity but diverge significantly at their C-termini (Caron & Wang, 1993). Both gyrase and topo IV are heterotetramers, while topo II is a heterodimer whose monomer is essentially a fusion of the two subunits of the other enzymes (Lynn et al., 1986). One of the principal differences between gyrase and the conventional type II enzymes is in the mode of interaction with the DNA. The relaxing and decatenating enzymes bind 25-35 bp of DNA without wrapping it around their core (Lee et al., 1989, Peng & Marians, 1995), while gyrase binds ~130 bp in a positive-superhelical manner (Liu & Wang, 1978, Orphanides & Maxwell, 1994); this interaction is probably the basis of their mechanistic differences. Topo II does not prescribe the orientation for the DNA segment to be translocated. The enzyme will perform strand passage with any transport segment presented to the DNA gate, thus permitting both intra- and intermolecular strand-translocation events. By contrast, the mechanism of supercoiling by gyrase requires the enzyme to distinguish between different DNA segments, so that subsequent strand passage will result in the introduction of two negative turns. In order for this to be achieved, a positive node on the DNA has to be inverted. In the case of gyrase, this node is introduced by the wrapping of DNA around the enzyme. The C-terminal domain of the A protein of DNA gyrase, which exhibits significant sequence diversity from the other topoisomerases, has been shown to be responsible for the right-handed wrapping of the DNA (Reece & Maxwell, 1991a). We proposed that removal of this domain will give rise to an enzyme that will function by a similar mechanism to that of other type II enzymes.

For the purpose of these experiments, we have used a 59-kDa N-terminal fragment that contains the first 523 residues of GyrA, thus removing the C-terminal DNA-binding domain. This fragment contains all the residues required for topoisomerase activity since it can perform quinolone-induced DNA cleavage when complexed with GyrB. The complex between the 59-kDa protein and GyrB (A59B2) is unable to catalyse the supercoiling of DNA and can only support low levels of ATP-independent relaxation. The mode of binding of this enzyme to DNA is distinctly different to that of gyrase and is very similar to the other type II enzymes. With A59B2 no significant wrapping of DNA occurs and the binding of the enzyme appears to cause a slight unwinding. The extent of this change in the linking number
CONVERSION OF DNA GYRASE INTO A CONVENTIONAL TYPE II TOPOISOMERASE

is very small and was estimated to be less than -0.03 turns per enzyme molecule. Most DNA-binding proteins cause some distortion of the helix upon binding (e.g. polymerases) and such a small unwinding is not unexpected. The inability to introduce a positive node on the DNA is consistent with the failure of this enzyme to supercoil DNA. Hydroxyl-radical footprinting of A642B2 bound to DNA (in the presence of CFX, in order to stabilise the protein-DNA complex) shows that this complex protects approximately 20-25 bp around the cleavage site (G. Orphanides, unpublished results). The 59-kDa protein is not much smaller than the 64-kDa domain and is likely to give a similar protection pattern. Similar experiments involving nuclease digestion with topo IV or eukaryotic topo II have shown a protection of 30-34 and 25-28 bp respectively (Lee et al., 1989, Peng & Marians, 1995). Taken together, these results indicate that the mode of DNA binding by A592B2 is very similar to that of topo II and topo IV.

Given the similarities in the DNA binding between A592B2 and the typical type II enzymes, the ATP-dependent relaxation of supercoiled DNA by the A592B2 complex does not come as a surprise. The enzyme seems not to distinguish between transport segments since it is able to relax both negatively- and positively-supercoiled DNA. By contrast, gyrase clearly dictates the direction of strand passage since it converts the positively-supercoiled substrate to the negatively-supercoiled form. The reaction of A592B2 is shown to be catalytic in nature as indicated by the number of superhelical turns removed by each molecule. The ATP dependency of this reaction was shown by the lack of activity in the absence of ATP or in the presence of ADPNP. Moreover, the reaction was completely stopped upon the addition of novobiocin, a competitive inhibitor of ATP hydrolysis by gyrase.

The affinity of A592B2 for DNA has been significantly affected by the removal of the C-terminal domains. Nitrocellulose-filter binding experiments showed that the binding of A592B2 to DNA is much weaker than that of gyrase. Moreover, the truncated enzyme exhibits a preference for supercoiled DNA, unlike gyrase. This preference can be explained on the basis of its preferential binding to DNA crossovers. This was supported by the observation that topo I is unable to fully relax the supercoiled DNA-A592B2 complex in the absence of ATP, i.e. the enzyme stabilises DNA crossovers and prevents them from being removed by topo I. When the complex was stabilised by quinolones the effect was even more pronounced. The small unwinding observed upon binding of the complex to DNA cannot account for this change since it can only result in less than one turn at the highest enzyme concentration used. A similar mode of binding was observed in the case of yeast topo II (Roca et al., 1993). Binding at crossovers stabilises the weak binding of the enzyme to the DNA and stimulates ATP hydrolysis. Removal of the crossovers during relaxation destabilises the enzyme-DNA complex and reduces the rate of hydrolysis (Fig. 2.4). Binding of the enzyme to crossovers gives an insight into the mechanism of DNA relaxation. Such a mode of binding presents one of the two helixes to the DNA gate formed on the other. Translocation of this segment through the gate will invert the node and change the linking number by 2. In a negatively-supercoiled substrate most nodes are negative while the
opposite applies to a positively-supercoiled molecule. Inversion of these nodes will result in substrate relaxation. Destabilisation of the enzyme-DNA complex subsequent to strand passage favours dissociation and binding to another crossover, as is indicated by the distributive nature of relaxation.

If the crossover is between a pair of helices that are part of different molecules, as in the case of catenated circles, then the relaxing enzyme will preferably bind to the crossover point and unlink the two molecules by translocating one helix. This was found to be the case with the A592B2 complex which has an approximately 30-fold higher efficiency in decatenating k-DNA than gyrase. When gyrase is presented with this substrate it will mainly perform DNA supercoiling. The wrapping of the DNA in the case of gyrase favours the translocation of a segment coming from the same DNA molecule, close to or within the wrapped segment, instead of an interlinked DNA helix. Gyrase has been shown to support catenation of DNA circles under conditions of DNA condensation (Kreuzer & Cozzarelli, 1980). The inability of A592B2 to perform this reaction is probably a result of its lower affinity for DNA. The equilibrium between the catenation and decatenation reactions should significantly favour unlinking since crossovers between interlinked molecules are clearly more probable than points of transient intermolecular contact in unlinked circles.

Deletion of the C-terminal domain of gyrase results in an enzyme with significantly different characteristics. On the basis of its altered mode of DNA binding, this enzyme is able to perform in vitro like topoisomerase II or IV. To test this possibility in vivo, the ability of the 59-kDa protein to complement a ParC-deficient strain was investigated. The temperature-sensitive phenotype was successfully complemented by the 59-kDa domain while expression of GyrA failed to maintain cell viability. The action of the 59-kDa protein is likely to be through its association with endogenous GyrB rather than ParE, since the addition of ParE to GyrA failed to show in vitro activity (Kato et al., 1992).

These results give an insight into the mechanism of action of topoisomerases. The mechanism of ATP-dependent reactions catalysed by the conventional type II enzymes and the A592B2 complex is characterised by the indiscriminating nature of strand passage. By contrast, gyrase dictates the direction of ATP-dependent strand passage by means of its mode of binding to DNA which creates a node and presents the transport segment to the enzyme gate. This difference in the mechanism is reflected both in the intra- and intermolecular reactions. Conventional topoisomerases are better decatenating enzymes, while DNA gyrase favours the intramolecular reaction because of the high probability of the transport segment to be part of the same molecule as the gate segment.

Gyrase also catalyses the relaxation of negative supercoils in the absence of nucleotide cofactor (Gellert et al., 1977, Sugino et al., 1977), a reaction also carried out by topoisomerase II, the form of gyrase that results after proteolytic removal of the ATPase domains (Gellert et al., 1979). This reaction must proceed through a distinct mechanism from that of ATP-dependent topoisomerisation. The limit of the supercoiling reaction catalysed by gyrase has been shown to be thermodynamic rather than steric (Bates & Maxwell, 1989,
Cullis et al., 1992). In this case, the enzyme must be able to prevent translocation of the DNA segment through the DNA gate if the increase in free energy of the resulting topoisomer is higher than that released by ATP hydrolysis. This could be accomplished if the transport segment was able to translocate back through the gate when the ATP-released energy is not sufficient. It is possible that the ATP-independent relaxation is a result of such a backward reaction. Incubation of highly negatively-supercoiled DNA (\(\sigma < -0.11\)) with gyrase in the presence of ATP results in relaxation of the substrate until it reaches a superhelical density of approximately -0.11, the limit of supercoiling by gyrase (A. Maxwell, unpublished observations). Similarly, when a supercoiling reaction catalysed by gyrase is allowed to reach the limit of supercoiling and then ADP is added so that the sum of [ATP] and [ADP] is constant but the ratio of [ATP] to [ADP] is varied, the reaction reaches another limit which is characterised by lower absolute superhelical density. Given that the free energy of ATP hydrolysis depends on the ratio of [ATP] to [ADP], in these experiments, a thermodynamic equilibrium seems to have been reached between two opposing reactions, ATP-dependent supercoiling and ATP-independent relaxation.

Considering the two-gate model for the mechanism of type II enzymes (Berger et al., 1996, Roca & Wang, 1994), the ATP-dependent reactions proceed by the translocation of the transport-segment first through the ATP gate and then through the DNA gate. However, ATP-independent strand passage is likely to proceed in the opposite direction. Gyrase cannot relax positively supercoiled DNA in the absence of nucleotide (Bates et al., 1996), and it has recently been shown that the probability of ADPNP-driven strand passage is high for positively-supercoiled DNA but gradually decreases with decreasing linking number (Bates et al., 1996). On the basis of the mode of binding of gyrase it is evident that positively-supercoiled DNA would facilitate an orientation of the transport segment such that it presents itself to the ATP gate. Negatively-supercoiled DNA will not as readily wrap itself around the enzyme and present itself to the ATP gate, therefore, the probability of strand passage with this substrate is significantly lower (Bates et al., 1996). At the same time, this substrate will have a higher possibility of assuming a conformation that presents a DNA segment to be translocated through the DNA gate from the other direction. Transportation of this segment through the DNA gate first and then through the ATP gate will result in one round of relaxation. This reaction is feasible by the escape mechanism described above since the supercoiled substrate has higher free energy than the relaxed form. On the contrary, positively-supercoiled DNA will not favour this reaction, despite it being thermodynamically feasible, because of the conformation of the enzyme-DNA complex. The ATP-independent reaction does not occur in the case of the conventional type II enzymes and could be due to an intrinsic feature of gyrase, maybe reflecting a weaker interface between the two A subunits which can now become transiently open thus allowing the admission of a DNA segment through the bottom gate. Alternatively, the possibility also exists that such a backward reaction could proceed via a one-gate mechanism.

These experiments are a demonstration of the common evolutionary origins of type II...
topoisomerases. It is likely that all type II topoisomerases share a common ancestor but have evolved to perform more specialised roles in different organisms. In the case of DNA gyrase the C-terminus is involved in the wrapping of the DNA around the enzyme, a mode of binding that creates a positive node and strongly favours strand passage in the direction of reduction of the linking number. In the case of eukaryotic topo II, the C-terminus performs a different function, being thought to have a regulatory role (Watt & Hickson, 1994).

2.4. References

Abstract
Type II DNA topoisomerases accomplish the formidable task of maintaining the topological state of DNA, which is constantly being entangled and twisted during processes such as transcription and replication. The mechanism of these enzymes involves the formation of an enzyme-operated gate on one DNA segment and the passage of another segment through this gate. DNA gyrase is the only type II topoisomerase able to introduce negative supercoiling into DNA, a uniqueness that requires the enzyme to prescribe the directionality of strand passage. Although it is known that this is a consequence of its characteristic mode of binding to DNA, the detailed mechanism by which the transported DNA segment is captured and directed through the DNA gate is largely unknown. We addressed this mechanism by probing the topology of the bound DNA segment at distinct steps of the catalytic cycle. We propose a model in which gyrase captures a contiguous DNA segment with high probability, irrespective of the superhelical density of the DNA, while the efficiency of strand passage depends on the superhelical free-energy. This mechanism is concerted, in that capture of the transported segment induces opening of the DNA gate, which in turn, stimulates ATP hydrolysis.

3.1. A model for the mechanism of DNA gyrase

DNA gyrase, like other type II topoisomerases, is believed to operate as a molecular clamp (Berger et al., 1996, Roca et al., 1996). The enzyme, which consists of two GyrA and two GyrB subunits, binds ~130 bp of DNA in a positive superhelical sense. Double-stranded cleavage of the DNA and covalent attachment of the phosphate backbone to Tyr122 of each GyrA forms a transient DNA gate (Reece & Maxwell, 1991). The two GyrB subunits form a protein clamp, which dimerises in the presence of ATP, trapping a DNA segment (the T segment). In a process coupled to ATP hydrolysis, the T segment is transported through the DNA gate. Indiscriminate capture of a T segment is sufficient to account for the reactions of...
A model for the mechanism of strand passage by DNA gyrase

The conventional type II enzymes (such as the eukaryotic topoisomerase II or the prokaryotic topoisomerase IV), i.e. DNA relaxation and decatenation. However, the supercoiling reaction performed by gyrase requires that the enzyme dictates the orientation of the T segment with respect to the DNA gate. This derives from the unique right-handed wrapping of the DNA by the enzyme, since deletion of the C-terminal DNA-binding domain of GyrA, which is responsible for the wrap, abolishes supercoiling in favour of relaxation and decatenation (Kampranis & Maxwell, 1996). However, the mechanism by which this interaction directs the T segment to the ATP-operated clamp and the details of the strand-passage reaction are still unclear.

Binding of a non-hydrolysable ATP analogue (5'-adenylyl-β,γ-imidodiphosphate; ADPNP) traps the clamp in the dimerised form and supports stoichiometric strand passage (Sugino et al., 1978). The efficiency of this reaction was found to depend on the topological state of the DNA; with positively supercoiled DNA the efficiency of strand passage is almost 100%, decreasing to ~25% with relaxed, and to minimal levels with negatively supercoiled DNA. It has been proposed that the efficiency of strand passage depends on the efficiency by which the T segment is captured (Bates et al., 1996). Thus, positively supercoiled molecules, that readily form the positive node to be inverted, are better substrates than relaxed or negatively supercoiled ones. Moreover, once the T segment is trapped, it is passed through the DNA gate with high probability, while nucleotide hydrolysis is required only for enzyme turnover. However, a number of observations appear to contradict this model. Firstly, it has been suggested that the presence of a DNA segment in the ATP-operated clamp stimulates the rate of ATP hydrolysis (Maxwell & Gellert, 1984, Tingey & Maxwell, 1996). Therefore, if capture of the T segment depended on the topological state of the DNA, so would be the rate of ATP hydrolysis. However, the rate of hydrolysis is the same in the presence of either linear, relaxed or negatively supercoiled DNA (Bates et al., 1996, Kampranis & Maxwell, 1996). Secondly, the thiophosphate ATP analogue ATPβS(Rp) cannot support efficient supercoiling although it is hydrolysed equally well to ATP, suggesting that nucleotide binding by itself is not sufficient for strand passage (Cullis et al., 1997). Thirdly, the above mechanism suggests that the limit of supercoiling is steric, in contrast to experimental evidence (Bates & Maxwell, 1989, Cullis et al., 1992, Westerhoff et al., 1988) suggesting that the limit is thermodynamic. To address the mechanism of strand passage we used DNA relaxation by topoisomerase I (topo I) as a topological probe. Topo I is an ATP-independent topoisomerase which relaxes DNA in the direction of the thermodynamic drive (Wang, 1996). Treatment of a protein-bound closed-circular DNA molecule with topo I relaxes the unconstrained DNA but not the part stabilised by the protein. Therefore, if protein binding induces changes in the topology of the DNA, analysis of the topo I-treated DNA after the removal of the protein can provide useful information on the topology of the protein-DNA complex.

To test whether the efficiency of T-segment capture depends on the topological state of the DNA, gyrase was incubated with relaxed, negatively and positively supercoiled
A MODEL FOR THE MECHANISM OF STRAND PASSAGE BY DNA GYRASE

pBR322 DNA (superhelical density ($\sigma$) = 0. -0.06 and +0.03 respectively) and the complexes were treated with topo I. Analysis of the DNA after removal of the enzyme, revealed that binding of gyrase induces a change in the linking number ($\Delta Lk$) of the bound segment of $\sim+0.8$ per enzyme ($\Delta Lk/\text{enz} = +0.8$), irrespective of the topological state of the starting material (Fig. 3.1a and Table 3.1). However, after formation of the enzyme-DNA-ADPNP complex and treatment with topo I, the DNA was found to be almost fully relaxed ($\Delta Lk/\text{enz} < +0.05$), again independent of the initial topological state of the substrate (Fig. 3.1a and Table 3.1). When the ability of the enzyme to perform ADPNP-induced supercoiling was investigated, each gyrase tetramer introduced 0.55 negative turns in relaxed DNA, in good agreement with previous observations (Bates et al., 1996). To study the relationship between strand passage and the linking number changes observed, we used an active-site mutant of gyrase (GyrA$^{\text{Ser122}}$) that cannot cleave DNA and thus cannot perform strand passage (Critchlow & Maxwell, 1996). With all three different topological forms, $A_2^{\text{Ser122}}B_2$ was found to introduce $\sim0.7$ positive turns per enzyme in the absence of ADPNP, i.e. very similar to wild-type. In all cases, $\sim+0.2$ turns were introduced when ADPNP was bound (Table 3.1).

![Image](https://via.placeholder.com/150)

**Fig. 3.1.** a, Typical topological assay showing the relaxation by topo I of the gyrase-DNA and gyrase-DNA-ADPNP complexes formed on relaxed DNA. b, Trapping of the T segment in the ATP-operated clamp is demonstrated by a similar experiment using $A_6^{\text{Ser122}}B_2$. 200 $\mu$M ciprofloxacin was present in this experiment to stabilise the weak enzyme-DNA complex (Kampranis & Maxwell, 1996), however similar results were obtained in the absence of the quinolone. $A_6^{\text{Ser122}}B_2$ also produced similar results with or without the drug.

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<th>Table 3.1. Summary of the results of the topo I topological assays</th>
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To test whether the T segment can be trapped inside the dimerised clamp when the DNA gate is not allowed to open, the active-site mutant of the C-terminal truncated version of gyrase (A642B2) was used. This enzyme does not wrap DNA, thus making it easier in a topo I experiment to identify if a DNA crossover has been trapped. Binding of A642^phe122B2 to negatively supercoiled DNA stabilised ~0.20 negative crossovers per enzyme (Fig. 3.1b). However, when ADPNP was bound to the mutant complexes, the number of negative crossovers trapped increased to ~0.45 per enzyme (Fig. 3.1b; A642^Ser122B2 produced similar results). Although the two helices stabilised in the absence of the nucleotide analogue do not necessarily represent a pair of G and T segments (Roca et al., 1993, Rybenkov et al., 1997), the additional crossovers stabilised in the presence of ADPNP must be due to the trapping of the T segment inside the dimerised clamp.

These results contradict the model for the mechanism of strand passage which predicts that, depending on the topological form of the DNA, there would be a different population of the enzyme that has trapped the T segment (Bates et al., 1996). In order to reconcile the results obtained with the wild-type enzyme with this model, one has to assume that the complexes that have trapped and transported the T segment have a similar conformation to those where clamp closure failed to trap a segment and that in this conformation the overall wrapping of the DNA is almost zero. However, this interpretation cannot account for the results obtained with A2^Ser122B2. This mutant should trap the T segment within the ATP-operated clamp. The topology of the node formed by the trapped T segment and the segment that contains the DNA gate must be positive in order for gyrase to perform supercoiling. Had T-segment capture depended on the topological state of the DNA, treatment with topo I of the A2^Ser122B2-ADPNP complex formed on positively supercoiled DNA would have been expected to produce a relatively large positive change in the overall linking number (ΔLk/enz. ~+1.0) while in the complex formed on negatively supercoiled DNA ΔLk/enz. would have been approximately zero.

To account for these observations, we propose that the T segment is captured with high probability irrespective of DNA topology. When ADPNP is used as a cofactor, the T segment is not released from the enzyme after it has passed the DNA gate, instead it is able to equilibrate between the two sides of the DNA gate. This equilibrium must depend on the superhelical free-energy of the DNA substrate. Thus, with positively supercoiled substrates the T segment would equilibrate predominantly beyond the gate, and stopping of the reaction at that point would reveal efficient strand passage. With negatively supercoiled substrates however, the T segment would be predominantly before the DNA gate, and very little strand passage would be observed. Treatment of gyrase-ADPNP complexes formed with DNA of different superhelical densities with topo I would relax the part of the DNA that is not constrained by the enzyme. Thus, the T segment would reach an equilibrium that is the same, independent of the topological state of the starting material.

At the structural level the information on gyrase is partial. The structure of the 59-kDa N-terminal domain of GyrA and the 43-kDa N-terminal domain of GyrB have been solved.
A model for the mechanism of strand passage by DNA gyrase independently (Morais Cabral et al., 1997, Wigley et al., 1991) while the structure of a fragment of yeast topoisomerase II homologous to the N-terminal domain of GyrA and the 47-kDa C-terminal domain of GyrB has also been solved (Berger et al., 1996). Taken together with electron microscopy studies on yeast topoisomerase II (Schultz et al., 1996), the spatial arrangement of the gyrase subunits can be deduced to be similar to that shown in Fig. 3.2. In this model the unknown structure of the 47-kDa domain of GyrB is replaced by that of the homologous domain of yeast topoisomerase II. The structure of the 33-kDa C-terminal domain of GyrA is unknown. This domain is the major component of the DNA-binding domain of gyrase and contributes ~100 bp of positive wrap, while its deletion abolishes the ability of gyrase to dictate the direction of strand passage (Kampranis & Maxwell, 1996). We suggest that the 33-kDa domain is located in such a way that would direct the T segment to the ATP-operated clamp. Moreover, in order to achieve this high efficiency of trapping, the T segment must be very close to or part of the wrapped segment. This is supported by hydroxyl-radical footprinting experiments that revealed an extension in the protection of one of the two DNA arms in the presence of ADPNP (Orphanides & Maxwell, 1994). We propose that the 33-kDa domain extends from the DNA gate towards the entrance of the clamp as shown in Fig. 3.2. In this complex the DNA is wrapped in a positive configuration, which however, does not include the pre-strand passage positive node formed by the DNA gate and the T segment (Fig. 3.2). In order for this to be formed, large movements of enzyme and DNA must accompany the binding of the nucleotide (Fig. 3.2). These are reflected in the changes in DNA topology observed in these experiments. Moreover, comparison of the crystal structures of the 59-kDa N-terminal domain of GyrA and the 92-kDa fragment of yeast topo II suggested that there are large conformational changes upon gate opening.

In the case of A₂₅SER₁²₂B₂ the overall wrapping drops to +0.2 turns per enzyme after the binding of the nucleotide. Since in this complex a positive node is always trapped, a negative distortion must occur in the rest of the molecule. To account for this, in the model shown in Fig. 3.2 we propose that the conformation of the DNA arm that has not been trapped is that of a negative superhelix. From the efficiency of ADPNP-induced strand passage we can estimate that in equilibrium ~27% of the complexes would be in the conformation where the T segment is beyond the DNA gate. Assuming that in the rest of the complexes the DNA has the same conformation as in A₂₅SER₁²₂B₂, in order for the overall Δlk to be ~0, the Δlk of the complexes that have passed the T segment must be ~0.55. We believe that is achieved by a change in the conformation of the DNA arm that has not been trapped from that of a negative superhelix when the T segment is in the ATP-operated clamp, to a positive superhelix, when the T segment has passed the DNA gate (Fig. 3.2). From these calculations it appears that nucleotide binding lowers the overall Δlk for strand passage from -2.0 to -0.75, thus lowering the overall activation energy for the reaction. Nevertheless, after release of the T segment from the post-strand passage cavity and reset of the enzyme to its starting conformation (Fig. 3.2), the overall linking number change would be -2.0. ATP hydrolysis is required for this process. However, the exact mechanism by which the ATP binding and hydrolysis steps co-
ordinate in order to couple the free energy produced to superhelical free energy cannot be deduced from these experiments. It is possible that the free energy change upon ATP binding is sufficient to stabilise the $\Delta Lk$ of $-0.75$ we have observed here, and the free-energy released upon ATP hydrolysis is required to complete the strand transport process.

Fig. 3.2. A model for the organisation of the gyrase tetramer and the mechanism of strand passage. On the left, the nucleotide-free conformation is shown, consisting of the 59-kDa domain of GyrA, the 43-kDa domain of GyrB and the $B'$ subdomain of yeast topoisomerase II, their relative positions deduced by electron microscopy observations. The structure and location of the 33-kDa domain is unknown, however a representation is shown here in an orientation that agrees with the results obtained in this paper. The conformational changes that are depicted to take place upon nucleotide binding are discussed in the text. In the gate-open conformation, the structure of the 59-kDa domain has been substituted with that of the $A'$ subdomain of yeast topoisomerase II.
The above model suggests that the presence of the T segment in the ATP-operated clamp might affect the DNA cleavage-religation equilibrium established between gyrase and the DNA segment that is bound across the DNA gate. To test this, the dependence of ADPNP-induced cleavage on DNA topology was investigated.

![Fig. 3.3. The presence of the T segment in the ATP-operated clamp induces DNA cleavage.](image)

| Table 3.2. Dependence of ADPNP-induced cleavage on DNA topology |
|-------------------------------|-------------------|-------------------|-------------------|
| DNA                           | - ADPNP cleavage efficiency % | + ADPNP cleavage efficiency % | Ratio |
| -ve s/c DNA                   | 0.16±0.07          | 1.22±0.20          | 7.6±2.0          |
| Relaxed DNA                   | 0.19±0.04          | 0.58±0.10          | 3.1±0.5          |
| +ve s/c DNA                   | 0.49±0.20          | 0.68±0.02          | 1.4±0.3          |

In the absence of nucleotide the DNA cleavage-religation equilibrium is predominantly in the ligated form, since interrupting the enzyme-DNA complex with a protein denaturant reveals only minimal amounts of cleavage. However, binding of ADPNP shifts this equilibrium, increasing the extent of cleavage (Fig. 3.3). With negatively supercoiled DNA, ADPNP induced cleavage was ~8 times more efficient than in the absence of the nucleotide, while with relaxed DNA the efficiency of cleavage increased by a factor of ~3 (Fig. 3.3 and Table 3.2). However, with positively supercoiled DNA the extent of ADPNP-induced cleavage was similar to that observed in the absence of the nucleotide (Fig. 3.3 and Table 3.2). These results provide further support for the above model and suggest that the presence of the T segment inside the ATP-operated clamp induces DNA cleavage. Moreover, due to the relatively small levels of DNA cleavage observed in these experiments, it is unlikely that the open-gate is the predominant enzyme conformation when ADPNP is...
bound. This suggests that the T segment equilibrates between the top and bottom cavities via a transient opening of the gate.

![Graph showing DNA binding to the active-site mutant A2Ser122B2](image)

**Fig. 3.4.** DNA binding to the active-site mutant A2Ser122B2 is unable to stimulate the rate of ATP hydrolysis. Reactions contained 20 nM gyrase, 2 mM ATP and the indicated amounts of DNA and the rate was measured at 25°C.

It has been proposed that the presence of a DNA segment in the ATP-operated clamp stimulates the rate of ATP hydrolysis (Maxwell & Gellert, 1984, Tingey & Maxwell, 1996). Therefore, T-segment capture appears to be related to both DNA cleavage and ATPase stimulation. We investigated the relationship between DNA cleavage and ATP hydrolysis by studying the ATPase activity of the active-site mutant A2Ser122B2. In this mutant, DNA binding did not stimulate the rate of ATP hydrolysis (Fig. 3.4). This was not due to any inhibition of the intrinsic ATPase reaction of the mutant, since the kinetics of ATP hydrolysis in the presence or absence of DNA are identical (data not shown). It appears therefore, that DNA cleavage is directly related to ATPase stimulation. Taken together these results suggest that a concerted mechanism operates in DNA gyrase, in which, trapping of a T segment induces cleavage of the DNA at the gate, an event that triggers ATP hydrolysis, which in turn is coupled to the transport and release of the T segment.

### 3.2. References


A MODEL FOR THE MECHANISM OF STRAND PASSAGE BY DNA GYRASE


Conformational changes in DNA gyrase revealed by limited proteolysis

Abstract
We have used limited proteolysis to identify conformational changes in DNA gyrase. Gyrase exhibits a proteolytic fingerprint dominated by two fragments, one of ~62 kDa, deriving from the A protein, and another of ~25 kDa from the B protein. Quinolone binding to the enzyme-DNA complex induces a conformational change which is reflected in the protection of the C-terminal 47-kDa domain of the B protein. An active site mutant (Tyr^{122} to Ser in the A protein) that binds quinolones but cannot cleave DNA still gives the quinolone proteolytic pattern, while stabilisation of a cleaved-DNA intermediate by calcium ions does not reveal any protection, suggesting that the quinolone-induced conformational change is different from an "open-gate" state of the enzyme. A quinolone-resistant mutant of gyrase fails to give the characteristic quinolone-associated proteolytic signature. The ATP-induced dimerisation of the B subunits is a key step of the gyrase mechanism. The proteolytic fingerprint of this conformation (stabilised by the non-hydrolysable ATP analog 5'-adenylyl-β,γ-imidodiphosphate) shows a protection of the 43-kDa N-terminal domain of the B subunit. The presence of quinolones does not prevent dimerisation since incubation of the enzyme-DNA complex with both 5'-adenylyl-β,γ-imidodiphosphate and quinolones gives rise to a complex whose proteolytic pattern retains the characteristic signature of dimerisation but has lost the quinolone-induced protection. As a result, the quinolone-gyrase complex can still hydrolyse ATP, albeit with different kinetic characteristics. We interpret the proteolytic signatures observed in terms of four complexes of gyrase, each representing a particular conformational state.

4.1. Introduction
DNA gyrase is a type II topoisomerase responsible for the manipulation of the topological state of DNA in bacteria (reviewed in (Reece & Maxwell, 1991b)). Catalysis by type II topoisomerases requires the hydrolysis of ATP and involves the passage of a segment
of DNA through a double-stranded break in another segment held open by the enzyme. Gyrase is distinct in its catalytic mechanism from the other enzymes of its class in that conventional type II enzymes (such as yeast topoisomerase II) cannot discriminate between DNA segments to be transported, thus favoring DNA relaxation or intermolecular strand passage (catenation/decatenation reactions). By contrast gyrase is able to dictate the direction of strand passage and, by so doing, is the only topoisomerase able to perform DNA supercoiling.

Gyrase consists of two proteins which combine to form an A$_2$B$_2$ complex that binds approximately 128 bp of DNA (Orphanides & Maxwell, 1994) in a positive superhelical sense around its core (Liu & Wang, 1978). When the A protein (GyrA, 97 kDa) is treated with either trypsin or chymotrypsin two large fragments are generated with approximate masses of 64 and 33 kDa (Reece & Maxwell, 1989). The C-terminal 33 kDa fragment, comprising the residues from 572 to 875, has been shown to bind DNA in positive superhelical sense but is unable to catalyze any of the reactions of gyrase (Reece & Maxwell, 1991a). The N-terminal fragment contains the active site tyrosine and fragments in the size range 58-64 kDa have been shown to possess catalytic activity (Reece & Maxwell, 1991c). When complexed to the B protein (GyrB), the N-terminal domain is able to perform ATP-dependent relaxation and increased DNA decatenation compared to the full length enzyme, thus behaving like a conventional type II topoisomerase (Kampranis & Maxwell, 1996). Therefore, the positive wrapping of DNA caused by the 33-kDa domains appears to be largely responsible for the unique properties of gyrase.

GyrB (90 kDa) also consists of two domains: a 43-kDa N-terminal and a 47-kDa C-terminal domain (Reece & Maxwell, 1991b). In the presence of GyrA, the 47-kDa domain cannot supercoil DNA but is able to perform all the ATP-independent reactions of gyrase (Gellert et al., 1979). The 43-kDa N-terminal domain has been produced as a separate gene product and was found to hydrolyse ATP (Ali et al., 1993). This domain has been crystallized in the presence of a non-hydrolysable ATP analog, 5'-adenyllyl-$\beta$-$\gamma$-imidodiphosphate (ADPNP), and the structure has been solved at 2.5 Å resolution (Wigley et al., 1991). As suggested by the crystal structure as well as by biochemical data (Ali et al., 1993, Ali et al., 1995), the protein forms a dimer in the presence of ATP. This dimerisation appears to be a key step in the mechanism of supercoiling. Briefly, gyrase binds DNA and wraps it in an interaction mainly supported by the 33-kDa domains of GyrA. This positions the transported segment in the right orientation for strand passage. Binding of ATP causes dimerisation of the B proteins which captures the transport segment and directs it through the double-stranded break made by the enzyme on the gate segment. ATP is then hydrolysed allowing the enzyme-DNA complex to return to its starting conformation.

Gyrase is inhibited by a number of antibacterial agents including the quinolones (Maxwell, 1997). Addition of a protein denaturant, e.g. sodium dodecyl sulphate (SDS), to a quinolone-arrested gyrase-DNA complex results in DNA cleavage (Gellert et al., 1977, Sugino et al., 1977). Cleavage does not require ATP, but ATP or ADPNP can change the...
efficiency of cleavage and alter the pattern of preference between cleavage sites (Morrison et al., 1980, Sugino et al., 1978). Efficient DNA cleavage by gyrase can also occur in the absence of quinolones if Mg\(^{2+}\) is substituted by Ca\(^{2+}\) (Reece & Maxwell, 1989).

The application of limited proteolysis can often provide useful information about the conformational changes resulting from the interaction of a protein with a substrate or effector molecule. Structured regions of a protein are usually very resistant to attack by low concentrations of endopeptidases while cleavage of the peptide backbone can occur in less structured regions or in loops linking structural domains of the protein. Using this approach it is often possible to separate functional domains of a protein and investigate their activity e.g. (Abdel-Meguid et al., 1984, Reece & Maxwell, 1989, Shiozaki & Yanagida, 1991, Stewart et al., 1996). The susceptibility of a region of a protein to cleavage can be altered when a conformational change occurs, resulting in cleavage sites being protected or new sites being revealed. Comparing the proteolytic pattern of a protein in the presence or the absence of a ligand it is possible to investigate conformational changes associated with this interaction (Lindsley & Wang, 1991, Lindsley & Wang, 1993). We have used this approach to study the interaction of DNA gyrase with quinolones and ADPNP, and we have identified characteristic proteolytic signatures for these complexes.

4.2. Results

The gyrase-DNA complex (complex I). To study the conformational changes associated with the interaction of DNA gyrase with a number of effector molecules a selection of endopeptidases was used: trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease, giving a wide range of sequence-specific cleavage sites. Trypsin is a serine protease that cleaves at the C-terminal side of arginine and lysine residues. Chymotrypsin cleaves proteins at the C-terminal side of tyrosine, phenylalanine and tryptophan, while V8 cleaves C-terminal to glutamic acid residues.

Treatment of the A subunit of DNA gyrase with 10 µg/mL of trypsin gives rise to one major product of an apparent molecular weight of ~62 kDa (Fig. 4.1 and 4.2A). In earlier times of this reaction a rather unstable band of ~33 kDa could be observed which probably represents the C-terminal domain of the protein (Reece & Maxwell, 1989). Similar treatment of GyrB produced one major fragment with an estimated size of ~25 kDa (Fig. 4.1 and 4.2A). Incubation of the individual subunits with DNA did not alter the proteolytic fingerprints (data not shown). When the heterotetramer (A\(_2\)B\(_2\)) was formed and treated with trypsin the fingerprint consisted of both the above main fragments (Fig. 4.1 and 4.2A). Formation of the enzyme-DNA complex (complex I) did not reveal any significant change in the proteolytic pattern (Fig. 4.2A), although a low level of resistance to proteolysis was evident in earlier times of the reaction. This resistance can be attributed to the stabilisation of the A\(_2\)B\(_2\) complex in the presence of DNA. N-terminal peptide sequencing of the two proteolytic products has identified the ~62 kDa fragment as the N-terminal part of GyrA starting at residue 18 (the sequence SSYLDY reflects residues 18-23 of GyrA). The smaller ~25-kDa
fragment was found to belong to the C-terminal part of GyrB. Peptide sequencing of this band revealed the sequence GKQEQY which starts at residue 540 of GyrB.

**Dimerisation of GyrB is indicated by protection of the 43-kDa domains (complex II).** An important step in the catalytic cycle of gyrase is the dimerisation of the B subunits in the presence of ATP (Ali et al., 1993, Ali et al., 1995). When the non-hydrolysable ATP analog ADPNP is used the two B subunits are locked in the dimeric form (Wigley et al., 1991). When GyrB or the A_2B_2 complex were incubated with ADPNP prior to treatment with 10 μg/mL trypsin, a protection of the N-terminal 43 kDa domain of GyrB was observed (Fig. 4.1). With time the 43-kDa fragment was slowly converted to a smaller 33 kDa product. This fragment was identified by peptide sequencing as the N-terminal part of the 43-kDa domain (N-terminal sequencing revealed the sequence SNSYDSSSIIK for both fragments). When chymotrypsin was used instead of trypsin, the 43-kDa domain was very stable to further digestion (data not shown). There was no observable difference in the ADPNP-induced fingerprint between the gyrase-DNA complex and the DNA-free enzyme (Fig. 4.1). Earlier experiments with the 43 kDa domain alone have shown that in the presence of ADPNP the protein is cleaved by trypsin at lysine 307 to give an N-terminal 33 kDa fragment which is protected from further digestion (Ali et al., 1995). It appears that residue 307 is not readily accessible by proteases when the full B protein is present since digestion of the 43 kDa domain to give a 33 kDa fragment occurs only in later time points or higher concentrations of protease.

**Fig. 4.1. The ADPNP-induced conformational change.** GyrA, GyrB, A_2B_2, or the gyrase-DNA complex were incubated for 1 hr at 25°C, with 2 mM ADPNP added to the samples where indicated. The samples were then treated with 10 μg/mL trypsin for 1 hr at 37°C and the products analysed by SDS-PAGE. On the right is a diagrammatic representation of the gyrase fragment corresponding to each band. The shaded part represents the position of the protected fragment in the respective subunit. The letter denotes the subunit (A or B) and the number indicates the approximate size of the fragment in kDa.

We addressed the relationship between DNA cleavage and ADPNP-induced dimerisation using a gyrase mutant, A_2^{Ser122}B_2, that cannot cleave DNA (Critchlow & Maxwell, 1996). When the A_2^{Ser122}B_2-DNA complex was incubated with ADPNP prior to trypsin treatment the characteristic protection of the 43-kDa domain was revealed (data not shown). These results suggest that DNA cleavage is not required for the closing of the ATP-operated clamp.

**Quinolone binding induces a conformational change which protects the 47-kDa domain of GyrB (complex III).** When the enzyme-DNA complex was incubated with 100 μM
ciprofloxacin (CFX) prior to treatment with protease, one more fragment was produced, with an approximate size of 47 kDa (Fig. 4.2A). Peptide sequencing identified this fragment as the C-terminal 47-kDa domain of the B protein starting at residue 395 (KGALD). It is interesting to note that the quinolone-resistance mutations associated with the B subunit map to the same domain of the protein (Yamagishi et al., 1986). When gyrase was incubated with CFX in the absence of DNA no protection of the 47-kDa domain was observed (Fig. 4.2A), consistent with the fact that very little binding of quinolones to gyrase is observed in the absence of DNA (Shen et al., 1989, Willmott & Maxwell, 1993, Yoshida et al., 1993).

The same digestion pattern was observed when oxolinic acid (500 µM) was used instead of CFX (data not shown) suggesting a similar mode of action between the first-generation quinolones (oxolinic acid) and the fluoroquinolones (CFX). The 47-kDa domain is very resistant to further digestion by high protease concentrations. When the enzyme-DNA-quinolone complex was treated with 100 µg/mL trypsin, little further degradation of the 47-kDa domain was observed (data not shown). The form of gyrase that is deleted for the 33-kDa domains (A642 B2), which is capable of efficient quinolone-induced DNA cleavage, also gives the characteristic complex III proteolytic signature (data not shown). Therefore, DNA wrapping by the 33-kDa domains of GyrA appears not to be necessary for the enzyme to undergo this conformational change.

This effect of quinolones was investigated further using chymotrypsin and Staphylococcus aureus V8 protease. Treatment of the enzyme-DNA complex with chymotrypsin produced a combination of a ~64-kDa product of GyrA and a ~41-kDa fragment of GyrB, to which the 47-kDa domain was added when the complex was incubated
with CFX (data not shown). V8 gave a proteolytic pattern very similar to that of trypsin (data not shown). The fact that the protection of the 47-kDa domain was observed with a variety of proteases and quinolones suggests that it is not caused by the drug directly but is a result of a conformational change. When the gyrase-DNA-quinolone complex is disrupted by SDS, the DNA is found to be cleaved and covalently attached to the enzyme (Gellert et al., 1977, Sugino et al., 1977, Tse et al., 1980). It is therefore possible that this fingerprint represents the conformation of the enzyme where the DNA is cleaved and the two segments have been pulled apart. To investigate this possibility an active-site mutant of gyrase (GyrA^{Ser122}) was used. This mutant binds quinolones as well as wild-type, but is unable to support DNA cleavage (Critchlow & Maxwell, 1996). Proteolysis of A\textsubscript{2}$^{Ser122}$B\textsubscript{2} in the presence of CFX showed the characteristic quinolone signature (Fig. 4.2B). This suggests that the quinolone-induced fingerprint does not reflect an open-gate state of the enzyme but is a result of a quite different conformational change involving the B subunits.

The specificity of the interaction between the drugs and the enzyme-DNA complex that results in the protection of the 47-kDa domain was addressed using a quinolone-resistant gyrase mutant. A variant of GyrA in which Ser\textsuperscript{83} is substituted by Trp binds quinolones very inefficiently (Willmott & Maxwell, 1993). Gyrase reconstituted with GyrA^{Trp83} was compared with wild-type gyrase for its ability to exhibit the characteristic quinolone proteolytic signature. The mutant failed to show any protection of the 47-kDa domain when incubated with concentrations of CFX in the range 0.2 – 200 μM (Fig. 4.3B). Under these conditions the protection of the wild-type enzyme was clearly seen (Fig. 4.3A).

**Calcium-induced cleavage.** When gyrase and DNA are incubated with calcium instead of magnesium ions in the absence of quinolones and the reaction is stopped by the addition of SDS and proteinase K, the DNA is cleaved (Reece & Maxwell, 1989). We have tried to distinguish between calcium- and quinolone-induced cleavage using limited proteolysis. DNA and gyrase were incubated for 2 hr under conditions that give calcium cleavage (4 mM Ca\textsuperscript{2+}), and then the mixture was treated with 10 μg/mL trypsin at 37°C.
Before the addition of protease, aliquots were removed from the proteolysis reactions and treated with SDS and proteinase K to reveal calcium-induced DNA cleavage (data not shown). Although under these conditions the level of Ca\(^{2+}\)-induced cleavage was comparable to that caused by quinolones, we have been unable to detect any protection of the 47-kDa domain in the presence of either linear or supercoiled DNA (Fig. 4.4). This suggests that the quinolone-gyrase-DNA complex (complex III) has a different conformation than the calcium-enzyme-DNA complex, in support of the above results suggesting that complex III fingerprint does not reflect a cleaved DNA complex but a quinolone-associated conformational change.

**Quinolones do not prevent dimerisation of the B subunits (complex IV).** Formation of the enzyme-DNA-quinolone complex (complex III) and incubation with ADPNP prior to protease treatment reveals protection of the 43-kDa but not the 47-kDa domains (Fig. 4.5). Similarly, pre-incubation of the gyrase-DNA complex with ADPNP followed by addition of quinolones results in the same tryptic fingerprint.

The ability of gyrase to bind quinolones in the presence of ADPNP was tested using rapid-gel filtration in the presence of a 147 bp DNA fragment based around the preferred 990 pBR322 cleavage site (Dobbs et al., 1992). The gyrase-DNA-CFX complex was compared...
to the complex formed when ADPNP was incubated with the enzyme after incubation with CFX and it was found that both these complexes bound the same amount of CFX (Fig. 4.6A). The stoichiometry of binding was close to two quinolone molecules per enzyme complex, as observed previously (Critchlow & Maxwell, 1996). When the gyrase-DNA complex was incubated with ADPNP prior to addition of the quinolone, binding of the drug was slightly reduced (Fig. 4.6A). When ADPNP was added to the gyrase tetramer in the absence of DNA, to induce closure of the ATP-operated clamp, and DNA and CFX were then added to the complex, it was found that gyrase could still bind the drug, albeit with low efficiency (Fig. 4.6A).

The ability of gyrase to cause quinolone-directed cleavage of the 147 bp fragment in the presence of ADPNP was investigated. Addition of ADPNP to the gyrase-DNA-quinolone complex resulted in a small decrease in the level of cleavage, while a greater decrease was observed when ADPNP was added before the quinolone (data not shown). When the ATP-operated clamp was dimerised in the absence of DNA and then the 147 bp fragment was

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**Fig. 4.6. Binding of CFX and ADPNP to the gyrase-DNA complex.**

**A. CFX binding.** The reactions were carried out in the presence of 5 pmol of 147 bp fragment, 15 pmol A₄₂B₂, 2 mM ADPNP and 25 μM ³²P-CFX at 25°C, but the order of addition of the ligands or DNA was varied as indicated. The gyrase-DNA complex was incubated with CFX alone for 2 h (sample labeled CFX) or, 1 hr with CFX prior to addition of ADPNP and further incubation for 1 hr (sample labeled CFX before ADPNP) or, 1 hr with ADPNP prior to addition of CFX and a further 1 hr incubation (sample labeled ADPNP before CFX). Another sample contained gyrase that had been incubated for 1 hr with ADPNP without DNA and then DNA and CFX was added and incubated for 1 hr further. **B. ADPNP binding.** Reactions were carried out at the same concentrations of enzyme and DNA as above with 200 μM CFX and 2 mM ³²P-ADPNP. The binding to gyrase and the gyrase-DNA complex was measured after a 2 hr incubation with the nucleotide at 25°C. The gyrase-DNA-CFX complex was allowed to form at 25°C for 1 hr and then radiolabelled nucleotide was added and the reaction was incubated for 1 more hr. Similarly, the gyrase-DNA-ADPNP complex was formed first and then CFX was added. Both incubations were at 25°C for 1 hr. Control experiments with ³²H-CFX or ³²P-ADPNP in which the enzyme was omitted showed that background radioactivity eluting from the column was no more than 3% of the maximum amount eluted when the enzyme was present. The values shown here have been corrected for this amount of background radioactivity.
added together with CFX, gyrase could still perform DNA cleavage, albeit at a low level (similar results were also obtained by M. O'Dea and M. Gellert (NIH, Bethesda, USA) and A. Howells (Leicester University, UK), personal communications). Using rapid-gel filtration the effect of quinolones on the binding of ADPNP was investigated (Fig. 4.6B). Quinolones did not inhibit ADPNP binding; gyrase was able to bind similar amounts of the nucleotide analog irrespective of the presence or absence of DNA or CFX. Addition of the quinolone to the complex prior to or following to the addition of ADPNP did not affect the binding of the nucleotide. In each case, the stoichiometry of binding was approximately one ADPNP molecule per GyrB monomer (Fig. 4.6B).

The fact that ADPNP binding to gyrase is slow (Tamura et al., 1992) can be used to observe the formation of the gyrase-CFX-ADPNP complex. ADPNP (250 μM) was added to the pre-formed quinolone-enzyme complex and samples were removed at certain times and treated with trypsin. Under these conditions, the slow conversion of complex III to this new conformation, complex IV, was evident (data not shown). Similarly, complex III was incubated with increasing low concentrations of ADPNP (0.1-1.0 mM) for the same amount of time and then treated with trypsin. A mixture of complex III and IV fingerprints was obtained with complex III predominating at low ADPNP concentrations and complex IV at higher (data not shown). These results are indicative of a conformational change where the quinolone-trapped gyrase-DNA complex is converted to a conformation in which the B subunits are dimerised due to the binding of ADPNP. This complex, (IV), has the same tryptic signature as complex II and its formation is independent of the order of addition of the effectors. We addressed the state of the DNA gate in complex IV by studying the proteolytic behaviour of the active-site mutant (GyrA\textsuperscript{Ser122}). Complex III was formed by incubating A\textsubscript{2}S\textsubscript{er122}B\textsubscript{2} and CFX and the ability of the active-site mutant to form complex IV was tested. We found that DNA cleavage is not required for the conversion from complex III to complex IV (data not shown).

Gyrase can hydrolyse ATP in the presence of quinolones. Since the gyrase-DNA-quinolone complex has not lost its ability to close the N-terminal clamp in the presence of ADPNP, it is likely that it can still do ATP hydrolysis. Indeed, we found that gyrase that has been pre-incubated with quinolones and DNA is still able to hydrolyse ATP albeit at a lower rate than that of the drug-free enzyme-DNA complex (Fig. 4.7). This could be due to either the gyrase-quinolone complex having different kinetic characteristics of ATPase, or the quinolones completely abolishing ATP hydrolysis but trapping only a fraction of the enzyme molecules. This second possibility was explored using the truncated form of the GyrA protein. A\textsubscript{592}B\textsubscript{2} (a version of gyrase deleted for the 33-kDa domains) does not exhibit DNA-stimulated ATPase, probably due to its weaker binding to DNA, but can perform quinolone-induced cleavage almost as efficiently as the full-length enzyme (Kampranis & Maxwell, 1996, Reece & Maxwell, 1991c). When the A\textsubscript{592}B\textsubscript{2}-DNA-quinolone complex is formed it can turn over ATP at a rate similar to that of the full-length enzyme in the presence of the drugs (Fig. 4.7). These results indicate that the quinolone-arrested gyrase-DNA complex has
adopted a conformation which still enables it to hydrolyse ATP but the kinetic characteristics of this reaction are different from those of the reaction of the drug-free enzyme.

**Fig. 4.7.** The gyrase-quinolone complex can hydrolyse ATP. ATPase reactions contained 1.5-fold excess of GyrA or GyrA59 over GyrB and 800 bp of linear pBR322 per enzyme heterotetramer. The concentration of enzyme on the abscissa is determined by the limiting concentration of GyrB. The assay was performed at 2 mM ATP and 200 µM CFX was added where indicated. The complexes were allowed to form for 2 hr at 25°C prior to the addition of ATP and the rates were measured at 25°C. The turnover numbers of the various complexes were measured to be, 0.81 s⁻¹ for the A₂B₂-DNA complex, 0.51 s⁻¹ for the A₂B₂-DNA-CFX complex, 0.13 s⁻¹ for the A₅₉₂B₂-DNA complex and 0.35 s⁻¹ for the A₅₉₂B₂-DNA-CFX complex.

**4.3. Discussion**

Limited proteolysis has previously been used in gyrase for the identification of functional domains in GyrA (Reece & Maxwell, 1989). Two domains were identified and their properties are now well established (Kampranis & Maxwell, 1996, Morais Cabral et al., 1997, Reece & Maxwell, 1991a). *In vivo* GyrB is found to be proteolysed into two fragments that have lately been shown to be different functional domains (Reece & Maxwell, 1991b). When applied to eukaryotic topoisomerase II, limited proteolysis revealed a conformational change associated with the binding of ADPNDP (Lindsley & Wang, 1991, Lindsley & Wang, 1993). This result was considered as evidence of the dimerisation of the N-terminal domains of topoisomerase II in the presence of ATP. The mechanism of supercoiling by type II topoisomerases involves the translocation of a DNA segment through an enzyme-stabilized DNA gate. This is accomplished by passing the DNA through the enzyme itself (Berger et al., 1996, Roca et al., 1996). This mechanism requires the enzymes undergoing significant conformational changes. Therefore, we decided to use limited proteolysis to identify conformational states that are involved in the catalytic cycle of gyrase as well as proteolytic fingerprints for inhibitor-trapped enzyme conformations.

**Complex I - The enzyme-DNA complex.** The first step was to establish the proteolytic signatures of the individual subunits and the holoenzyme. Treatment of GyrA with trypsin yielded only one product fragment. Due to the much higher protease concentrations used in these experiments than used previously (Reece & Maxwell, 1989), the 33-kDa domain of GyrA appeared only in earlier times of the reaction and was quickly degraded. Presumably due to the same reason, the N-terminal tryptic site was at lysine 17 rather than arginine 6, as found previously (Reece & Maxwell, 1989). The size of the product fragment was estimated to be ~62 kDa, supposing that the C-terminal tryptic site is at arginine 571, as suggested before (Reece & Maxwell, 1989). Nevertheless, it is possible that due to the higher
concentration used, trypsin was able to cleave at the stretch of arginine and lysine residues at the region 561-567. This would have resulted in a fragment with size of 61 kDa. Indeed, in some cases the band corresponding to this fragment appeared as a doublet in SDS PAGE, possibly representing a mixture of the 62- and 61-kDa fragments (Fig. 4.1-4.5). It seems unlikely that cleavage could have occurred at any other Arg or Lys residue because this would have resulted in a significant reduction in the size of this fragment which would have been evident by SDS-PAGE.

Treatment of GyrB produced only one fragment which started at Gly⁵⁴⁰. Judging by the position of this fragment on SDS PAGE and comparing with size markers, we estimated the mass of this peptide to be approximately 25 kDa. Examination of the amino-acid sequence of GyrB revealed that a fragment of that size could be produced only if cleavage had occurred in a region containing four possible tryptic sites between residues Arg⁷⁶⁰ and Lys⁷⁶⁸. Cleavage at these sites would result in peptides with sizes between 25-26 kDa. We used secondary structure and solvent accessibility prediction for GyrA and GyrB in an attempt to identify which of those residues was more likely to be accessed by proteases. Almost all the above possible cleavage sites appear to be part of loop structures making discrimination between them impossible. Using a recent sequence alignment of type II topoisomerases we identified homologous residues of those possible cleavage sites in yeast topoisomerase II (Caron & Wang, 1994). None of these residues is present in the known crystal structure of yeast topoisomerase II (Berger et al., 1996). For simplicity we decided to call this peptide a 25-kDa fragment and the one produced from GyrA a 62-kDa fragment. When the gyrase subunits were mixed to form the holoenzyme (A₂B₂) the same proteolytic fingerprint was observed suggesting that the formation of the complex did not cause any changes in the structure of the two proteins that could be detected with the proteases used in these experiments.

Formation of the gyrase-DNA complex results in the positive wrapping of ~130 bp of DNA by the enzyme (Liu & Wang, 1978, Orphanides & Maxwell, 1994). This mode of binding is believed to direct a contiguous DNA segment to the ATP-operated clamp formed by the B subunits (Kampranis & Maxwell, 1996). In this first step of the mechanism a DNA cleavage-religation equilibrium is established. DNA cleavage proceeds by the means of a transesterification reaction between a pair of tyrosine residues, one in each A subunit, and a pair of phosphates four base-pairs apart. The covalent link is with the 5' ends of the DNA while the 3' ends are left with a free hydroxyl group. Religation is the reversal of this reaction. In the absence of any cofactor this cleavage-religation equilibrium is shifted to the ligated form since treatment of the complex with SDS and proteinase K reveals only minimal levels of cleavage (chapter 3). This conformation of the gyrase-DNA complex, prior the binding or after the release of any cofactor, we term complex I (Fig. 4.8). Complex I exhibits a signature identical to that of the DNA-free enzyme, consisting of a combination of the 62-kDa and the 25-kDa fragments.
Complex II - A nucleotide-dependent conformational change. An essential subsequent step in the mechanism of supercoiling involves the closure of the ATP-operated clamp and the trapping of the transported segment inside the protein tetramer. This is effected by the dimerisation of the B subunits caused by ATP binding. The DNA gate then opens and the DNA segment is transported through the double-stranded break. ATP hydrolysis is not required for this translocation since stoichiometric supercoiling can be observed in the presence of the non-hydrolysable ATP analog ADPNP (Bates et al., 1996, Sugino et al., 1978). ADPNP binding is very tight and results in the trapping of the protein clamp in the dimerised form (Ali et al., 1995, Tamura et al., 1992, Wigley et al., 1991). This conformation of the enzyme, which is a major intermediate in the catalytic cycle, we term complex II (Fig. 4.8). ATP hydrolysis is then required to allow the enzyme to return to its native conformation (complex I). The proteolytic fingerprint of complex II is consistent with that seen before with only the 43-kDa domains of GyrB (Ali et al., 1995). Indeed, dimerisation of the 43 kDa proteins gave a resistant 33-kDa fragment which is also present in the case of the holoenzyme (A2B2). However, in the case of the holoenzyme the whole 43-kDa domain is protected as a result of the dimerisation, and the 33-kDa fragment is produced at later times. Presumably the presence of the rest of GyrB restricts access to the tryptic site at residue 307. There was no significant difference in the fingerprint of the gyrase-DNA-ADPNP and the DNA-free gyrase-ADPNP complexes. This is in agreement with previous observations suggesting that DNA is not necessary for the dimerisation of the B subunits (Ali et al., 1993) and that the presence of DNA does not affect the rate or the stoichiometry of ADPNP binding to the gyrase holoenzyme (Tamura et al., 1992). DNA cleavage is not required for the dimerisation of the B subunits since the active-site mutant A2Ser122B2, that is unable to cleave DNA, can still reveal the complex II-characteristic fingerprint.

Complex III - A quinolone-induced conformational change. Trapping of the gyrase-DNA complex by quinolones gives rise to a characteristic proteolytic fingerprint. This could be due to either a conformational change or a direct protection of a proteolytic cleavage site by the drug. The latter seems unlikely since all three proteases used in this study were able to reveal this characteristic protection. Being that a total of six different amino-acids could be targeted by all three enzymes it seems quite unlikely that a molecule the size of a quinolone (~360 Da) could have such a direct effect. Moreover, this protection was observed with more than one member of this class of compounds (CFX and OXO). The quinolone-protected fragment seems to comprise the whole C-terminal domain of GyrB since it runs next to purified 47-kDa domain on SDS-PAGE (data not shown). Nevertheless, it is still possible that cleavage could have occurred close to the C-terminus, after lysine 798, releasing a 650 Da fragment. A difference of that size would be quite difficult to determine by SDS-PAGE. This characteristic protection of the 47-kDa domain does not appear to be complete. This is evident both by the lower relative intensity of the 47-kDa band and the high proportion of 25-kDa fragment still present (Fig. 4.2A and 4.2B). Since the 25-kDa fragment derives from the 47-kDa domain, protection of this domain would result in decreased intensity of the 25-kDa
Conformational changes in DNA gyrase

band. Although significant reduction in the intensity of this band is observed (Fig. 4.2B), this never completely disappears. It is possible that a proportion of inactive protein exists in the GyrB preparation and does not participate in the formation of the gyrase-DNA-quinolone complex.

To test the specificity of the interaction between the drugs and the enzyme that leads to the protection of the 47-kDa domains, we examined the effect of quinolone-resistance mutations in the proteolytic signature. A mutation in GyrA able to significantly decrease quinolone binding, also prevented the appearance of the complex III-characteristic fingerprint. It has been shown previously that the C-terminal truncated version of gyrase (A592B2 or A642B2) is almost as efficient as the full enzyme in performing quinolone-induced DNA cleavage (Kampranis & Maxwell, 1996, Reece & Maxwell, 1991c). We tested the significance of the wrapping of the DNA around the enzyme to the proteolytic signature of the enzyme-quinolone complex. We found that the presence of the 33-kDa domains is not required for the enzyme to undergo the quinolone-associated conformational change.

Denaturation of the quinolone-trapped gyrase-DNA complex reveals covalent linkage of the enzyme to the DNA (Gellert et al., 1977, Sugino et al., 1977, Tse et al., 1980). It is therefore believed that quinolones (as well as other topoisomerase-targeting agents) interfere with the DNA cleavage-religation equilibrium by trapping the complex in a cleaved DNA conformation. To test whether the conformational state observed here reflects the structure of the complex when the DNA is cleaved, an active site mutant was used. It has been shown previously that DNA cleavage is not required for quinolone binding, since active-site mutants of gyrase that have lost their ability to cleave DNA can still bind quinolones as well as wild-type (Critchlow & Maxwell, 1996). We found that the mutation of Tyr122 to Ser does not prevent the appearance of the quinolone-characteristic proteolytic signature. Moreover, formation of the gyrase-DNA complex in the presence of calcium ions does not result in the protection of the 47-kDa domains. It is likely that calcium ions shift the cleavage-religation equilibrium to the open form, thus inducing DNA cleavage in the presence of a protein denaturant. It is therefore clear that simply stabilisation of the cleaved-DNA conformation cannot account for the conformational change observed here.

Complex IV – A strand-passage incapable intermediate. Quinolone binding does not prevent dimerisation of the ATP-operated clamp. When ADPNP is used to trap the gate in the closed form, a fingerprint similar to that of complex II is revealed. The lack of the expected protection on the 47-kDa domain is not due to any weakening of quinolone binding, as manifested by the ability of gyrase to bind the drug in the presence of the nucleotide (Fig. 4.6A). The small reduction in the level of quinolone binding observed when the complex was pre-incubated with ADPNP cannot account for the complete disappearance of the protection on the 47-kDa domain. This reduction in binding can be attributed to the trapping of DNA-free gyrase molecules by ADPNP. Such molecules would not be able to bind DNA efficiently, thus reducing the overall number of molecules able to bind quinolones. Indeed, closing of the clamp by ADPNP prior to the addition of DNA to the complex resulted in
significant reduction in the level of quinolone binding (Fig. 4.6A). Similar results were obtained when quinolone-induced DNA cleavage was studied. Cleavage was not affected by addition of ADPNP while little reduction in cleavage efficiency was observed when ADPNP was added to the gyrase-DNA complex before the drug. Closing of the ATP-operated clamp does not abolish binding and cleavage of a linear DNA fragment, although the efficiency of this process is low. Such a result is consistent with the finding of Roca and Wang (Roca & Wang, 1992) that topoisomerase II could bind and cleave linear DNA even when pre-incubated with ADPNP. It has been proposed that in this case the DNA threads through a hole in the enzyme and finds its way to the binding site surrounding the active-site tyrosines (Roca & Wang, 1992).

Despite having the same proteolytic signature, complex IV is different from complex II because of the presence of the drug. Although the characteristic protection of the 47-kDa domains has disappeared, the enzyme would still have to adopt a conformation that would prevent catalytic strand passage, which now may not be as resistant to proteases as the one in complex III. Both complexes III and II can give rise to complex IV upon incubation with the appropriate molecule (ADPNP or CFX). The $A_2^{\text{Ser122}}B_2$ complex can undergo the conformational change required to convert complex III to complex IV, suggesting that both complexes III and IV reflect conformations of the enzyme where the DNA gate is closed (although the DNA might be in the cleaved state). Moreover, this mutant revealed the characteristic complex II fingerprint when incubated with ADPNP in the absence of the drugs, indicating that DNA cleavage is not required for GyrB dimerisation and that the enzyme-operated DNA gate is probably in the closed form in the presence of the nucleotide analog. Although it is formally possible that binding of ADPNP traps the DNA gate in the open state and such a conformation does not have an observable effect on the proteolytic signature, it is suggested from DNA cleavage experiments that in the presence of the nucleotide analog the DNA is predominantly in the ligated form (chapter 3).

There is one more implication from these results. This regards the ability of the enzyme-DNA complex to remain associated during the protease treatment, despite its fragmentation. In previous work where GyrA was treated with trypsin to yield the 64- and 33-kDa fragments, these remained tightly associated after cleavage. This was evident from gel filtration experiments where the two fragments eluted from the column as one complex (Reece & Maxwell, 1989). When these two fragments were made as separate gene products and mixed together, supercoiling activity could be restored (Reece & Maxwell, 1991a). In other experiments, the 43-kDa domain of GyrB was treated with trypsin and the fragments were denatured with urea; the refolded peptides were still able to assemble and bind coumarin drugs (manuscript in preparation). Examination of the results presented here suggests a similar behavior. It is clear that had the complex not been stable, the disappearance of the 47-kDa domain protection in complex IV would not have been observed. If fragments were dissociating from the gyrase-DNA complex as soon as they were produced, then release of the 43-kDa domains would have resulted in the enzyme reverting back to the complex III
Conformational changes in DNA gyrase. This would have produced a combination of 43- and 47-kDa domain protection (we assume here that the 395 tryptic site would still be the most sensitive GyrB site in complexes III and IV, as it is in complex I). Moreover, it seems reasonable that the presence of DNA (i.e. the ~130 bp wrap) greatly improves the stability of the complex.

Quinolones do not abolish ATP hydrolysis, but in their presence gyrase hydrolyses the nucleotide in a slower rate (Fig. 4.7). Interpretation of this as incomplete binding of the drug is not plausible since under the conditions of these experiments full inhibition of supercoiling occurs. An additional piece of evidence comes from studying the truncated form of the enzyme, A592B2. In the absence of quinolones, this enzyme does not exhibit DNA-dependent ATPase in the presence of linear DNA (Kampranis & Maxwell, 1996). In the presence of the quinolones A592B2 is able to hydrolyse ATP in a rate similar to that of the drug-inhibited full-length enzyme (Fig. 4.7). The confirmation by proteolysis that dimerisation of the B subunits can still occur in the presence of the drugs suggests that the quinolone-trapped complex (complex III) hydrolyses ATP through the formation of complex IV. This pathway is shown in figure 4.8. The characteristics of this catalytic pathway are studied in the accompanying paper (Kampranis & Maxwell, 1998).

Fig. 4.8. Summary of the proteolytic signatures and their relationship to the gyrase catalytic cycle. On the left, a diagrammatic representation is given of the various fragments produced by the tryptic treatment of gyrase in the presence of ligands. On the right, a summary is shown of the four conformational states identified in these experiments together with a representation of their proteolytic signatures. The relationship of these complexes with the gyrase catalytic pathway or the non-productive cycle undergone by the enzyme in the presence of the drugs is also demonstrated.
Mechanistic implications. The blocking of supercoiling by quinolones contrasts with their ability to induce DNA cleavage. Moreover, quinolone-directed DNA cleavage is sequence-specific in that under certain conditions full cleavage occurs only at certain sites while cleavage at other locations takes place with less efficiency (Kirkegaard & Wang, 1981, Morrison et al., 1980, Sugino et al., 1978). It appears that there would be complexes formed between gyrase and certain DNA sites in which the cleavage-religation equilibrium in the presence of the quinolones is shifted to the ligated form. The drugs have to fulfill the dual role of blocking strand passage from all these complexes but allowing the DNA to be in the cleaved form in some of them. We believe that the mechanism of inhibition of gyrase by quinolones has its basis on the conformational change seen here. Quinolones could block supercoiling by stabilising a conformation of the enzyme where, irrespective of whether the DNA is cleaved or not, the DNA gate cannot open sufficiently to allow strand passage. We believe that complexes III and IV reflect such a conformation. It is possible that in the presence of quinolones the 47-kDa domains of GyrB come close together, stabilising the closed DNA gate conformation and blocking strand-passage. Such a conformational change could result in the proteolytic protection of these domains. The fact that DNA cleavage is not required for quinolones to induce this conformational change explains how quinolones block supercoiling from all gyrase-DNA complexes, irrespective of the state of DNA cleavage at those sites.

Conclusions. We have been able to identify a number of proteolytic signatures of DNA gyrase. These fingerprints have been interpreted in terms of four different complexes each representing a different conformation of the enzyme. Two of these complexes are part of the normal catalytic cycle of gyrase while the other two are part of an non-productive cycle undergone by the enzyme in the presence of quinolone drugs (Fig. 4.8). Quinolone binding stabilises a conformational state of the enzyme that is responsible for the inhibition of its activity. DNA cleavage is not required for the binding of the drugs or the induction of this conformational change. We suggest that inhibition of supercoiling by quinolones is the consequence of a drug-induced conformational change involving the 47-kDa domains of GyrB and that DNA cleavage is a subsequent slower step. Further evidence for this proposal is presented in the accompanying paper (Kampranis & Maxwell, 1998).

4.4. References
CONFORMATIONAL CHANGES IN DNA GYRASE

The DNA gyrase-quinolone complex: ATP hydrolysis and the mechanism of DNA cleavage

Abstract
Quinolone binding to the gyrase-DNA complex induces a conformational change that results in the blocking of supercoiling. Under these conditions gyrase is still capable of ATP hydrolysis which now proceeds through an alternative pathway involving two different conformations of the enzyme. The kinetics of ATP hydrolysis via this pathway have been studied and found to differ from those of the reaction of the drug-free enzyme. The quinolone-characteristic ATPase rate is DNA-dependent and can be induced in the presence of DNA fragments as small as 20 bp. By observing the conversion of the ATPase rate to the quinolone-characteristic rate, the formation and dissociation of the gyrase-DNA-quinolone complex can be monitored. Comparison of the time dependence of the conversion of the gyrase ATPase with that of DNA cleavage reveals that formation of the gyrase-DNA-quinolone complex does not correspond to the formation of cleaved DNA. Quinolone-induced DNA cleavage proceeds via a mechanism consisting of two cleavage events that is modulated in the presence of a nucleotide cofactor. We demonstrate that quinolone binding and drug-induced DNA cleavage are separate processes constituting two sequential steps in the mechanism of action of quinolones on DNA gyrase.

5.1. Introduction
DNA gyrase is the intracellular target of the quinolone group of antibacterial agents. The addition of quinolone drugs to an in vitro reaction containing DNA gyrase, relaxed closed-circular DNA, and ATP leads to the inhibition of supercoiling (Gellert et al., 1977). If this reaction is terminated by the addition of a protein denaturant, like sodium dodecyl sulphate (SDS), the DNA is found to be cleaved in both strands with a gyrase A subunit (GyrA) covalently attached to each 5'-phosphoryl terminus (Tse et al., 1980). Quinolones
also inhibit the relaxation, catenation and decatenation reactions of gyrase (Gellert et al., 1977, Hallett & Maxwell, 1991).

The molecular details of the interaction of the drugs with the gyrase-DNA complex are unknown. When the binding of the drugs to the enzyme was investigated, insignificant binding of quinolones to the A or B subunit (GyrB) or to the gyrase holoenzyme (A₂B₂) was detected (Shen et al., 1989b, Willmott & Maxwell, 1993, Yoshida et al., 1993). Binding of the drugs to the DNA is weak, and more efficient in the case of single- compared to double-stranded DNA (Shen et al., 1989a, Shen & Pernet, 1985). Quinolones were found to bind strongly to the complex formed by gyrase and DNA, and ATP appeared to assist this interaction (Shen et al., 1989b). These results led to the proposal that the drugs bind to the single-stranded DNA region that is revealed when gyrase cleaves DNA during turnover (Shen et al., 1989c). This proposal was addressed by site-directed mutagenesis of the gyrase active-site. Mutation of tyrosine 122 to serine or phenylalanine abolished supercoiling and quinolone-induced DNA cleavage but the mutants could still bind the drugs equally well as wild-type (Critchlow & Maxwell, 1996). Clearly DNA cleavage is not required for drug binding. Similar results have also been obtained with topoisomerase IV (Marians & Hiasa, 1997).

Using limited proteolysis we found that quinolones stabilise a conformational change in the gyrase-DNA complex (Kampranis & Maxwell, 1998). DNA cleavage is not required for the enzyme to undergo this conformational change since a mutant that binds quinolones but cannot cleave DNA gives the same proteolytic fingerprints in the presence of the drugs as the wild-type enzyme. We suggested that this quinolone-stabilised conformation is responsible for the inhibition of the gyrase functions. At the molecular level this conformation was interpreted as a state of the enzyme where the DNA gate is trapped in the closed form irrespective of the DNA cleavage-religation state. ATP hydrolysis could still occur when gyrase was in the quinolone-trapped state but the complex exhibited a rate of hydrolysis that was lower than that of the drug-free enzyme. In order to shed more light on the interaction of quinolones with gyrase we have studied the characteristics of ATP hydrolysis by the enzyme-drug complex. We show that the rate of ATP hydrolysis can provide a sensitive tool for the monitoring of the formation of the gyrase-quinolone complex and we use this tool to probe the relationship between drug binding and drug-induced DNA cleavage.

5.2. Results

The kinetics of ATP hydrolysis in the presence of quinolones. In the preceding paper (Kampranis & Maxwell, 1998), we described the formation of two different complexes in the presence of quinolones and suggested that the drug-inhibited enzyme can hydrolyse ATP by undergoing a different catalytic cycle entailing these two complexes. To provide further support for the existence of this alternative pathway, we have analysed the kinetics of ATP hydrolysis in the presence of the drugs (Fig. 5.1).
The interaction of gyrase with ATP leading to nucleotide hydrolysis is far from simple, and it has been shown that the Michaelis-Menten paradigm cannot describe sufficiently the experimental data obtained (Lindsley & Wang, 1993, Maxwell et al., 1986). This is mainly due to the presence of two binding sites for ATP and the requirement for B subunit dimerisation in order for nucleotide hydrolysis to take place (Ali et al., 1993). A more comprehensive model describing the ATP hydrolysis by DNA gyrase is shown below:

\[
E + ATP \rightleftharpoons _{K_d} E.ATP + ATP \rightleftharpoons _{K_d} E.ATP, \quad \rightarrow _{k_3} E + 2ADP + 2Pi
\]  

(1)

There is presently no experimental evidence for ATP hydrolysis taking place when the nucleotide is bound to only one of the two ATP-binding sites. Moreover, when the A₂B₂ tetramer was reconstituted with a mixture of wild-type GyrB and a mutant that cannot bind the nucleotide it was found that both GyrB subunits must be functional in nucleotide binding in order for catalytic supercoiling to occur (O'Dea et al., 1996). Therefore, in this mechanism we assume that both ATP-binding sites have to be filled before hydrolysis can take place. The steady state equation describing this interaction is shown in the appendix. In scheme 1 \(k_3\) reflects the apparent rate constant of the ATP hydrolysis/product release step of the mechanism. Because of the experimental setup used in these experiments these two steps are indistinguishable. Our assay measures changes in ADP concentration and therefore the rate of hydrolysis measured depends on the rate by which ADP is released from the complex. By fitting the data in Fig. 5.1 to eq. 15 we obtained values of \(k_3\) of 0.56 ± 0.03 s⁻¹ in the absence of CFX and 0.27 ± 0.04 s⁻¹ in the presence of the drug, i.e. the final step of the ATPase mechanism is inhibited by the drugs by a factor of ~2. The first steps of the ATPase mechanism, namely the two successive nucleotide binding steps, are represented in this model by the equilibrium dissociation constants \(K_{d1}\) and \(K_{d2}\) respectively. The interaction of ATP with the two binding sites in the drug-free complex appears to exhibit positive co-operativity.
with \( K_{d2} \) being approximately one order of magnitude lower than \( K_{d1} \). The values of these constants were estimated to be \( K_{d1} \approx 590 \mu M \) and \( K_{d2} \approx 70 \mu M \). The gyrase-drug complex, exhibits a similar equilibrium dissociation constant for the first step, \( K_{d1} \approx 530 \mu M \), but the second step has a \( K_{d2} \) of \( \approx 260 \mu M \); it seems that co-operativity between the two ATP sites is significantly reduced in the presence of quinolones.

The quinolone-characteristic rate is DNA-dependent. The dependence of the quinolone-characteristic ATPase rate on DNA concentration was investigated. \( A_2B_2 \) and the version of gyrase that has been deleted for the 33-kDa domains, \( A_{592}B_2 \), were incubated with increasing concentrations of linear pBR322 and the ATPase rate in the presence of CFX was measured (Fig. 5.2). Drug-free gyrase showed the well documented increase in the rate of hydrolysis with increasing DNA concentrations (Maxwell & Gellert, 1984). \( A_{592}B_2 \) was unable to show any increased ATPase in the absence of CFX in the range of DNA concentrations used in these experiments. This characteristic of \( A_{592}B_2 \) has been seen previously and was attributed to the weak DNA binding of this enzyme (~10-fold weaker than \( A_2B_2 \) (Kampranis & Maxwell, 1996)). In the presence of the drug, the ATPase activity of \( A_2B_2 \) exhibited a steep dependence on DNA concentration at low DNA:enzyme ratios and reached a plateau after \( \approx 200 \text{ bp:enzyme} \). The plateau rate was \( \approx 0.63 \text{ s}^{-1} \). As described previously (Kampranis & Maxwell, 1998), when incubated with quinolones and DNA, \( A_{592}B_2 \) exhibits a rate of hydrolysis similar to that of the drug-bound full-length enzyme-DNA complex. The dependence of this rate to DNA concentration is not as steep as in the case of \( A_2B_2 \). At the DNA concentrations used in this experiment the \( A_{592}B_2 \)-DNA-CFX rate seemed to reach a plateau value which approaches the maximum \( A_2B_2 \)-DNA-quinolone rate.

![Figure 5.2](image_url)

**Figure 5.2.** The dependence of the quinolone-characteristic rate on DNA concentration. The reactions contained 25 nM GyrB dimer, 37.5 nM GyrA or GyrA59 dimer and 200 \( \mu M \) CFX where indicated. The indicated concentrations of linear pBR322 DNA were added and the samples were incubated for 1 hr at 25°C. After the incubation, ATP was added to a final concentration of 2 mM and the rate of ATP hydrolysis was measured at 25°C.

We investigated the effect of DNA length on the quinolone rate. Fragments in the range 20-198 bp based on the preferred cleavage site of pBR322 were constructed as described in Experimental Procedures. Large DNA fragments, such as the 198 bp and the 147 bp fragment, when incubated with \( A_2B_2 \) and CFX, showed the quinolone characteristic rate at stoichiometric ratios of enzyme:DNA (Fig. 5.3A). In the case of smaller size fragments, such as a 90-mer or a 80-mer, higher concentrations of DNA were required for reaching the
maximum rate. We found that DNA fragments as short as 20 bp are sufficient for inducing the quinolone-complex characteristic rate (Fig. 5.3B). The ability of short fragments to form the enzyme-DNA-quinolone complex was observed for both $A_2B_2$ and the truncated form $A59_2B_2$ (Fig. 5.3B). Again the concentration of 20-mer or 40-mer required for reaching the maximum quinolone-rate was higher than stoichiometric.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{The effect of DNA length on the formation of the gyrase-quinolone complex. 20 nM $A_2B_2$ or $A59_2B_2$ complex (reconstituted with 20 nM GyrB dimer and 30 nM GyrA or GyrA59 dimer) was mixed with the indicated concentrations of DNA fragments in the presence of 200 $\mu$M CFX. The DNA fragments had lengths from 20-198 bp and were all based on the pBR322 sequence surrounding the preferred quinolone cleavage site at position 990 (Dobbs et al., 1992). The complexes were allowed to form for 3 hrs at 25°C and the ATPase rate was measured at 2 mM ATP. A, The interaction between $A_2B_2$ and the 198, 147, 90, 80 bp fragments. B, the interaction of $A_2B_2$ and $A59_2B_2$ with the 40 bp and the 20 bp DNA fragments.}
\end{figure}

Conversion to the quinolone-characteristic rate monitors enzyme-drug complex formation. DNA gyrase and linear pBR322 were incubated with increasing concentrations of CFX (1 nM - 10 $\mu$M) and the ATPase rate was measured. The rate of hydrolysis was reduced from 0.98 s$^{-1}$ in the absence of CFX to 0.6 s$^{-1}$ at the highest drug concentration (Fig. 5.4A). We fitted these data to a rectangular hyperbola (see eq. 24 in the appendix) and estimated the inhibitory effect of CFX. An inhibition constant ($K_{i,q}$) was defined as the drug concentration at which half-maximal conversion to the quinolone rate occurs. This constant was measured to be $K_{i,q} = 31 \pm 5$ nM$^{-1}$. As seen before, $A59_2B_2$ does not have a DNA-dependent ATPase

\footnote{The gyrase concentration present in this determination (20 nM) is close to the measured value of $K_{i,q}$, and at low CFX concentrations the concentration of added drug would not be equal to the concentration of free drug in solution. For this, the above results are better interpreted using eq. 26 which takes into account this effect (see appendix). Analysis of the above data with eq. 26 yielded a value of $K_{i,q} = 8.4 \pm 1.3$ nM.}
activity, while in the presence of quinolones it exhibits the quinolone-characteristic rate. When the same experiment was performed with the A592B2 complex the rate increased with increasing concentrations of drug (Fig. 5.4A inset). The quinolone-induced rate reached a maximum at ~0.5 s\(^{-1}\). The A592B2-DNA-quinolone complex appeared to be significantly less stable than the respective A2B2 complex requiring much higher concentrations of the drug for its formation. The inhibition constant for this complex was determined to be \(K_{iq} = 36 \pm 8 \, \mu M\). As seen previously, the impaired affinity of short DNA fragments for gyrase requires higher DNA concentrations for the formation of the complex. We reported earlier that the affinity of A592B2 for DNA is approximately 10-times lower than the A2B2 complex (Kampranis & Maxwell, 1996). Therefore, the result obtained with A592B2 is likely to be a manifestation of the weaker stability of the A592B2-DNA complex.

**Figure 5.4.** The rate of ATP hydrolysis monitors the formation of the gyrase-quinolone complex. The formation and dissociation of the gyrasequinolone complex was studied by observing the rate of ATP hydrolysis. A, 20 nM A2B2 (reconstituted with 20 nM GyrB dimer and 30 nM GyrA dimer) was mixed with 16 \(\mu M\) bp linear pBR322 DNA and the indicated concentrations of CFX and incubated for 1 hr at 25°C. ATP was then added at 2 mM and the ATPase rate was measured at 25°C. The result were fitted to a rectangular hyperbola described in equation (24) in the appendix and the concentration of CFX where half maximal conversion of the ATPase occurs was determined. Inset, 20 nM A592B2 complex (reconstituted with 20 nM GyrB dimer and 30 nM GyrA59 dimer) was treated identically to the A2B2 complex in the presence of the indicated concentrations of CFX. B, The dissociation of the quinolones from the enzyme-DNA-drug complex. 4 pmol A2B2 (reconstituted with 4 pmol GyrB dimer and 6 pmol GyrA dimer) was mixed with 3 nmol bp of linear pBR322 and 40 nmol CFX and incubated for 2 hr at 25°C. The enzyme-DNA complex was then separated from the free drug by gel filtration through a Sephadex G-50 spin-column (Nick spin-columns, Pharmacia). The eluted complex was introduced into an ATPase reaction and the rate was monitored. A similar sample in which CFX had been omitted was treated in parallel (control sample). The rate of hydrolysis measured for the control sample was stable with time. To account for any loss in enzyme in the gel-filtration step, the rate of the control sample was set arbitrarily to 1 s\(^{-1}\). The rate of hydrolysis of the quinolone containing sample was measured for 10 min periods and the turnover numbers were then estimated relative to the control sample and displayed versus time. The times at the middle of each 10 min period were used as time-points for each of these rates. The apparent dissociation rate constant was determined by fitting the results to a simple exponential equation.

We studied the dissociation of quinolones from the enzyme-drug complex. The gyrase-DNA-quinolone complex was formed and the enzyme-DNA complexes were separated from the free drug by gel filtration through a spin-column. The enzyme complex
was then introduced into an ATPase reaction and the rate was monitored. Under these conditions the rate of ATP hydrolysis reverted from the quinolone rate to the drug-free one. The rate of hydrolysis was plotted against time and this plot was used to estimate the dissociation rate constant of the drug from the complex (Fig. 5.4B). The apparent dissociation rate constant was measured to be $k_{\text{obs off}} = 3 \times 10^{-4} \text{ s}^{-1}$. As we will discuss later, the interaction of quinolones with gyrase appears to involve a number of different steps. Therefore, $k_{\text{obs off}}$ is an observed dissociation rate constant representing the overall dissociation process. The value of $k_{\text{obs off}}$ determined by this method represents a lower limit in the apparent dissociation rate since the drug was not fully removed from the reaction after it had dissociated from the complex. Rebinding would result in a slower apparent dissociation phase.

*The interaction of gyrase with small DNA fragments is slow.* We examined the time-dependence of the appearance of the quinolone-characteristic ATPase rate. With large fragments (198 and 147 bp) or pBR322 DNA the interaction between the enzyme-DNA complex and the drugs was completed quickly after the addition of the drug so no change in the ATPase rate could be observed (data not shown). When we studied the time-dependence of the interaction of gyrase with a 40-mer we found that the appearance of the quinolone-characteristic rate was a slow process (Fig. 5.5). Similar results were found for a 20 bp fragment.

![Fig. 5.5. The formation of the A592B2-DNA-quinolone complex in the presence of short DNA fragments is slow. 20 nM A592B2 complex (reconstituted with 20 nM GyrB dimer and 30 nM GyrA59 dimer) was incubated with the indicated concentrations of the 40 bp fragment in the presence of 200 μM CFX at 25°C. The rate was measured over 30 min periods and was plotted against the time at the middle of this period. The results are fitted to eq. 34 described in the appendix.](image)

This phenomenon was common for both A₂B₂ and A₅₉₂B₂ and the appearance of the quinolone rate showed the same time-dependence for both complexes (data not shown). We measured the rate of ATP hydrolysis over a period of 3 hr and plotted the values against time (Fig. 5.5). From this plot we could estimate the apparent rate of the formation of the enzyme-drug complex by fitting to a simple exponential. The half-life of the conversion to the quinolone complex was ~20 min at 300 nM 40-mer and 20 nM A₅₉₂B₂. When the concentration of the 40 bp fragment was varied (50 – 300 nM) the apparent rate of complex formation increased with DNA concentration (Fig. 5.5). However, the relationship between rate of complex formation and DNA concentration was not linear. A three-fold increase in
DNA concentration, from 100 nM to 300 nM, resulted only in 50% increase in the rate of complex formation, from 4x10^4 s⁻¹ to 6x10^4 s⁻¹.

Quinolone binding does not correspond to the formation of the cleaved DNA complex. We investigated the time dependence of the conversion to the quinolone ATPase rate and that of drug-induced DNA cleavage using linear or negatively supercoiled pBR322 as substrate and concentrations of CFX in the range 50 nM – 15 µM. Conversion to the quinolone-characteristic rate was always immediate. Even at the lowest concentration of CFX used (50 nM) no evidence for a slow conversion of the ATPase rate was apparent. By contrast, when the time dependence of DNA cleavage was studied, the formation of the cleaved complex was very slow (data not shown). Similar experiments with gyrase and enoxacin also suggested that the formation of the cleaved complex is slow (Scheirer & Higgins, 1997). The rate of quinolone-directed cleavage increased with drug concentration but the slow formation of the product was clearly observable throughout the range of drug concentration used (up to 15 µM).

We set out to test the slow formation of the cleaved complex using relaxed DNA as substrate and substoichiometric ratios of enzyme to DNA (enzyme:DNA = 0.9). Under these conditions, on average less than one cleavage event is expected per plasmid. Therefore substrate linearisation can be viewed as the measure of double-stranded DNA cleavage. Using relaxed DNA as substrate presents distinct advantages. First, gyrase would bind almost equally well to all the topological isomers present, unlike the case of supercoiled DNA where binding to the nicked species would be favoured (investigation of the reaction using negatively supercoiled DNA would require high purity nick-free DNA); preferential binding would result in more than one cleavage event per DNA molecule. Second, using relaxed DNA the time dependence of the inhibition of supercoiling could be followed.

Figure 5.6A shows the formation of the cleaved complex at 5 µM CFX in the absence of nucleotide cofactor (note that the gel contains chloroquine, so that the relaxed DNA runs as positively supercoiled and is thus resolved from the nicked band). The slow appearance of the linear form is clearly evident. Under these conditions, the intensity of the open circular band increases in the first stages of the reaction, reaches a maximum after 15 min, and then decreases to return back to a level similar to that in the beginning of the reaction. Control experiments carried out in the absence of either the drug or the enzyme showed that this phenomenon was caused by the enzyme-drug complex. Singly-nicked DNA could be produced by the gyrase-quinolone complex if cleavage occurred only in one of the two DNA strands. Accumulation of the singly-nicked species in the early stages of the cleavage reaction suggests that this is an intermediate in the cleavage reaction. The amount of the singly- and doubly-nicked products was quantified and plotted against time (Fig. 5.6B).

The plot is typical of a reaction proceeding through an intermediate product. We can describe the cleavage reaction using the following simple scheme:

\[
\text{closed - circular DNA} \xrightarrow{k_1} \text{singly - nicked DNA} \xrightarrow{k_2} \text{doubly - nicked DNA} \tag{2}
\]
where $k_1$ and $k_2$ are the apparent rate constants for the formation of the singly- and doubly- nicked DNA products respectively. 

A.

![Image](https://example.com/image.jpg)

**Fig. 5.6. DNA cleavage is a slow reaction proceeding via two single-strand scission events.** Cleavage experiments were performed as described in the methods section. A, Typical experiment showing the cleavage of relaxed pBR322 at 5 μM CFX in the absence of ATP. The 1% agarose gel contains 3 μg/mL chloroquine. B, The intensity of the nicked and linear bands was quantified and the extent of single- or double stranded cleavage caused by the enzyme-quinolone complex was estimated. The results are plotted as percentage of maximum total cleavage. Filled rectangles (■) indicate the singly-nicked product, open circles (○) represent the doubly-nicked product and filled circles (•) represent the sum of the two products. C, The results of a similar experiment carried out in the presence of 2 mM ATP and 5 μM CFX. D, The enzyme-DNA complex was incubated with 2 mM ADPNP for 1 hr and then 500 nM CFX was added and the cleavage reaction was monitored with time. The results were fitted to eq. 4-6 by allowing both rate constants to fluctuate.

The concentration of each of these species at any time is described by the following equations:

$$[ccDNA] = [ccDNA]_0 e^{-k_2 t}$$

(3)

$$[snDNA] = [ccDNA]_0 \frac{k_1}{k_2 - k_1} (e^{-k_2 t} - e^{-k_1 t})$$

(4)

$$[dnDNA] = [ccDNA]_0 (1 - \frac{k_1 e^{-k_1 t} - k_2 e^{-k_2 t}}{k_2 - k_1})$$

(5)

$$[snDNA] + [dnDNA] = [ccDNA]_0 (1 - e^{-k_1 t})$$

(6)
The abbreviations ccDNA, snDNA and dnDNA represent the closed-circular, singly-nicked and doubly-nicked forms of DNA described in eq. 2. \([\text{ccDNA}]_0\) is the concentration of the cleavage-proficient gyrase-quinolone complexes at the beginning of the reaction. By fitting the data obtained to the above kinetic model we were able to determine the values of the two apparent rate constants. The apparent rate constant for the first cleavage event was readily measured from eq. 6 to be \(k_1 = 1.6 \pm 0.1 \times 10^{-3}\) \(s^{-1}\). However, the apparent rate constant for the second step could not be uniquely defined when \(k_1\) was fixed at the above value. For this reason, the interaction was simulated using the program KFitSim (D. Thomas., N. Millar) and the value of \(k_2\) giving the best fit was determined to be \(k_2 \sim 1.9 \times 10^{-3}\) \(s^{-1}\).

We studied the cleavage reaction in the presence of ATP (Fig. 5.6C). In this experiment the fast binding and inhibition of gyrase by the drugs was clearly evident; the strand-passage reaction was blocked immediately after addition of the drug and no supercoiling was observed (data not shown). DNA cleavage was again slow and proceeded via a singly-nicked intermediate as seen previously. The relative amounts of the three DNA species were quantified and the apparent rate constants for the two cleavage steps were determined. The cleavage reaction in the presence of ATP was faster than in the absence of the nucleotide. At 5 \(\mu\)M CFX the values of the rate constants for the two cleavage steps were \(k_1 = 2.2 \pm 0.5 \times 10^{-3}\) \(s^{-1}\) and \(k_2 \sim 4.2 \times 10^{-3}\) \(s^{-1}\). These rates are faster than those observed in the absence of nucleotide at the same drug concentration (Fig. 5.6A,B) suggesting that the presence of the nucleotide increases the rate of the reaction. We examined the cleavage reaction in the presence of ATP over a range of drug concentrations. It was found that the rate of cleavage increased with quinolone concentration. Values for \(k_1\) were determined to be \(2.5 \pm 0.8 \) and \(3.8 \pm 0.5 \times 10^{-3}\) \(s^{-1}\) at 10 \(\mu\)M and 15 \(\mu\)M CFX respectively while \(k_2\) increased to \(\sim 5.3\) and \(\sim 7.1 \times 10^{-3}\) \(s^{-1}\) at these drug concentrations. A similar investigation was carried out in the presence of the non-hydrolysable ATP analog, 5'-adenylyl-\(\beta,\gamma\)-imidodiphosphate (ADPNP). The two steps of the cleavage reaction were again apparent in the presence of the nucleotide analogue (Fig. 5.6D). The rate constants representing the two cleavage steps were also dependent on drug concentration and at 0.5 \(\mu\)M CFX were determined to be \(k_1 = 7.5 \pm 0.5 \times 10^{-4}\) \(s^{-1}\) and \(k_2 \sim 3.2 \times 10^{-3}\) \(s^{-1}\).

5.3. Discussion

The kinetics of ATP hydrolysis. Using limited proteolysis we identified two conformational states of DNA gyrase in the presence of quinolone drugs (complex III and complex IV) and suggested that ATP hydrolysis in this case proceeds through an alternative pathway involving complexes III and IV (Kampranis & Maxwell, 1998). We have addressed this argument further by investigating the kinetic characteristics of hydrolysis by the drug-bound complex. The kinetics of hydrolysis can be described by the scheme shown in eq. 1. Essentially the two nucleotide-binding steps are represented with the respective equilibrium dissociation constants (\(K_{d1}\) and \(K_{d2}\)) and the hydrolysis/product release step with the apparent
first order rate constant $k_3$. Drug binding reduces the rate of the overall hydrolysis step ($k_3$) by a factor of $\sim 2$. The equilibrium dissociation constants determined by this method should be treated as estimates as the data are inadequate to uniquely define these values. Nevertheless, the results suggest that the two successive nucleotide binding steps exhibit positive cooperativity. By contrast, in the case of the drug-bound enzyme, cooperativity seems to be less pronounced. This effect can be rationalised on the basis of the conformational change induced by the drug. For cooperativity to take place a mechanism of allosteric interaction must operate between the two GyrB proteins. As the conformational change observed by proteolysis has its major effect on the C-terminal domain of GyrB (Kampranis & Maxwell, 1998), it is not difficult to imagine how such a change in protein conformation could affect allosteric communication between the two subunits.

*The DNA dependence of the quinolone characteristic ATPase rate.* We studied the DNA dependence of the formation of the gyrase-DNA-quinolone complex by looking at the conversion of the ATPase rate. The formation of the complex was very efficient at low DNA concentrations and reached a plateau when the DNA to enzyme ratio exceeded 200 bp per gyrase molecule. The footprint of gyrase is $\sim 130$ bp (Orphanides & Maxwell, 1994). Nevertheless, two enzyme molecules that are bound at sites that are separated by a stretch of DNA smaller than the footprint of the protein exclude this part of the DNA from being bound, so that the effective length of DNA occupied by a DNA binding protein is much larger than its footprint (McGhee & von Hippel, 1974). Therefore, the "critical" concentration of $\sim 200$ bp per enzyme, for the drug-bound complex, represents here the minimum amount of DNA required for all the gyrase molecules to be bound. These results indicate that at low DNA concentration the formation of the enzyme-DNA-drug complex is limited by the amount of DNA present in the reaction and as soon as there is enough DNA there for all the enzyme molecules to be bound, the maximum rate is reached. The fact that the quinolone rate plateaus at this critical DNA concentration suggests that the gyrase-DNA-quinolone complex does not form only at certain preferred sites on DNA. If this was the case, a lot more DNA would have been required for the ATPase rate to level out. In contrast to quinolone-induced DNA cleavage that takes place at preferred DNA sequences (Kirkegaard & Wang, 1981, Morrison *et al*., 1980, Sugino *et al*., 1978), any sequence of DNA long enough to accommodate a gyrase molecule can give rise to the enzyme-quinolone complex. The dependence of the formation of the enzyme-DNA complex on DNA concentration is steeper in presence of the drug. This can be explained in terms of the higher stability of the quinolone-gyrase-DNA complex as opposed to the drug-free one. Being that the footprint of A59$_2$B$_2$ is much smaller than that of full-length gyrase (Kampranis & Maxwell, 1996), it would be expected that the rate of this complex in the presence of the drugs would have reached a plateau at DNA concentrations lower than those required in the case of the A$_2$B$_2$-quinolone complex. As we will discuss below the stability of the A59$_2$B$_2$-DNA-quinolone complex is impaired due to the weaker affinity of this enzyme for DNA.
higher DNA concentrations for reaching the maximum ATPase rate in this case is probably a manifestation of this poor stability.

The effect of DNA length. We found that a DNA fragment as short as 20 bp is sufficient for inducing the quinolone-characteristic rate. This is in agreement with the results of Cove et al. and Gmunder et al. who found that gyrase can cleave DNA fragments as small as 20 bp in the presence of quinolones (Cove et al., 1997, Gmunder et al., 1997). DNA fragments smaller than 100 bp were shown to be unable to stimulate efficiently the ATPase of drug-free gyrase and when they did so, the need for DNA binding at two sites on the enzyme was evident (Maxwell & Gellert, 1984). By contrast, the results obtained here with the 20 bp fragment suggest that DNA wrapping or occupation of those two sites is not required for the induction of the quinolone complex-characteristic rate.

The affinity of the enzyme for DNA seems to be an important factor affecting the stability of the gyrase-quinolone complex. Firstly, induction of the quinolone-characteristic rate in the presence of small DNA fragments (<100 bp), that are known to have reduced affinity for the enzyme (Maxwell & Gellert, 1984), requires much higher DNA concentrations than when larger (>147 bp) fragments were used (Fig. 5.3A and 5.3B). Secondly, deletion of the 33-kDa domains in the case of A592B2 results in significant reduction in the ability of this enzyme to bind DNA (Kampranis & Maxwell, 1996). When we looked at the appearance of the quinolone-characteristic rate in the case of A592B2 we found that the A592B2-DNA-quinolone complex requires much higher concentrations of DNA and drug for its formation than the full length gyrase (Fig. 5.2 and 5.4B). We reported above that gyrase requires approximately 200 bp of a linear DNA molecule for quinolone-complex formation which appears to contradict the requirement for only 20 bp found with short fragments. However, being that complexes formed with small DNA fragments are less stable than the complexes formed with larger fragments, it seems reasonable that although only 20 bp are sufficient for complex formation, this complex is not stable enough to be the major species when linear pBR322 DNA is used.

The gyrase-quinolone complex. Taking into account the association of the enzyme-DNA complex and the slow cleavage step seen in the result section, the formation of the gyrase-quinolone complex can be described by the following simple scheme:

\[ E + DNA \rightleftharpoons E.DNA + Q \rightleftharpoons E.DNA.Q \rightleftharpoons E.DNA.Q^* \]

In this scheme, \( K_D \), \( K_Q \) and \( K_C \) describe the apparent equilibrium dissociation constants for the DNA binding, quinolone binding and DNA cleavage steps respectively. \( E.DNA.Q \) and \( E.DNA.Q^* \) represent the drug-bound complex in the DNA ligated and cleaved form respectively. A similar scheme has been proposed by Scheirer and Higgins (Scheirer & Higgins, 1997). In this case only a single form of the enzyme-DNA-quinolone complex was proposed. We believe we have evidence for the existence of at least 2 distinct quinolone-bound complexes.
For simplicity, the binding of quinolones to the enzyme-DNA complex is represented in scheme (7) by a single step although the interaction is clearly more complicated. Although no definitive experimental evidence exists for the exact stoichiometry of the complex, recent data suggest that this is probably two quinolone molecules per enzyme-DNA complex (Critchlow & Maxwell, 1996, Kampranis & Maxwell, 1998). In the recent crystal structure of GyrA59 the known quinolone resistance mutations in GyrA are located in a discrete region in the proximity of the active-site tyrosine (Morais Cabral et al., 1997). This region probably represents part of the quinolone-binding site in the enzyme-DNA complex; one such region is present in each GyrA59 monomer. Therefore, we expect that binding of the drugs would include at least two steps and that cooperativity might exist between these two binding events.

In the above scheme, $K_Q$ represents the apparent equilibrium dissociation constant describing the overall quinolone binding process. We showed in this paper that DNA cleavage is a slow reaction that consists of two independent cleavage events. Therefore, $K_C$ here is the apparent equilibrium constant for the overall cleavage process. We should stress here that scheme 7 represents an equilibrium and E.DNA.Q* (the cleaved complex) is not an end-product. The above reaction is fully reversible as indicated by the ability of the gyrase-DNA complex to revert back to the quinolone-free ATPase rate once the drug is removed (Fig. 5.4C). A similar experiment to the one described in Fig. 5.4C, where drug-induced DNA cleavage was monitored instead of ATPase, also revealed that cleavage is fully reversible upon drug dissociation (C. Willmott, unpublished results). Based on the above scheme, a steady-state equation can be derived that relates the rate of ATP hydrolysis to the formation of the gyrase-quinolone complex (see appendix). In deriving this equation we consider that all quinolone-bound complexes, irrespective of the cleavage-religation state of the DNA, exhibit the quinolone-characteristic rate. In support of this we found that, firstly, the quinolone-induced rate in the case of short fragments containing a preferred cleavage site (147, 198, 90, 80 bp fragments) is similar to the rate exhibited by the gyrase-quinolone complex formed on linear pBR322 (a substrate where cleavage occurs with much less efficiency). Secondly, studying the time dependence of cleavage we found that the quinolone-characteristic rate appeared immediately after the addition of the drug and remained constant throughout the slow process of DNA cleavage. Thirdly, when the dependence of the quinolone-induced rate on DNA concentration was investigated in the presence of pBR322 DNA it was found that as soon as sufficient amounts of DNA were present for the gyrase-DNA-quinolone complex to be formed the rate reached a maximum that remained constant at higher DNA concentrations. This argues that the gyrase-quinolone complex can be formed at any DNA sequence, irrespective whether these are strong cleavage sites or not, and still show the same ATPase rate. The rate of hydrolysis therefore must be independent of DNA sequence or the state of the cleavage-religation equilibrium.

The steady-state equation relating the rate of ATP hydrolysis to the concentrations of quinolone and DNA is shown in the appendix (eq. 23). When the dependence of the formation of the enzyme-quinolone complex on quinolone concentration was investigated we
determined an inhibitory constant $K_{iq}$ as the concentration of quinolone required for half-maximal conversion of the ATPase rate. The relationship between $K_{iq}$ and the apparent equilibrium association constant for the enzyme-quinolone complex $K_Q$ can be determined from the above steady-state equation (see appendix):

$$K_q = \frac{1 + \frac{K_D}{[DNA]} K_Q}{1 + \frac{1}{K_C}} \quad (8)$$

In the experiment described in Fig. 5.4A we studied the quinolone concentration dependence of the interaction of A$_2$B$_2$ with linear pBR322. Given the experimentally determined value of the equilibrium constant for the binding of gyrase to DNA ($K_D \sim 0.5 \times 10^{-9}$ M, (Maxwell & Gellert, 1984)) and the concentrations of enzyme and DNA present, most of the enzyme would be in the DNA bound form. Therefore, $[E]/[E.DNA] = K_D/[DNA] << 1$. Examination of the cleavage reaction of gyrase with pBR322 at conditions of full inhibition of the enzyme supercoiling activity reveals that the amount of enzyme-quinolone complexes that have induced DNA cleavage is much lower than the amount of enzyme molecules present in the reaction (Fig. 5.6A and SCK, unpublished observations). Therefore, in this case, $[E.DNA.Q^*]/[E.DNA.Q] = 1/K_C < 1$ and $1 + 1/K_C \sim 1$. Under these conditions (9) becomes:

$$K_q \equiv K_Q \quad (9)$$

Thus, in the particular case of the interaction of A$_2$B$_2$ with linear pBR322, $K_{iq}$ reflects the apparent equilibrium constant for the dissociation of the enzyme-quinolone complex. Clearly this is not true in the case of A$_{59}$B$_2$ where the affinity of the complex for DNA ($K_D$) is weaker (Kampranis & Maxwell, 1996). Using the same system (A$_2$B$_2$ and linear pBR322) we found that the apparent dissociation rate constant for this complex is $k_{off}^{obs} = 3 \times 10^{-4}$ s$^{-1}$ (Fig. 5.4B). From the values of the equilibrium inhibitory constant, $K_{iq}$, and the observed dissociation rate constant, $k_{off}^{obs}$, we can estimate the overall association rate constant of the formation of the gyrase-linear DNA-quinolone complex to have a lower value of $k_{on}^{obs} > 1 \times 10^{4}$ M$^{-1}$ s$^{-1}$.

The slow interaction of gyrase with short DNA fragments. When we studied the interaction of gyrase with small DNA fragments we found that the conversion to the quinolone-characteristic ATPase rate was slow. We consider this to be direct evidence for the slow formation of the enzyme-cleaved DNA complex. In the presence of long DNA fragments, conversion to the quinolone-characteristic rate is immediate since both the covalent and non-covalent enzyme-DNA-quinolone complexes are very stable and the conversion of one complex to the other cannot be observed as they both exhibit the same rate of hydrolysis. However with short fragments the non-covalent complex is very unstable and the appearance of the quinolone-characteristic rate depends mainly on the formation of the stable covalent complex. To describe these results a pre-steady state equation was derived
based on scheme 7. In this model, DNA cleavage is considered to be the rate-limiting step of the reaction and the first two steps of this scheme are considered to be significantly faster so that equilibrium between them is always established (see appendix). This equation takes into account the effect of DNA concentration on the rate of complex formation and explains the observation that their relationship is not linear (see “Results” and factor F in equation 34 in the appendix). Application of this equation to the data obtained in these experiments allows us to determine values for the apparent equilibrium constant for the DNA cleavage step ($K_C$) and the apparent rate constants ($k_{C+1}$, $k_{C-1}$) for the interconversion between the cleaved and ligated forms of the enzyme-DNA-quinolone complex. Under these conditions, $K_C$ had an estimated value of $\approx 5.9 \times 10^{-2}$ while the apparent rate constant for DNA cleavage $k_{C+1}$ was found to be $\approx 2.2 \times 10^{-3}$ s$^{-1}$ ($k_{C-1} \approx 1.3 \times 10^{-4}$ s$^{-1}$). This ($k_{C+1}$) is in good agreement with the values of the apparent rate constants ($k_1$ and $k_2$) obtained for the two successive cleavage events, although the difference in the substrate used does not allow for further comparison. The value of the equilibrium constant $K_C$ suggests that, in this particular situation, most of the complexes in equilibrium would be in the cleaved state (E.DNA.Q*). This is in agreement with experimental data on the cleavage of this site. Cleavage of the 147 bp fragment used in this study (which is centred around the same preferred cleavage site as the 20 and 40 bp fragments) is full at stoichiometric concentrations of gyrase and 25 $\mu$M CFX (data not shown). However, the fact that the gyrase-quinolone complex can form on different DNA sequences but only a number of these complexes would reveal DNA cleavage (this work and (Kirkegaard & Wang, 1981, Morrison et al., 1980, Sugino et al., 1978)), suggests that the state of the cleavage-religation equilibrium ($K_C$) depends on the sequence of the DNA segment on which the complex is formed.

**Quinolone-induced DNA cleavage consists of two separate scission events.** From the results presented in this paper it is clear that quinolone binding and DNA cleavage are two different processes. Drug binding is quite fast ($k_{obs} > 10^4$ M$^{-1}$s$^{-1}$) and largely independent of DNA sequence, while drug-induced cleavage is relatively slow and occurs with different efficiency at different DNA sites. The DNA cleavage reaction appears to consist of two independent cleavage steps. Examination of the time-courses of quinolone-induced cleavage under various conditions revealed that double-stranded cleavage proceeds by two successive scission events, one on either strand of the DNA substrate. This resulted in the appearance of both singly- and doubly-nicked DNA species in the process of the cleavage reaction (Fig. 5.6). The overall interaction leading to DNA cleavage can be summarised in the scheme showed in Fig. 5.7. By measuring the concentration of the different cleaved DNA species we were able to determine rate constants representing these two cleavage events. Due to the complicated nature of this interaction the constants determined by this method, $k_1$ and $k_2$, represent apparent rate constants for the conversion between the three DNA species in Fig. 5.7, rather than any specific pathway in this scheme. We found that the value of these rate constants was dependent on quinolone concentration. From the scheme in Fig. 5.7 the involvement of quinolone-dependent steps in the overall interaction is evident. The nature of
this interaction suggests that cooperativity could play an important role in the process of drug binding. Moreover, the possibility of a stoichiometry of more than two quinolone molecules per complex or the existence of different modes of drug binding cannot be ruled out. Although these experiments were performed under conditions where only one cleavage event occurs per plasmid, this does not necessarily involve the same site for all the enzyme-DNA-quinolone complexes in the reaction. Indeed, when a similar experiment was performed using linear pBR322, a number of sites appeared to be cleaved more efficiently than others (data not shown). Therefore, the rates observed here represent the overall result of DNA cleavage events occurring at a number of different complexes which are likely to exhibit different kinetic parameters.

\[
\begin{align*}
&\text{BA-AB} \\
&+\text{DNA} \\
\downarrow
\end{align*}
\]

\[
\begin{align*}
\text{Closed circular DNA} &\quad E-E &\quad +Q &\quad E-EQ &\quad +Q &\quad QE-EQ \\
\downarrow &\quad k_1 \\
\text{Singly-nicked DNA} &\quad E-E^* &\quad +Q &\quad E-EQ^* &\quad +Q &\quad QE-EQ^* \\
\downarrow &\quad k_2 \\
\text{Doubly-nicked DNA} &\quad *E-E^* &\quad +Q &\quad *E-EQ^* &\quad +Q &\quad *QE-EQ^*
\end{align*}
\]

Despite these complexities, a number of conclusions can be drawn from these results. Firstly, cleavage on one DNA strand seems to have a positive effect on the cleavage of the other strand. Being that the first cleavage event has the possibility to occur in either DNA strand while the second step could only occur in the strand that has not been already cleaved, the rate constant describing the first step of the reaction should be divided by a factor of 2 in order to accurately represent this interaction. This makes the second cleavage step from 2 to 8 times faster in all the cases studied in these experiments. Secondly, ATP appears to increase the rate of the cleavage reaction. Comparison of the rate of ATP hydrolysis in the presence of the drugs (0.6 s\(^{-1}\)) with the rate of cleavage in the presence of the nucleotide
(2.2×10³ s⁻¹) suggests that ~300 ATP molecules have been hydrolysed by the time one cleavage event has been completed (the numbers quoted here reflect the situation at 5 μM CFX where inhibition of the enzyme is complete and fast). This suggests that during that time the gyrase-quinolone complex interconverts between the conformations of complexes III and IV undergoing the uncoupled cycle of ATP hydrolysis suggested in the previous paper (Kampranis & Maxwell, 1998). It is possible that the deformation imposed on the enzyme-bound DNA during this interchange between conformational states plays an important role in quinolone-induced DNA cleavage.

A model for the action of quinolones. Based on the results of this work, a model can be devised to describe the interaction of quinolones with the gyrase-DNA complex. Examination of the recent crystal structure of GyrA59 reveals that a certain extent of unwinding may occur upon binding of the DNA to gyrase (Morais Cabral et al., 1997). It can therefore be envisaged that formation of the gyrase-DNA complex results in the deformation of the part of the DNA that is bound across the active site so that a “DNA-bubble” is formed (Fig. 5.8). Quinolones bind more favourably to negatively supercoiled or single-stranded DNA (Shen et al., 1989a, Shen & Pernet, 1985). Moreover, binding of the quinolones to the gyrase-DNA complex formed with negatively supercoiled DNA is stronger than when relaxed DNA is used (Shen et al., 1989b). Therefore it is possible that the combination of these unwound DNA bases (DNA-bubble) with a number of residues on the protein, that may interact directly with the drug, make up a specific binding site. The molecular details of this binding site are yet unknown and high-resolution structural studies are probably the best method for its elucidation. Nevertheless, important information for its location has been revealed by mutational analysis of the cleavage of a preferred site for T4 topoisomerase which suggested that the drugs interact directly with the two bases on either side of the scissile phosphodiester bond (Freudenreich & Kreuzer, 1993). In the case of gyrase, Cove et al. found that gyrase shows a preference for a pair of guanine bases being present on either side of the cleaved bond (Cove et al., 1997). These results indicate that the binding-site for the drugs may be situated in the internucleotide space between the bases that flank the scissile bond, and the bases on either side of this bond interact directly with the drug. This interaction of the drugs with the enzyme and the DNA results in the stabilisation of a certain conformation of the enzyme and blocks strand-passage (Kampranis & Maxwell, 1998). This quinolone-trapped conformation hydrolyses ATP with a characteristic rate.

The interaction of quinolones with DNA is known to cause unwinding (Tornaletti & Pedrini, 1988). Recent work on the interaction of norfloxacin with DNA suggests that drug binding distorts the DNA and that this structural alteration (probably helix unwinding) is exacerbated by the presence of topoisomerase IV (Marians & Hisa, 1997). Moreover, ligation of singly-nicked plasmid DNA in the presence of A592 B2 and CFX results in slightly negatively supercoiled DNA (Kampranis & Maxwell, 1996). These results suggest that the binding of the drugs and the subsequent conformational change would cause further deformation in the already unwound DNA.
Fig. 5.8. A model describing the action of quinolones on DNA gyrase. Top, A schematic representation of the conformational changes undergone by gyrase in the presence of quinolones and their relationship to ATP hydrolysis and DNA cleavage (the graphic is adapted from (Hammonds & Maxwell, 1997)). On the left (unshaded part), the normal pathway of ATP hydrolysis is shown. On the right (shaded part), the complexes formed as a result of quinolone binding are demonstrated. The catalytic cycle shown in the shaded part is responsible for the quinolone-characteristic ATPase rate. The conformational change in the 47-kDa domain that is caused by the binding of the drugs is shown by a slightly different shape of the GyrB representation (compare complex I and III or complex II and IV). The position of the quinolone molecules in the gyrase-quinolone complex is arbitrary. Bottom, A representation of the conformation of the DNA around the active-site, corresponding to the complexes shown above, as this is described in the model for DNA cleavage proposed in the Discussion section. The shaded background represents the enzyme and the change in its shape reflects the quinolone-induced conformational change.

Because of this conformational change, the DNA cleavage-religation equilibrium possibly now occurs in a different molecular environment than when it takes place in the drug-free enzyme (complex I). We found that the quinolone-induced cleavage reaction is a slow reaction. By contrast the Ca$^{2+}$-induced cleavage reaction is much faster (unpublished results). We believe that Ca$^{2+}$ ions act by shifting the normal (complex I) cleavage-religation equilibrium to the cleaved form without this being a result of a profound effect on the conformation of the complex (Kampranis & Maxwell, 1998). This suggests that the cleavage-religation equilibrium in the absence of the drugs is relatively fast, with the complex
predominantly in the ligated form. In contrast, the binding of quinolones alters the enzyme conformation around the active site and disfavours the cleavage reaction. In certain cases, when this is supported by the DNA sequence bound at the active site, the deformation of the DNA molecule would be such that the cleavage reaction would be promoted. Drug-induced cleavage is therefore a slow reaction that occurs only when the molecular composition of the DNA in the active-site is appropriate and is followed by an even slower religation step. We found that DNA cleavage proceeds via two scission events. Cleavage on one DNA strand may allow the other strand to deform more readily thus making the second cleavage event faster. It has been shown that ATP or ADPNP alter the efficiency and site-specificity of quinolone-induced cleavage (Morrison et al., 1980, Sugino et al., 1978). This could be explained by a mechanism where different DNA sequences, because of their different molecular composition, would be subjected to different structural deformations when the enzyme is in the complex IV state. Therefore, some sites would be deformed in a way that prevents DNA cleavage and thus being protected while others would have resumed a conformation that supports the cleavage reaction.

**Conclusions.** Quinolone binding is followed by a conformational change in the gyrase-DNA complex that is responsible for the inhibition of the enzyme. When the complex is in the quinolone-trapped state, it can hydrolyse ATP with a characteristic rate. We have studied this quinolone-characteristic ATPase rate and used it as a sensitive tool for monitoring the formation of the gyrase-quinolone complex. We found that the binding of the drugs to the gyrase-DNA complex is a relatively fast reaction while DNA cleavage is a subsequent slow step. The cleavage reaction consists of two scission events, one in each DNA strand. Based on these results we suggest that DNA cleavage is the result rather than a pre-requisite of quinolone binding.

### 5.4. Appendix

The steady-state equation describing ATP hydrolysis is derived based on the following model:

\[
E + ATP \rightleftharpoons^{k_{+1}}_{k_{-1}} E.ATP + ATP \rightleftharpoons^{k_{+2}}_{k_{-2}} E.ATP_2 \rightarrow E + 2ADP + 2Pi
\]  

(1)

In steady state:

\[
K_{d1} = \frac{[E][ATP]}{[E.ATP]} \quad (10)
\]

\[
K_{d2} = \frac{[E.ATP][ATP]}{[E.ATP_2]} \quad (11)
\]

and \[ [E]_0 = [E] + [E.ATP] + [E.ATP_2] \quad (12) \]

where \([E]_0\) is the total concentration of enzyme in the reaction.
From (10), (11) and (12) ⇒ \[ [E.ATP_2] = \frac{[E]_0[ATP]^2}{[ATP]^2 + K_{d2}[ATP] + K_{d1}K_{d2}} \] (13)

The rate of ATP hydrolysis (\(v\)) is:

\[
v = \frac{d[ADP]}{dt} = \frac{d[Pi]}{dt} = 2k_3[E.ATP_2] \quad (14)
\]

In the experiments described in this paper the rate of ATP hydrolysis is expressed as the rate of change in ADP concentration per enzyme complex (determined by the limiting concentration of GyrB):

\[
\frac{v}{[E]_0} = \frac{d[ADP]}{dt} \quad (15)
\]

The steady-state equation describing the binding of quinolones is derived from the following scheme:

\[
E + DNA\rightleftharpoons_{K_D}^{} E.DNA + Q\rightleftharpoons_{K_Q}^{} E.DNA.Q \rightleftharpoons_{K_R}^{} E.DNA.Q^* \quad (7)
\]

At equilibrium:

\[
K_D = \frac{[E][DNA]}{[E.DNA]} \quad (16), \quad K_Q = \frac{[E.DNA][Q]}{[E.DNA.Q]} \quad (17), \quad \text{and} \quad K_R = \frac{[E.DNA.Q]}{[E.DNA.Q^*]} \quad (18)
\]

\[
[E]_0 = [E] + [E.DNA] + [E.DNA.Q] + [E.DNA.Q^*] \quad (19)
\]

From (16), (17), (18) and (19) ⇒

\[
[E.DNA.Q] = \frac{[Q][DNA][E]_0}{(1+1/K_R)[Q][DNA] + K_Q[DNA] + K_QK_D} \quad (20)
\]

from (18) ⇒ \([E.DNA.Q] + [E.DNA.Q^*] = (1+1/K_R)[E.DNA.Q] \) ⇒

\[
[E.DNA.Q] + [E.DNA.Q^*] = \frac{(1+1/K_R)[Q][DNA][E]_0}{(1+1/K_R)[Q][DNA] + K_Q[DNA] + K_QK_D} \quad (21)
\]
The gyrase-quinolone complex

In the experiments shown in Fig. 5.4 the concentration of linear pBR322 DNA is constant while the concentration of drug is varied. In this case the observed rate of ATP hydrolysis (v) will be the sum of the rates of the DNA-free enzyme, the DNA-bound-enzyme and the gyrase-quinolone complex:

\[ v = v_E + v_{E.DNA} + v_{E.DNA.Q+E.DNA.Q^*} \]

If \( k_E \), \( k_{E.DNA} \), \( k_Q \) are the turnover numbers of the DNA-free enzyme, the enzyme-DNA complex and the drug bound enzyme-DNA complexes respectively, then:

\[ v = k_E[E] + k_{E.DNA}[E.DNA] + k_Q[E.DNA.Q + E.DNA.Q^*] \]

Figure 5.4A describes the interaction of A2B2 with CFX. Due to the high affinity of A2B2 for DNA, the concentration of DNA-free enzyme would be very small compared to the concentration of the DNA-bound form (i.e. \( v_E + v_{E.DNA} \equiv v_{E.DNA} = k_{E.DNA}[E.DNA] \)). In the case of the A592B2 complex (Fig. 5.4A inset), linear DNA cannot stimulate the ATPase of this enzyme (Kampranis & Maxwell, 1996), i.e. \( v_E + v_{E.DNA} \equiv v_E = k_0[E] \). If the turnover number of the drug-free complexes is \( k_0 \) (either A2B2 or A592B2) then the rate of hydrolysis at any [CFX] would be,

\[ \frac{v}{[E]} = k_0 \frac{[E]+[E.DNA]}{[E]_0} + k_Q \frac{[E.DNA.Q]+[E.DNA.Q^*]}{[E]_0} \]  

(22)

From (19) and (21), (22) becomes:

\[ \frac{v}{[E]} = k_0 + (k_Q - k_0) \frac{(1+\frac{1}{K_C})[Q][DNA]}{(1+\frac{1}{K_C})[Q][DNA]+K_Q[DNA]+K_Q'K_D} \]  

(23)

Equation (23) can be written in the form of the following rectangular hyperbola:

\[ \frac{v}{[E]} = k_0 + (k_Q - k_0) \frac{[Q]}{[Q]+\frac{1}{K_Q}[DNA]} K_Q \]  

(24)

The quinolone concentration resulting in 50% inhibition (\( K_{iq} \)) is therefore,

\[ K_{iq} = \frac{1+K_Q[DNA]}{1+\frac{1}{K_C}K_Q} \]  

(8)

Because at low quinolone concentrations (close to the concentration of enzyme) the concentration of free drug [\( Q \)] cannot be approximated to the total drug concentration [\( Q \)], the interaction is better described if in eq. 21 [\( Q \)] is substituted by [\( Q \)] \([E.DNA.Q]+[E.DNA.Q^*]\)). Thus,
\[ [E.DNA.Q] + [E.DNA.Q^*] = \]
\[
\frac{(1 + 1/K_c)([Q] - ([E.DNA.Q] + [E.DNA.Q^*]))[DNA][E]_0}{(1 + 1/K_c)([Q] - ([E.DNA.Q] + [E.DNA.Q^*]))[DNA] + K_d[DNA] + K_d K_D}
\]

This can be written in the form,
\[
([E.DNA.Q] + [E.DNA.Q^*])^2
\]
\[-(K_w + [Q] + [E]_0)([E.DNA.Q] + [E.DNA.Q^*]) + [Q][E]_0 = 0 \quad (25)
\]

From the solutions of this equation only one has a finite limit (note that \([E.DNA.Q] + [E.DNA.Q^*]\) cannot exceed \([E]_0\)). This is,
\[
[E.DNA.Q] + [E.DNA.Q^*] = \frac{K_w + [Q] + [E]_0 - \sqrt{(K_w + [Q] + [E]_0)^2 - 4[E]_0[Q]}}{2}
\]

Substituting back to (22),
\[
\frac{v}{[E]_0} = k_0 + (k_q - k_0) \frac{K_w + [Q] + [E]_0 - \sqrt{(K_w + [Q] + [E]_0)^2 - 4[E]_0[Q]}}{2} \quad (26)
\]

On the pre-steady state level \(K_c = k_{c-1} / k_{c+1}\) where \(k_{c-1}\) and \(k_{c+1}\) represent the apparent first order rate constants for the quinolone induced DNA cleavage and religation reactions respectively. Supposing that the DNA and quinolone binding steps are much faster than the DNA cleavage event, as illustrated by experimental data, then the equilibrium involving the first two steps of this reaction would be established very fast compared to any conversion to the cleaved form. Then at any time (16), (17) and (19) would obtain. By combining those three equations,
\[
[E.DNA.Q] = \frac{[Q][DNA]([E]_0 - [E.DNA.Q^*])}{([Q] + K_q)[DNA] + K_q K_D} \quad (27)
\]

When \([DNA]\) and \([Q]\) are in excess over \([E]_0\) then they can be considered to remain constant throughout the reaction. In this case,
\[
\frac{[Q][DNA]}{([Q] + K_q)[DNA] + K_q K_D} = F \quad \text{is constant} \quad (28)
\]

In this case (27) becomes,
\[
[E.DNA.Q] = F([E]_0 - [E.DNA.Q^*]) \quad (29)
\]
The rate of formation of the cleaved complex is,

\[
\frac{d[E\cdot DNA\cdot Q^*]}{dt} = k_{c+1}[E\cdot DNA\cdot Q] - k_{c-1}[E\cdot DNA\cdot Q^*] \Rightarrow
\]

\[
\frac{d[E\cdot DNA\cdot Q^*]}{dt} = k_{c+1}F([E]_0 - [E\cdot DNA\cdot Q^*]) - k_{c-1}[E\cdot DNA\cdot Q^*] \Rightarrow
\]

\[
\frac{d[E\cdot DNA\cdot Q^*]}{dt} + (k_{c+1} + k_{c-1})[E\cdot DNA\cdot Q^*] = k_{c+1}F[E]_0 \quad (30)
\]

This is a linear first order differential equation whose solution is:

\[
[E\cdot DNA\cdot Q^*] = e^{-(k_{c+1} + k_{c-1})t} \left( \frac{k_{c+1}F[E]_0}{k_{c+1}F + k_{c-1}} e^{(k_{c+1} + k_{c-1})t} + c \right) \quad (31)
\]

At \( t = 0, [E\cdot DNA\cdot Q^*] = 0 \) and from (31)

\[
c = -\frac{k_{c+1}F[E]_0}{k_{c+1}F + k_{c-1}}
\]

By replacing \( c \) back to (31):

\[
[E\cdot DNA\cdot Q^*] = \frac{k_{c+1}F[E]_0}{k_{c+1}F + k_{c-1}} (1 - e^{-(k_{c+1} + k_{c-1})t}) \quad (32)
\]

The total concentration of quinolone bound complexes is:

\[
[E\cdot DNA\cdot Q] + [E\cdot DNA\cdot Q^*] = F[E]_0 + F[E\cdot DNA\cdot Q^*] + [E\cdot DNA\cdot Q^*] \Rightarrow
\]

\[
\frac{[E\cdot DNA\cdot Q] + [E\cdot DNA\cdot Q^*]}{[E]_0} = F + (1 + F) \frac{k_{c+1}F}{k_{c+1}F + k_{c-1}} (1 - e^{-(k_{c+1} + k_{c-1})t}) \quad (33)
\]

Since a short DNA fragment, at these concentrations, is unable to stimulate the ATPase of gyrase (i.e. \( k_E = k_{EDNA} \)), the rate of ATP hydrolysis at any time would be:

\[
\frac{v}{[E]_0} = k_0 + (k_0 - k_E)(F + (1 + F) \frac{k_{c+1}F}{k_{c+1}F + k_{c-1}} (1 - e^{-(k_{c+1} + k_{c-1})t})) \quad (34)
\]

Where \( k_0 \) is the turnover number of the drug-free complexes (\( k_0 = k_E = k_{EDNA} \)) while \( k_E \) is again the turnover number of the drug-bound complexes.
5.5. References


Hydrolysis of one ATP is sufficient to promote supercoiling by DNA gyrase

Abstract
Mutation of Glu$^{42}$ to Ala in the B subunit of DNA gyrase abolishes ATP hydrolysis but not nucleotide binding. Gyrase complexes that contain one wild-type and one Ala$^{42}$ mutant B protein were formed and the ability of such complexes to hydrolyse ATP was investigated. We found that ATP hydrolysis was able to proceed independently only in the wild-type subunit, albeit at a lower rate. With only one ATP molecule hydrolysed at a time, gyrase could still perform supercoiling but the limit of this reaction was lower than that observed when both subunits can hydrolyse the nucleotide.

6.1. Introduction
The three-dimensional structure of DNA plays a key role in many biological processes. Reactions such as replication, transcription or recombination, not only are regulated by, but also have a profound effect on the topology of the DNA molecule. The enzymes responsible for maintaining the topological state of DNA are DNA topoisomerases. One such enzyme is DNA gyrase, a bacterial topoisomerase that introduces negative supercoils into DNA in a reaction coupled to ATP hydrolysis. The action of gyrase involves the creation of a double-stranded break in one DNA segment and the passage of another segment through this enzyme-stabilised DNA gate.

The ability of gyrase to negatively supercoil DNA is unique amongst topoisomerases and is based on its mode of DNA binding (Kampranis & Maxwell, 1996, Liu & Wang, 1978). Gyrase wraps DNA in a right-handed manner (Liu & Wang, 1978), resulting in the positioning of two segments of DNA in the right orientation for supercoiling. Binding of ATP
closes a protein clamp which traps the DNA segment to be transported. The nucleotide is then hydrolysed and the free energy is coupled to the supercoiling reaction. After hydrolysis, the enzyme is reset for another round of supercoiling. The limit of supercoiling is believed to be thermodynamic rather than steric since gyrase can supercoil very small DNA circles, while a nucleotide analog (ATPαS) with higher free-energy of hydrolysis than ATP is capable of taking the limit of the supercoiling reaction to higher negative-superhelical density (Bates & Maxwell, 1989, Cullis et al., 1992).

Gyrase is a heterotetramer in which two A (GyrA, 97 kDa) and two B (GyrB, 90 kDa) subunits constitute an A\(_2\)B\(_2\) complex (Reece & Maxwell, 1991). There is one ATP-binding site per GyrB, which is situated in the 43-kDa N-terminal domain of the protein. The structure of this domain complexed with the ATP analog 5'-adenylyl-β,γ-imidodiphosphate (ADPNP) was solved by x-ray crystallography and was found to be a dimer (Wigley et al., 1991). Study of the ATPase reaction of this domain revealed that dimerisation is an essential step for ATP hydrolysis (Ali et al., 1993). It is very likely that dimerisation of the 43-kDa domain also occurs in the ATPase reaction of intact gyrase (Kampranis & Maxwell, 1998a). The rate of ATP hydrolysis by gyrase is stimulated by the presence of DNA (Maxwell & Gellert, 1984), while the kinetics of hydrolysis show positive cooperativity between the two ATP-binding sites (Kampranis & Maxwell, 1998b, Maxwell et al., 1986, Tamura et al., 1992).

A number of issues concerning the mechanism of ATP hydrolysis are still unclear. These include the mechanism of cooperativity between the two sites and the coupling of the free energy produced by these two reactions to a single strand-passage event. Directly related to these issues is the question of whether ATP hydrolysis can take place only in one of the two sites and the capacity of such a reaction to support supercoiling. At the molecular level the mechanism of hydrolysis of ATP by gyrase involves nucleophilic attack by water on the γ-phosphate of ATP with Glu\(^{42}\) of GyrB acting as a general base (Jackson & Maxwell, 1993). Mutation of Glu\(^{42}\) to Ala in GyrB, abolishes ATP hydrolysis but not nucleotide binding (Jackson & Maxwell, 1993). We formed heterogeneous gyrase tetramers containing one wild-type and one mutant GyrB\(^{Ala42}\) subunit and used these complexes to address the above questions.

### 6.2. Results

**ATP traps A\(_2\)B\(_2\)\(^{Ala42}\) in the dimerised form (complex II).** Limited proteolysis has been used as a sensitive probe for detecting conformational changes in DNA topoisomerases (Kampranis & Maxwell, 1998a, Lindsley & Wang, 1993). Treatment of gyrase with trypsin produces two major fragments, one of ~62 kDa, derived from the A protein, and another of ~25 kDa from the B protein (Fig. 6.1); this proteolytic fingerprint has been termed complex I (Kampranis & Maxwell, 1998a). Binding of ADPNP induces dimerisation of the B subunits resulting in a conformation that protects the 43-kDa domains of GyrB from tryptic digestion. The characteristic fingerprint of this conformation (termed complex II) consists, apart from
the two above mentioned fragments, of another two bands, corresponding to the 43-kDa domain and a 33-kDa fragment of this domain (Fig. 6.1; (Kampranis & Maxwell, 1998a)). ATP cannot trap the wild-type enzyme in this conformation since hydrolysis resets the enzyme to its native form (Fig. 6.1). We probed the A2B2Ala42 complex using limited proteolysis to determine the state of the ATP-operated clamp in the presence of ATP or ADPNP. The characteristic complex II signature was apparent after treatment with trypsin in the presence of both nucleotides (Fig. 6.1). These results suggest that, upon incubation of the A2B2Ala42 complex with ATP, the nucleotide binds to the enzyme and induces dimerisation of the B subunits but, due to the inability of this complex to hydrolyse the nucleotide, it becomes trapped in the dimerised conformation (complex II).

ATP hydrolysis can proceed only at one site. We addressed the possibility of ATP hydrolysis occurring only at one of the two ATP-binding sites in the case where both sites are occupied by the nucleotide. To achieve this we formed heterotetramers of gyrase that contained, apart from the two GyrA protomers, one wild-type GyrB subunit and one mutant GyrBAla42 subunit. Such complexes can be formed by reconstituting the gyrase heterotetramer in the presence of both wild-type and mutant GyrB proteins (O'Dea et al., 1996). Electron microscopy and crosslinking studies have failed to detect any dimeric forms of GyrB in the absence of GyrA, suggesting that a significant proportion of GyrB is monomeric in solution (Kirchhausen et al., 1985, Klevan & Wang, 1980). Therefore, we expect that by allowing a mixture of the wild-type and mutant GyrB proteins to equilibrate and then adding GyrA, a
random distribution of gyrase complexes containing two wild-type, two mutant, or one of each kind of GyrB proteins would be produced. Indeed, in similar experiments, O'Dea and co-workers found no bias for the formation of any particular one of the above complexes (O'Dea et al., 1996). It is unlikely that heterogeneous tetramers could be studied separately due to the uncertainties concerning the stability of the gyrase tetramer (i.e. such an isolated population would probably re-equilibrate to a mixture of heterogeneous and homogeneous tetramers). Therefore, we decided to study the behaviour of complexes formed at different ratios of wild-type to mutant subunit and analyse the results obtained in terms of the predicted population of each tetrameric species, assuming random association.

We tested the ability of heterogeneous tetramers to hydrolyse ATP. Mixtures containing varying amounts of homogeneous and heterogeneous tetramers were formed by mixing a constant concentration of the wild-type subunit with increasing concentrations of the mutant protein (up to a 12-fold excess). GyrA was always in excess of the total B protein and the ATPase reaction of both the DNA-free enzyme and the gyrase-DNA complex were studied. Increasing concentrations of the mutant protein resulted in inhibition of the rate of hydrolysis (Fig. 6.2). However, this inhibition was not full, and, in the case of the gyrase-DNA complex, the ATPase rate seemed to plateau at approximately 40% of the rate exhibited by the sample containing only wild-type enzyme-DNA complex. In the absence of DNA, increasing concentrations of the mutant resulted in a decrease in the rate of hydrolysis that levelled out at ~ 65% of the rate of the reaction containing only the DNA-free wild-type enzyme (Fig. 6.2).

The ATPase rates observed were specific to the gyrase complexes and not due to any contamination, since they were completely inhibited by the addition of novobiocin to the reaction. We calculated the amount of tetramers with two wild-type subunits at the concentrations of wild-type and mutant protein used in these experiments and were able to estimate the theoretical rate of hydrolysis in the case where the only complexes that were able to hydrolyse the nucleotide were those that contained two wild-type B subunits (see Appendix). The theoretical curve representing this situation is shown in Fig. 6.2. The
deviation of the experimental data from this theoretical curve is clear. These results suggest that ATP can be hydrolysed independently only at one site but that the rate of hydrolysis is lower than when both sites are capable of undergoing this reaction. The turnover number of the wild-type enzyme, in the absence of DNA, was measured to be $k_{\text{BB}}^{-\text{DNA}} = 0.12 \text{ s}^{-1}$. By estimating the concentration of the different species in the above mixtures, the turnover number exhibited by the heterogeneous complexes can be determined. The equation that relates the observed rate of hydrolysis to the concentration of the wild-type and mutant proteins, in terms of the turnover numbers of the wild-type enzyme and the heterogeneous tetramer, is shown in the Appendix. By fitting the experimental data to eq. 2 (see Appendix) the turnover number of the heterogeneous complex was determined to be $k_{\text{BB}}^{*^{-\text{DNA}}} = 0.04 \text{ s}^{-1}$. In the presence of DNA, the wild-type enzyme was measured to hydrolyse the nucleotide with a turnover number of $k_{\text{BB}}^{+\text{DNA}} = 1.06 \text{ s}^{-1}$, compared to an estimated $k_{\text{BB}}^{*+\text{DNA}} = 0.18 \text{ s}^{-1}$ in the case of the heterogeneous tetramer-DNA complex.

In another experiment, mixtures of tetramers were formed where the total concentration of GyrB protein (wild-type and mutant) was kept constant, while the ratio of the wild-type to mutant subunit was varied from 100% GyrB\text{Ala42} to 100% wild-type GyrB. The rate of hydrolysis for both the enzyme-DNA and the DNA-free complexes was measured; the results obtained from this experiment were plotted together with two theoretical curves (Fig. 6.3A). One of these curves describes the situation where heterogeneous tetramers cannot hydrolyse ATP (complete inhibition), while the other shows the situation where wild-type GyrB subunits can hydrolyse the nucleotide at the same rate irrespective of them being part of heterogeneous or homogeneous tetramers (no inhibition). The experimental data fall between those two lines. The rate of the enzyme-DNA complex is the one affected more by the formation of the heterogeneous tetramer and is closer to the curve describing complete inhibition, while the rate of the DNA-free complex is affected less and is closer to the theoretical curve of no inhibition, consistent with the results in Fig. 6.2. By fitting these results to eq. 3 the turnover number of the heterogeneous complexes can be determined. The values obtained in this experiment ($k_{\text{BB}}^{*-\text{DNA}} = 0.03 \text{ s}^{-1}$ for the DNA-free complex and $k_{\text{BB}}^{*+\text{DNA}} = 0.20 \text{ s}^{-1}$ for the DNA-bound one) are in very good agreement with those determined in the experiment described in Fig. 6.2, giving an average of $k_{\text{BB}}^{*-\text{DNA}} = 0.035 \text{ s}^{-1}$ and $k_{\text{BB}}^{*+\text{DNA}} = 0.19 \text{ s}^{-1}$ for the two experiments.

**ATP hydrolysis at one site is sufficient for strand passage.** To test the ability of heterogeneous complexes to support supercoiling, samples were removed from the above ATPase reactions at regular intervals and the extent of supercoiling was determined (Fig. 6.3B; the reactions were performed in the presence of relaxed pBR322 DNA and in the absence of spermidine). The wild-type $A_2B_2$ complex produced a distribution of topoisomers which had a centre that differed from the centre of the distribution of the relaxed substrate by a linking number ($\Delta \text{Lk}$) of 20. The limit of supercoiling lowered gradually as the ratio of mutant to wild-type increased. At 90% GyrB\text{Ala42} the centre of the distribution had a $\Delta \text{Lk}$ of 17 (Fig. 6.3B). At 100% GyrB\text{Ala42} supercoiling was virtually abolished (the limited amount
of supercoiling observed in this case is probably due to low levels of contamination of the GyrA preparation with wild-type GyrB and does not affect the results of this experiment).

A.

Fig. 6.3. ATP-hydrolysis at one site is sufficient for strand passage. A, Mixtures of heterogeneous and homogeneous tetramers were formed by keeping the total GyrB (wild-type and mutant) concentration, [B]_tot, constant at 40 nM while the ratio of wild-type to mutant was varied from 100% GyrB_{Ala42} to 100% wild-type GyrB. The concentration of GyrA was 60 nM, and 20 nM relaxed pBR322 was present where appropriate. The rates shown here are the average of three determinations. Results are presented as the ratio of the measured rate to the rate of the sample containing only wild-type gyrase. B, Samples were removed from the above reactions at 30 min intervals and were analysed by agarose gel electrophoresis to measure the limit of supercoiling. The 1% agarose gel contains 1 μg/mL chloroquine. At this concentration of chloroquine, the relaxed topoisomers appear positively supercoiled on the gel, while the negatively supercoiled products of the reaction appear still as negatively supercoiled but with lower absolute superhelical density.

The levels of supercoiling observed in the case of the 90% mixture was not due to the low levels of wild-type A2B2 complexes present in the reaction (calculated to be 1%). This was confirmed by directly comparing the level of supercoiling supported from the heterogeneous mixture to that supported by an amount of wild-type enzyme equal to the predicted concentration of wild-type complexes in this mixture. In this experiment, at the time when the heterogeneous mixture had reached the limit of the supercoiling reaction, the wild-type mixture had been unable to support significant levels of supercoiling (data not shown). This extent of supercoiling is due to a catalytic reaction, since the levels of enzyme used are not sufficient to ascribe this result to a stoichiometric interaction. It appears from these results that heterogeneous tetramers have not lost their ability to supercoil DNA but that the limit of this reaction is lower. The experiments reported here with GyrB_{Ala42} were also performed with the GyrB_{Gin42} mutant, which also binds but does not hydrolyse ATP (Jackson & Maxwell, 1993), yielding similar results (data not shown).
6.3. Discussion

The $A_2B_2^{Ala42}$ complex is unable to hydrolyse ATP (Jackson & Maxwell, 1993) and this is manifested by the trapping of this protein in the dimerised conformation (complex II) in the presence of the nucleotide (Fig. 6.1). This result also reveals that the complex II-characteristic proteolytic signature is not an attribute of the particular complex formed with ADPNP but reflects the conformation of the catalytic intermediate formed in the presence of ATP. Thus, the structure of complex II formed with ATP or ADPNP is indistinguishable, at least at the level detectable by limited proteolysis.

The mechanism of ATP hydrolysis by DNA gyrase can be summarised in the scheme shown in Fig. 6.4. The central point in this mechanism is the dimerisation of the B subunits (formation of complex II). ATP binds to the monomerised subunits within the $A_2B_2$ complex, and dimerisation has to occur before hydrolysis can take place (Ali et al., 1993). Binding of ATP in only one of the two subunits is sufficient for dimerisation (Ali et al., 1995, Lindsley & Wang, 1993). Although, once the clamp is in the dimerised form, binding of a second ATP molecule appears not to be possible (Wigley et al., 1991), no direct experimental evidence exists on this issue.

It has recently been reported that when ATP binding at one of the two sites was abolished, gyrase could not perform catalytic supercoiling (O'Dea et al., 1996). However, it is not clear whether this is because the complex with one ATP has poor or no ATPase activity or whether hydrolysis in this case is uncoupled from strand-passage. Nevertheless, with wild-type enzyme, occupation of the two ATP-binding sites is cooperative, therefore the complex

![Fig. 6.4. The mechanism of ATP hydrolysis by DNA gyrase. Schematic diagram showing the steps involved in the mechanism of hydrolysis by DNA gyrase. The upper shaded part encloses the pathways undergone by the heterogeneous tetramers. E-E is used here to represent the dyadic symmetry of the gyrase molecule. EE indicates the conformation of gyrase where the B subunits are in the dimerised (clamp-closed) form (complex II). The rate constants are discussed in the text while the equilibrium dissociation constants $K_{d1}$ and $K_{d2}$ are according to (Kampranis & Maxwell, 1998b).](image-url)
with two ATP molecules bound should be the predominant species at high ATP concentrations (Kampranis & Maxwell, 1998b, Maxwell et al., 1986, Tamura et al., 1992). Moreover, experiments with the 43-kDa domain of gyrase showed that dimerisation of two ATP-bound domains is favoured over dimerisation of one bound and one free domain by two orders of magnitude (Ali et al., 1995). ADP does not stabilise the dimerised form (Ali et al., 1993), resulting in the monomerisation of the subunits after hydrolysis (Fig. 6.4). Hydrolysis in the doubly-occupied complex comprises two hydrolysis reactions. In the experiments described in this paper, we set out to address the issue of interdependence between these two hydrolysis events and their coupling to strand-passage.

We found that complexes containing only one ATPase-proficient GyrB subunit hydrolyse the nucleotide at a lower rate than the wild-type enzyme. The pathway that describes reactivity at one of the two sites is shown in the upper shaded part of Fig. 6.4. The spectrophotometric assay used in these experiments for determining the rate of hydrolysis measures changes in the concentration of free ADP, therefore the rate of the actual hydrolysis reaction cannot be measured separately from that of the monomerisation and product release steps. However, experiments with the 43-kDa domain of GyrB suggested that the rate-limiting step in the mechanism of ATP hydrolysis by gyrase is either a conformational change associated with monomerisation or product release (Ali et al., 1993). Thus, due to the 2 ATP molecules hydrolysed per round, the apparent rate constant of the monomerisation/product release step in the wild-type complex can be determined to be $k_{\text{app}}^{\text{wt}} = k_{\text{BB}+\text{DNA}}^{\text{BB}} / 2 = 0.53 \text{ s}^{-1}$.

In the case of the heterogeneous tetramer, the mutant site would contain the unhydrolysed nucleotide after hydrolysis on the other site had occurred. This would likely result in stabilisation of the dimerised form, inhibiting the rate of the monomerisation/product release step. Therefore, in the heterogeneous tetramer-DNA complex, the rate-limiting step slows-down to $k_{\text{app}}^{\text{h}} = k_{\text{BB}+\text{DNA}}^{\text{BB}} = 0.19 \text{ s}^{-1}$ (note that only one ATP is hydrolysed per heterogeneous tetramer). In the absence of DNA, gyrase exhibits a much lower turnover number, $k_{\text{BB}+\text{DNA}}^{\text{BB}} = 0.12 \text{ s}^{-1}$ in our experiments. This is because binding of gyrase to DNA stimulates the ATPase activity of the enzyme (Maxwell & Gellert, 1984). It is not yet clear what is the rate-limiting step in the reaction of the DNA-free complex. If monomerisation or product release is again the rate-limiting step then the results obtained here with the heterogeneous tetramer could be explained in terms of the inhibition of the monomerisation/product release step by the bound nucleotide. If this is the case, DNA binding should stimulate the ATPase activity by accelerating the monomerisation/product release part of the mechanism. However, it is possible that in the absence of DNA, the rate-limiting step is the actual hydrolysis reaction. DNA binding would stimulate the rate of this step thus making monomerisation/product release rate-limiting in the enzyme-DNA complex.

The limit of the supercoiling reaction in the presence of the heterogeneous tetramers was $\Delta Lk_{17}$ in contrast with $\Delta Lk_{20}$ observed in the case of wild-type gyrase. The value of $\Delta Lk_{20}$ is in good agreement with that determined previously for the limit of supercoiling in the absence of spermidine (Westerhoff et al., 1988). Assuming that the limit of the
supercoiling reaction is directly related to the free-energy of ATP hydrolysis (Cullis et al., 1992), the lower absolute superhelical density reached in the case of the heterogeneous complex should reflect the reduction in the free-energy released when only one ATP is hydrolysed. However, the free-energy released by the extra ATP molecule does not reflect the difference in the free-energy of the products, if this is determined according to the relationship described by Cullis et al. (Cullis et al., 1992). The dependence of the limit of supercoiling on the free-energy of ATP hydrolysis was studied by varying the ratio of [ATP]/[ADP] present in the reaction whilst keeping the total concentration of nucleotides constant (Westerhoff et al., 1988). It has been suggested that, especially in the absence of spermidine, the relationship between linking number change and phosphate potential is not proportional due to significant ATPase slip and ATP-independent DNA relaxation (Westerhoff et al., 1988). We believe that the results obtained here reflect a complicated equilibrium established between the ATP-dependent supercoiling and ATP-independent relaxation reactions of the wild-type and heterogeneous complexes. Therefore, making any correlation between the limit of supercoiling and the free-energy of ATP-hydrolysis is beyond the capacity of these experiments.

In conclusion, we found that gyrase can hydrolyse ATP at one active-site and can supercoil DNA even with only one ATP hydrolysed at a time. Although this reaction is unlikely to occur in vivo, due to the physiological ATP concentration being relatively high, the results obtained here provide us with useful information on the mechanism of energy coupling in DNA gyrase.

6.4. Appendix

If \([B]\) is the concentration of wild-type and \([B^*]\) is the concentration of the mutant GyrB protein, then the total concentration of B protein in a mixture of the above two will be:

\[
[B]_{\text{tot}} = [B] + [B^*]
\]

Considering random mixing of the two GyrB proteins and an excess of GyrA over \([B]_{\text{tot}}\), the concentrations of the various species of heterotetramer formed would be:

\[
[A_2 B_2] = \frac{[B]^2}{2[B]_{\text{tot}}}
\]
\[
[A_2 BB^*] = \frac{[B][(B)_{\text{tot}} - [B]}}{[B]_{\text{tot}}}
\]
\[
[A_2 B^*_2] = \frac{([B]_{\text{tot}} - [B])^2}{2[B]_{\text{tot}}}
\]

The rate of hydrolysis \(v = \frac{d[ADP]}{dt}\) exhibited by one such mixture would be the sum of the rates exhibited by the \(A_2 B_2\) and \(A_2 BB^*\) complexes (\(A_2 B^*_2\) does not hydrolyse the nucleotide):

\[
v = v_{BB} + v_{BB^*}
\]

If \(k_{BB} (= v_{BB}/[A_2 B_2])\) is the turnover number of the wild-type complex \((A_2 B_2)\) and \(k_{BB^*} (= v_{BB^*}/[A_2 BB^*])\) is the turnover number of the mutant complex \((A_2 BB^*)\), then by combining the above equations, we can estimate the rate of hydrolysis in terms of the starting concentrations of the wild-type and mutant proteins. Thus,
\[ v = (k_{BB} - 2k_{BB^*}) \frac{[B]^2}{2[B]_{tot}} + [B]k_{BB^*} \]  

(1)

In the experiments described in Fig. 6.2, the concentration of wild-type GyrB, \([B]\), is kept constant whilst titrating mutant GyrBA \(^{Ala42}\) protein. In this case the rate exhibited by the wild-type complex in the absence of any mutant protein is \(v_{wt} = k_{BB}[B]/2\). The ratio of the rate exhibited by the heterogeneous mixture (\(v\)) to the rate exhibited by the sample containing only wild-type (\(v_{wt}\)) can be expressed in terms of the ratio of the concentration of mutant to the concentration of wild-type protein present in the reaction ([\(B^*\)]/[\([B]\)]):

\[ \frac{v}{v_{wt}} = \frac{1 + 2 \frac{k_{BB^*}}{k_{BB}} \left( \frac{[B^*]}{[B]} \right)}{\left( \frac{[B^*]}{[B]} \right) + 1} \]  

(2)

In the experiment described in Fig. 6.3, the total concentration of B protein, \([B]_{tot}\), is constant while the ratio of wild-type to mutant subunit is varied. In this case, the rate exhibited by the sample containing only the wild-type complex is \(v_{wt} = k_{BB}[B]_{tot}/2\), since in this particular sample \([B] = [B]_{tot}\). From this and eq. 1 the ratio \(v/v_{wt}\) can be written in terms of the ratio of the concentration of the wild-type subunit to the total concentration of B protein ([\(B]\)/[\([B]_{tot}\)])

\[ \frac{v}{v_{wt}} = (1 - 2 \frac{k_{BB^*}}{k_{BB}} \left( \frac{[B]}{[B]_{tot}} \right)^2 + 2 \frac{k_{BB^*}}{k_{BB}} \left( \frac{[B]}{[B]_{tot}} \right) \]  

(3)

The theoretical lines describing the situations of complete inhibition or no inhibition can be derived from the above equations by substituting \(k_{BB^*}\) by 0 or \(k_{BB}/2\) respectively.

6.5. References


7 Conclusions

7.1. Mechanistic aspects

Work mainly on the mechanism of the eukaryotic type II topoisomerases has established that these enzymes operate as molecular clamps. Briefly, the enzyme binds and cleaves a DNA segment (termed the G segment) creating a DNA gate through which another segment (the T segment) is passed. Indiscriminate transport of a T segment is sufficient to account for the relaxation and decatenation reactions performed by these enzymes. However, DNA gyrase is an atypical type II topoisomerase as it performs DNA supercoiling rather than relaxation. On the basis of biochemical data, significant sequence similarity between gyrase and the other type II enzymes, and structural similarities revealed by crystallographic studies of fragments of these enzymes, it is believed that gyrase also operates as a molecular clamp. However, in order to perform the supercoiling reaction, gyrase has to transport the T segment only at a certain orientation with respect to the G segment. Gyrase differs from the other enzymes on the mode of DNA binding. Conventional topoisomerases, bind ~25-30 bp of DNA while gyrase binds ~130 bp in a positive superhelical sense. We studied a version of gyrase that had been deleted for the C-terminal domain of GyrA, which has been implicated in DNA wrapping, in order to probe the importance of this interaction for the mechanism of supercoiling. This truncated version of gyrase (A592B2 or A642B2) had a similar mode of binding to the conventional enzymes but it had lost its ability to supercoil DNA. However, it supported efficient relaxation and decatenation and could replace a conventional type II topoisomerase in vivo. These results clearly suggest that gyrase shares the same basic mechanism with the other type II enzymes and that its unique properties are attributable to its characteristic interaction with the DNA.
However, these results do not reveal the exact mechanism by which the T segment is directed to the ATP-operated clamp. Previous experiments had suggested that nucleotide binding was sufficient for strand-passage since binding of a non-hydrolysable ATP analogue could support stoichiometric levels of supercoiling. The efficiency of this reaction was found to depend on the topological state of the DNA substrate, leading to the conclusion that the efficiency by which the T segment was trapped depended on DNA topology. However, this model failed to explain a number of observations such as the ability of gyrase to hydrolyse ATP at the same rate irrespective of the superhelical density of the DNA present in the reaction. Being that the trapping of a T segment was found to directly related to the stimulation of the rate of ATP hydrolysis, it seemed striking that the rate of hydrolysis was independent of DNA topology. Moreover, if the efficiency of strand-passage depended on the topology of the substrate, the limit of the supercoiling reaction would have been steric rather than thermodynamic, as it has been suggested by experiments using ATP analogues or very small DNA circles. To clarify these issues, we probed the topological state of the DNA in the enzyme-DNA complex formed on DNA substrates of varying superhelical density. We found that the conformation of the bound DNA segment after the binding of the nucleotide was the same irrespective of the topological state of the DNA. This suggested that the T segment was captured with high efficiency independent of DNA topology. This conclusion together with structural information on fragments of gyrase enabled us to propose a model for the organisation of the gyrase-DNA complex and the mechanism of supercoiling. In this model the DNA is wrapped around the enzyme in a way that directs a contiguous DNA segment to the ATP-operated clamp. Nucleotide binding traps this segment inside the enzyme and results in large changes in the topology of the bound segment. The efficiency by which the T segment is transported through the DNA gate depends on the superhelical free energy of the substrate and thus positively supercoiled molecules are better substrates than negatively supercoiled ones. However, nucleotide binding is not sufficient for the release of the passed T segment and the free-energy of ATP hydrolysis is required for the opening of the exit gate. The exact mechanism that couples the free energy produced by nucleotide binding and hydrolysis to DNA superhelical free energy is not clear but it is possible that these two events represent distinct steps.

The mechanism of energy coupling was studied by forming gyrase complexes that contain one wild-type and one mutant GyrB subunit that can bind but not hydrolyse ATP. This heterogeneous complex could hydrolyse ATP in the wild-type subunit, albeit with lower rate. This reaction was sufficient to enable this complex to support supercoiling; a reaction which reached lower limits of superhelical density than the one supported by the wild-type complex. These results are in support of the existence of a thermodynamic limit in the supercoiling reaction, however, in these experiments this was found not to be directly related to the free-energy released from ATP hydrolysis. Although the nature of these experiments is such that does not allow for further conclusions, its is tempting to speculate that this result is
CONCLUSIONS

a manifestation of the existence of two steps in the mechanism of energy coupling, one associated with nucleotide binding and one with hydrolysis.

7.2. The interaction of DNA gyrase with quinolones

Quinolones have long been used as potent antibacterial agents and their target has been identified as DNA gyrase. However, the mechanism of inhibition of gyrase and the molecular details of this interaction are largely unknown. Binding experiments made it clear that both gyrase and DNA have to be present in order for efficient quinolone binding to occur, suggesting that the complex is a ternary one. Disruption of the gyrase-DNA-quinolone complex with a protein denaturant reveals that the enzyme is covalently linked to the DNA via its active-site tyrosines, indicating that the drugs arrest the enzyme-DNA complex in a conformation in which the DNA is cleaved. Due to the high affinity of quinolones for single stranded DNA and the ability of ATP to facilitate the binding of quinolones to the enzyme-DNA complex, it has been proposed that the drugs bind to a single-stranded pocket created upon cleavage of the DNA by gyrase during catalytic turnover. However, this model was put into doubt when it was discovered that DNA cleavage was not a requirement for efficient quinolone binding.

We probed the gyrase-DNA-quinolone complex using limited proteolysis. Quinolone binding induces a conformational change in the enzyme-DNA complex that is independent of the ability of the enzyme to cleave DNA. We propose that the ability of quinolones to inhibit the gyrase activities has this conformational change as its basis. When the enzyme-DNA complex is in the quinolone-bound conformation, it can still close the ATP-operated clamp and hydrolyse the nucleotide. However, the kinetic characteristics of ATP hydrolysis in the presence of the drug are different to those of the drug-free complex. The conversion of the ATPase rate from the drug-free to the quinolone-bound one has been used as a sensitive probe for monitoring the binding of the drugs. Although drug binding has been closely associated with DNA cleavage, comparison of the time-scales of those two processes reveals that quinolone binding is a relative fast reaction while drug-induced cleavage is slow. Moreover, DNA cleavage occurs via two scission events, one on each DNA strand, and is modulated by the presence of ATP. These results led to the proposal of a model in which quinolones bind in the partially unwound region of DNA that is bound across the enzyme active site and stabilise a certain enzyme conformation which is unable to perform strand-passage. DNA distortions due to drug binding and the associated conformational change, alter the enzyme-DNA cleavage-religation equilibrium resulting in the slow accumulation of the cleaved complex. The nucleotide sequence of the bound DNA segment and nucleotide-induced conformational changes play an important role in modulating the efficiency of quinolone-induced cleavage.
8 Experimental procedures

8.1. Enzyme preparation

GyrA, GyrA<sup>Ser122</sup>, GyrAT<sup>Trp83</sup> (gift of C. Willmott), GyrA59 (gift of C. Smith),
GyrA64, GyrA64<sup>Phe122</sup>, GyrA64<sup>Ser122</sup>, GyrA33 (gifts of S. Critchlow), GyrB (gifts of A.
Maxwell and A. Howells), were purified as described previously (Maxwell & Howells, 1998,
Reece & Maxwell, 1991b). Mutant GyrB<sup>Ala42</sup> or GyrB<sup>Gln42</sup> proteins were a gift of Dr. A. P.
Jackson and were made as described previously (Jackson & Maxwell, 1993). The general
methods describing these preparations are given below.

**GyrA.** 500 mL of Terrific broth (tryptone 12 g/L, yeast extract 24 g/L, glycerol 0.8%
(v/v), 17 mM KH<sub>2</sub>PO<sub>4</sub>, 72 mM K<sub>2</sub>HPO<sub>4</sub>) containing 100 µg/mL ampicillin was inoculated
with 5 mL of an overnight culture of *E. coli* JM<sup>MacA</sup> (JM109 containing plasmid pPH3). The
culture was grown at 37°C in a shaking incubator and when the absorbance at 600 nm has
reached 0.5, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added and the growth
was continued for 6 hr. The cells were harvested by centrifugation at 6,000 rpm for 10 min at
4°C in a Beckman JA-10 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended in
a minimum volume of 50 mM Tris·HCl (pH 7.5), 10% (w/v) sucrose and then quick frozen in
liquid nitrogen and stored at -70°C. Upon thawing the cell suspension was adjusted to 2 mM
DTT, 20 mM EDTA and 100 mM KCl. The cells were then passed three times through a
pre-cooled French press operating at a pressure between 8,000-12,000 psi. Immediately the
cell extract was centrifuged at 34,000 rpm for 1 hr at 4°C in a TFT 50.38 rotor in a Sorvall
centrifuge. The pellet contains cell debris and the supernatant was transferred into a fresh
tube and 0.314g of solid ammonium sulphate was added per g of solution. The mixture was
stirred for 30 min in an ice bath in the cold room and the insoluble material containing the
partially purified gyrase was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C in a SS34 rotor in a Sorvall centrifuge. The pellet was resuspended in a small volume of TED (50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 4 mM DTT) and both the supernatant and the pellet were analysed by SDS PAGE. The protein fraction was then extensively dialysed overnight in TED. The A subunit was then purified on a Hi Load Q Sepharose FPLC column (Pharmacia) at a flow rate of 1 mL/min using a very shallow (minimum 250 mL) 0-450 mM NaCl gradient in TED. Before application to the column the conductivity of the sample was measured and adjusted to the 10-18 µS range. The fractions were analysed by SDS PAGE and the correct fractions pooled together. For extra purity the sample was then purified on a Mono Q 10/10 FPLC column (Pharmacia) as above. The correct fractions were pooled and dialysed against Enzyme Buffer (EB: 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 4 mM DTT and 10% (w/v) glycerol). The protein solution was then aliquoted out, fast frozen in liquid nitrogen and stored at -70°C.

GyrB. The gyrase B protein was purified from strain JM109, which contains the plasmid pAG111 (or mutated derivative) which has the gyrB gene under the tight control of the tac promoter. Cell growth and preparation of cell extract were as described for the GyrA protein. The cell extract was dialysed against TED buffer and loaded onto a Heparin-Sepharose column (Pharmacia) which had been pre-equilibrated with TED buffer. The cell extract was loaded onto the column at a flow rate of 1 mL/min and recirculated for approximately 1 hr to ensure complete binding. The column was then washed with 3 column volumes of TED buffer containing 0.2 M NaCl before protein was eluted with a linear NaCl gradient of 0-0.5 M NaCl in TED. GyrB protein elutes at 0.4 M NaCl. The protein was purified further on a MonoQ 5/5 column (Pharmacia) at a flow rate of 1 mL/min and protein was eluted with a linear salt gradient (0-0.4 NaCl). GyrB was eluted at 0.3 M NaCl. Fractions were pooled, dialysed against EB and stored at -70°C.

GyrA64 or GyrA59. The N-terminal domain of GyrA (or its mutants) was purified from cell extracts of JM109 which contained the over-expressed protein. The extract was diluted with 0.5 x TGED buffer (25 mM Tris.HCl (pH 7.5), 5% glycerol (w/v), 0.5 mM EDTA and 1 mM DTT) to reduce the conductivity of the sample to below 10 µS. The diluted extract was then applied to a heparin Sepharose column pre-equilibrated in 0.5 x TGED for 30-40 min at a flow rate of 3 mL/min. The buffer containing cell extract was recirculated for 30 min until all of the protein had bound. The column was then washed with 100 mL of 0.5 x TGED to remove unbound proteins. A linear salt gradient (0-0.6 M) was applied to the column and fractions containing the 64 kDa protein were found to elute at approximately 250 mM NaCl. Fractions of interest were dialysed against TED buffer and then finely ground ammonium sulphate was added to a concentration of 1.0 M whilst stirring gently at 4°C. Any precipitate formed was removed by centrifugation in a SS-34 rotor using a Sorvall Superspeed centrifuge. The sample was loaded onto a Phenyl Sepharose FPLC column (Pharmacia) pre-equilibrated with TED buffer containing 1.0 M ammonium sulphate. Proteins were eluted with a linear 1.0-0.0 M ammonium sulphate gradient. The 64 kDa
protein elutes at the end of the gradient. Pure samples (>95% pure) were dialysed against EB and stored at -70°C.

GyrA33. The C-terminal domain of GyrA was purified from JM109 containing the plasmid pRJR79 which encodes the 33-kDa protein. Cells were grown as before and disrupted using a French press. The resulting extract was spun at 34,000 rpm in a TFT 50.38 rotor using a Sorvall ultracentrifuge. The 33-kDa protein was insoluble and was found in the pellet which was resuspended in Enzyme Buffer containing 6 M guanidine hydrochloride. After 20 min on ice, this suspension was again spun at 34,000 rpm as before to remove any cell debris. The supernatant containing the 33-kDa protein was dialysed against Enzyme Buffer overnight at 4°C. The protein was then purified by heparin Sepharose chromatography. The protein was eluted with a 0.0-1.0 M NaCl gradient, the 33 kDa domain was eluted at approximately 0.5-0.6 M NaCl. Fractions were pooled, dialysed against TGED and loaded onto a FPLC mono Q column. The 33 kDa protein eluted at approximately 0.5 M NaCl. Peak fractions were pooled and dialysed into Enzyme Buffer overnight, frozen in liquid nitrogen and stored at -70°C.

Other enzymes. Chicken erythrocyte topoisomerase I was provided by Mrs. A. J. Howells. E. coli DNA ligase was purchased by New England Biolabs.

8.2. DNA preparation

Negatively supercoiled and relaxed forms of plasmid pBR322 were provided by Mrs. A. J. Howells (University of Leicester). Negatively supercoiled form was prepared according to the general procedure outlined below, while the relaxed form was prepared from the supercoiled form by relaxation with topoisomerase I. Linear pBR322 was prepared by digestion of the supercoiled form with EcoRI.

Large-scale plasmid preparation. 400 mL cultures of E. coli JM109 containing the appropriate plasmid were grown in Luria Bertani broth at 37°C for 24 hr. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C in a Beckman J2-21 centrifuge with a Beckman JA-10 rotor. The cells were resuspended in 48 mL of Solution I (25 mM Tris-HCl (pH 8.0), 50 mM sucrose, 10 mM EDTA, 1.7 mg/mL lysozyme), and incubated at room temperature for 10 min. The cells were then lysed in an alkaline environment by the addition of 120 mL of Solution II (0.2 M NaOH, 1% (w/v) SDS) and gentle stirring (not vortexing). After 10 min incubation on ice, the solution was neutralised by the addition of 60 mL of pre-cooled Solution III (3 M sodium acetate (pH 5.2)) and incubated again on ice for 15 min. Centrifugation at 8,000 rpm for 30 min at 4°C leads to precipitation of proteins, chromosomal DNA and high molecular RNA, leaving plasmid DNA in solution. The supernatant was then filtered through a glass wool into a fresh Beckman polycarbonate bottle and 150 mL of isopropanol was added. Centrifugation at 8,000 rpm for 30 min precipitates the nucleic acids. The pellet was resuspended in 4 mL TE buffer and the sample was weighed accurately. Two rounds of CsCl density gradient centrifugation are needed to further purify the covalently closed circular DNA from contaminating open circular DNA, linear DNA and
low molecular weight RNA. 1.019 g of CsCl and 0.11 mL of a 10 mg/mL solution of ethidium bromide was added per gram of sample. The sample was then pipetted into a 39 mL Beckman polyallomer tube and centrifuged at 45,000 rpm in a VTi 50 rotor in a Beckman centrifuge overnight (minimum 16 hr). Using a long-wave UV light, the bands with the different DNA isoforms could be visualised and with a sterile syringe and needle the band that contained the covalently closed circular DNA (lower band) was transferred to a 5.1 mL Beckman polyallomer tube. The samples were carefully weighed and balanced and centrifuged in a VTi 65.2 rotor at 48 K overnight (minimum 16 hr). The lower plasmid band was then removed as described previously and extracted with an equal volume of water-saturated butanol for as many times necessary for the colour to disappear. Equal volumes of water and isopropanol were then added and the DNA was left to precipitate at room temperature for at least 2 hr. The DNA was pelleted in a clean Corex tube by centrifugation at 8,000 rpm for 30 min in a Sorvall ultracentrifuge with an HB4 rotor. The pellet was resuspended in 0.3 M sodium acetate, 2.5 volumes of ethanol was added and the DNA was precipitated by centrifugation as above. The DNA was resuspended in TE and the concentration was determined by its absorption at 260 nm.

Small-scale plasmid preparation. Plasmid DNA from bacterial cultures of up to 5 mL was prepared using the Wizard™ miniprep DNA purification kit (Promega) as described in the manufacturers instructions. Briefly, a cleared lysate was prepared in a similar way to the method described for large-scale plasmid preparation. The resultant lysate was then combined with a suspension of silica-based resin which preferentially binds DNA and can be subsequently rapidly rehydrated by the addition of water or TE buffer allowing plasmid DNA to be released.

Singly-nicked DNA. Singly-nicked pBR322 was prepared by digestion of the supercoiled form with DNAase I in the presence of EtBr (Greenfield et al., 1975). Supercoiled pBR322 DNA (190 μg) was incubated with 180 μg EtBr and 88 U DNAse I (Boehringer) in 5 mM Tris.HCl (pH 7.5), 125 mM NaCl, 20 mM MgCl₂, 0.1 mg/mL BSA in a final 400 μL reaction volume at 25°C for 30 min. The reaction was terminated and the EtBr removed by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform/isoamyl alcohol extraction and ethanol precipitation.

Positively-supercoiled DNA. Positively-supercoiled DNA was prepared from singly-nicked pBR322 by incubation with gyrase and ligase. A 300 μL reaction containing 7.5 pmol nicked DNA, 150 pmol gyrase and 50 U E. coli DNA ligase (NEB) in "Ligase Buffer" (NEB), was incubated at 16°C overnight and terminated as described above. Alternatively, positively supercoiled DNA was prepared by incubating relaxed pBR322 with gyrase and topo I under the conditions described for the topo I relaxation assay.

Highly negatively-supercoiled DNA. Highly negatively-supercoiled DNA (σ ~ -0.13) was prepared by relaxation of supercoiled pBR322 by chicken erythrocyte topoisomerase I in the presence of EtBr. A 400 μL reaction containing 190 μg supercoiled pBR322 and 34.2 μg EtBr in 20 mM Tris.HCl (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 4% (w/v) glycerol, 0.1
mg/mL BSA and topoisomerase I was incubated in the dark at 25°C for 2 hr. The DNA was purified as described above for the preparation of nicked pBR322.

**Preparation of short (198-20 bp) DNA fragments.** The 198 bp fragment was amplified by the polymerase chain reaction (PCR) using pBS147 as a template (Orphanides & Maxwell, 1994). The 147, 90, 80 bp fragments containing the major gyrase cleavage site of plasmid pBR322 (Dobbs *et al.*, 1992) were prepared by PCR using pBR322 as a template. The polymerase chain reaction (PCR) was performed in a Perkin-Elmer Gene Amp 9600 system. Each 100 μL reaction contained 3 μM of each primer, 300 μM of each dNTP, 10^{2}-10^{3} copies of the template and 10 μL of 10x polymerase buffer. The reactions were pre-incubated at 94°C for 5 min and then 1 μL of a 6:1 (Unit:Unit) mixture of Taq and Pfu polymerase was added and the reactions allowed to cycle under the following protocol.

<table>
<thead>
<tr>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>10</td>
</tr>
<tr>
<td>89°C</td>
<td>55°C</td>
<td>72°C</td>
<td>20</td>
</tr>
</tbody>
</table>

The products were then purified using a QIAGEN PCR Purification Kit. The following oligodeoxynucleotides were used in the PCR reactions.

147L 5' biotin TCG GGG AAT TCG CAT 3'  
147R 5' CCG AGG AAT TCT GGA C 3'  
198L 5' biotin TCG AGG TCG ACG GT 3'  
198R 5' AAC TAG TGG ATC CCC C 3'  
90L 5' biotin CGC TGG GCT ACG TCT TGC 3'  
90R 5' GCA TCC CGA TGC CGC CGG AAG C 3'  
80L 5' biotin GGC TAC GTC TTG CTG G 3'  
80R 5' CCG ATG CCG CCG GAA GC 3'

The 40 bp and 20 bp fragments, based on the same pBR322 site, were prepared by the annealing of complementary chemically synthesised oligonucleotides that had been purified by HPLC (PNACL, University of Leicester). The sequence of these oligonucleotides was:

40T 5' CGC GAC GCG AGG CTG GAT GGC CTT CCC CAT TAT GAT TCT T 3'  
40B 5' AAG AAT CAT AAT GGG GAA GGC CAT CCA GCC CGC CGT G 3'  
20T 5' GGC TGG ATG GCC TTC CCC AT 3'  
20B 5' ATG GGG AAG GCC ATC CAG CC 3'

**Preparation and purification of oligodeoxynucleotides.** Oligonucleotides were synthesised at 1 μmol scale using an Applied Biosystems 394 DNA synthesiser (PNACL, University of Leicester). Phosphoramidite-synthesised oligonucleotides require a final reaction step with concentrated ammonia for their complete deprotection. The resulting organic by-products (e.g. benzamides) need to be removed from the oligonucleotides before
these are used in enzymatic processes. This was achieved using the method of Sawadogo and Van Dyke (1991). Briefly, 100 μL of the nucleotide solution was mixed with 1 mL of n-butanol, vortexed for 15 seconds and finally centrifuged for 1 min at 13,000 rpm. The single H₂O-containing butanol phase is discarded and the pellet is dried under vacuum and resuspended in H₂O. Sometimes the purification is not complete in one step and it may be necessary to repeat the butanol extraction to achieve pure products. In this case, the pellet is resuspended in 100 μL H₂O and the procedure repeated. Biotinylated oligonucleotides were synthesised with the incorporation of a trityl group allowing for tritylated full length sequences to be separated from untritylated failure sequences by exploiting the intrinsic hydrophobicity of the 4,4'-dimethoxytrityl group. For the purification the Cruachem Oligonucleotide purification (COP) cartridges were used. 2 mL of de-ionised H₂O was added to the ammonium hydroxide solution of the ‘trityl-on’ oligonucleotide and the mixture was divided to 5 equal parts which were then purified separately. A 5 mL syringe was fixed onto the cartridge and 2 mL of acetonitrile was gently pushed through the cartridge at a rate of 1-2 drops per second. The cartridge was then flushed with 2 mL of 2 M triethylamine acetate (pH 7.0) (TEAA) at the same rate. The oligonucleotide solution was then applied to the cartridge using the above procedure. The eluate was collected and loaded on the column for a second time. The cartridge was then flushed first with 3 mL of 1:10 dilution of ammonium hydroxide and then with 2 mL of de-ionised H₂O. The purified oligonucleotides were then eluted with 1 mL of 20% (v/v) acetonitrile/water, dried under vacuum and resuspended in H₂O.

Phenol Extraction. In order to remove proteins from DNA samples, an equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1) was added and the mixture was vortexed and centrifuged at 13,000 for 5 min. The top aqueous phase contains the purified DNA and is removed carefully.

Ethanol Precipitation of DNA. Sodium acetate (pH 5.2) was added to a DNA solution to a final concentration of 0.3 M and then 3 volumes of cold (-20°C) absolute ethanol was added and the mixture was incubated on dry ice for 15 min and then centrifuged for 30 min at 13,000 rpm at 4°C. The supernatant was discarded and the pellet was washed with cold (-20°C) 70% ethanol, centrifuged as above and the new pellet was dried under vacuum and resuspended in the buffer required.

Determination of DNA Concentration. The concentration of a DNA solution was determined spectrophotometrically by measuring the absorbance of the solution at 260 nm. A 50 μg/mL solution of double-stranded DNA or a 33 μg/mL solution of single-stranded DNA has an absorbance of 1 cm⁻¹ at this wavelength. For the determination of the concentration of the primer solutions the extinction coefficient of the primer, ε₂₆₀, can be calculated as the sum of the respective extinction coefficient of each deoxyribonucleotide in the primer. The coefficients employed for the four deoxyribonucleotides are: ε₈=15.4 mM⁻¹cm⁻¹, ε₅=9.7 mM⁻¹cm⁻¹, ε₉=11.4 mM⁻¹cm⁻¹, ε₆=9.2 mM⁻¹cm⁻¹.
8.3. Enzyme assays

**Agarose gel electrophoresis.** Agarose at a concentration of 1% (w/v) was melted in TAE (40 mM Tris acetate, 2 mM EDTA) and poured into a suitable former (the appropriate amount of chloroquine was added when necessary). After the gel had set it was transferred to a horizontal electrophoresis tank and submerged in TAE buffer (containing the necessary concentration of chloroquine). One third volume of STEB loading buffer (40% (w/v) sucrose, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mg/mL bromophenol blue) was added to the DNA samples before loading on the gel. Electrophoresis was performed at 30-120 V. The gel was then stained in a 1 μg/mL solution of ethidium bromide in TAE and destained in TAE buffer. When chloroquine was present the staining and destaining steps were repeated until the chloroquine was almost completely removed. The DNA was then visualised using a UV transilluminator and photographed using Kodak T_{max} film. Alternatively, the image was captured to file using a UVP Gel Documentation System (UVP Ltd., Cambridge, UK).

**Polyacrylamide gel electrophoresis of DNA.** Polyacrylamide gels were used for the detection of short DNA fragments. The gels contained 5% acrylamide (19:1 acrylamide: bis-acrylamide) in TBE buffer (90 mM Tris borate (pH 8.3), 2 mM EDTA) and were polymerised upon the addition of 0.1% (w/v) ammonium persulfate and 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED). Polyacrylamide gels were run vertically in a Bio-Rad apparatus at 80-120 V and stained and visualised as above.

**Filter-binding assays.** Binding of proteins to DNA can be detected by using a nitrocellulose filter binding assay. The principle of the technique is that protein-free nucleic acids pass freely through membrane filters, whereas protein-DNA complexes are retained. The assay can be quantitated by using radioactively labeled DNA to form the DNA-protein complex and determining the amount of radioactivity retained on the filter by scintillation counting. Nitrocellulose filter binding assays have been used previously to quantify the gyrase-DNA complex (Higgins & Cozzarelli, 1982, Maxwell & Gellert, 1984). Filter binding was performed using a 25 mm Millipore glass filtration apparatus and 25 mm diameter, 0.45 μm pore nitrocellulose filters (Schleicher and Schuell). Experiments were carried out in 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 5 mM DTT, 6.5% glycerol and 0.36 mg/mL BSA. Filters were soaked in this buffer for at least 1 hr prior to use. Samples containing ³H-labeled pBR322 DNA (provided by Mrs. A. Howells, Leicester University) were filtered at a flow rate of 1 mL/min through a nitrocellulose filter previously washed in 0.5 mL of buffer. After loading the sample, the membrane was washed with 0.5 mL of buffer. Filters were dried under infra-red heat lamps and the amount of DNA retained in each was determined by scintillation counting. Only very low levels of non-specific retention of DNA was found in the control samples that did not contain gyrase.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).** Separating gels contained 0.1% (w/v) SDS, 375 mM Tris-HCl (pH 8.8), 12% or 15% (w/v) acrylamide, and 2.67% (w/v) N,N'-bis-methylene-acrylamide, and were polymerised by adding 0.05% (w/v) ammonium persulphate and 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine.
(TEMED). The stacking gels contained 0.1% SDS (w/v), 125 mM Tris-HCl (pH 6.8), 4% (w/v) acrylamide, and 0.9% N'N'-bis-methylene-acrylamide, and were polymerised by adding 0.05% (w/v) ammonium persulphate and 0.1% (v/v) TEMED. Protein samples were prepared by the addition of an equal volume of 2x Sample Application Buffer (SAB), leading to a final concentration of 62 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, and 0.001% (w/v) bromophenol blue, followed by heating in a 95°C water bath for 5 minutes. Gels were cast and then run for approximately 40 minutes at 40 mA in a Biorad Minigel apparatus. Gels were stained for protein using Coomassie brilliant blue (0.1% w/v) in an aqueous 30% (v/v) methanol, 12% (v/v) trichloroacetic acid, 10% (w/v) sulphosalicylic acid solution and were destained using an aqueous 5% methanol, 7.5% acetic acid solution. Gels were stored in the destaining solution.

**Rapid gel filtration.** In order to investigate the binding of ligands to the gyrase-DNA complex the method of rapid gel filtration was used. $^3$H-CFX (Amersham) and $^3^2$P-ADPNP (ICN) binding assays were performed in 50 mM Tris.HCl (pH 7.5), 50 mM KCl, 4 mM MgCl$_2$, 4 mM dithiothreitol, 6.5% (w/v) glycerol at 25°C and enzyme-bound and free ligand molecules were separated by gel filtration through a spin-column (Nick spin-columns, Pharmacia) (Ali et al., 1995, Tamura et al., 1992). The reactions (50 μL) were applied on an G50 Sephadex spin-column (Pharmacia) which has been pre-equilibrated on the appropriate binding buffer. Prior to the addition of sample to the spin column, the top of the Sephadex bed was rehydrated by the addition of 100 μL of buffer. The columns were centrifuged at 1,700 rpm for 4 min in an MSE centrifuge. As a result macromolecules and bound ligand pass through the column and collected in an Eppendorf tube while free ligand is retained in the Sephadex beads. Samples were collected in screw-capped 1.5 mL tubes and the exact volume of eluate determined by weighing each collection tube before and after centrifugation. 100 μL of each column eluate was added to 4.5 mL of the liquid scintillant OptiPhase (Fisons) and counted on a Tri-Carb 2000CA Liquid Scintillation Analyser. The amount of enzyme that eluted from the column was estimated by determining the protein concentration of the sample prior and after centrifugation using the Bradford method (Bradford, 1976).

**Limited proteolysis.** Samples contained 0.3 mg/mL of GyrA and/or GyrB, 0.4 mg/mL linear pBR322 DNA (where indicated) and various effector molecules (as described in the figure legends) in 50 mM Tris.HCl (pH 7.5), 50 mM KCl, 4 mM MgCl$_2$, 4 mM dithiothreitol, 6.5% (w/v) glycerol. After incubation for at least 1 hr at 25°C, protease was added and the reaction was allowed to proceed at 37°C. Samples were taken at various times and quenched by adding an equal volume of 2x SAB (SAB: 62 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue, and boiling for up to 15 min. The products were analysed by SDS polyacrylamide gel electrophoresis (PAGE).

**Supercoiling assays.** Gyrase activity was determined using a DNA supercoiling assay, essentially as described by Mizuuchi et al (Mizuuchi et al., 1984). Reaction volumes of 30 μL contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl$_2$, 1.8 mM spermidine, 0.36
mg/mL BSA, 9 μg/mL tRNA, 6.5% (w/v) glycerol, 5 mM DTT, 1.4 mM ATP, 10 μg/mL relaxed pBR322* DNA and appropriate concentrations of the two subunits. Reactions were incubated for 1 hr at 25°C and the protein was extracted by the addition of 30 μL chlorophorm:isoamyl alcohol (24:1). One unit of supercoiling activity was defined as the amount of the enzyme required to convert 50% of the relaxed DNA to the supercoiled form in 1 hr at 25°C. Activities were expressed as units/mg of protein. Activities of the individual subunits were determined by performing supercoiling assays where the subunit being assayed was serially diluted into reaction mixture containing an excess of the other subunit.

**ATP-independent relaxation.** ATP-independent relaxation assays were performed under the same conditions as the supercoiling assays but for the omission of ATP. Also, negatively supercoiled pBR322 DNA was used instead of the relaxed form.

**ATP-dependent relaxation.** ATP-dependent relaxation was carried out initially under standard supercoiling conditions but was subsequently optimised to 35 mM Tris.HCl (pH 7.5), 12.5 mM KCl, 8 mM MgCl₂, 0.36 mg/mL BSA, 9 μg/mL tRNA, 6.5% (w/v) glycerol, 5 mM DTT, 1.4 mM ATP and 10 μg/mL negatively supercoiled pBR322.

**DNA wrapping.** The extent of DNA wrapping around GyrA and the 59-kDa domain was determined by incubating nicked pBR322 (26.6 pg/mL) with different concentrations of the enzyme and resealing the nick with 5 Units *E. coli* ligase (NEB) as described previously (Reece & Maxwell, 1991a).

**Topo I relaxation assays.** Topo I relaxation assays contained 0.8 μg of the appropriate topological form of pBR322 and the indicated amounts of enzyme in 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 1.8 mM spermidine, 0.36 mg/mL BSA, and 9 μg/mL tRNA. ADPNP was added to 1.4 mM and the reactions were incubated for 30 min at 25°C. Samples were then treated with chicken erythrocyte topo I (A. J. Howells, Leicester University) for 30 min at 37°C. The final concentration of topo I was a six-fold excess of the concentration required to fully relax the same amount of negatively supercoiled pBR322 (σ = -0.06) under the above conditions. Reactions were stopped by 0.2% SDS and treated with 0.1 mg/mL proteinase K for 30 min at 37°C. The DNA was extracted with chloroform / isoamyl alcohol 25:1 and analysed by agarose gel electrophoresis. Experiments with A642B2 were all performed in ATP-dependent relaxation conditions (Kampranis & Maxwell, 1996). Linking number changes were measured by determining the centre of the topoisomer distribution after capture of the gel image by camera and analysis of the image using the program ImageQuant (Molecular Dynamics Inc.).

**Catenation and decatenation assays.** Decatenation reactions were performed using 13.3 μg/mL kinetoplast DNA (Topogen) under ATP-dependent relaxation conditions at 37°C. Catenation reactions were performed in 25 mM Tris.HCl (pH 7.5), 15 mM KCl, 2 mM MgCl₂, 5 mM spermidine, 0.36 mg/mL BSA, 9 μg/mL tRNA, 6.5% (w/v) glycerol, 5 mM DTT, 0.5 mM ATP and 6.7 μg/mL negatively supercoiled pBR322, at 37°C.

**ADPNP-induced strand passage experiments.** ADPNP-driven single-strand passage experiments were performed in a similar manner to the topo I relaxation assays but for the
omission of the topo I relaxation step. Linking number changes were measured by determining
the centre of the topoisomer distribution after capture of the gel image by camera and analysis
of the image using the program ImageQuant (Molecular Dynamics Inc.).

**ADPNP-induced DNA cleavage.** ADPNP-induced cleavage was revealed by disrupting
the enzyme-DNA complexes with 0.2% SDS and removal of the protein with 0.1 mg/mL
proteinase K at 37°C for 30 min. The efficiency of cleavage and was determined by
quantifying the relative intensity of the linear band using the program ImageQuant (Molecular
Dynamics Inc.) and was expressed as percentage of enzyme molecules that have induced
cleavage.

**Drug-induced cleavage assays.** Quinolone-induced cleavage assays were carried out
under the same conditions as the supercoiling assays except that appropriate concentrations
of quinolone was included and ATP was present only when indicated. The reactions were
incubated for times up to 3 hr at 25°C and the reaction was stopped by the addition of SDS to
0.2% (w/v) and proteinase K (Sigma) to 0.1 µg/mL final concentrations. The sampled were
then incubated for another 30 min at 37°C and then extracted and analysed similarly to the
supercoiling assay reactions. In quinolone-induced DNA cleavage timecourses, 10 nM relaxed
pBR322 was incubated with 18 nM GyrA, 54 nM GyrB (giving a final concentration of A_2B_2
of 9 nM) and the indicated concentrations of ciprofloxacin (CFX) at 25°C for the times
shown. Cleavage was revealed by addition of 0.2% SDS and 0.1 mg/mL proteinase K and
incubation at 37°C for 30 min. The results were analysed by electrophoresis on 1% agarose
gels containing 3 µg/mL chloroquine where indicated, and the cleavage was quantified by
analysis of the captured image with the program ImageQuant (Molecular Dynamics Inc.).

**Ca^{2+}-induced cleavage assays.** Ca^{2+}-induced cleavage was performed under regular
supercoiling conditions but for the substitution of Mg^{2+} with 4 mM Ca^{2+}. Cleavage was again
revealed by the addition of 0.2% SDS and 0.1 mg/mL proteinase K and incubation at 37°C for
30 min.

**ATPase assays.** ATPase assays were performed using a pyruvate kinase-lactate
dehydrogenase linked assay as described previously (Ali et al., 1993, Hammonds & Maxwell,
1997), in 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM MgCl_2, 5 mM DTT, 6.7% w/v
glycerol, 2 mM ATP at 25°C. 200 µL reactions contained 400 µM phosphoenolpyruvate, 250
µM nicotinamide adenine dinucleotide (NADH) and 3.5 µL of pyruvate kinase/lactate
dehydrogenase mix (in 50% (w/v) glycerol, 100 mM KCl, 10 mM HEPES (pH 7.0), 0.1 mM
EDTA; Sigma). ATP was added as a Mg^{2+} complex to ensure that the free [Mg^{2+}] in the
assay was maintained. Reactions were initiated by the addition of ATP and the decrease in
A_{340} measured as a function of time. The change in absorbance was related to [NADH]
decrease / [ADP] production using an extinction coefficient ε_{340} = 6.22 mM^{-1} cm^{-1}.

**Peptide sequencing.** N-terminal peptide sequencing was carried out by Dr. K. Lilley
(PNAACL, Leicester University). Proteolysis reactions containing 100 pmol of each subunit
were treated as described previously and the product fragments were separated by SDS
PAGE. The polyacrylamide gel was used by PNAACL to complete the analysis.
EXPERIMENTAL PROCEDURES

Formation of heterogeneous tetramers. Heterogeneous tetramers were formed by mixing the indicated amounts of wild-type and mutant GyrB proteins and allowing for the mixture to equilibrate at 25°C for 1 hr. GyrA was then added in excess of the total B protein and the gyrase complexes were allowed to form for 1 hr at 25°C. After DNA was added (where indicated), reactions were incubated at 25°C for a further 30 min. 2 mM ATP was then added to initiate the reactions.

Curve-fitting. Curve-fitting was performed using the programs KaleidaGraph 2.1.3 (Adelbeck software) and MacCurveFit v. 1.3 (Kevin Raner software). Kinetic simulations were performed with KFitSim (D. Thomas, N. Millar).

Image Analysis. Images of DNA gels were captured in TIFF format using a UVP system and program (UVP Ltd., Cambridge UK). Image files were analysed with the program ImageQuant (Molecular Dynamics Inc.). Linking number changes were measured by quantifying the intensity of the DNA bands across the length of a lane and determining the centre of the distribution by inspection of the produced bell-shaped curve. DNA cleavage was quantified as a percentage of overall DNA present by comparing the intensity of the linear band to the intensity of the total DNA present in each reaction. Quantitation of the intensity of the nicked band was corrected for the amount of nicked DNA already present in the DNA preparation.

Secondary structure prediction. Secondary structure and solvent accessibility predictions were performed using the programs PHDsec and PHDacc (B. Rost, EMBL Heidelberg, (Rost & Sander, 1993, Rost & Sander, 1994)).

8.4. References
EXPERIMENTAL PROCEDURES
