# DNA Cleavage Is Not Required for the Binding of Quinolone Drugs to the DNA Gyrase−DNA Complex†

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ABSTRACT: The primary target for the quinolone group of antibacterial agents is DNA gyrase. One model for the interaction of quinolone drugs with gyrase and DNA suggests that the drugs bind to the single-stranded regions revealed following DNA cleavage by the enzyme. We have tested this hypothesis by using mutants which have the active-site tyrosine in the gyrase A subunit altered to phenylalanine or serine. We have found that proteins bearing these mutations are still able to bind drug, suggesting that DNA cleavage is not a prerequisite for drug binding. We have also found that the blocking of transcription by RNA polymerase *in vitro* by the gyrase−quinolone complex on DNA does not occur when the active-site tyrosine is mutated to serine; i.e., polymerase blocking requires DNA cleavage.

The intracellular target of the quinolone and coumarin groups of antibacterial agents is DNA gyrase. Gyrase is a bacterial type II topoisomerase which couples the free energy of ATP hydrolysis to the introduction of negative supercoils into closed-circular DNA (Reece & Maxwell, 1991b; Wigley, 1995). The enzyme from *Escherichia coli* consists of two subunits, A and B, of molecular masses 97 and 90 kDa, respectively, in an $A_2B_2$ complex.

The mechanism of DNA supercoiling is thought to involve the following steps. The enzyme binds to DNA, and a segment of ∼130 bp[1] (base pairs) is wrapped around the protein tetramer in a positively-supercoiled sense. This wrapped DNA is cleaved in each strand at sites separated by 4 bp. This results in the covalent attachment of the 5′-phosphate groups at the break sites to the active-site tyrosines, *viz.* $Tyr^{122}$ of the A subunits. Another segment of DNA is transported through this double-stranded break and through the enzyme itself. Resealing of the break results in the reduction of the linking number of the DNA by two. At some point during this reaction cycle, ATP is bound and hydrolyzed. If ATP is replaced by the nonhydrolyzable ATP analog ADPNP (5′-adenylyl $\beta,\gamma$-imidodiphosphate), only limited supercoiling is observed, consistent with nucleotide binding promoting one round of supercoiling, and hydrolysis being required for enzyme turnover.

A number of lines of evidence demonstrate that the A and B proteins contain distinct functional domains. The gyrase A protein (GyrA) comprises an N-terminal domain (59−64 kDa), which is responsible for DNA breakage−reunion and contains residues involved in the interaction with quinolones, and a 33-kDa C-terminal domain involved in DNA wrapping (Reece & Maxwell, 1989, 1991a,c). The gyrase B protein (GyrB) comprises an N-terminal domain (43 kDa), which contains the ATPase site and the coumarin drug-binding site, and a 47-kDa C-terminal domain involved in the interaction with the A protein and DNA (Brown et al., 1979; Gellert et al., 1979; Adachi et al., 1987; Ali et al., 1993).

Gyrase is the target of a number of antibacterial agents including the quinolone and coumarin drugs (Drlica & Coughlin, 1989; Rádl, 1990; Reece & Maxwell, 1991b; Maxwell, 1992, 1993). The coumarin antibiotics (e.g., novobiocin) block DNA supercoiling by inhibiting the ATPase reaction of gyrase (Mizuuchi et al., 1978); their target has been shown to be the B protein. Their mode of action has been suggested to be competitive with ATP (Sugino et al., 1978; Sugino & Cozzarelli, 1980), a suggestion now supported by recent data from X-ray crystallographic studies (Wigley et al., 1991; Lewis et al., 1996). The quinolones (e.g., nalidixic acid, oxolinic acid, and ciprofloxacin) inhibit supercoiling by interrupting the DNA breakage−reunion cycle. This mode of action resembles that of a number of anti-tumor agents whose target is the eukaryotic enzyme related to gyrase, DNA topoisomerase II (Liu, 1989; Corbett & Osheroff, 1993). Incubation of gyrase and DNA in the presence of a quinolone drug and termination of the reaction with SDS lead to the production of double-stranded DNA breaks with the A proteins covalently bound to the newly-formed 5′-phosphates *via* the active-site tyrosines. The bound protein may be removed by proteolysis, releasing the DNA fragments (Gellert et al., 1977; Sugino et al., 1977; Tse et al., 1980). There are preferred sites of DNA cleavage, and weak consensus sequences have been derived (Lockshon & Morris, 1985). ATP (or ADPNP) is not required in the cleavage reaction but can modulate the efficiency of cleavage at certain sites (Morrison et al., 1980). DNA cleavage by gyrase can also be stimulated when $Mg^{2+}$ is replaced by $Ca^{2+}$ (Reece & Maxwell, 1989).

Although quinolone drugs are known to inhibit the DNA supercoiling reaction of gyrase *in vitro*, their mode of action *in vivo* is thought to be *via* another mechanism. A number of lines of evidence, including work on topoisomerase II and

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[1] Abbreviations: ADPNP, 5′-adenylyl $\beta,\gamma$-imidodiphosphate; bp, base pair(s); CFX, ciprofloxacin; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; $Nal^r$, nalidixic acid-resistant; PCR, polymerase chain reaction; ts, temperature sensitive.

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anti-tumor drugs, suggest that the gyrase−quinolone complex on DNA can form a barrier to the passage of polymerases and that this can lead to cell death (Kreuzer & Cozzarelli, 1979; Liu, 1989; Maxwell, 1992). Indeed, experiments *in vitro* show that either gyrase or quinolone alone has no effect on transcription by RNA polymerase but that the gyrase−quinolone complex on DNA blocks transcription (Willmott et al., 1994).

One of the unresolved issues concerning DNA gyrase is the mode of interaction of the enzyme with quinolones. The drugs have been reported to interact with DNA, and the presence of gyrase has been shown to stimulate this interaction (Shen & Pernet, 1985; Shen et al., 1989a,b). A number of models have been proposed for the structure of the quinolone complex with gyrase and DNA (Shen et al., 1989c; Palumbo et al., 1993; Fan et al., 1995). One of these suggests that the drugs interact with the exposed edges of the DNA bases revealed upon cleavage of DNA by gyrase; i.e., quinolones hydrogen-bond to the four-base single-strand extensions at the enzyme's active site (Shen et al., 1989c). Binding studies have confirmed that quinolones bind preferentially to the complex between gyrase and DNA rather than to the protein or DNA alone (Willmott & Maxwell, 1993; Yoshida et al., 1993). In this paper, we have tested whether the cleavage of DNA by gyrase is required for the binding of quinolones to the DNA gyrase−DNA complex.

## EXPERIMENTAL PROCEDURES

*Drugs.* Ciprofloxacin (CFX) was a gift from Bayer (Germany). $^3$H-CFX was custom-synthesized by Amersham International (U.K.) from a precursor (1-cyclopropyl-1,7-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid) generously provided by Bayer. Drug purity was assessed by HPLC (Barbato et al., 1994).

*Enzymes and DNA.* The DNA gyrase A and B proteins were prepared from overproducing strains JMtacA and JMtacB as described previously (Hallett et al., 1990). The 64- and 33-kDa fragments of GyrA were prepared as described by Reece & Maxwell (1991a,c). Plasmids containing the genes encoding the gyrase A proteins bearing the mutations Phe$^{122}$ and Ser$^{122}$ (Wilkinson & Wang, 1990) were generous gifts from Dr. A. J. Wilkinson (University of York, York, U.K.). These mutations were moved into plasmids overexpressing the GyrA protein (pPH3) and the 64-kDa domain (pRJR242) by replacing a 582-bp *Sac*I/*Xma*I restriction fragment with the corresponding fragment carrying the mutation. Briefly, the mutation sites were amplified from plasmids pDH24(Ser122) and pDH24(Phe122) by the polymerase chain reaction (PCR) using oligonucleotides 5′-GAGCGACCTTGCTCGAGAAA-3′ and 5′-TCAACTTC-CACTTCTGCGCG-3′. The amplified DNA fragments were digested with the restriction endonucleases *Sac*I and *Xma*I (New England Biolabs), and the resultant 582-bp fragments were cloned into both pPH3 and pRJR242 and the sequence was determined using either CircumVent thermal cycle dideoxy DNA sequencing (New England Biolabs) or automated sequencing using a *Taq* DyeDeoxy Terminator Sequencing Kit (Applied Biosystems). Relaxed plasmid pBR322 and a 147-bp DNA fragment were prepared as described previously (Bates & Maxwell, 1989; Dobbs et al., 1992). Linear pBR322 was prepared by digestion of the supercoiled form with *Eco*RI.

*Enzyme Assays.* Gyrase-catalyzed supercoiling of relaxed pBR322 DNA was carried out as described by Reece and Maxwell (1989). Quinolone-induced cleavage of DNA by gyrase was carried out under supercoiling conditions except that ciprofloxacin was included at 5 $\mu$g/mL (13.5 $\mu$M) and ATP was omitted. Incubations were for 1 h at 25 °C and were terminated by the addition of SDS and proteinase K, to 0.2% and 0.1 mg/mL, respectively, and incubated for a further 30 min at 37 °C. For $Ca^{2+}$-directed cleavage, CFX was omitted, and 4 mM $CaCl_2$ replaced 4 mM $MgCl_2$. Transcription assays using T7 RNA polymerase were carried out as described previously (Willmott et al., 1994). The binding of gyrase to DNA was measured by a gel-retardation assay described previously (Dobbs et al., 1992) and by nitrocellulose filter binding (Maxwell & Gellert, 1984). Filter binding was performed using a 25 mm Millipore glass filtration apparatus and 25 mm diameter, 0.45 $\mu$m pore nitrocellulose filters (Schleicher & Schuell). Prior to use, filters were soaked in binding buffer [35 mM Tris·HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 5 mM DTT, 6.5% glycerol, and 0.36 mg/mL BSA] for at least 1 h. Reaction mixtures (30 $\mu$L) were as described for supercoiling assays, except that 5′-end-labeled 147-bp DNA replaced pBR322, and ATP was omitted. Samples were incubated for 1 h at 25 °C, and then 170 $\mu$L of binding buffer was added to each sample. A 200 $\mu$L aliquot was immediately filtered at a flow rate of 1 mL/min through a filter previously washed in 0.5 mL of binding buffer. After the sample was loaded, the membrane was washed with 0.5 mL of binding buffer. Filters were dried under infrared lamps, and the amount of DNA retained was determined by scintillation counting. This procedure was found to yield low levels of nonspecific retention of DNA.

*Drug-Binding Studies.* The binding of $^3$H-CFX to gyrase and DNA was measured using a spin-column assay described previously (Willmott & Maxwell, 1993). Briefly, gyrase and/or DNA was incubated with radiolabeled ciprofloxacin in binding buffer (80 $\mu$L) for 3 h at 25 °C; 75 $\mu$L of each sample was then passed through a prepacked Sephadex G50 column (Nick Spin Columns, Pharmacia) equilibrated in binding buffer. Prior to the addition of sample to the spin column, the top of the Sephadex bed was rehydrated by the addition of 75 $\mu$L of binding buffer. The columns were centrifuged at 1700 rpm for 4 min in an MSE Centaur 2 centrifuge at 4 °C. Samples were collected in screw-capped 1.5 mL tubes, and the exact volume of eluate was determined by weighing each collection tube before and after centrifugation. The amount of labeled drug in each sample was determined by scintillation counting and corrected for the amount of eluate recovered.

## RESULTS

*Generation and Characterization of Active-Site Mutants.* The cleavage of DNA by DNA gyrase is thought to involve the nucleophilic attack of a phosphodiester bond in DNA by Tyr$^{122}$ of GyrA (Horowitz & Wang, 1987). Support for this idea has been provided by site-directed mutagenesis experiments in which the active-site tyrosine was replaced by phenylalanine or serine (Wilkinson & Wang, 1990). Expression of either protein in a *gyrA$^{ts}$* (temperature-sensitive) strain failed to complement the ts mutation at the nonpermissive temperature. Moreover, the purified Ser$^{122}$

protein was found to be catalytically inactive (Wilkinson & Wang, 1990).

An important feature of the model for quinolone binding to the gyrase−DNA complex proposed by Shen and coworkers is that the drugs bind to the exposed edges of the bases following DNA cleavage by gyrase (Shen et al., 1989c). Therefore, DNA cleavage should be a prerequisite for drug binding. We sought to test this idea by employing GyrA proteins bearing the $Ser^{122}$ and $Phe^{122}$ mutations. When we moved these mutations into plasmid pPH3 [which overexpresses GyrA; Hallett et al. (1990)], we obtained two unexpected results. First, strains expressing the $Phe^{122}$ protein grew very poorly and yielded very little GyrA upon induction. Second, expression of the $Ser^{122}$ protein yielded purified GyrA with significant supercoiling activity (~10% of wild type; data not shown). A similar observation to the latter was also made by Wilkinson and Wang (1990) and can be attributed either to significant contamination by chromosomal wild-type GyrA or to supercoiling activity intrinsic to the $Ser^{122}$ protein.

To circumvent these problems, we moved the mutations into plasmids expressing the 64-kDa domain of GyrA. We have previously shown that this domain, when complexed with GyrB, can support quinolone-induced cleavage of DNA as efficiently as the intact GyrA protein (Reece & Maxwell, 1991c). We found that the 64-kDa protein containing either the $Phe^{122}$ or the $Ser^{122}$ protein can be overproduced in a similar manner to the wild-type protein (data not shown). When these proteins and the wild-type 64-kDa domain were purified, they showed no detectable DNA supercoiling activity in the presence of GyrB (Figure 1A). However, when complexed with the 33-kDa C-terminal domain of GyrA, only the wild-type protein gave supercoiling activity. When assayed for DNA cleavage activity, only the wild-type protein supported quinolone-dependent cleavage activity in the presence of GyrB (Figure 1B). DNA cleavage by DNA gyrase can also be stimulated by $Ca^{2+}$ (Reece & Maxwell, 1989). We have found that, when complexed with GyrB, the wild-type 64-kDa fragment does not cleave DNA in the presence of $Ca^{2+}$ (Figure 2). However, if the 33-kDa domain is also included in this reaction, cleavage activity is apparent. When the mutant 64-kDa proteins were assayed for $Ca^{2+}$-stimulated cleavage, no activity was found either in the absence or in the presence of the 33-kDa domain. These results suggest that neither the $Phe^{122}$ nor the $Ser^{122}$ proteins are catalytically active.

DNA binding by the wild-type and mutant 64-kDa proteins was measured by gel retardation and filter binding. We found that the complexes of the 64-kDa proteins with GyrB bind DNA weakly and that DNA binding by the both the wild-type and mutant 64-kDa proteins is enhanced by the presence of CFX (Table 1). This result suggests that the $Phe^{122}$ and $Ser^{122}$ proteins are able to interact with the quinolone drug. Addition of the 33-kDa protein to complexes between the wild-type and mutant 64-kDa proteins with GyrB and DNA leads to stabilization of the DNA−protein complex. Results from DNA-binding studies (and other experiments) are summarized in Table 1.

*Drug-Binding Studies.* To measure the binding of CFX to gyrase and DNA, we have employed the technique of rapid-gel filtration (spin columns) which has previously been used in drug-binding experiments (Willmott & Maxwell, 1993; Gormley et al., 1996). In this assay, drug is incubated

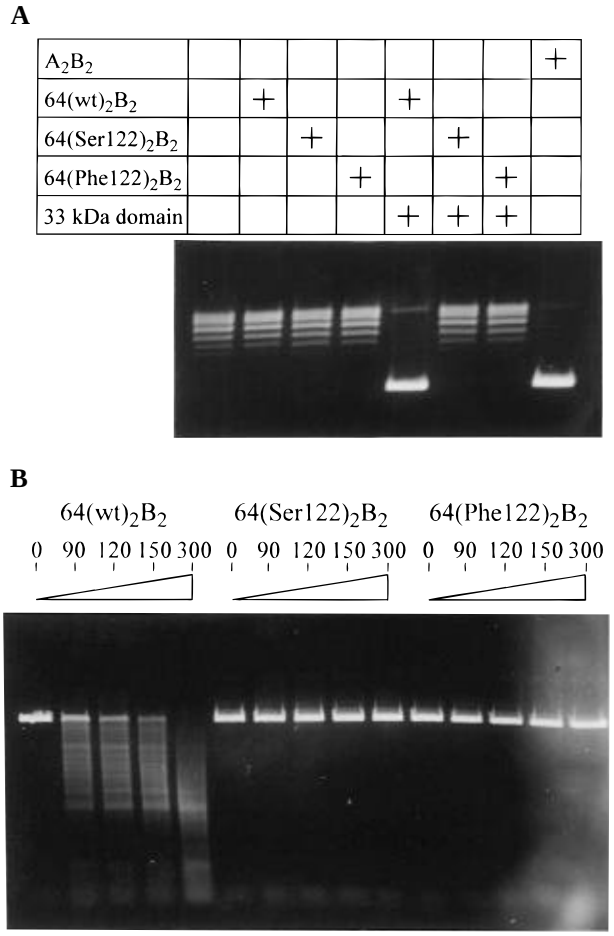


FIGURE 1: Activities of wild-type and mutant 64-kDa proteins. (A) Supercoiling activities of wild-type and mutant $64_2B_2$ complexes in the presence and absence of the 33-kDa domain of GyrA. Relaxed pBR322 DNA (10 $\mu$g/mL) was incubated with each gyrase mix (10 nM) for 1 h at 25 °C and analyzed on a 0.8% agarose gel. (B) Quinolone-induced cleavage reactions of wild-type and mutant $64_2B_2$ complexes. Linear pBR322 DNA (30 $\mu$g/mL) was incubated with each gyrase mix (concentrations as indicated in nM) in the presence of 5 $\mu$g/mL (13.5 $\mu$M) ciprofloxacin for 1 h at 25 °C. After addition of SDS and proteinase K and incubation for 30 min at 37 °C, samples were analyzed on a 1% agarose gel.

with gyrase and/or DNA and then rapidly spun through a column of Sephadex G50 in a centrifuge tube. Drug bound to macromolecule is eluted from the column. Using this technique, we have analyzed the binding of $^3$H-CFX to complexes between a 147-bp DNA fragment containing the preferred gyrase cleavage site from plasmid pBR322 (Dobbs et al., 1992) and wild-type and mutant gyrases (Figure 3). In all experiments, we found insignificant binding of the drug to DNA and only low levels of binding to protein alone. With the 64-kDa fragment complexed with GyrB and DNA, we found lower levels of drug binding to the mutant complexes than to wild type (Figure 3A). However, in the presence of the 33-kDa GyrA domain, binding to the mutant proteins was increased to at least two-thirds that of wild type (Figure 3B). In the case of the $Ser^{122}$ mutation, the full-length GyrA protein could be used in a drug-binding assay, and we found that the level of binding to the wild-type and mutant complexes was very similar (Figure 3C). When this binding experiment was carried out at a range of protein concentrations, the binding of drug to the wild type and the mutant was again found to be very similar (Figure 4).

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $A_2B_2$ | | + | | | | | | |
| $64(wt)_2B_2$ | | | + | | | + | | |
| $64(Ser122)_2B_2$ | | | | + | | | + | |
| $64(Phe122)_2B_2$ | | | | | + | | | + |
| 33 kDa domain | | | | | | + | + | + |

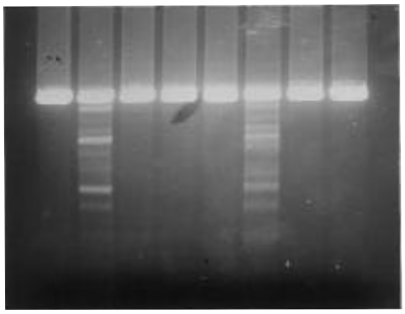

FIGURE 2: $Ca^{2+}$-induced cleavage by wild-type and mutant gyrase complexes. Linear pBR322 DNA (10.5 nM) was incubated for 1 h at 25 °C with each gyrase mix (120 nM protein) as indicated, in the presence of 4 mM $CaCl_2$. Cleavage was revealed by the addition of SDS and proteinase K, and the samples were analyzed as described in Figure 1.

*Transcription Assays.* In previous work, we have shown that the complex between gyrase and quinolone drugs on DNA prevents the passage of RNA polymerase and leads to premature transcription termination (Willmott et al., 1994). We have now tested the GyrA(Ser$^{122}$) protein in such experiments (Figure 5). We have found that, although this protein binds quinolone drug when complexed with GyrB and DNA (Figures 3C and 4), it fails to block transcription; i.e., transcription blocking requires the enzyme to be able to cleave DNA. In addition, we have found that a complex between the 64-kDa fragment bearing the Phe$^{122}$ mutation, the 33-kDa fragment, and GyrB fails to block transcription by T7 RNA polymerase in the presence of ciprofloxacin, whereas the equivalent wild-type complex efficiently blocked transcription (data not shown). These data indicate that DNA cleavage by gyrase is required for a complex between gyrase, quinolone, and DNA to form a barrier to the passage of RNA polymerase.

## DISCUSSION

An important question concerning the quinolone drugs is the nature of their binding site within the gyrase−DNA complex. The occurrence of quinolone-resistance mutations in *gyrA* led to the expectation that the drugs bind directly to GyrA (Gellert et al., 1977; Sugino et al., 1977). However, the occurrence of mutations in *gyrB* suggested that the binding site may encompass both subunits (Yamagishi et al., 1986). Binding studies by Shen and co-workers have led to a somewhat different conclusion. Their initial binding data showed no binding to either GyrA or GyrB, or to the intact enzyme ($A_2B_2$), but significant binding to DNA (Shen & Pernet, 1985). Further binding data showed that gyrase could stimulate the binding of quinolones to double-stranded DNA (Shen et al., 1989b). These and other data have been incorporated into a model for the interaction of quinolones with gyrase and DNA (Shen et al., 1989c). In this model, gyrase cleaves double-stranded DNA with a four-base stagger, and the exposed single-strand regions are proposed to constitute the binding sites for the drugs, which interact with the bases *via* hydrogen bonding to the 3-carboxy and 4-oxo groups common to nearly all quinolones. Drug binding is thought to be cooperative with at least four molecules binding per site, associating with each other by ring stacking and hydrophobic interactions. Interaction with gyrase is proposed to occur *via* the group at the C-7 position of the quinolone (Shen et al., 1989c). One potential difficulty with this model is that the C-7 group of the drug is quite variable in quinolones (Maxwell, 1992).

We have sought to apply a direct test to this model by determining whether mutants which lack the active-site tyrosine, responsible for DNA cleavage, can bind the drugs. Two site-directed mutations at Tyr$^{122}$ of GyrA had already been reported (Wilkinson & Wang, 1990). In this earlier work, it was found that GyrA with either the Phe or the Ser mutation at position 122 would, as expected, not complement a GyrA(ts) strain. However, unexpected results were obtained in other experiments. Nalidixic acid sensitivity is dominant over resistance (Hane & Wood, 1969); i.e., expression of an episomal wild-type *gyrA* gene in a nalidixic acid (Nal$^r$)-resistant strain leads to a sensitive phenotype. It was found that expression of an episomal *gyrA* gene encoding the Phe$^{122}$ mutation in a *gyrA* (Nal$^r$) background led to a resistant phenotype, whereas expression of the Ser$^{122}$ mutation conferred sensitivity, although the cells were still somewhat resistant compared to those expressing wild-type enzyme (Wilkinson & Wang, 1990). When the GyrA(Ser$^{122}$) was purified, it was found to possess ~1% of the supercoiling activity of preparations of the wild-type enzyme. It was suggested that this level of activity resulted from contamination from chromosomal (wild-type) GyrA. In DNA cleavage experiments, the GyrA(Ser$^{122}$) protein was found to support only low-level cleavage in the presence of GyrB and oxolinic acid (Wilkinson & Wang, 1990).

In our experiments, we found that GyrA(Phe$^{122}$) could not be easily expressed and that GyrA(Ser$^{122}$), which could be readily expressed, exhibited significant supercoiling activity. In light of these data and those from Wilkinson and Wang, it is likely that the residual supercoiling activity found for the GyrA(Ser$^{122}$) protein is due to contamination by chromosomal GyrA. The relatively high level of contamination we observed (~10%) is suprising but might be a consequence of elevated expression of chromosomal (wild-type) GyrA in response to a drop in the intracellular level of supercoiling (Menzel & Gellert, 1983), caused by GyrA(Ser$^{122}$) titrating out the intracellular GyrB and making inactive gyrase complexes. The observation of reduced nalidixic acid resistance when GyrA(Ser$^{122}$) was expressed in a *gyrA* (Nal$^r$) background could be explained by the formation of heterodimers which, although catalytically inactive, could still lead to the formation of a cleavable complex (Wilkinson & Wang, 1990). In the case of GyrA(Phe$^{122}$), it seems likely that this protein is produced in an inactive form such that it is deleterious to growth, as observed in our experiments, and fails to complement a *gyrA* (Nal$^r$) mutation in the experiments of Wilkinson and Wang.

To get around these problems with the mutant GyrA proteins, we made the 64-kDa N-terminal domain of GyrA bearing these mutations. The properties of the wild-type and mutant gyrase complexes are summarized in Table 1. In previous work, it had been shown that this fragment was capable of catalyzing DNA cleavage when complexed with GyrB (Reece & Maxwell, 1989, 1991c), and low-level supercoiling activity had also been detected. In the present

Table 1: Summary of the Properties of Wild-Type and Mutant Gyrase Proteins

| protein[a] | supercoiling | cleavage | | DNA binding | | CFX binding |
|---|---|---|---|---|---|---|
| | | +CFX | $+Ca^{2+}$ | −CFX | +CFX | |
| GyrA(wt) | +++ | +++ | +++ | +++ | +++ | +++ |
| 64(wt) | − | +++ | − | + | ++ | +++ |
| 64(wt) + 33 | ++[b] | +++[b] | +++ | NT | NT | +++ |
| GyrA($Ser^{122}$) | + | + | − | +++ | +++ | +++ |
| 64($Ser^{122}$) | − | − | − | + | ++ | ++ |
| 64($Ser^{122}$) + 33 | − | − | − | NT | NT | +++ |
| GyrA($Phe^{122}$)[c] | NT[d] | NT | NT | NT | NT | NT |
| 64($Phe^{122}$) | − | − | − | ++ | +++ | ++ |
| GyrA($Phe^{122}$) + 33 | − | − | − | NT | NT | +++ |

[a] All assays are in the presence of GyrB and DNA. Each (+) represents a factor of ~3 in activity; (−) indicates no activity. [b] Data from Reece and Maxwell (1991a,c). [c] No protein available. [d] NT = not tested.

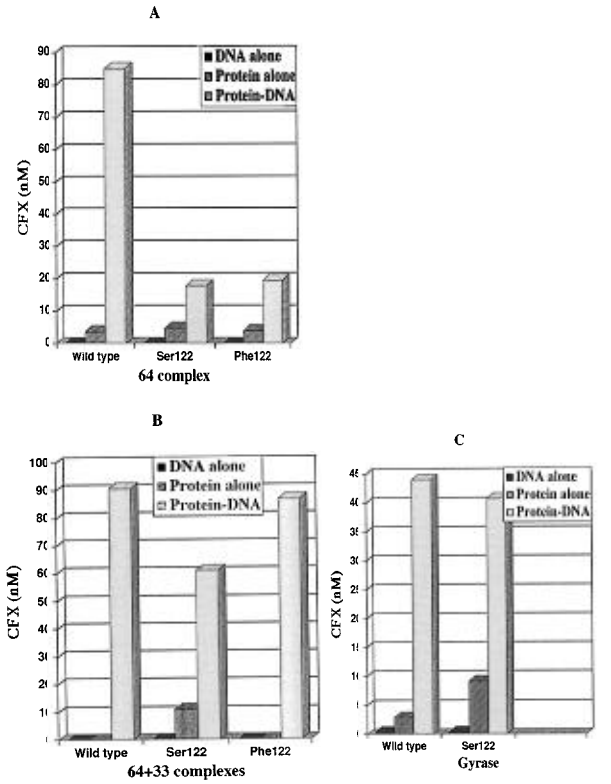


FIGURE 3: Binding of ciprofloxacin to DNA−gyrase complexes. The binding of $^3$H-CFX to wild-type and mutant complexes was measured by spin columns. Samples containing the 147-bp DNA fragment (40 nM) and protein as indicated were incubated for 3 h at 37 °C before being passed through a spin column. (A) $64_2B_2$ complexes (960 nM) with 27 $\mu$M CFX. (B) $(64+33)_2B_2$ complexes (600 nM) with 27 $\mu$M CFX. (C) $A_2B_2$ complexes (200 nM) with 13.5 $\mu$M CFX.

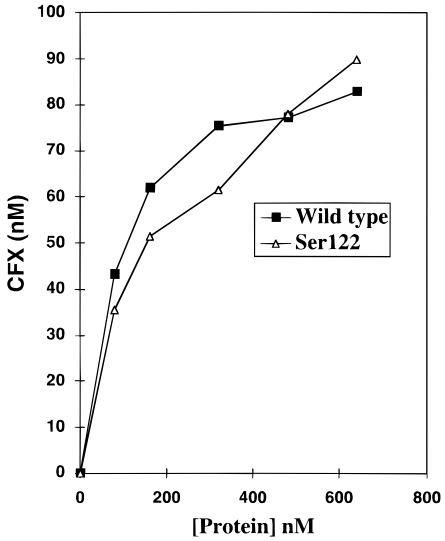


FIGURE 4: Binding of ciprofloxacin to wild-type and GyrA($Ser^{122}$) gyrase−DNA complexes. Samples containing the 147-bp DNA fragment (40 nM), $^3$H-CFX (300 $\mu$M), and wild-type and mutant gyrases ($A_2B_2$) at a range of concentrations were incubated for 3 h at 37 °C before being passed through a spin column.

work, we were unable to detect supercoiling with the wild-type 64-kDa fragment complexed with GyrB, unless the 33-kDa domain was also present. It is possible that in the earlier work the supercoiling detected was attributable to low-level contamination by GyrA (Reece & Maxwell, 1989, 1991c). In contrast to the wild-type protein, the mutant 64-kDa proteins showed no supercoiling activity in the presence of the 33-kDa C-terminal domain of GyrA and GyrB. In addition, the mutant 64-kDa proteins showed no quinolone-induced DNA cleavage activity in the presence of GyrB (Figures 1 and 2). One slightly suprising result was that in contrast to quinolone-induced cleavage, which is very efficient with the wild-type 64-kDa fragment complexed to GyrB (Reece & Maxwell, 1989, 1991c), $Ca^{2+}$-induced cleavage only occurred in the presence of the 33-kDa fragment (Figure 2). This result could reflect the lower stability of the complex between the 64-kDa fragment, GyrB, and DNA, but in view of the ability of this complex to cleave DNA in the presence of quinolones, may imply that $Ca^{2+}$-induced cleavage is mediated *via* the 33-kDa domains of GyrA. One possibility is that the presence of $Ca^{2+}$ stabilizes the interaction between the 33-kDa domains and DNA and in so doing influences conformational changes within the gyrase−DNA complex such as to interrupt the normal supercoiling cycle.

One potential interpretation of the lack of catalytic activity of the mutant 64-kDa fragments is that they are misfolded. However, we have shown that, in the presence of GyrB, both mutant proteins bind DNA; indeed, in the absence of the 33-kDa domains, the complexes formed between the mutant 64-kDa proteins, GyrB, and DNA are apparently more stable than that with the wild-type 64-kDa protein. Moreover, the binding of both mutant and wild-type proteins was stabilized by the presence of ciprofloxacin, a result that implies that the $Phe^{122}$ and $Ser^{122}$ mutants can bind drug. [In the filter-binding experiments reported by Wilkinson and Wang (1990), increased retention of the complex between GyrA-($Ser^{122}$), GyrB, and DNA was observed in the presence of oxolinic acid, a result that also implies that the $Ser^{122}$ mutant can bind drug.]

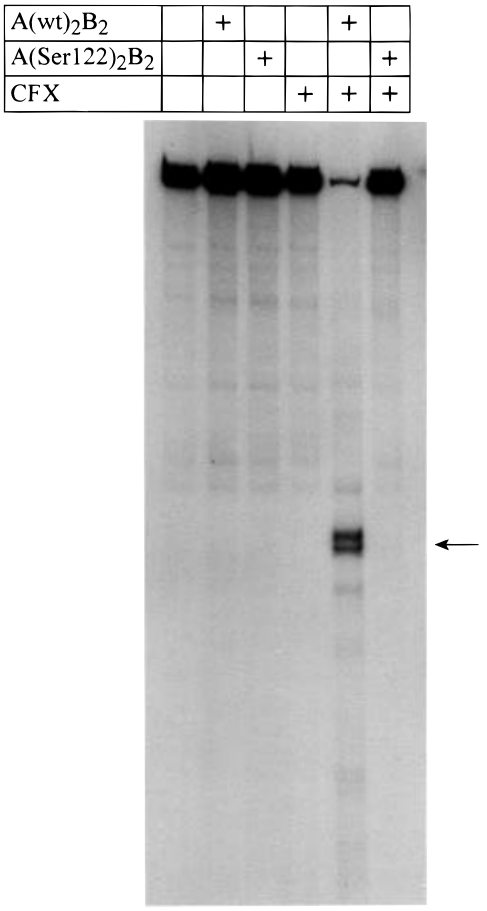


FIGURE 5: Transcription assays in the presence of wild-type and GyrA(Ser$^{122}$) gyrase−DNA complexes. T7 template DNA (4 nM) was incubated with wild-type or mutant gyrase (32 nM) and 5 $\mu$g/mL (13.5 $\mu$M) ciprofloxacin for 3 h at 37 °C. Transcription was then initiated by the addition of T7 RNA polymerase and the incubation continued for a further 30 min. Samples were analyzed on a 6% denaturing polyacrylamide gel. The arrow shows the positions of blocked transcripts.

In drug-binding experiments with radiolabeled ciprofloxacin, we found no detectable binding to DNA, as previously reported (Willmott & Maxwell, 1993), and little binding to the protein alone. With the 64-kDa N-terminal GyrA fragment in the presence of GyrB and DNA, we found that the binding of drug to the wild-type protein was significantly greater than to the mutant proteins (Figure 3). The presence of the 33-kDa C-terminal GyrA domain increased the binding of drug to the mutant proteins, and the full-length wild-type and Ser$^{122}$ GyrA proteins showed very similar levels of drug binding. Clearly, there are differences between the binding to wild-type and mutant $64_2B_2$−DNA complexes which must be addressed. First, it is possible that there are two modes of binding of quinolones to a gyrase−DNA complex: either prior to or after DNA cleavage, and the binding to a complex where the DNA has been cleaved is tighter than to the complex where cleavage has not occurred. In such a situation, the binding to the active-site mutants would be weaker because of the inability of these enzymes to cleave DNA. Second, the discrepancy may be due to the stability of the $64_2B_2$−DNA complex during the gel-filtration procedure. Binding of quinolone to the wild-type $64_2B_2$−DNA complex may induce the formation of a covalent complex between gyrase, quinolone, and DNA which is more stable on the spin-column. With the mutants, a covalent complex cannot form with the possible result that the mutant $64_2B_2$−DNA complex dissociates on the column, reducing the amount of drug bound to the mutant complex. The fact that addition of the 33-kDa domains (which confer stability on the gyrase−DNA complex) increases the amount of drug bound to the mutant complexes provides support for this idea.

Analysis of the binding of [$^3$H]ciprofloxacin to wild-type and the Ser$^{122}$-mutant gyrase−DNA complexes has revealed that both exhibit very similar levels of drug binding, providing strong evidence for quinolones being able to bind to a gyrase−DNA complex in which the DNA is not cleaved. By using the full-length GyrA protein in combination with GyrB, it is possible to estimate the stoichiometry of quinolone binding to a gyrase−DNA complex. The gyrase holoenzyme is known to form a 1:1 complex with the 147-bp DNA fragment, as determined by gel-retardation experiments (Dobbs et al., 1992). When assessing the amount of drug binding to a fixed concentration of DNA, in the presence of a range of gyrase concentrations, it can be seen that binding plateaus at approximately twice the concentration of DNA (Figure 4). This implies that the stoichiometry of drug binding is 2 molecules of drug per gyrase−DNA complex. However, this result is only an estimation of the stoichiometry of drug binding because the rapid gel-filtration technique used to measure binding is not a true equilibrium technique and it is possible that the binding of weakly-interacting drug molecules is disrupted during this procedure.

The fact that quinolones can still bind to active-site mutants of gyrase when complexed with DNA, and that quinolones stabilize the binding of mutant $64_2B_2$ complexes to DNA, provides strong evidence that DNA cleavage by gyrase is not a prerequisite for drug binding. This is in conflict with the Shen model for the interaction of quinolones with gyrase and DNA (Shen et al., 1989c). Although our experiments suggest that DNA cleavage is not required for quinolone interaction with gyrase and DNA, it is possible that a precleavage complex between gyrase, quinolone, and DNA exists which is converted to a, perhaps more stable, complex following cleavage. Perhaps a way in which this could be achieved is if quinolones interact with a gyrase−DNA complex in a similar manner as that suggested for the interaction of eukaryotic topoisomerase II inhibitors with topoisomerase II and DNA (Freudenreich & Kreuzer, 1993). The planar ring system of the quinolone drug could intercalate into the internucleotide space next to the cleaved phosphodiester bond and interact with residues of the gyrase protein(s). This interaction would be very similar in the presence or absence of DNA cleavage, but the presence of the quinolones may push the cleavage equilibrium in the direction of cleavage.

In previous work, we have shown that the complex between gyrase and quinolones on DNA forms a barrier to transcription (Willmott et al., 1994). When gyrase containing GyrA(Trp$^{83}$), which confers quinolone resistance and fails to bind quinolone drugs (Willmott & Maxwell, 1993), was used in these experiments, transcription was inhibited only at very high drug concentrations; i.e., polymerase blocking requires drug binding (Willmott et al., 1994). Given that GyrA(Ser$^{122}$) can bind quinolone drugs in the presence of GyrB and DNA, we tested whether this complex could also block transcription. We found that it could not, suggesting that DNA cleavage is required for polymerase blocking. The implications of this result are that in the complex between wild-type gyrase, DNA, and quinolone drug, the DNA is

cleaved and covalent bonds exist between the GyrA proteins and the 5′-phosphates of the broken DNA, whereas in the absence of drug the complex is chiefly noncovalent, allowing passage of the polymerase. In the case of the GyrA($Ser^{122}$) mutant, the binding of the drug does not stabilize the cleaved complex and hence does not block the passage of polymerases.

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