

# A Single Point Mutation in the DNA Gyrase A Protein Greatly Reduces Binding of Fluoroquinolones to the Gyrase-DNA Complex

CHRISTOPHER J. R. WILLMOTT AND ANTHONY MAXWELL\*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, United Kingdom

Received 3 August 1992/Accepted 27 October 1992

**Binding of the quinolone drug norfloxacin to gyrase and DNA has been investigated. We have detected binding to gyrase-DNA complex but find no significant binding to either gyrase or DNA alone. Enzyme containing gyrase A protein with the mutation Ser-83 to Trp (conferring quinolone resistance) showed greatly reduced drug binding.**

The target of the quinolone antibacterial agents is thought to be DNA gyrase. The enzyme from *Escherichia coli* consists of two A and two B subunits (the products of the *gyrA* and *gyrB* genes, respectively) and is responsible for catalyzing topological changes in DNA (for a review, see reference 11). A recent model for the mechanism by which quinolones inhibit gyrase envisages the binding of drug by hydrogen bonding to a single-stranded DNA pocket at the active site of the enzyme (13).

We have used a rapid gel-filtration (spin column) technique (7) as a means of studying binding; free ligand is retained on the column, and ligand bound to macromolecule is eluted. This approach has previously been used to address the question of quinolone interaction with gyrase and DNA (12). [<sup>3</sup>H]norfloxacin ([<sup>3</sup>H]NFX), a gift from L. L. Shen (Abbott Laboratories), was repurified by reverse-phase high-pressure liquid chromatography according to the recommended procedure (12). Gyrase subunits were prepared from overproducing strains (5) and preincubated together in equimolar concentrations for 30 min at 25°C to form the A<sub>2</sub>B<sub>2</sub> complex. Typical specific activities were ~10<sup>6</sup> U/mg for GyrA proteins and ~6 × 10<sup>4</sup> U/mg for GyrB (10). In addition to the wild-type proteins, GyrA containing the mutation Ser-83 to Trp, which is known to lead to high-level resistance to quinolones (1, 16), was also prepared. Plasmid pMECS5 encoding GyrA<sub>Trp-83</sub> was kindly donated by L. M. Fisher (University of London), and the section encoding residues 20 to 369 (a *SacI*-*Bst*BI fragment) was exchanged with the corresponding region of the wild-type *gyrA* gene in expression vector pPH3 (5).

The DNA used was a 147-bp fragment containing the major gyrase cleavage site from pBR322 (3), which has been shown to be cleaved by gyrase at a single site into fragments of 69 and 78 bp (2). Incubation of the 147-bp fragment (86 nM) with gyrase (700 nM) and NFX (2.5 μg/ml; 7,800 nM) at 25°C for 3 h, followed by the addition of sodium dodecyl sulfate (SDS) and proteinase K and a further 30 min of incubation at 37°C, leads to complete cleavage of this fragment at the expected site (2, 3, 14). The 147-bp fragment was prepared from plasmid pSTD147 as described previously (2). Gel retardation assays (9) confirmed that the 147-bp DNA fragment was bound by one gyrase only and that an eightfold excess of enzyme resulted in complete

binding (data not shown). A molar excess of gyrase over DNA is generally required to completely bind the DNA, in part because of a proportion of inactive enzyme in the preparation (2).

Binding reactions were carried out in 120 μl of 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 4 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 5% (wt/vol) glycerol, and 0.36 mg of bovine serum albumin per ml. The drug was incubated with gyrase and/or DNA for 3.5 h at 25°C before two 50-μl samples were withdrawn from each reaction, applied to columns of G50 Sephadex beads preformed in a 1-ml syringe, and centrifuged at 1,500 × g for 2 min. The volumes of the eluants were measured, and the amount of [<sup>3</sup>H]NFX was determined by scintillation counting. The averaged results (from two to four experiments) are given in Table 1.

No binding of [<sup>3</sup>H]NFX to the 147-bp fragment was detected. At 10-fold-higher DNA concentrations, binding of the drug was still insignificant (data not shown). In addition, very little binding to either the wild-type DNA gyrase or the enzyme carrying the GyrA<sub>Trp-83</sub> mutation was found. Significant binding to the complex of wild-type gyrase and DNA occurred (Table 1), in agreement with a previous result (12). Crucially, this binding was virtually abolished for complexes of the GyrA<sub>Trp-83</sub> enzyme and DNA. Other experiments showed that enzyme-DNA complex prepared with another quinolone-resistant protein, GyrA<sub>Ala-83</sub> (6), bound approximately threefold less drug than wild-type complex did (data not shown).

In a second series of experiments, DNA cleavage (4) was used as an indirect monitor of binding. When the related quinolone ciprofloxacin (CFX) was used, no binding to either wild-type gyrase or the DNA alone was found (Table 2). A series of assays was performed in which one component (gyrase, DNA, or CFX) was omitted from the initial reaction mixture and added after the other two components had been incubated for 2 h and passed through a spin column. The new reaction mixture was then incubated for a further 2 h prior to the addition of 0.2% SDS and 1 mg of proteinase K per ml and 30 min of incubation at 37°C. When gyrase and DNA were incubated together and CFX (20 μg/ml; 60 μM) was added following centrifugation, cleavage was observed (Table 2), but when gyrase alone was incubated with quinolone and DNA was added following centrifugation, no cleavage was detected. Similarly no cleavage was observed when DNA and CFX were incubated together and enzyme was added subsequently.

\* Corresponding author. Electronic mail address: ONY@UK.AC.LE.VAX.

TABLE 1. Binding of [<sup>3</sup>H]NFX to the gyrase-DNA complex

DNA	Concn (nM) of:			
	A <sub>2</sub> B <sub>2</sub> <sup>a</sup>		NFX	
	Wt	Trp-83	Total	Bound <sup>b</sup>
86	700	0	7,800	11.9 (1.6)
0	700	0	7,800	0.5 (0.2)
86	0	700	7,800	0.2 (0.2)
0	0	700	7,800	0.4 (0.2)
86	0	0	7,800	0

<sup>a</sup> Wt, wild-type (quinolone-sensitive) gyrase; Trp-83, gyrase carrying the quinolone resistance mutation Ser-83 to Trp in the A subunit.

<sup>b</sup> Standard deviations are in parentheses.

It should be pointed out that the centrifugation involved in a spin column assay means that the experiments are not performed under true equilibrium conditions. These results do not, therefore, rule out the possibilities that some weak interaction may be occurring between quinolones and either gyrase or DNA alone and that dissociation may occur during centrifugation.

Control experiments have revealed comparable extents of DNA cleavage with and without centrifugation. The stability of the gyrase-DNA-quinolone complex following centrifugation was also confirmed. After incubation of gyrase and DNA with unlabelled CFX for 2 h, the reaction mixture was passed through a spin column and incubation continued for a further 0 to 3 h prior to the addition of SDS and proteinase K. The extent of cleavage in samples 3 h after incubation was identical to that in samples treated immediately after centrifugation, implying that the ternary complex is stable throughout this period.

A recent model proposes that quinolones bind to a single-stranded DNA pocket at the active site of the enzyme (13). Yoshida and coworkers have commented that this model underplays the significance of quinolone resistance arising from gyrase mutations (15); a fuller critique is given elsewhere (8, 11). Supercoiling assays reveal that gyrase with GyrA<sub>Trp-83</sub> subunits is able to perform the introduction of negative supercoils into closed circular DNA as efficiently as the wild-type enzyme (14). In so doing it would be expected to open up the DNA in an identical way to its quinolone-

sensitive counterpart. In view of the results reported here, we support the proposal that both the enzyme and the DNA are required for stable interaction with quinolones but contend that efficiency of binding is primarily determined by the gyrase A subunits.

We thank Andy Bates and Jane Gilbert for their comments on the manuscript.

We thank the MRC (UK) for a postgraduate studentship to C.J.R.W. A.M. is a Lister Institute Jenner Fellow.

## REFERENCES

- Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fisher. 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. *Antimicrob. Agents Chemother.* **33**:886-894.
- Dobbs, S. T., P. M. Cullis, and A. Maxwell. 1992. The cleavage of DNA at phosphorothioate internucleotidic linkages by DNA gyrase. *Nucleic Acids Res.* **20**:3567-3573.
- Fisher, L. M., K. Mizuuchi, M. H. O'Dea, H. Ohmori, and M. Gellert. 1981. Site-specific interaction of DNA gyrase with DNA. *Proc. Natl. Acad. Sci. USA* **78**:4165-4169.
- Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa. 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. USA* **74**:4772-4776.
- Hallett, P., A. J. Grimshaw, D. B. Wigley, and A. Maxwell. 1990. Cloning of the DNA gyrase genes under *tac* promoter control: overproduction of the gyrase A and B proteins. *Gene* **93**:139-142.
- Hallett, P., and A. Maxwell. 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. *Antimicrob. Agents Chemother.* **35**:335-340.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maxwell, A. 1992. The molecular basis of quinolone action. *J. Antimicrob. Chemother.* **30**:409-414.
- Maxwell, A., and M. Gellert. 1984. The DNA dependence of the ATPase activity of DNA gyrase. *J. Biol. Chem.* **259**:14472-14480.
- Reece, R. J., and A. Maxwell. 1989. Tryptic fragments of the *Escherichia coli* DNA gyrase A protein. *J. Biol. Chem.* **264**:19648-19653.
- Reece, R. J., and A. Maxwell. 1991. DNA gyrase: structure and function. *CRC Crit. Rev. Biochem. Mol. Biol.* **26**:335-375.
- Shen, L. L., W. E. Kohlbrenner, D. Weigl, and J. Baranowski. 1989. Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. *J. Biol. Chem.* **264**:2973-2978.
- Shen, L. L., L. A. Mitscher, P. D. Sharma, T. J. O'Donnell, D. T. W. Chu, C. S. Cooper, T. Rosen, and A. G. Pernet. 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. *Biochemistry* **28**:3886-3894.
- Willmott, C. J. R., and A. Maxwell. Unpublished data.
- Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura. 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* **35**:1647-1650.
- Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura. 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. *Mol. Gen. Genet.* **211**:1-7.

TABLE 2. Ciprofloxacin-induced cleavage of DNA by gyrase<sup>a</sup>

Gyrase (700 nM)	Incubation prior to centrifugation (2 h)		Subsequent addition (2 h of incubation)	Result
	DNA (60 nM)	CFX (60 μM)		
+	+	+	None	Cleavage
+	+	-	CFX	Cleavage
+	-	+	DNA	No cleavage
-	+	+	Gyrase	No cleavage

<sup>a</sup> After centrifugation, the mixture was passed through a spin column (2 min) before the subsequent addition. After the second 2-h incubation, SDS and proteinase K were added; the final incubation was at 37°C for 30 min.