

Quinolone binding to DNA is mediated by magnesium ions

(norfloxacin/plasmid DNA/ternary complex)

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ABSTRACT The binding of plasmid DNA to norfloxacin, a quinolone antibacterial agent, was investigated by fluorescence, electrophoretic DNA unwinding, and affinity chromatography techniques. The amount of quinolone bound to DNA was modulated by the concentration of Mg^{2+} . No interaction was evident in the absence of Mg^{2+} or in the presence of an excess of Mg^{2+} , whereas maximum binding was observed at a Mg^{2+} concentration of 1–2 mM. The experimental data can be fitted to the formation of three types of Mg adducts: a binary adduct with norfloxacin and Mg^{2+} , a binary adduct with DNA and Mg^{2+} , and a ternary adduct with quinolone, plasmid, and Mg^{2+} . We propose a model for the ternary complex, in which Mg acts as a bridge between the phosphate groups of the nucleic acid and the carbonyl and carboxyl moieties of norfloxacin. Additional stabilization may arise from stacking interactions between the condensed rings of the drug and DNA bases (especially guanine and adenine), which may account for the preference exhibited by quinolones for single-stranded and purine-rich regions of nucleic acids. Other possible biochemical pathways of drug action are suggested by the observation that norfloxacin binds Mg^{2+} under conditions that are close to physiological.

Conflicting literature reports have been accumulating on the role played by DNA in the mechanism of action of quinolone compounds. Although a large amount of biological data has indicated that DNA gyrase was the target for quinolone compounds (1–4), recent reports dismissed DNA gyrase as the target and pointed to DNA as the direct binding species (5). In fact, a cooperative interaction was proposed to occur between quinolones and supercoiled DNA. Subsequent publications by the same laboratory have modified this view extensively (6–8). In particular, Shen *et al.* (7) have proposed that in the presence of ATP bound gyrase induces a specific quinolone binding site in the relaxed DNA substrate. Gel-electrophoresis experiments by Tornaletti and Pedrini (9) showed that norfloxacin (Nor) is able to unwind the DNA double helix in the presence of Mg^{2+} . On the other hand ^{19}F NMR measurements failed to show any direct DNA–quinolone interaction (10). We were also unable to detect binding using fluorescence spectroscopy techniques (11).

Even if reconsidered in terms of affinity, the interaction with DNA is still of great concern because of the possible long-term genotoxicity of quinolone compounds, which are increasingly adopted as first-choice antibiotics for the treatment of many infections, and because it addresses the real mechanism of action of this class of molecules. To shed some light on this cumbersome problem, we have focused our attention on the role of Mg^{2+} in the binding of the model quinolone drug Nor to plasmid DNA. Our approach includes fluorescence and affinity chromatography measurements and electrophoretic DNA-unwinding assays.

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MATERIALS AND METHODS

Chemicals. Nor and [^{14}C]Nor (specific activity, 46.5 $\mu Ci/mg$; 1 Ci = 37 GBq) were a kind gift of Merck Sharp & Dohme. Magnesium chloride and perchlorate were purchased from Fluka.

Substrate DNAs. pAT153 and pBR322 were propagated by conventional methods (12). Naturally supercoiled DNA was purified on CsCl and sucrose gradients as described (12).

DNA-cellulose resins were prepared by absorbing plasmid DNA onto the cellulose matrix and covalently linking it by UV-irradiation as described (13). The DNA-cellulose derivative contained 2.1–5.3 mg of DNA per gram of resin as described (13, 14).

DNA Unwinding. Unwinding experiments in the presence of quinolone compounds were performed using a DNA circle-ligation assay essentially as described (15). Briefly, 100 ng of nicked circular pAT153 substrate was exposed to 1 unit of T4 DNA ligase in 66 mM Tris-HCl/ various amounts of $MgCl_2$ /10 mM dithioerythritol/0.7 mM ATP, pH 7.5, in the presence of known drug concentrations. After incubation at 0°C for 10 min, the reaction was stopped by addition of 20 mM EDTA. Samples that had been extracted with butanol were loaded onto 1% agarose gels containing chloroquine (0.94 $\mu g/ml$). The average linking number of each ligated population of topoisomers was calculated after densitometric scanning of ethidium bromide-stained gels (15).

DNA Affinity Chromatography. [^{14}C]Nor (5000 cpm/nmol) was run through a DNA-cellulose column prepared as described above. The loading buffer contained 10 mM Tris-HCl (pH 7.0), 20 mM NaCl, and various concentrations of Mg^{2+} . The concentration of the bivalent metal ion was determined by atomic absorption spectrometry measurements. The amount of quinolone bound to the column was calculated from the radioactivity retained in the column after repeated washings with loading buffer. Control experiments were done to exclude aspecific drug absorption onto the column. The data were further confirmed by elution of the column-bound drug with the loading buffer containing an excess (50–100 mM) of either Mg^{2+} or EDTA.

Some of the measurements were also performed using the fluorescence response of Nor to determine quantitatively the eluted quinolone. Standard solutions of Nor were used as a reference, containing between 0.1 and 1 μM drug and known amounts of Mg^{2+} .

Fluorometric Titrations. These measurements were made in the same buffer systems used for affinity chromatography with a MPF66 Perkin-Elmer apparatus, interfaced to a Perkin-Elmer 7500 data station. The drug concentration normally ranged from 0.1 to 1 μM . Alternatively, quinolone solutions contained various amounts of Mg^{2+} at a fixed plasmid concentration or various amounts of DNA at a fixed ion concentration. Excitation wavelength was 330 nm, and the examined range of emission was 360–540 nm.

Abbreviation: Nor, norfloxacin.

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RESULTS

Nor Binds to Mg^{2+} Ions. The fluorescence emission of Nor is remarkably increased by addition of Mg^{2+} ions at neutral pH (Fig. 1). Saturation occurs at a Mg^{2+} concentration of ≈ 10 mM and is slightly influenced by ionic strength at room temperature. As the fluorescence response of Nor and of its Mg complex at 423 nm is linear with concentration in the range we examined (up to a few micromolar), the binding constant can be evaluated, by assuming a 1:1 complex, as suggested by the large excess of Mg^{2+} used with reference to Nor. The association constant K_1 in 20 mM NaCl/10 mM Tris·HCl, pH 7.0, is $990 \pm 36 M^{-1}$. Increasing ionic strength causes a moderate decrease in K_1 to $\approx 890 M^{-1}$ in 150 mM salt.

Nor Binding to Plasmid DNA Is Mediated by Mg^{2+} . Fluorescence experiments. The fluorescence intensity of Nor between 0.1 and 1 μM in the presence of excess pBR322 DNA remains practically unaffected in the absence of Mg^{2+} or in an excess of Mg^{2+} . This is in agreement with our previous findings (11) and indicates that no evidence for binding to DNA is obtained under the above experimental conditions. However, at a Mg^{2+} concentration in the millimolar range, a DNA-concentration-dependent quenching of Nor fluorescence occurs. Fig. 1 shows the fluorescence response in the presence and absence of plasmid as a function of Mg^{2+} concentration. Quenching of the fluorescence has been reported by Shen (29) as a result of DNA binding by Nor. If this fact is considered, it is possible to evaluate the amount of Nor engaged in the complex formation with DNA from fluorescence experiments. In fact if I_0 is the fluorescence response of Nor at a Nor concentration denoted N_t in the absence of Mg^{2+} , if I_{max} is the corresponding value in the presence of excess Mg^{2+} , and if the drug-DNA complex (T) is not fluorescent, then the band intensity observed at a given (free) magnesium concentration $[M]$ will be given by the equation:

$$I = [N](I_0 + K_1[M]I_{max})/N_t,$$

from which the free drug concentration $[N]$ can be calculated. The concentration of drug-Mg complex $[NM]$ is then immediately available, as $[NM] = K_i[N][M]$. Subtracting $[N]$ and $[NM]$ from the total drug concentration yields $[T]$.

According to our data, the maximum quinolone bound at a total Nor concentration of 1 μM and a total plasmid concentration of 0.77 mM (on a phosphate basis, which corresponds

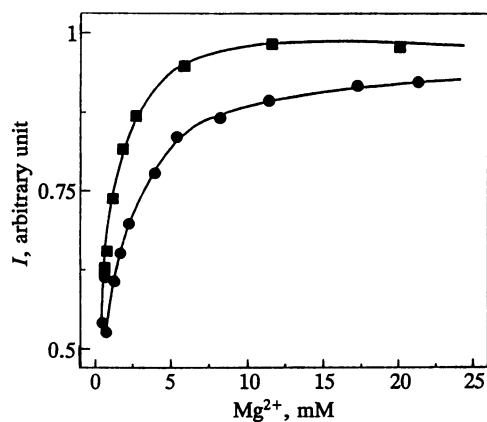


FIG. 1. Effects of Mg^{2+} and pBR322 plasmid DNA on the fluorescence response (423 nm, I) of 1 μM Nor in 10 mM Tris/20 mM NaCl, pH 7.0, at 25°C. Squares, fluorescence emission readings in the absence of the plasmid; circles, readings in the presence of 0.77 mM plasmid (calculated on a phosphate basis).

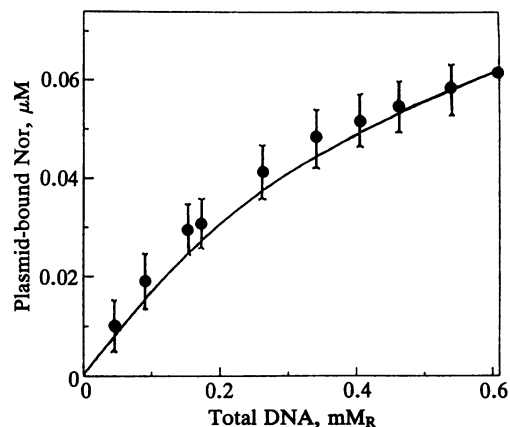


FIG. 2. Amount of Nor bound to plasmid DNA (T) as a function of total DNA concentration (per base molar residue, mM_R) in 10 mM Tris/20 mM NaCl, pH 7.0, at 25°C. Nor ranged between 0.26 and 0.21 μM , and total Mg^{2+} was 1 mM. The solid line corresponds to the plot of T vs. total DNA according to Eq. 5, using K_2 and K_4 values of $2.5 \times 10^4 M^{-1}$ and $2.4 \times 10^3 M^{-1}$, respectively.

to 88 nM plasmid) is $\approx 0.16 \mu M$, which corresponds to ≈ 2 molecules per plasmid.

Binding measurements were also performed as a function of DNA concentration at a fixed Mg^{2+} concentration (1 mM). The data are presented in Fig. 2 and show the increase in T as a function of total DNA concentration. Values of T at approximately constant DNA concentration and metal ion concentrations are linearly related to the concentration of Nor up to 1 μM drug.

DNA unwinding. Results from DNA-unwinding experiments are presented in Fig. 3. Nor was capable of unwinding pAT153 DNA as shown by a reduction of the topoisomer helical density with increasing drug concentration. Again the process is clearly modulated by Mg^{2+} concentration since unwinding sharply increases up to an optimal Mg^{2+} concentration of ≈ 2 mM and then drops to lower values as the Mg^{2+} concentration is increased. Virtually no unwinding is detected in the presence of 30 mM $MgCl_2$ and 133 μM Nor. These experiments, performed at high drug concentration, show essentially the same features as those performed at 0.1–1 μM Nor, which suggests that we are dealing with the same type of complex. If unwinding of the double helix is $\approx 7.2^\circ$ per Nor molecule bound (9), then the number of Nor molecules bound per plasmid can be evaluated from the

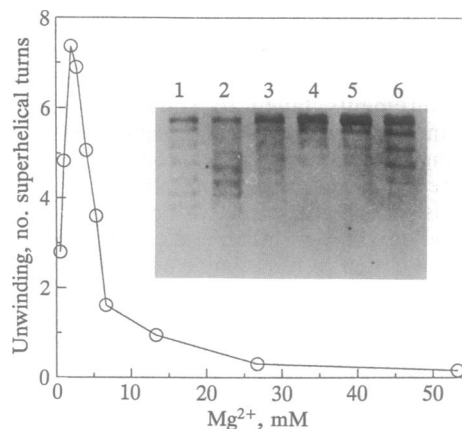


FIG. 3. Unwinding (superhelical turns) of pAT153 DNA (100 ng) produced by 133 μM Nor in the circle ligation assay as a function of Mg^{2+} concentration. (Inset) Electrophoretic pattern is shown in the presence of the following amounts of Mg^{2+} . Lanes: 1, control; 2, 0.5 mM; 3, 1.0 mM; 4, 1.5 mM; 5, 2.0 mM; 6, 4.0 mM.

unwinding results. At 5 mM Mg^{2+} and 133 μM Nor, the total unwinding angle is close to 1250° , which corresponds to some 175 molecules per plasmid DNA. This is in good agreement with the data of Shen *et al.* (6). In fact according to the Hill equation reported by them, at a Nor concentration of 133 μM , the number of drug molecules bound per plasmid would be 180.

DNA affinity chromatography. The amount of drug bound to the plasmid DNA resin was also evaluated as a function of Mg^{2+} concentration (Fig. 4). In confirmation of the data presented above, no appreciable binding was observed at low and high concentrations of bivalent metal ion, whereas the maximum level of drug binding occurs at 2–3 mM Mg^{2+} , at which about three molecules of Nor were bound per plasmid molecule under our experimental conditions. A further indication of the role played by Mg^{2+} in mediating the quinolone–DNA interaction is the observed ability of an excess of Mg^{2+} or EDTA (≈ 50 mM) to elute the bound drug efficiently and quantitatively. Experiments were also performed to characterize whether the interaction between Nor and plasmid DNA was taking place in a cooperative manner. The radiolabeled Nor could be varied over a wide range of concentrations at a constant Mg^{2+} concentration and no evidence of cooperativity was observed, as shown in Fig. 5, where the amount of resin-bound Nor is reported as a function of loaded drug. In fact a linear relationship was found that eventually reached a plateau under saturation conditions.

DISCUSSION

An extensive investigation has been carried out by Shen and coworkers (5–8) on the mechanism of inhibition of DNA gyrase and DNA binding by quinolone antibacterial agents. They proposed that a form of specific, saturable, and highly cooperative binding takes place with supercoiled DNA or with relaxed DNA–DNA gyrase complexes in the presence of ATP. The quinolone would bind to separated DNA strands through hydrogen bonds between the bases and its carbonyl and carboxyl groups. High binding affinity would be acquired via a cooperative mechanism whereby (as for Nor) four drug molecules interact by π – π ring stacking of the quinolone aromatic moiety and tail-to-tail hydrophobic interactions involving the N-ethyl groups.

In a preliminary investigation using fluorometric and radiometric measurements, we were unable to detect the occurrence of quinolone–DNA interaction (11). Hence, we have tried to clarify the reasons for this discrepancy and also to further analyze the proposed binding model. We realized that the major difference in the experimental conditions between our work and that of Shen and coworkers (6–8) was Mg^{2+} concentration. We had investigated the binding process

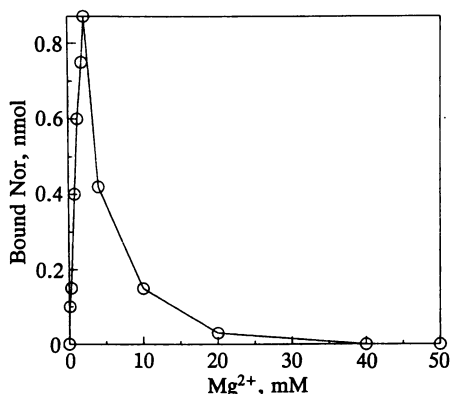


FIG. 4. Binding of ^{14}C -labeled Nor to the pBR322-cellulose resin (containing 0.79 mg of DNA) as a function of Mg^{2+} concentration.

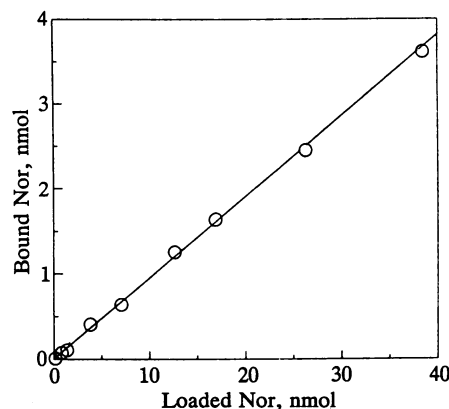


FIG. 5. Relationship between loaded and bound ^{14}C -labeled Nor in the affinity chromatography experiments using pBR322-cellulose resin at 4 mM Mg^{2+} .

either with no Mg^{2+} or with excess Mg^{2+} (≈ 50 mM), whereas Shen and coworkers (6–8) generally used a fixed intermediate concentration of Mg^{2+} (≈ 5 mM). This fact and the finding that DNA-unwinding experiments had to be performed in the presence of Mg^{2+} (9) suggested that the concentration of Mg^{2+} could play a role in the formation of the quinolone–DNA complex. This proved to be the case as we found that the extent of Nor binding is modulated by the presence of Mg^{2+} . The modulation was not simply the result of a competition between metal ion and plasmid for binding to Nor, as the extent of drug–DNA complex exhibited a maximum as a function of Mg^{2+} . Thus the first conclusion that can be drawn is that Mg^{2+} participates in the binding of quinolone to DNA; i.e., a ternary complex forms involving the drug, the metal ion, and the nucleic acid. We showed that Mg^{2+} is able to bind to Nor with a stability constant of $\approx 10^3 M^{-1}$. The metal ion binding site probably involves the carbonyl and carboxyl moieties of the quinolone that form a chelate structure.

On the other hand the tendency of Mg^{2+} to bind to DNA is well documented (17, 18). The simplest way for the metal ion to generate a ternary complex would be to form a bridge between the quinolone moiety and the nucleic acid. In fact at physiological conditions the drug is mainly zwitterionic (19) and the presence of a carboxylate group could interfere with the negative charge density of DNA when the drug approached the nucleic acid; however, the Mg –drug complex is positively charged, which would greatly facilitate its reaching the nucleic acid, and the Mg edge of the Mg –drug complex should interact electrostatically with phosphate groups (20, 21). This is in agreement with the observed decrease in unwinding and binding on increasing ionic strength (5, 9). However, this would not account for the preference of quinolones for single-stranded regions of the nucleic acid. The fact that binding is poorer for a linear double-helical sequence as compared to a single-stranded sequence suggests that bases not involved in pairing stabilize the drug– Mg complex. Although the drug is not able to intercalate into a DNA double helix (7, 9), it could form stacking interactions with the bases in a single-stranded region or a distorted B-form in the plasmid, where high unwinding energy is not required. In addition, an increased conformational flexibility of the base could allow optimization of both electrostatic binding through Mg^{2+} and π – π interactions with the planar aromatic system of the drug. Considering the overlapping surface, stacking should be more effective for purine than for pyrimidine bases. As a matter of fact, poly(dG) or poly(G) and poly(A) or poly(dA) bind more tightly than other sequences (6). Our point is strengthened by the observation that enhanced stability of ternary metal ion–ATP–phenanthroline complexes is caused by stacking interactions involving the

aromatic portions of ATP and phenanthroline (22). Indeed, similar behavior is observed when phenanthroline is replaced with Nor in the above system (data not shown).

The fact that binding reaches a maximum as a function of Mg^{2+} concentration deserves comment.

Equilibria that can be written in the presence of quinolone, Mg, and DNA are as follows:



where D is DNA and DM the DNA-Mg complex. Eqs. 2-4 relate to ligand interactions with a macromolecule, and hence, they were treated as described by McGhee and Von Hippel (23). The exclusion parameter n was considered to be 2 in Eqs. 2 (18) and 3 (like Mg, the Nor-Mg complex is doubly charged) and 1 in Eq. 4, which refers to the binding of Nor to the Mg-DNA complex. If the total Nor concentration is the sum of $[N]$, $[NM]$, and $[T]$ and the latter two terms are negligible in the mass equilibria involving DNA and Mg^{2+} (the nucleic acid and the metal ion are always in large excess with reference to Nor), then the following equations can be written:

$$N_t = [N] + [NM] + [T],$$

$$M_t = [M] + [DM],$$

and

$$D_t = [D] + 2[DM],$$

where the subscript t refers to the total concentration of each component, and the DNA concentration is given per base molar residue. Since $K_2 = [DM](D_t - [DM])/([M](D_t - 2[DM])^2)$,

$$[D] = D_t/(1 + 4K_2[M])^{0.5}$$

and

$$[N] = (N_t - [T])/(1 + K_1[M]).$$

Since $[DM] = (D_t - [D])/2$,

$[T] =$

$$\frac{2K_2K_4N_tD_t}{[1 + 4K_2[M] + (1 + 4K_2[M])^{0.5}]/([M] + K_1) + 2K_2K_4D_t} \quad [5]$$

According to this equation, T is 0 at $[M] = 0$ and at $[M] = \infty$. Fits of binding data from fluorescence experiments as a function of DNA and Mg^{2+} (Figs. 3 and 6) and Nor concentration (data not shown) are clearly satisfactory. Considering the value found for K_1 , the results are best fit with a K_2 value of $2.2 \pm 0.4 \times 10^4 M^{-1}$ and a K_4 value of $1.9 \pm 0.5 \times 10^3 M^{-1}$ (per phosphate residue). The value found for K_2 is in good agreement with data available in the literature for the binding of Mg^{2+} to double-stranded DNA (17, 18). Since from the above equilibria it follows that $K_1K_3 = K_2K_4$, K_3 can be evaluated to be $\approx 4.2 \times 10^4 M^{-1}$. Accordingly, the most favored binding equilibrium corresponds to the interaction between the quinolone- Mg^{2+} complex and the plasmid. No cooperativity factors are required to fit the data. This is at variance with the model proposed by Shen *et al.* (7). It is, however, puzzling that in some experiments cooperativity factors well above the maximum number of Nor molecules bound per plasmid were reported (6). In fact the Hill param-

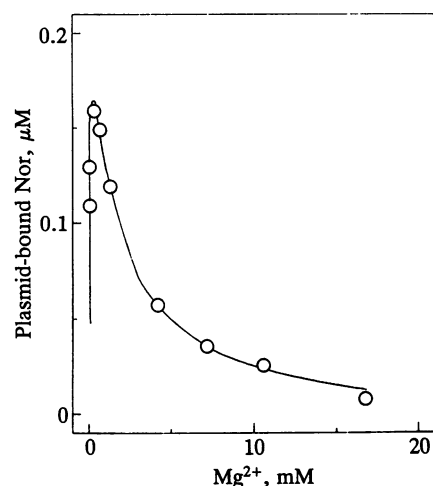


FIG. 6. Amount of ternary complex (plasmid-bound Nor) formed under the experimental conditions described in Fig. 1 as a function of Mg^{2+} concentration. The solid curve represents the best least-squares fit using Eq. 5. Values for K_2 and K_4 are $2.2 \times 10^4 M^{-1}$ and $1.7 \times 10^3 M^{-1}$, respectively.

eter cannot exceed the number of ligand-binding sites (24). The reason for this discrepancy is not apparent at the moment.

Cooperativity could possibly arise from bridging of two Nor molecules by Mg^{2+} , similar to the case reported (16) for the binding of chromomycin to DNA. If the above reaction was occurring, we expect that the amount of ternary complex would depend on the square of Nor concentration, which we did not observe.

The optimal concentration of Mg^{2+} for ternary complex formation falls in the range of intracellular Mg^{2+} concentrations in bacteria, so that this mode of binding could be biologically relevant. However, the model proposed here does not necessarily imply a correspondence to the situation *in vivo* in the presence of gyrase. In fact effects at the enzyme level are certainly pertinent to the quinolone mode of action, as indicated by a vast amount of genetic data including sequencing of the mutated enzyme (25-27). It is also relevant that the impairment of even very few gyrase molecules could affect the function of the whole chromosome, where a limited number of gyrase binding sites are available (28).

In addition to a revised DNA-binding model including a role for Mg^{2+} , the formation of Nor-metal ion complexes suggests other interactions, some of which could be pharmacologically relevant. A further step to fully understand the molecular mechanism of action of quinolone drugs is an investigation of ternary gyrase/quinolone/ Mg^{2+} and quaternary gyrase/DNA/quinolone/ Mg^{2+} systems in the presence or absence of ATP.

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