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NMR investigation of pefloxacin-cation-DNA interactions: the essential role of Mg^{2+}

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Abstract

^{19}F-NMR spectra show that, in the absence of magnesium, pefloxacin binds poorly to DNA and preferentially to single-stranded rather than to double-stranded DNA. In the presence of a fixed concentration of magnesium (10 mM), the competition between double-stranded DNA species and pefloxacin for binding to magnesium is more important with linear DNA than with supercoiled plasmidic DNA. Only the ^{19}F signals of pefloxacin free and bound to magnesium are observed, but the formation of the ternary complex pefloxacin/Mg^{2+}/DNA is indicated by the decrease in the integration of the proton decoupled ^{19}F signal at the high concentrations of double-stranded (linear and plasmidic) DNA. The variations of the ^{19}F NOE demonstrate that this formation depends strongly on the magnesium concentration, the optimum concentration being ≈ 2 mM in the presence of *Escherichia coli* DNA (4 mM). The ^{19}F NMR data show that the quinolone ring of pefloxacin has little mobility in the ternary complex. In this complex, pefloxacin could be bound to two magnesium ions, each cation acting as a bridge between this molecule and a phosphate group of the DNA backbone.

Keywords: NMR; Fluoroquinolone; Magnesium; DNA

1. Introduction

Fluoroquinolones, such as pefloxacin (Fig. 1), are antibacterial agents strongly effective against a broad spectrum of Gram-positive and Gram-negative bacteria (Smith, 1984; Cornett and

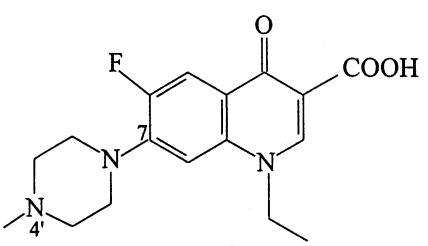

Fig. 1. Structure of pefloxacin.

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Wentland, 1986; Fernandes and Chu, 1987; Chu and Fernandes, 1989). Their activity is due to the inhibition of the supercoiling of DNA catalyzed by the enzyme DNA gyrase. Conflicting reports have been accumulating in the literature on the molecular details of drug-DNA and drug-enzyme interactions. Shen and co-authors (Shen et al., 1989a,b) have proposed the first drug-DNA model, which implies hydrogen-bond type interactions between the DNA unpaired bases and the quinolone, as well as a stacked dimerization of the drug. Very recently, they have modified this model and included a possible interaction between the C7 substituent and the quinolone pocket on the B subunit of DNA gyrase (Morrissey et al., 1996). Palumbo and co-workers have pinpointed the role played by magnesium in the quinolone-DNA interaction (Palù et al., 1992; Palumbo et al., 1993). These authors have suggested that Mg^{2+} acts as a bridge between the quinolone and the phosphate groups of the DNA, and that this complex is stabilized by stacking interactions between the condensed rings of the drug and the DNA bases in a single-stranded region or a distorted B-form in plasmid. Recently, Llorente et al. (1996) have proposed another model based on the intercalation of quinolone into the double helix of DNA. The structure is stabilized by the binding of the magnesium ion with the sp^2 oxygens present in quinolone, a phosphate and a purine base of the DNA. However, the results obtained by Hurley and co-workers (Fan et al., 1995) in their parallel study of quinobenzoxazines and norfloxacin have led these authors to state that, in the presence of magnesium, only the quinobenzoxazines are able to form a stable intercalated complex with DNA and that direct evidence for an intercalative role for norfloxacin is lacking.

In order to have a better understanding of the role of magnesium and to try to clarify the discrepancies between the several models of the quinolone/DNA complexes, we have investigated the pefloxacin-Mg^{2+}-DNA interactions by vibrational and NMR spectroscopies (Lecomte, 1995). The results of the Surface-Enhanced Raman Scattering (SERS) study have already been published (Lecomte and Baron, 1997). By using mainly ^{19}F-NMR, we have first determined the pK_a values of pefloxacin and evaluated its self-association constant (Lecomte et al., 1996). Then we have shown that, in the pefloxacin/magnesium complex, the binding sites are first, the carbonyl and carboxylate groups, then, the N4′-piperazinyl atom, and we have calculated the association constants with this divalent ion (Lecomte et al., 1994; Lecomte and Chenon, 1996). Finally, we have studied the pefloxacin interaction with several DNA species in the presence and absence of magnesium, and the results are reported in this paper.

NMR of the fluorine nucleus offers several advantages, (i) the possibility to work in buffered aqueous solutions, (ii) the large range of chemical shifts of this nucleus, which makes it very sensitive to weak interactions, (iii) the large gyromagnetic ratio of the ^{19}F nucleus and its 100% natural abundance, what allowed us to study solutions of pefloxacin diluted down to 10^{-4} M, concentration where pefloxacin is monomeric (Lecomte et al., 1996). This concentration is in the range of those found in the biological fluids of patients under pefloxacin treatment (from 2×10^{-5} M in serum up to 10^{-3} M in urine). Therefore, it was used for the study of the pefloxacin-DNA interaction, either in the absence or presence of magnesium. Since the linewidth of ^{19}F pefloxacin signal strongly depends on the pH (Lecomte et al., 1996) we have chosen to hold it at 8.0, which is still very close to the physiological pH, and gives a ^{19}F linewidth suitable for the NMR studies. Therefore, the percentages of the different species are the following: anionic 67%, zwitterionic 30%, neutral 2% and cationic 1% (Lecomte et al., 1996). It is important to note that the low percentages of the neutral and cationic species are highly favorable to the formation of the pefloxacin/Mg^{2+} complex (Lecomte et al., 1994; Lecomte and Chenon, 1996).

We report and discuss first the results obtained on the pefloxacin-DNA solutions, then those induced by the presence of magnesium in these solutions.

2. Materials and methods

2.1. Materials

Pefloxacin (Fig. 1) was a gift from Rhône Poulenc Rorer, France and used without further purification. Double-stranded herring sperm DNA and *Escherichia coli* (W3110 strain) DNA were purchased from Sigma. The single-stranded herring sperm DNA was obtained from the double-stranded DNA by thermal denaturation monitored by following the absorbance at 260 nm.

The supercoiled pBR322 plasmid DNA was isolated from *E. coli* (DH5α) (Hanahan, 1983). Bacterial culture was grown in Luria broth at 37°C in a gyratory shaker. Ten milliliters of an overnight culture was added to 1 l of medium supplemented with 17 μg/ml ampicillin and 15 μg/ml tetracycline. When the A_{600} reached 0.6, chloramphenicol was added up to a final concentration of 200 μg/ml and incubation was continued for 16 h. Then, the plasmid was purified using a Kit Flexipret, Pharmacia, France. One liter of bacterial culture yielded $\approx$1 mg of pBR322 DNA.

2.2. NMR studies

Pefloxacin (0.1 mM) was dissolved in buffer containing Tris–HCl (20 mM), KCl (20 mM), ethylene glycol (10% v/v), and EDTA (1 mM). The pH and the temperature were adjusted to 8.0 and 292 K, respectively. The salt of magnesium used ($MgCl_2$) was from Sigma, like all compounds used for the buffer. The concentration of magnesium varied from 0 to 50 mM.

^{19}F-NMR spectra were recorded at 282.2 MHz on a Bruker AM-300 spectrometer (7.0 T). A capillary containing a solution of trifluoroacetic acid (TFA) in D_2O was used for the field-frequency lock of the NMR spectrometer. In the buffer used and at 292 K, the fluorine chemical shift of pefloxacin is -48.646 ppm from TFA (external reference). In addition, the CF_3 signal was used to normalize the integration data and to control the temperature and homogeneity. The acquisition parameters (recycle time 1.22 s, flip angle $\approx$60°) were carefully adjusted in order to prevent any saturation of the pefloxacin signal. In the presence of *E. coli* (4 mM) and at the optimum magnesium concentration (2 mM), each spectrum needed $\approx$300 000 scans to give reliable data (O.D. samples 5 mm).

^{1}H-NMR spectra of all the solutions were also recorded on a Bruker AM-300 spectrometer in order to check the influence of the increase in the viscosity, induced by the high concentrations of DNA, on the linewidth of the signal of ethylene glycol. No significant variations of this linewidth were observed, even at the highest DNA concentrations used.

3. Results and discussion

3.1. Pefloxacin-DNA interaction in the absence of magnesium

Two double-stranded DNA species (circular supercoiled pBR322 and linear herring sperm) and one single-stranded DNA (thermally denatured herring sperm) were studied. Throughout this paper, the DNA concentrations are given per base molar residue. The maximum nucleotide concentration was 18 and 20 mM for the native and denatured herring sperm DNA, respectively, and 5.6 mM for pBR322 DNA.

Fig. 2 shows the variations of the chemical shift (δ) and linewidth (LW) of the ^{19}F signal with the DNA concentration. These variations are similar for the two native DNA studied and larger for the denatured DNA. They indicate clearly an interaction between pefloxacin and DNA. Similar variations of the ^{19}F spectra of difloxacin upon addition of denatured calf thymus DNA have been observed by Shen and co-workers (Shen, 1993).

The LW variations are difficult to interpret rigorously. In fact, when a small molecule exchanges between solution and a rigidly bound site on a macromolecule of large molecular weight, the increase in the linewidth of its NMR signals is induced by two simultaneous processes (i) the increase in the rotational correlation time τ_c of the complex with respect to that of the free ligand, (ii) the exchange rate between the free and bound

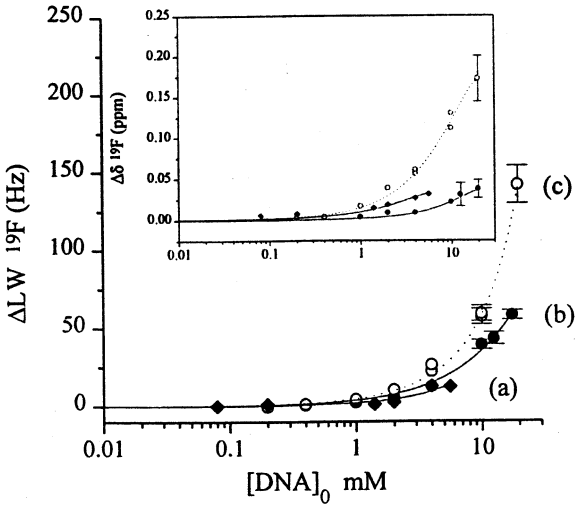

Fig. 2. Variations of the ^{19}F linewidth (Hz) of pefloxacin (10^{-4} M) with the concentration (per base molar residue) of (a) plasmid pBR322 DNA, (b) native herring sperm DNA, and (c) thermally denaturated herring sperm DNA, at pH 8.0 and 292 K. Inset: variations of the ^{19}F pefloxacin chemical shift (282 MHz) (same conditions as for the linewidth data).

species. Chenon et al. (1990) have shown that the ^{19}F linewidth of pefloxacin bound to bovine serum albumin (BSA) is ≈ 245 Hz at 292 K and a magnetic field strength of 7 T. Since the molecular weight of pBR322 DNA (2.88×10^6 Da) is 43 times that of BSA (6.63×10^4 Da), the linewidth of the ^{19}F signal of the pefloxacin/pBR322 DNA complex is expected much larger than 250 Hz. For the second process, when the exchange is very 'fast' on the chemical shift time-scale, the linewidth of the signal is the weighted average of the linewidths of the free and bound species. When this exchange is 'intermediate', the observed linewidths depend on several factors: the linewidths of the free and bound species, the percentages of these two species, the difference between their chemical shifts (Hz), and the rate constant of the dissociation of the complex (Feeney et al., 1979). In the present study, the variations observed in the linewidths seem mainly due to the exchange process since the integration of the proton decoupled ^{19}F signal is nearly constant whatever the DNA concentration, what indicates that the nuclear Overhauser effect (NOE) and then τ_c do not change noticeably. That allows the formation of large amounts of complexes to be ruled out, what is consistent with the small values determined for the binding constants per nucleotide of pefloxacin to several DNA species (Shen et al., 1989b; Bazile-Pham Khac and Moreau, 1994) Therefore, in the absence of magnesium, pefloxacin binds weakly to DNA. The drug binds preferentially to single-stranded (thermally denatured) DNA rather than to double-stranded DNA (linear herring sperm or circular supercoiled pBR322), confirming what was already reported by Shen and Pernet (1985).

The increase in DNA concentration induces a slight increase in the ^{19}F chemical shifts. Since the increase in pefloxacin concentration induces a similar trend in the ^{19}F chemical shift (Lecomte et al., 1996), that could corroborate the model proposed by Shen and co-workers (Shen et al., 1989a,b) for the pefloxacin-DNA complex that involves a stacked dimerization of the drug as well as hydrogen-bond type interactions between single-stranded DNA and the quinolone. However, at the concentration 10^{-4} M, pefloxacin is monomeric (Lecomte et al., 1996). Furthermore, such a model is not consistent with the results obtained by SERS (Lecomte and Baron, 1997) because the bands assigned to the NH vibrations of the bases are not modified upon addition of pefloxacin, what rules out their involvement in hydrogen bonding with this drug. Therefore, the changes in the ^{19}F spectra could be explained by a stacking interaction between the condensed rings of the drug and the DNA bases in the case of the single-stranded DNA. If the interaction with the double-stranded DNA is of the same kind, that would imply a local distortion of this DNA.

3.2. Pefloxacin-Mg^{2+}-DNA interaction

When pefloxacin, magnesium, and DNA are present in the solution, competition between the formation of the three binary and the ternary complexes occurs. In an attempt to gain insight into the formation and the structure of this ternary complex, the concentration of pefloxacin and either magnesium or DNA was held constant, when varying the concentration of the third component.

3.2.1. Magnesium concentration is constant (10 or 50 mm)

Two native double-stranded DNA species (herring sperm and pBR322), were chosen. Their maximum nucleotide concentration were 11.8 and 5.6 mM, respectively. Magnesium concentration $[Mg^{2+}]_0$ was first hold at 10 mM since, at this concentration and in the absence of DNA, pefloxacin can be considered to be complexed more than 99% to magnesium, mainly as a 1:2 (drug:magnesium) complex (Lecomte and Chenon, 1996). Furthermore, this magnesium concentration is low enough to not induce the change from the *B*- to the *Z*-form of DNA (Saenger, 1984; Fan et al., 1995).

Fig. 3 shows the variations observed for the chemical shift and the linewidth of the ^{19}F signal of pefloxacin under these conditions. The chemical shift has no significant variations with the DNA concentration and its average is very close ($+0.02$ ppm) to its value in the presence of magnesium only (-47.30 ppm). In contrast, notable variations are observed for the linewidth, much larger for the linear than for the circular supercoiled DNA, and larger, for a given DNA

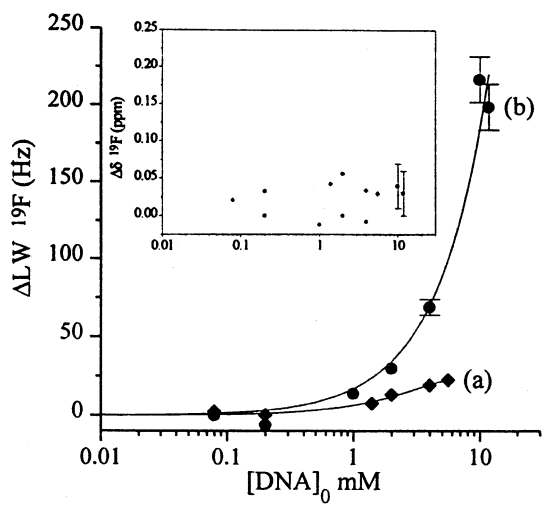

Fig. 3. Variations of the ^{19}F linewidth (Hz) of pefloxacin (10^{-4} M) with the concentration (per base molar residue) of (a) plasmid pBR322 DNA and (b) native herring sperm DNA, in the presence of $MgCl_2$ (10 mM), at pH 8.0 and 292 K. Inset: variations of the ^{19}F pefloxacin chemical shift (282 MHz) (same conditions as for the linewidth data).

species, than in the absence of magnesium (compare Fig. 2 and Fig. 3). Furthermore, the integration of the proton decoupled fluorine signal decreases by 25 and 15% for the highest concentrations of herring sperm and pBR322 DNA, respectively.

These integration data indicate clearly the formation of the ternary complex pefloxacin/Mg^{2+}/DNA at the high DNA concentrations, since in the absence of DNA the integration of the proton decoupled fluorine signal is constant whatever the magnesium concentration. This ternary complex cannot be observed directly due to its expected very broad ^{19}F signal induced by its long correlation time and its efficient chemical shift anisotropy relaxation mechanism (Hull and Sykes, 1975). However, it can transfer (see below) its negative NOE to the species with which it is exchanging, i.e. pefloxacin free and bound to magnesium, thus inducing a decrease in the integration of their proton decoupled signals. The linewidth variations reflect the exchange between these latter pefloxacin species (Lecomte and Chenon, 1996). Since magnesium has more affinity for DNA ($22 \pm 4 \times 10^3$ M^{-1} for pBR322) (Palù et al., 1992) than for pefloxacin ($1.60 \pm 0.05 \times 10^3$ M^{-1}) (Lecomte and Chenon, 1996), an increase in DNA concentration induces a decrease in the amount of Mg^{2+} available for pefloxacin, then an increase in the free pefloxacin concentration. The variations of the linewidth indicate that the competition between double-stranded DNA species and pefloxacin for binding to magnesium is more important with linear DNA than with supercoiled plasmidic DNA.

The values of the ^{19}F chemical shifts and integrations indicate, without ambiguity, the presence of both pefloxacin/magnesium and ternary complexes in the solutions. Since the ^{19}F signal of the ternary complex cannot be observed, ^{19}F-NMR cannot give any direct information on the structure of this complex. However, we have shown that, in the binary complex, the binding sites are, first, the carbonyl and carboxylate groups, then the N4′-piperazinyl atom (Lecomte and Chenon, 1996). Besides, it is now well established that magnesium interacts with the phosphate groups of DNA and not with the base moieties (Wang et al.,

1996 and references therein). All these informations already allow us to conclude that Mg^{2+} acts as a bridge between quinolone and DNA, confirming the model suggested by Palù et al. (1992) for the ternary complex. The formation of the ternary complex at a lesser extent with pBR322 DNA than herring sperm DNA could be due to their different topological states, the magnesium access to the phosphate groups being more hindered in the supercoiled circular structure of the plasmid than in the linear structure of herring sperm DNA.

These data are at variance with those obtained by Gatto and reported by Palumbo et al. (1993). These authors stated that linear double-stranded DNA is not able to bind efficiently to the quinolones at any Mg^{2+} concentration. In another hand, our data agree with those obtained by Bazile-Pham Khac and Moreau (1994), who observed a significant increase in the affinity constant of pefloxacin for calf thymus DNA in the presence of magnesium.

Only the pefloxacin-pBR322 DNA solutions were studied with the magnesium concentration hold at 50 mM instead of 10 mM. This concentration is still much smaller than the limiting concentration 0.7 M which induces the change of conformation of DNA from the *B*- to the *Z*-form (Saenger, 1984). The observed ^{19}F chemical shift is again nearly constant whatever the DNA concentration (maximum 5.6 mM) and its average is close ($+0.03$ ppm) to its value in the presence of only 50 mM of magnesium (-47.28 ppm). However, in contrast to the previous results with $[Mg^{2+}]_0 = 10$ mM, the integration of the proton decoupled fluorine signal becomes nearly constant and only a slight increase in its linewidth is observed at the high DNA concentrations. All these results indicate that the competition between DNA and pefloxacin for binding magnesium is, as expected, much weaker when the Mg^{2+} concentration is high enough, and that the major species in solution are the binary complexes DNA/Mg^{2+} and pefloxacin/Mg^{2+}.

The differences between the spectra obtained for these two magnesium concentrations prompted us to investigate the influence of the magnesium concentration on the formation of the ternary complex.

3.2.2. DNA concentration is constant (4 mM)

For this series of experiments, we have preferred to use a chromosomic DNA rather than a supercoiled plasmidic DNA, and we have chosen *E. coli* W3110 more defined than herring sperm. The ratio $[DNA]_0/[pefloxacin]_0$ was held at 40 and the magnesium concentration $[Mg^{2+}]_0$ varied from 0 up to 50 mM. In order to obtain further information on the structure of the ternary complex, the fluorine spectra were obtained, with and without decoupling of the protons.

Fig. 4 summarizes the variations of some characteristic features of the ^{19}F signal with the magnesium concentration. On the basis of only the chemical shift variations (Fig. 4A), pefloxacin seems totally complexed to magnesium at $[Mg^{2+}]_0$ larger than 5 mM. However, when chemical exchange occurs, the linewidths are more sensitive than the chemical shifts to the presence of minor species. In fact, when the magnesium concentration further increases, the linewidth decreases towards its value observed in the absence of DNA (Fig. 4B). The integration of the coupled ^{19}F signal with respect to TFA shows a sharp decrease at the magnesium initial concentration of 2 mM (Fig. 4C). A few solutions were also studied at 284 K by ^{19}F-NMR without proton decoupling. Two broad signals were observed and assigned to pefloxacin free and bound to magnesium. Their integration shows also a sharp minimum at $[Mg^{2+}]_0 \approx 2$ mM. These decreases reflect the formation of the ternary complex pefloxacin/Mg^{2+}/DNA which cannot be observed directly, as already pointed out. This is clearly confirmed by the corresponding decrease observed in the NOE variations (Fig. 4D). When different species are in chemical exchange, it is well known (Neuhaus and Williamson, 1989) that the NOE of some species can be transferred to the other species. Since, as already mentioned, the formation of the pefloxacin/magnesium complex does not induce any change in the pefloxacin NOE, the observed decrease in the NOE of pefloxacin species, free and bound to magnesium, comes from their exchange with the ternary complex which has a negative NOE owing to its long correlation time. It is difficult to fit these NOE data in order to get the equilibrium constants, as Palù et al.

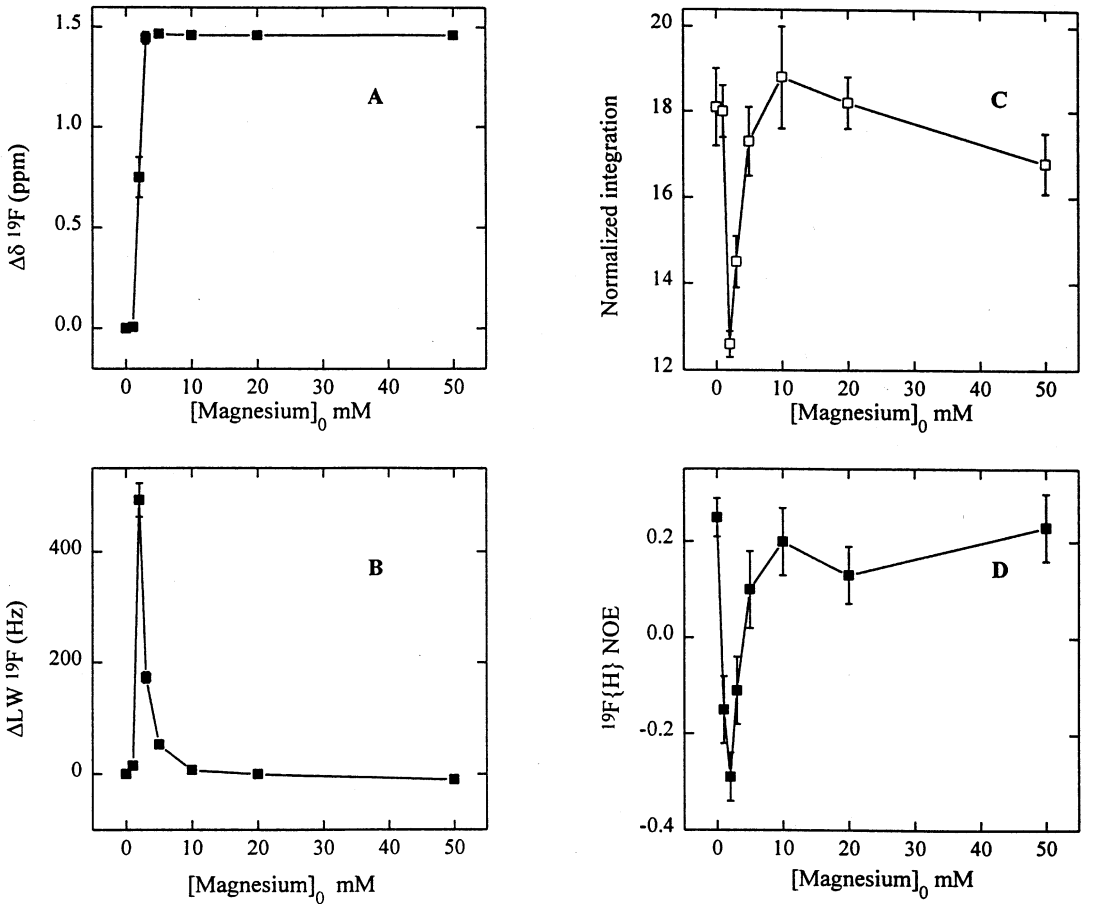

Fig. 4. Variations of some characteristic features of the ^{19}F pefloxacin (10^{-4} M) signal (282 MHz) with the magnesium concentration: (A) chemical shift, (B) linewidth, (C) integration of the coupled signal, normalized with respect to that of TFA (in a capillary), (D) NOE. DNA *E. coli* concentration was held at 4 mM.

(1992) did, since the amount of transferred NOE depends not only on the NOE of the different species but also on their exchange rates on the chemical shift and relaxation time scales (Neuhaus and Williamson, 1989).

Palù et al. (1992) have reported a similar optimum magnesium concentration (1—2 mM) for the formation of the ternary complex norfloxacin/magnesium/DNA when the quinolone concentration is either 1 μM (plasmid concentration 0.77 mM) or 133 μM (plasmid concentration not given).

The ^{31}P DNA signals have been observed whatever the magnesium concentration, in contrast to the ^{19}F signal of pefloxacin which becomes too broad to be observed when this drug is part of the ternary complex. That indicates that the quinolone ring has little mobility in this complex. Our present NMR data do not allow us to determine precisely its structure. Nevertheless, taking into account this reduced mobility and the binding sites of pefloxacin with magnesium (Lecomte and Chenon, 1996), this drug, in the ternary complex, could be bound to two magnesium ions, each cation being also in interaction with a phosphate group of the DNA backbone, in a way similar to that proposed by Hurley and co-workers for the norfloxacin molecule externally bound to DNA (Fan et al., 1995). Such a model implies some hindrance of the piperazinyl C7 substituent

which seems to have an important role in the activity of quinolones (see the article by Llorente et al., 1996 and references therein), although a nonaromatic nitrogen heterocyclic substituent is not an absolute requirement (cf. nalidixic and oxolinic acids, flumequine) (Smith, 1984; Cornett and Wentland, 1986; Fernandes and Chu, 1987; Chu and Fernandes, 1989).

4. Conclusion

^{19}F-NMR spectra show that, in the absence of magnesium, pefloxacin binds poorly to DNA and preferentially to single-stranded rather than to double-stranded DNA.

Our data show that the access of magnesium to the phosphate groups of DNA is, as expected, more favored in double-stranded linear than supercoiled plasmidic DNA. Thus, in the presence of a fixed concentration of this cation (10 mM), their competition with pefloxacin for binding to magnesium is stronger for the former than for the latter DNA species. Only the ^{19}F signals of pefloxacin free and bound to magnesium are observed, but the formation of the ternary complex pefloxacin/Mg^{2+}/DNA is indicated by the decrease in the integration of the proton decoupled ^{19}F signal at the high concentrations of double-stranded (linear and plasmidic) DNA.

The variations of the ^{19}F NOE demonstrate that this formation depends strongly on the magnesium concentration, the optimum concentration being ≈ 2 mM in the presence of *E. coli* DNA (4 mM). The ^{19}F-NMR data show that the quinolone ring of pefloxacin has little mobility in the ternary complex. In this complex, pefloxacin could be bound to two magnesium ions, each cation acting as a bridge between this molecule and a phosphate group of the DNA backbone. It appears essential to take into account the influence of Mg^{2+} concentration on the formation and the structure of the ternary complex, whatever its molecular details, in order to progress in the knowledge of the mechanism of action of the quinolones.

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