Binding Mode of Norfloxacin to Calf Thymus DNA

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Abstract: Norfloxacin, a quinolone antibacterial reagent, has been studied with respect to its binding to calf thymus DNA using fluorescence and linear dichroism techniques and unwinding of supercoiled DNA. The fluorescence of norfloxacin is strongly quenched in the presence of DNA and using this decrease in a fluorescence titration the equilibrium constant of the complex formation was established to be $2.8 \times 10^3\ M^{-1}$. The electric transition moments of the norfloxacin chromophore have been analyzed using fluorescence anisotropy, magnetic circular dichroism, and linear dichroism in stretched poly(vinyl alcohol) film and INDO/S calculations. These data are then used to interpret flow linear dichroism results for the norfloxacin−DNA complex. The transition moments for the long-wavelength transitions are found to be oriented at about 65.0−85.0° with respect to the DNA helix axis. A near perpendicular orientation of the norfloxacin chromophore plane makes it possible to exclude classical groove or surface binding modes. The possibility of a classical intercalation binding mode also can be ruled out from unwinding experiments. However, it is shown that the molecular plane of norfloxacin is near perpendicular relative to the DNA helix axis with a possibility of a bending of the DNA helix at the binding site.

Introduction

Quinolones are a group of extremely potent antibacterial agents[1] which are increasingly being used for the treatment of many infections. A large amount of biological data indicate that the functional target of these drugs is DNA gyrase, an essential type II DNA topoisomerase which catalyzes the conversion of relaxed supercoiled DNA into a negatively supercoiled form.[1] However, Shen et al. have reported that norfloxacin (Figure 1), one of the most potent DNA gyrase inhibitors of quinolone family, does not bind directly to DNA gyrase but binds to DNA itself.[2] This finding motivated the studies of direct binding of quinolones to various types of DNA and a DNA−gyrase complex. Subsequent studies[3,4] by the same group showed that norfloxacin binds preferentially to the single-stranded DNA. When DNA was paired, binding of norfloxacin was weak and exhibited no base preference. They also detected a saturable drug binding phase with supercoiled DNA, with the binding possibly enhanced by the addition of gyrase to relaxed DNA in the presence of a nonhydrolyzable ATP analogue. On the basis of these findings, a cooperative quinolone−DNA binding model for the inhibition of DNA gyrase has been proposed,[5] in which norfloxacins were bound in the specific

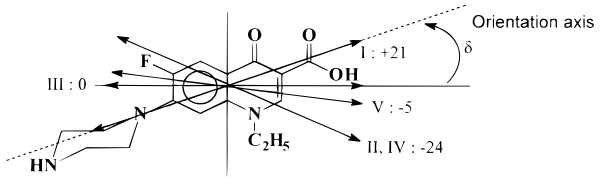

Figure 1. Molecular structure of norfloxacin and the electric dipole transition moments.

single-stranded DNA pocket which was induced by gyrase and were stabilized by the hydrogen bonding, the $\pi-\pi$ stacking of the norfloxacin rings, and the tail-to-tail hydrophobic interactions. Reports appeared which conflicted with the proposal of Shen et al. Although electrophoresis experiments[6] showed that norfloxacin is able to unwind the DNA double helix in the presence of Mg^{2+}, a ^{19}F NMR study[7] and fluorescence spectroscopic technique[8] failed to show any direct interaction between DNA and quinolones. Palù et al. showed that norfloxacin binds to plasmid DNA in the presence of an appropriate amount of Mg^{2+} but exhibits no interaction in either an absence or excess amount of Mg^{2+} ion by fluorescence technique, electrophoretic DNA unwinding, or affinity chromatography techniques.[9] These observations led them to propose a model for the ternary complex, in which the Mg^{2+} ion acts as a bridge between the phosphate groups of nucleic

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acids and the carbonyl and carboxyl moieties of norfloxacin.

Despite its importance, the nature of the binding mode of norfloxacin to DNA is still not known. Intercalation has been deemed unlikely[6] because the unwinding angle of norfloxacin is much smaller than those for classical intercalators such as ethidium bromide. We here used well-established spectroscopic techniques to study DNA−ligand complexes in solution to address the question of the binding geometry of the norfloxacin−DNA complex. We demonstrate that norfloxacin binds to mixed-sequence DNA, also in the absence of Mg^{2+} ions, and focus on the complex geometry under these simplified conditions.

Experimental Section

Materials. Supercoiled ϕX174 DNA was purchased from Pharmacia and used for the unwinding experiment without further purification. Double-stranded calf thymus DNA, purchased from Sigma, was dissolved in a 5 mM cacodylate buffer at pH 7.0 containing 100 mM NaCl and 1 mM EDTA and dialyzed several times at 4 °C against 5 mM cacodylate buffer at pH 7.0. This buffer was used throughout this work. Norfloxacin was purchased from Sigma and used without further purification. The concentrations of DNA and norfloxacin were determined spectrophotometrically using the molar extinction coefficients $\epsilon_{258\ nm} = 6700\ cm^{-1}\ M^{-1}$ and $\epsilon_{273\ nm} = 37\ 500\ cm^{-1}\ M^{-1}$ for DNA bases and norfloxacin, respectively.

Methods. Determining the Transition Moments of Norfloxacin. Complete information regarding the transition moment is essential in order to elucidate the binding geometry of a norfloxacin−DNA complex. The transition moments of the norfloxacin molecule can be determined by a combination of fluorescence polarization anisotropy (FPA),[10,11] magnetic circular dichroism (MCD),[12] LD^r,[13−16] and quantum mechanical calculations.

The FPA was measured in a 1,2-propanediol glass at −60 °C using an Aminco SPF-500 "corrected spectra" spectrofluorometer equipped with a polarizer for both excitation and emission. The fluorescence excitation spectra were measured at 415 nm emission with a band-pass of 15 nm for FPA measurement. The band-pass of the excitation monochromator was 4 nm. The excitation signal was averaged over an appropriate number of scans. The LD of norfloxacin in the stretched poly(vinyl alcohol) (PVA) film was measured by the method described by Kubista et al.[16] and the MCD was measured in a 15 mM phosphate buffer at pH 7.2 on a Jasco 720 spectropolarimeter equipped with horseshoe magnet, as described earlier.[12]

MO Calculations. To aid assignment of the electronic transitions, MO calculations were performed with the INDO/S model Hamiltonian[17−20] on neutral and cationic forms of norfloxacin. The two-center electron repulsion integrals were calculated using the Mataga−Nishimoto scheme.[21] In the configuration interaction (CI) calculation, 226 singly excited (singlet) configurations using the 15 highest occupied and 15 lowest unoccupied MO's were included. Expansion of the CI space was tested, but the effects on the calculated spectra were negligible. The effect of polar solvent (water) on the calculated spectrum was considered in the self-consistent reaction field (SCRF) calculations. For the SCRF calculation, the molecule was embedded in a spherical cavity surrounded by a solvent continuum which was characterized by the dielectric constant and refractive index of water ($\epsilon = 78$, $n = 1.333$). We used the SCRF model B[22] and a cavity radius of 5.0 Å in the SCRF calculation. The geometry used in the calculation was extracted from the crystal structure of 4-(3-carboxy-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-quinolyl)-1-methylpiperazinium methansulfonate dihydrate.[23]

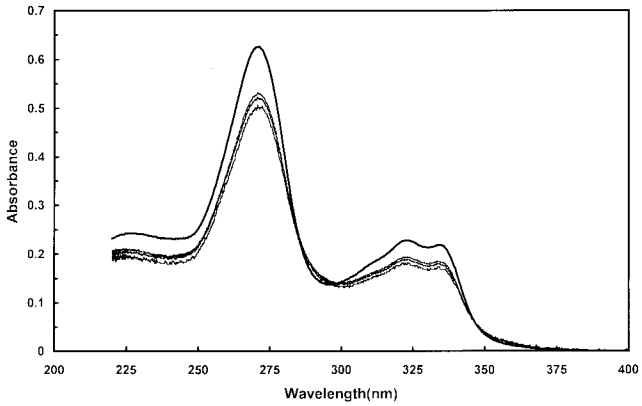

Figure 2. Absorption spectra of norfloxacin in the absence (thick curve) and presence of DNA. The data were collected for 197 μM DNA and a mixing ratio, R, of 0.017, 0.034, 0.050, 0.067, and 0.084. The absorption spectrum of norfloxacin-free DNA was subtracted, and the resulting spectrum was normalized to 16.5 μM. Only those for $R =$ 0.017, 0.050, and 0.084 (from bottom at 270 and 330 nm) are shown.

Unwinding of Supercoiled DNA. The unwinding of DNA by norfloxacin was followed by electrophoretic mobility of the supercoiled ϕX174 DNA in agarose gels, with the nicked circular form used as a control. Since the binding of norfloxacin to double-stranded DNA is too weak for the drug to remain bound during electrophoresis, a gel buffer was prepared to contain norfloxacin at the drug concentration necessary to maintain the desired binding ratio (as calculated from the binding constants determined here). Multigels[24] consisting of ten 1% agarose strips (8 mm wide and each with a separate loading well) were prepared to contain different concentrations of norfloxacin. Equal amounts of RFI and RFII DNA (20 μM base each in 20 μL), incubated at the desired binding ratio, were loaded in each well. The gel was covered by a glass plate and run into a horizontal electrophoresis cell in a non-submarine manner to avoid leakage of free norfloxacin from the gel strips. The positions of the two DNA bands were measured on the gels using the fluorescence of norfloxacin bound to DNA. To check the electrophoretic unwinding protocol and calibrate the degree of supercoiling of the RFI sample, the unwinding of the same DNA lot by ethidium bromide ($K = 10^6\ M^{-1}$) was also monitored by the multigel technique.

Results

Normal Absorption. The absorption spectra for different binding ratios of norfloxacin with DNA are shown in Figure 2. The data were collected for a constant DNA concentration (197 μM) and for mixing ratios ([norfloxacin]/[DNA base]), R, of 0.017, 0.034, 0.050, 0.067, and 0.084 and are shown with the pure DNA spectrum subtracted and normalized to a norfloxacin concentration of 16.5 μM. The spectra of free norfloxacin and those for $R =$ 0.017, 0.050, and 0.084 only are depicted to facilitate comparison. A 16−18% hypochromism in the 250−280 nm and 310−340 nm regions and three isosbestic points (at 285, 299, and 346 nm) were observed in the absorption spectra in the presence of DNA. Changes in the absorption

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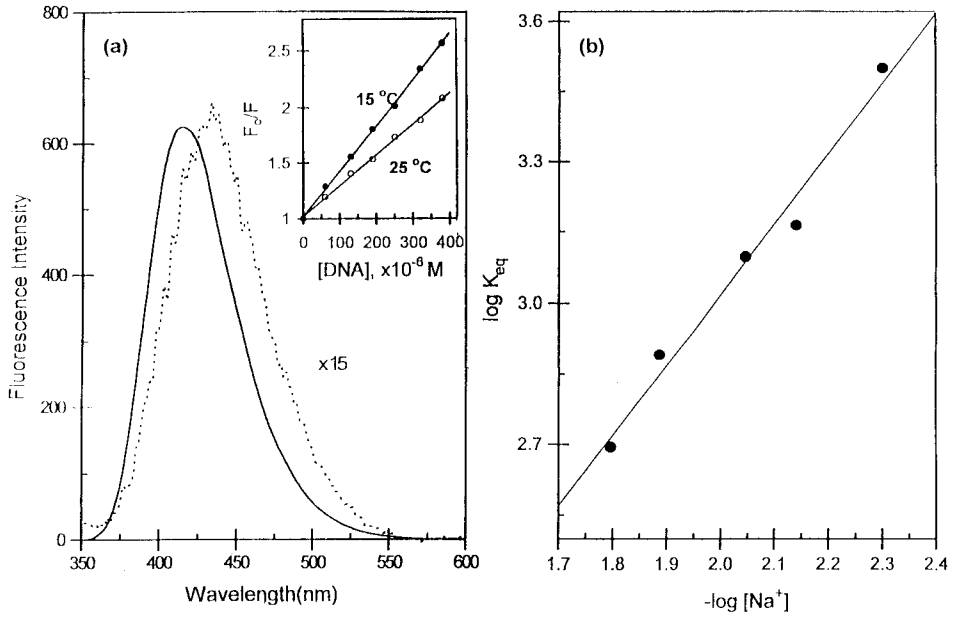

Figure 3. (a) Fluorescence emission spectra of norfloxacin in the absence (thick curve) and presence of DNA. [norfloxacin] = 1.0 μM and [DNA] = 443.4 μM in nucleotide base. The dotted curve represents the emission spectrum (enlarged 15 times) of the DNA-bound norfloxacin obtained by dialyzing the drug−DNA solution against a solution of drug and then subtracting the spectrum of the equilibrated drug solution from that of the drug−DNA solution. Excitation wavelength was 323 nm. Slit widths were 4 and 7 nm for excitation and emission window. Insert: Stern−Volmer quenching plot at 15 and 25 °C. The fluorescence intensity was measured at 323 nm for excitation and 415 nm for emission. (b) Plot of log K_{eq} vs −log [Na^+]. The slope is calculated to be 1.2.

spectrum of norfloxacin after mixing with DNA indicated that norfloxacin directly (without ATP or Mg^{2+} mediation) forms a complex with double-helical calf thymus DNA. Furthermore, three isosbestic points in the absorption spectra suggested that the conformation of the DNA-bound norfloxacin was homogeneous. Due to the low binding constant (see below), we may assume that not all the norfloxacin molecules were bound.

Fluorescence Measurement. The direct interaction between norfloxacin and DNA was also evidenced by changes in the fluorescence characteristics of the drug upon binding to DNA. As the DNA concentration increased, a strong decrease in the fluorescence intensity of norfloxacin was apparent (Figure 3a). This observation agrees with the report by Shen et al.,[25] but not with that of Palù et al.[8] We cannot explain these disparate results. The equilibrium constant for the norfloxacin−DNA complex formation can be approximated by the Stern−Volmer equation[10] ($F_o/F = 1 + K_{sv}[Q]$, where K_{sv} is the Stern−Volmer constant and F_o and F are the fluorescence intensity in the absence and presence of quencher), since the fluorescence intensity of norfloxacin in the presence of highly concentrated DNA is very low compared to that of free norfloxacin; the emission intensity of DNA-bound norfloxacin is less than 5% that of DNA-free norfloxacin. If F_o/F is plotted against the DNA concentration, the Stern−Volmer constant, which is equivalent to the equilibrium constant for the complex formation, can be calculated from the slope.[10] The Stern−Volmer plot for the norfloxacin−DNA mixture is depicted in Figure 3a (insert). The equilibrium constants for the norfloxacin−DNA complex were estimated to be approximately 4.1×10^3 M^{-1} at 15 °C and 2.8×10^3 M^{-1} at 25 °C. The increase in the equilibrium constant as the temperature is lowered, together with hypochromism in the absorption spectrum in the presence of DNA, supported a ground-state complex formation between norfloxacin and DNA. The emission spectrum of the bound species was obtained by dialyzing the drug−DNA solution (1 μM norfloxacin plus 443.4 μM DNA) against a solution of drug (1 μM norfloxacin) and then subtracting the spectrum of the equilibrated drug solution from that of the drug−DNA solution. The resulting spectrum (Figure 3a, dotted curve) exhibited a peak at 433 nm, a 19 nm red-shift compared to the DNA-free norfloxacin (at 414 nm).

When the log of equilibrium constants at various sodium concentrations was plotted (log K vs −log [Na^+]; Figure 3b), the slope was ∼1.2. Mono- and dicationic ethidium and propidium binding to DNA reportedly results in a 1 and 2 Na^+ ion release.[26] Considering that 0.2−0.3 sodium ions should be released in the intercalation conformational change,[27] norfloxacin may possess one positive charge at the binding pocket.

Norfloxacin Transition Moment Polarization Assignment. To determine the norfloxacin orientation on DNA from flow LD, we must know how the norfloxacin transitions are polarized. The electric transition moment can be determined by a combination of FPA, LD in a PVA film, quantum mechanical calculation, and MCD.[12−15] The absorption spectra of norfloxacin in water, in PVA film, and in 1,2-propanediol glass were similar, indicating negligible perturbation by the solvent on the electronic states of norfloxacin.

FPA reflects the moment directions relative to the emitting transition moment, while LD^r reflects transition moment directions relative to the molecular orientation axis. Both LD^r and FPA should be wavelength independent for a pure electronic transition, if vibronic effects are negligible. Therefore, the pronounced wavelength dependence observed in FPA (Figure 4a) in 1,2-propanediol glass at −60 °C and in LD^r (Figure 4b) in PVA film indicated overlaps between bands with different polarizations. Table 1 summarizes the results from the LD and FPA measurements. The number and positions of the transitions were confirmed by MCD measurements on norfloxacin in neutral phosphate buffer (Figure 5). To obtain moment direc-

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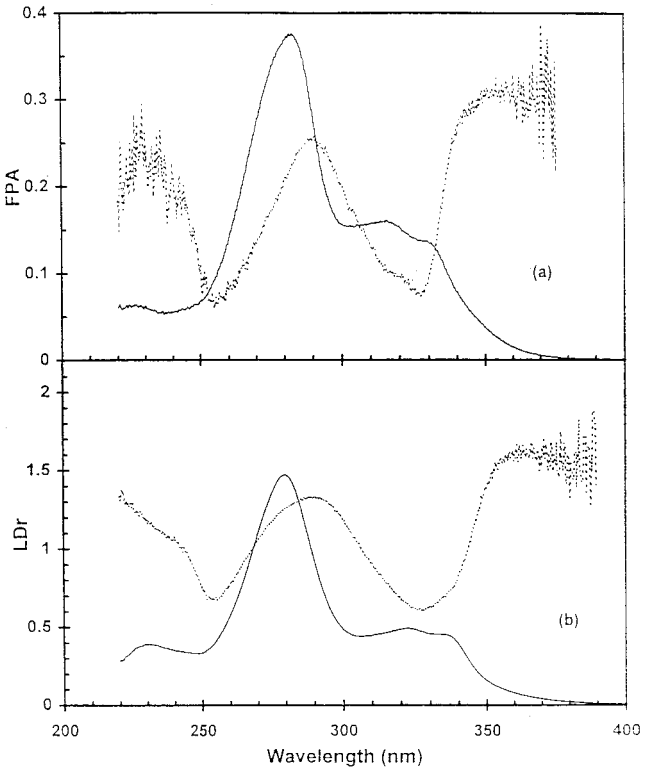

Figure 4. (a) FPA (dotted curve) and fluorescence excitation spectrum of norfloxacin in 1,2-propanediol glass at −60 °C. Excitation spectrum was taken with 425 nm emission. Slit widths were 4 and 15 nm for FPA measurement and 4 and 4 nm to record excitation spectrum for the excitation and emission windows. (b) LD^r (dotted curve) and isotropic absorption spectrum (solid curve) of norfloxacin in stretched PVA film.

Table 1. Observed Electronic Transitions of Norfloxacin

transition moment	λ (nm)	LD^r	α^a (deg)	FPA	β^b (deg)
I	360	1.60	0−10	0.31	
II	325	0.60	41−50	0.075	45
III	290	1.35	19−24	0.255	20
IV	255	0.67	39−48	0.06	47
V	230	1.1	28−33	0.225	26

[a] Angle relative to orientation axis. [b] Angle between *i*th transition and the emitting moment.

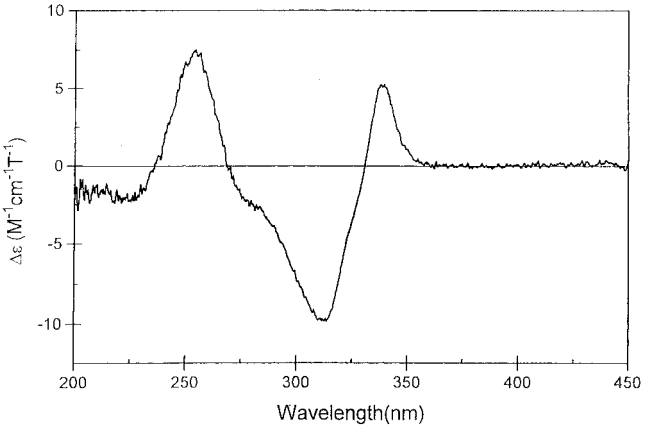

Figure 5. MCD spectrum of norfloxacin in a 15 mM phosphate buffer.

tions related to the molecular coordinate system (Figure 1), we must first assign the direction of the orientation axis. Previous measurements of molecules oriented in stretched polymer sheets demonstrated that the sample molecules tend to orient with their smallest cross section perpendicular to the macroscopic orientation direction (stretching direction).[28,29] The orientation axis of norfloxacin was therefore assumed to be parallel to the long axis of the molecule ($\delta = +21°$, Figure 1). The similar shapes of the LD^r and FPA spectra are characteristic of a rodlike molecule, where the lowest lying transition is oriented closely to the orientation axis.[13]

Table 2. INDO/S Spectrum for Neutral Norfloxacin

transition	λ (nm)	oscillator strength	δ^a (deg)
1 ($n \rightarrow \pi^*$)	379	0.00075	
2 ($\pi \rightarrow \pi^*$)	313	0.097	+45
3 ($\pi \rightarrow \pi^*$)	302	0.02	−11
4 ($n \rightarrow \pi^*$)	297	0.002	
5 ($\pi \rightarrow \pi^*$)	280	0.88	+28
6 ($\pi \rightarrow \pi^*$)	255	0.47	−64
7 ($\pi \rightarrow \pi^*$)	250	0.077	0

[a] See Figure 1 for definition.

We used the results from the INDO/S calculations (Table 2) to aid the choice of moment directions. Only the first seven transitions are shown, due to the complexity of the calculated spectrum below 240 nm. The calculated spectrum (not shown) of the cationic form of norfloxacin was very similar to the spectrum of neutral form because the chromophore was unaffected by this protonation. The lowest lying transition (1) is a $n \rightarrow \pi^*$ transition, which is calculated to be very weak. There was no indication from either LD or FPA experiments that the lowest lying transition had this character; therefore, we assumed that this very weak transition was overlapped by the much stronger $\pi \rightarrow \pi^*$ transition (2) found at 313 nm in the calculation. The energy difference between the calculation and experiment (measured transition I at 360 nm) was probably due to unsatisfactory treatment of the solvent effects for this transition. The polarization of this transition was almost along the long axis of the norfloxacin molecule, in good agreement with the observed angle (Table 1). The next calculated transition (3) is also a $\pi \rightarrow \pi^*$ transition, polarized with an angle of 56° away from transition 2 in good agreement with the angle obtained from the FPA analysis. Transition 4 from the calculation is a weak $n \rightarrow \pi^*$ transition. The next two transitions are $\pi \rightarrow \pi^*$ transitions (transition 5 at 280 nm and transition 6 at 255 nm) and are the strongest ones. They correspond probably to the composite's strong band, observed at 250−300 nm. Transition 5 has a long axis polarization which is in agreement with the experiment. Transition 6 is polarized away from the long axis. At 240 nm, there is a strong increase in both LD^r and FPA, indicating the presence of a $\pi \rightarrow \pi^*$ transition. There was a corresponding calculated transition (7) at 250 nm. The concluded experimental absolute polarizations are shown in Figure 1.

Flow LD of the Norfloxacin−DNA Complex. In addition to the changes in the absorption and fluorescence characteristics, the appearance of an LD spectrum in the norfloxacin absorption region is definitive evidence for the norfloxacin−DNA complex formation. If the norfloxacin molecules were not bound to DNA, they would not be oriented in the flow and, therefore, no LD signal in the norfloxacin absorption region would be observed. The magnitude of flow LD over the entire absorption region of norfloxacin appeared to be 0 when DNA was absent (data not shown), indicating that norfloxacin itself is not oriented in the flow. No LD signal could also appear, even though norfloxacin is bound to DNA, if the angle between the transition

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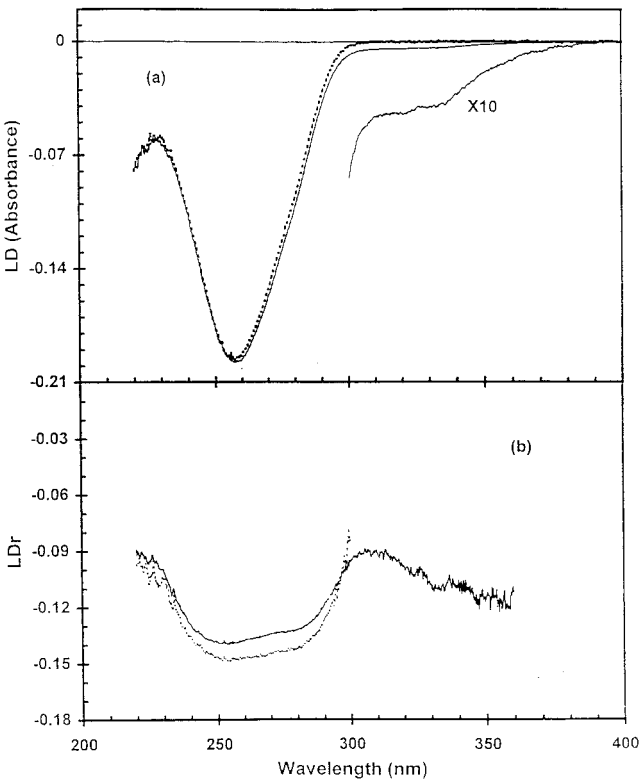

Figure 6. (a) Flow-oriented LD and (b) LD^r spectrum of norfloxacin complexed with DNA. DNA concentration was 197 μM, and that of norfloxacin was 16.5 μM. LD signal was averaged over five scans. The isotropic absorption spectrum of the norfloxacin−DNA complex to calculate LD^r was obtained by subtracting absorption spectrum of the free norfloxacin from the measured absorption spectrum of the norfloxacin−DNA mixture. LD^r spectra for other mixing ratios fell into a similar range. LD and LD^r spectrum of norfloxacin-free DNA is compared as the dotted curve.

moments of norfloxacin and DNA helix axis is equal to the magic angle value (54°45′), but this is not our case (see below). LD was measured for the norfloxacin−DNA complex at various mixing ratios; Figure 6a shows the LD spectrum at the mixing ratio of 0.084. The norfloxacin−DNA complex showed a dominantly negative LD signal at DNA absorption region, in accordance with the DNA bases being perpendicular to the helix axis. The small, negative LD signal in the drug absorption region (300−360 nm) indicated that the transition moments of norfloxacin were oriented, on average, more perpendicular than parallel to the helix axis. Negative LD in the DNA absorption region and positive in the drug absorption region are expected for the groove binding drugs,[30] and therefore, the possibility of a groove binding geometry of norfloxacin could be eliminated. The possibility that the positively charged norfloxacin would bind to the negatively charged phosphate groups of DNA was also ruled out because, in this case, the norfloxacin molecule would not be oriented in the same direction, resulting a zero LD magnitude in the drug absorption region.

The LD^r spectra were calculated by the method described by Nordén et al.[31,32] In the course of LD^r calculation, the isotropic absorption spectra of the norfloxacin−DNA complexes were obtained by subtracting the unbound norfloxacin absorption spectra, which are estimated from the equilibrium constant measured by the Stern−Volmer method. Although the amount of the free norfloxacin under our conditions was as much as ∼65%, the qualitative interpretation of LD^r spectra was unaffected by the percentage. Figure 6b shows the average LD^r of the norfloxacin−DNA complex (for the mixing ratio of 0.084, [DNA] = 197 μM) over five independent measurements. The angle between the transition moment I (at 360 nm) and the DNA helix axis is calculated to be 75.0°, and that of the transition II (at 325 nm), relative to the DNA helix axis, was 72.3°. The lower limits of the five measurements were 67.0° and 68.9° and the upper limits were 79.5° and 86.2°, respectively, for transition moments I and II. These variations in LD^r values arise from the small LD signal in the norfloxacin absorption region. The exact angle between the other transition moments and the DNA helix axis could not be calculated due to the overlapping of the LD^r signal between the other transition moments of norfloxacin and the DNA itself. The angles calculated from all other mixing ratios (R = 0.017, 0.034, 0.050, and 0.067, [DNA] = 197 μM) fell in the same category, suggesting the existence of a single binding mode of norfloxacin to DNA. The effective angles of 75.0° and 72.3° with respect to the DNA helix axis (15.0° and 17.7° with respect to the plane normal to the DNA helix axis) were within the range of the base inclination angle of B-form DNA.[33,34] It is note worthy that the magnitude of LD^r in the DNA absorption region, i.e. the orientability of DNA, is decreased with an increased drug load; for the classical intercalators, increasing in the LD^r magnitude in the DNA absorption region is expected due to the stiffness and elongation of the template DNA upon drug intercalation. Therefore, the observed decreases in the orientability of DNA upon norfloxacin binding may indicate a significant bending or duplex dissociation near drug binding site.

Unwinding of Supercoiled DNA. Figure 7a,b shows the mobility of RFI and RFII DNA for different concentrations of free norfloxacin in the gel. Both forms of DNA exhibit mobility shifts with increased norfloxacin concentration, indicating interactions with DNA under the present conditions. The binding to nicked circular DNA was weak, however, as evidenced by the lack of an observable mobility shift below 10 μM. The mobility shift for the supercoiled DNA was also very weak below 10 μM, but a more marked mobility shift than for the nicked circle was observed at higher concentrations of norfloxacin. The stronger effect of norfloxacin on supercoil mobility is more clearly seen by forming the ratio of the mobility of RFI and RFII (Figure 7b), which exhibited a weak but significant decrease when the norfloxacin concentration was increased to above 1 μM. There was no marked minimum, however, in contrast to classic intercalators such as ethidium bromide, where the mobility of the supercoiled DNA coincides with that of the nicked-circle control at an ethidium bromide concentration of about 0.04 μM under present conditions. With ethidium bromide, the nicked circle exhibited a steady decrease in mobility with an increase in the drug concentration throughout the whole concentration range studied, as expected from the positive charge and helix lengthening contributed by the more strongly bound and intercalated ethidium bromide.[35] The dye concentration of about 0.04 μM (0.02 μg/mL) that induced comigration of RFI and RFII was very similar to the value obtained by Poddar and Maniloff for ϕX174 DNA, using the same unwinding assay.[36]

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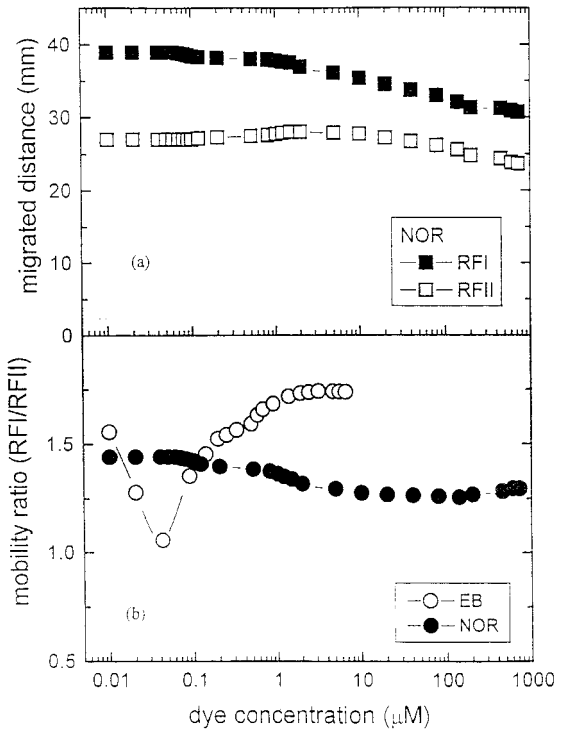

Figure 7. Unwinding of supercoiled ΦX174 DNA by norfloxacin (closed symbols) and ethidium bromide (opened symbols) in agarose gels followed by electrophretic mobility. [DNA] = 20 μM base for both RFI and RFII. (a) Migrated distance after 5 h at 2.6 V/cm. (b) Mobility ratio between super-coiled and nicked circular DNA.

Discussion

Norfloxacin Complexation with DNA in the Absence of Mg^{2+}. The observed hypochromism and three isosbestic points in the absorption spectra were indicative of the homogeneous binding mode of norfloxacin to DNA. These observations contrast with previous reports by Shen et al., in which one norfloxacin molecule per 6646 base pairs (of relaxed ColE1 DNA) was bound with a specific binding mode and the number of drug bound increases sharply in the nonspecific binding phase as drug concentration increases[2] and the drug did not bind to double-stranded DNA and homopolymers per se at the drug concentration comparable to ours.[3] Our observations also contrast with the report by Palù et al., in which no interaction between norfloxacin and plasmid DNA is evident[9] in the absence of Mg^{2+}. The gradual decrease in the 300−350 nm absorption band with a decreased mixing ratio suggested that a significant amount of norfloxacin in the system was not bound to DNA. From the equilibrium constant, ∼65% of norfloxacin was unbound when the mixing ratio was 0.017 ([DNA] = 197 μM) under our conditions. This did not significantly change with the mixing ratio.

Fluorescence spectra are extremely sensitive to the environment of fluorophore. After adding DNA, changes were apparent in both the shape and intensity of the emission spectrum of norfloxacin. This observation is similar to that reported for the norfloxacin−thermal denatured DNA mixture.[25] In addition to the hypochromism in the absorption spectrum, changes in the fluorescence emission spectrum strongly indicated an interaction between norfloxacin and DNA. The equilibrium constant, which is equivalent to the Stern−Volmer quenching constant in the static quenching mechanism, was obtained from the slope of Figure 3a as $2.8 \times 10^3\ M^{-1}$ at 25 °C, which is lower than those of the other intercalators by a factor of about 10^2-10^4. This may explain why previous studies failed to detect any interaction between norfloxacin and double-stranded DNA.

Flow LD and LD^r of the Norfloxacin−DNA Complex. The absorption band at 300−370 nm of norfloxacin is due to two electric dipole allowed transitions (transitions I and II, Table 1). Their transition moments were determined as reported above. In an aqueous solution, the maximum of the low-energy transition is found at 360 nm; this transition possesses a moment directed essentially parallel to the line between the carbon atom of the carboxylic acid and nitrogen atom of piperazine ring. The second transition, with a maximum absorption at 325 nm, has its moment at an angle of about 45° relative to the first one, rotated toward the carbonyl moiety (Figure 1). If norfloxacin is located along either the minor or the major groove, a positive wavelength-independent LD^r corresponding to an angle of 45° between the long-axis polarized norfloxacin transition moment and the DNA helix is expected. An angle of 45° between the transition moment of the drugs and the DNA helix axis for the minor groove binding drug, 4′,6-diamidino-2-phenylindole, and Hoechst 33258 has recently been reported.[37−39] When norfloxacin is bound to the phosphate group of the DNA stem, it is free to rotate, resulting in random orientation; therefore, a zero LD and LD^r in the 300−370 nm region is expected for such a binding mode. Both minor groove and external binding modes can be eliminated from the observed angles of 75.0° and 72.3° for the transitions I and II, transitions which are on the same molecular plane, separated by 45°, relative to the helix axis (Figure 6b). Remaining potential binding modes are intercalation and major groove binding; in the former case, the plane of the norfloxacin molecule is almost parallel to the DNA bases. The possibility of norfloxacin being parallel to the DNA bases in the minor groove can be ruled out because the minor groove is too narrow to facilitate the norfloxacin molecule in this direction. These factors suggest that the binding mode of norfloxacin to double-helical DNA is most likely intercalative.

However, deviations in the LD^r spectrum from the classical intercalation behavior should be noted. Upon drug intercalation between the nucleo-bases, DNA is unwound, stiffened, and elongated. These conformational changes of DNA would result in an increase in the LD^r magnitude in the DNA absorption region (∼260 nm) because stiffening and elongation of DNA would make the DNA's orientation in the flow more effective. We observed instead a decrease LD^r magnitude in DNA absorption region. This observation may imply a bending of the DNA stem near the norfloxacin binding site. Another possible reason for the decrease in LD^r magnitude might be local melting of the double-helical DNA due to norfloxacin binding; norfloxacin may locate itself at the denatured site of DNA. This agrees somewhat with the reports by Shen et al.[5] However, this is not likely to occur with double-helical DNA because, in that case, the norfloxacin chromophore would not itself be oriented and the LD^r in the norfloxacin absorption region would thus be zero. The angles of 75.0° and 72.3° for the transitions I and II also disagree with classical intercalation mode in which the plane of the intercalator is parallel to the DNA bases. The angles we observe indicate instead that the plane of the norfloxacin molecule be tilted at the binding site.

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Unwinding of Supercoiled DNA. Norfloxacin has been shown to bind very weakly to linear and nicked circular DNA but to have a stronger affinity for supercoiled DNA, probably by binding to locally denatured regions.[2,3] The binding constant for this binding mode is comparable to that of ethidium bromide.[6,8] We chose to investigate potential unwinding in the absence of Mg^{2+} ions (assumed here to be similar for linear and supercoiled DNA). In this condition norfloxacin indeed has different effects on the migration of supercoiled and nicked circles. A weak minimum in the mobility ratio (Figure 7b) was observed at drug concentrations 1000-fold higher than ethidium bromide, which is consistent with the affinity constant in the 10^3 range for norfloxacin and 10^6 for ethidium bromide. The minimum is much more shallow for norfloxacin, however, than for the intercalator ethidium bromide, where the two DNA forms comigrate at a certain binding ratio (the binding ratio which removes all supercoiling). The observed unwinding with norfloxacin therefore does not support classical intercalation; it is well-known that many other perturbations of the DNA helix result in unwinding,[40−42] such as the partial intercalation observed with the ruthenium−trisphenantroline complexes, as monitored by the supercoiled-DNA assay or the local denaturation caused by *cis*-diamminedichloroplatinum(II). In conclusion, the unwinding data indicate that the binding of norfloxacin perturbs the DNA helix, but not by classic intercalation. The fact that norfloxacin decreases the mobility of RFII cannot be due to unwinding but can be ascribed to a reduction of the net charge of the complex and/or to lengthening /bending of the helix.[43]

Binding Mode of Norfloxacin in Double-Helical Calf Thymus DNA. The spectroscopic properties of the norfloxacin−DNA complex in the absence of Mg^{2+} can be summarized by a 16−18% hypochromicity in the absorption spectrum, effective quenching in the fluorescence intensity, red-shift in the fluorescence emission spectrum, and the angle of about 75.0° between the lowest electric dipole transition and the DNA helix axis. In addition to the spectroscopic results, unwinding of supercoiled DNA by norfloxacin is much less effective than a classical intercalator, ethidium bromide. On the basis of these observations, we can rule out groove binding, classical intercalation, and surface binding of norfloxacin in DNA. However, it is clear that the planar polycyclic hydrocarbon part of norfloxacin is near perpendicular with respect to DNA helix axis. DNA bending near the norfloxacin binding site is conceivable. It should be noted that the observed spectroscopic properties may correspond to an average of several binding modes, although we consider this unlikely because of the isosbestic points observed in the absorption spectrum, of the independence of the LD^r spectrum on the mixing ratio, and of the shape of the fluorescence emission spectrum of bound norfloxacin.

Norfloxacin is zwitterionic at neutral pH. It has been argued[8] that Mg^{2+} promotes binding of norfloxacin to DNA by forming a complex with the carboxylic acid and carbonyl group of the drug, thereby converting a repulsive negative charge to a positive attracting charge, making the drug in effective dicationic. It is therefore note worthy that the mobility shifts we observe with the relaxed circular DNA in the absence of Mg^{2+} (Figure 7a) are consistent with the drug reducing the negative charge of DNA. The ionic strength dependent affinity constant (Figure 3b) suggests that the effective charge of the drug (in terms of the number of displaced counterions) was +1.0. A pertinent question is whether, in the absence of Mg^{2+}, there may be another cation (H^+) that contributes to make the drug positively charged. The absorption spectrum of the bound form (at pH 7.0) is very similar to that of the free drug (in the absence of DNA) below pH 4.0, when the carboxylic group is protonated[44] and is distinctly different from that of free drug at pH 7.0. This suggests that, in the absence of Mg^{2+}, protons may promote norfloxacin binding to DNA at a neutral pH by neutralizing the negative charge on the carboxylate group of the bound drug. If only one H^+ is involved, the effective charge should be +1.0. In addition to the ionic strength effect on the affinity constant and the fact that the absorption spectrum of the DNA bound norfloxacin (at pH 7.0) is very similar to that of the free drug (in the absence of DNA) below pH 4.0, we have also observed that the equilibrium constant for the norfloxacin−DNA complex formation increases at pH 5.0 by a factor of 5−7 over that at pH 7.0 (data not shown), which supports the importance of the protonation in the complex formation.

Conclusion

Norfloxacin obviously bind to double-stranded calf thymus DNA without requiring any mediation from ATP and Mg^{2+}. The equilibrium constant of the norfloxacin−DNA complex formation was estimated to $2.8 \times 10^3\ M^{-1}$ at 25 °C, which is quite low compared to the classical intercalators but high enough to permit a characterization of the binding geometry using spectroscopic methods. From LD^r and unwinding of supercoiled DNA results, possibilities of classical minor groove binding, surface binding, and intercalation can be ruled out. Also from the LD^r result, it can be concluded that the molecular plane of norfloxacin is near perpendicular relative to the DNA helix axis (parallel to nucleo-bases) with a strong possibility of bending of the DNA stem near the norfloxacin binding site.

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