Mechanism of Inhibition of DNA Gyrase by Quinolone Antibacterials: A Cooperative Drug–DNA Binding Model

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Received May 25, 1988; Revised Manuscript Received December 6, 1988

ABSTRACT: We have proposed a cooperative quinolone–DNA binding model for the inhibition of DNA gyrase. The essential feature of the model is that bound gyrase induces a specific quinolone binding site in the relaxed DNA substrate in the presence of ATP. The binding affinity and specificity are derived from two unique and equally important functional features: the specific conformation of the proposed single-stranded DNA pocket induced by the enzyme and the unique self-association phenomenon (from which the cooperativity is derived) of the drug molecules to fit the binding pocket with a high degree of flexibility. Supporting evidence for and implications of this model are provided.

We have extensively studied and analyzed the binding of quinolone compounds to DNA gyrase and various natural and synthetic DNAs in an attempt to elucidate the molecular mechanism of inhibition of this important DNA-modifying enzyme. Some crucial supporting evidence, such as the role of DNA gyrase in drug binding, has also been obtained. We summarize below all the published experimental evidence which is essential to the proposal of this binding model.

Summary of Available Evidence

Drug Does Not Bind to DNA Gyrase at Its Inhibitory Concentration. Both equilibrium dialysis and membrane filtration techniques failed to detect any appreciable level of drug binding to DNA gyrase near the drug's supercoiling inhibition constant (K_i),[1] i.e., around 3 μM for norfloxacin. On the other hand, the drug binds to DNA to varying degrees which depend on the structural form of the DNA (Shen & Pernet, 1985).

Drug Binds Poorly to Relaxed Double-Stranded DNA. Binding of quinolones to relaxed double-stranded DNA is weak and not saturable. Binding to linearized ColE1 DNA, to relaxed circular ColE1 DNA, or to the complex formed by adding gyrase to relaxed DNA in the absence of ATP gave molar binding ratios of ca. one drug molecule per DNA molecule (1 drug per 6646 base pairs)[2] at a drug concentration near K_i (Shen & Pernet, 1985). Studies with a synthetic double-stranded polydeoxynucleotide suggest that the drug does not bind to double-stranded DNA per se (Shen et al., 1989b).

Quinolones Bind Preferentially to Single-Stranded DNA, but in a Nonspecific and Noncooperative Manner. The amount of norfloxacin bound increased more than 10 times when DNA was denatured by a simple heating–cooling step (Shen & Pernet, 1985). When synthetic single-stranded homopolymer nucleic acids were used, the level of drug binding was approximately the same as that to denatured DNA, and the binding was found to be independent of the chain length of the homopolymer and was not saturable up to the drug solubility limit of about 1 mM. A quantitative comparison of the bindings to these synthetic homopolymers suggested that the drug binds to single-stranded DNA through hydrogen bonds which become available when the bases are unpaired. This type of binding was considered to be the prototype of specific drug binding to DNA (Shen et al., 1989b).

Drug Binds Specifically to a Saturable Site on Supercoiled DNA in a Highly Cooperative Manner. We detected a saturable drug binding phase with supercoiled DNA. Despite the presence of nonspecific drug binding, the drug saturation phase was clearly demonstrated at concentrations near the drug's K_i (Shen & Pernet, 1985). Binding of the drug to this site was found to be dependent on a high power of the drug concentration (fourth to sixth power); a cooperative binding mechanism was strongly suggested. It was proposed that the saturable drug binding site on supercoiled DNA is a small denatured bubble (such as that in the cruciform structure) or an easily denaturable region in the supercoil and that this type of binding represents the actual binding mode adopted by quinolones during inhibition of DNA gyrase (Shen et al., 1989b). The biological significance of this type of drug binding observed with supercoiled DNA in vitro is implied by the good correlation between the K_i and K_d values of several selected major quinolones (Shen & Pernet, 1985). The cooperativity of drug binding observed with supercoiled DNA may derive from strong intermolecular drug–drug interactions when they are bound to the localized bubble.

Gyrase Enhances Drug Binding to Relaxed Forms of DNA. Our preliminary publication (Shen & Pernet, 1985) has revealed that the drug binds poorly either to relaxed ColE1 DNA (a substrate for DNA gyrase) or to a complex formed between relaxed DNA and gyrase in the absence of ATP. Recently, we have demonstrated (Shen et al., 1989a) that drug binding may be enhanced by the addition of gyrase to relaxed DNA

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[1] Abbreviations: K_i, supercoiling inhibition constant; K_d, dissociation constant; bp, base pairs; IC_{50}, drug concentration to cause 50% inhibition of the supercoiling activity of DNA gyrase; M/E, ratio of K_i values of *M. luteus* DNA gyrase and *E. coli* DNA gyrase.

[2] ColE1 plasmid DNA has 6646 base pairs (Chan et al., 1985).

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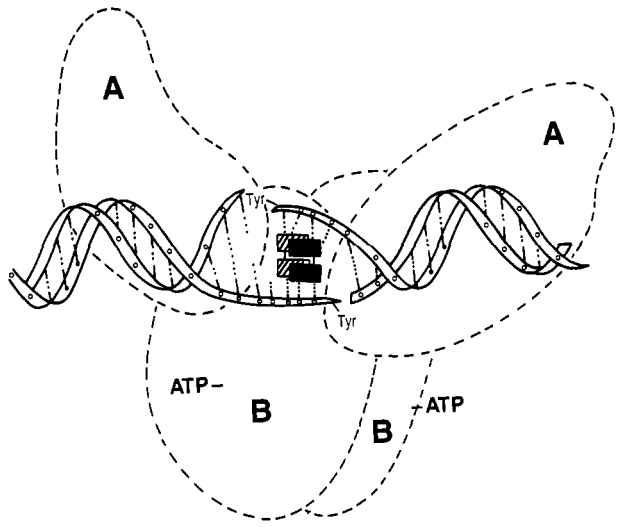

FIGURE 1: Model of the proposed quinolone–DNA cooperative binding in the inhibition of DNA gyrase. Quinolone molecules (filled and slashed rectangles) bind to a gyrase-induced DNA site during the intermediate gate-opening step of the DNA supercoiling process via hydrogen bonds (indicated by dotted lines) to the unpaired bases. Subunit A of gyrase forms covalent bonds between tyrosine-122 and the 5′-end of the DNA chain (Horowitz & Wang, 1987); the subsequent opening of the DNA chains along the four base pair staggered cuts results in a locally denatured bubble which is an ideal site for the drug to bind. When relaxed DNA substrate (represented by the short DNA segment in the diagram) is used, ATP is required for the induction of the drug binding site. Dashed curves mimic the shape of the DNA gyrase revealed by the electron microscopic image of the *M. luteus* enzyme (Kirchhausen et al., 1985).

in the presence of a nonhydrolyzable ATP analogue. When linear DNA was used, such a saturable drug binding phase was detectable with the enzyme–DNA complex either in the presence or in the absence of ATP. These saturable complex-dependent bindings resemble those with supercoiled DNA alone (Shen & Pernet, 1985) in terms of binding affinity and cooperativity (Shen et al., 1989b). These results suggest that the drug binds to a gyrase-induced DNA site, which was locally unwound during the intermediate gate-opening step. This step evidently requires the binding of ATP when covalently closed circular DNA is used but not when DNA has free rotating ends. Moreover, there is a close correlation of norfloxacin-induced cleavable complex formation with levels of norfloxacin bound to complexes of gyrase and DNA (Shen et al., 1989a; Gellert et al., 1977; Sugino et al., 1977). These results strongly support the model that the drug inhibits the enzyme by binding to a specific site on DNA created by DNA gyrase.

On the basis of the above evidence, the following model is proposed.

The Proposed Model

Previous observations and new evidence presented here allow us to propose a cooperative quinolone–DNA binding model for the inhibition of DNA gyrase. This model is illustrated in Figure 1. The essential feature of the model is that bound gyrase induces a binding site for the drug in the relaxed DNA substrate in the presence of ATP. We envision this to occur as follows: DNA gyrase acts initially by cutting both strands at four base pair staggered positions (Morrison & Cozzarelli, 1979). The cleaved DNA strands with the protruding four-base single-stranded segments have to be moved apart to allow the subsequent strand passing process to occur, and this single-stranded "DNA gate" formed during this intermediate step would serve as an ideal drug binding site. Binding of the drug locks the strands in place and indefinitely ties up the enzyme, preventing its turnover.

The cooperativity and the high binding affinity are derived from the strong intermolecular drug–drug interactions. The details of the proposed drug interactions at the DNA binding pocket are illustrated in Figure 2 where we arbitrarily chose TGTG as the nucleotide sequence at the binding site. The drug molecules have been assembled inside the single-stranded pocket through hydrogen bonds between the carbonyl groups on the quinolone rings and the hydrogen-bond donors of the DNA bases on the separated DNA strands. The drugs interact among themselves through two types of interactions: (i) stacking of two adjacent quinolone rings hydrogen bonded to the same separated DNA strand, with the orientation of the two rings in a flip-over position so that the 4-keto groups point in the same direction toward the DNA while the 3-carboxyl groups are located on the opposite sides to avoid charge repulsion; (ii) tail-to-tail hydrophobic interactions between the drug molecules hydrogen bonded to the two opposite DNA strands. These two types of interaction, which are commonly seen in the nalidixic acid crystal (see later in Figure 5), would render the drug a unique and stronger affinity to the binding site by acquiring extra effective binding domains. The binding of the first drug to the single-stranded DNA is essentially confined to a unidimensional space; the two types of interactions described above for subsequently bound drugs expand the binding to a multidimensional one and simultaneously increase the total available hydrogen-bond acceptors on the ligand as a whole. This proposed mode of drug self-association at the binding site is not unlike the formation of phospholipid micelles in solution with a hydrophobic core and hydrophilic surface.

This model explains the cooperative binding phenomenon as follows: after the first drug molecule binds to the DNA site through a hydrogen bond(s), the second drug molecule binds with greater ease since it binds not only through hydrogen bonds to DNA but also through the interactions with the neighboring bound drug molecule. The initial binding of the drug may also create a local conformational change at the DNA receptor site for additional drug molecules to bind until the receptor site is saturated. The model presented in Figure 1 does not rule out the possible interaction between the assembled drug and DNA gyrase molecules or the possibility of drug molecules located outside the assembled drug cluster to further interact with the DNA base through stacking. All these may further stabilize drug binding.

An alternative binding mode model is presented in Figure 3. In this model, the drugs bind mainly through stacking with the DNA bases, and the cooperativity is derived from the hydrophobic interactions between the N1 hydrophobic groups of the drugs bound to opposite strands. This model is not favored as it does not involve the hydrogen bonds which are suggested by our experiments with homopolymer nucleic acids. If the drug binds mainly via ring stackings to DNA bases, then it would be difficult to explain the fact that major quinolones do not have a DNA intercalating effect at high drug concentration (Shen et al., 1988; Tornaletti & Pedrini, 1988). It also cannot explain the drug's higher binding specificity to single-stranded DNA than to the double-stranded. Besides, such a drug binding mode would allow close contacts between the negatively charged carboxyl groups on the drugs and the negative charges on the DNA backbone.

Discussion and Implications of the Model

The supercoiling mechanism of DNA gyrase is well established (Brown & Cozzarelli, 1979; Cozzarelli, 1980; Gellert,

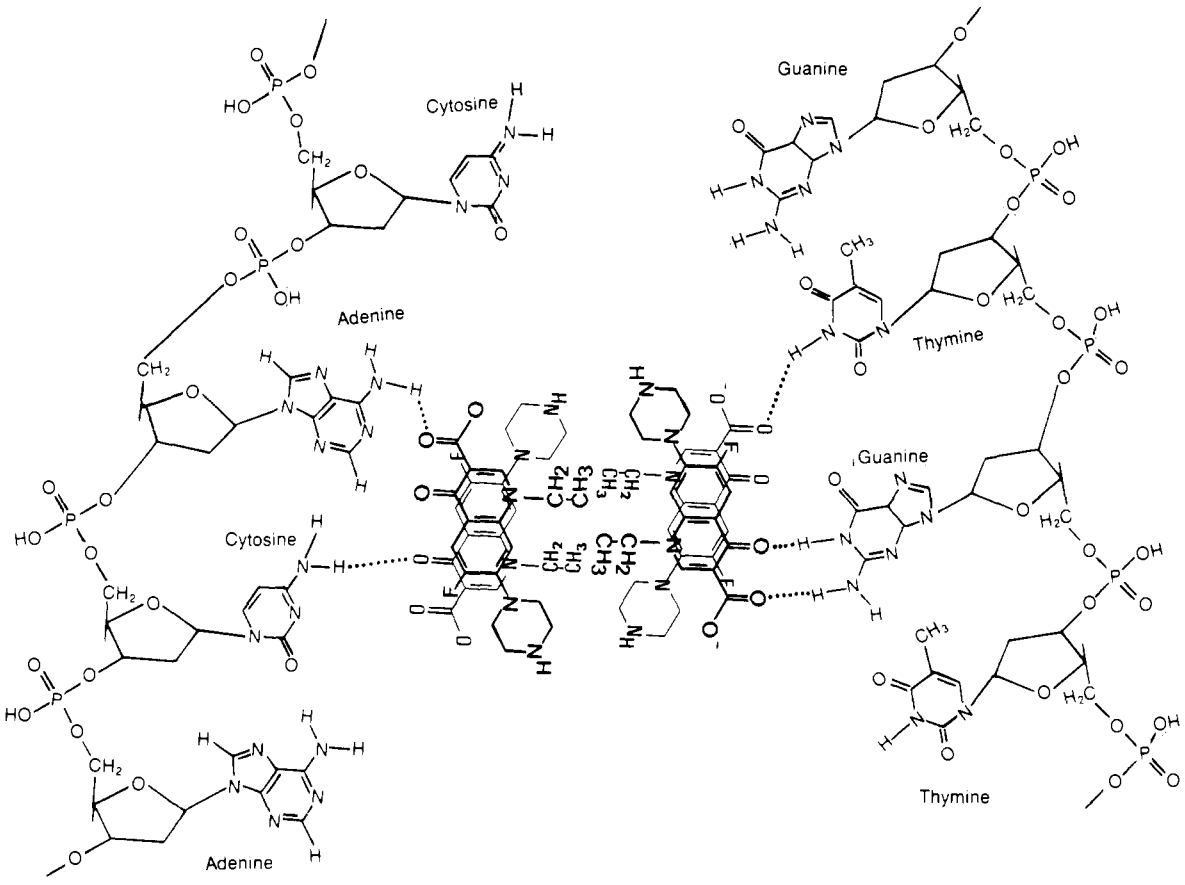

FIGURE 2: Illustration of the proposed drug self-association mode at the quinolone binding site in a single-stranded DNA pocket. Norfloxacin molecules (drawn separately by thick and thin lines located at the center of the diagram) are used for illustrating the two types of proposed interactions: the $\pi-\pi$ ring stackings of the quinolone rings and the tail-to-tail hydrophobic interaction between the *N*-ethyl groups. Only four drug molecules are shown in the diagram; the number of drug molecules involved may be higher depending on the size and the configuration of the binding site. Also, the angle between the two molecules interacting through N1 hydrophobic tails is not necessarily fixed at 180° so as to provide flexibility in bond pairing. Nucleotide sequence in the binding site was arbitrarily selected.

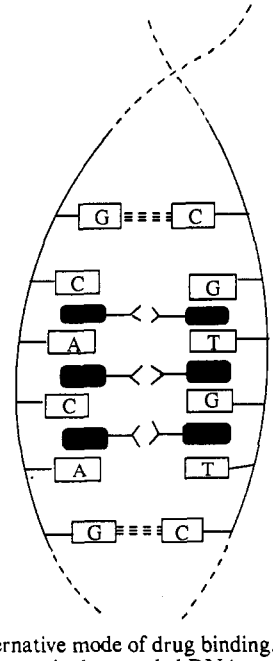

FIGURE 3: An alternative mode of drug binding. The drug molecules (filled boxes) bind to a single-stranded DNA pocket via ring stackings with the DNA bases (open boxes). The binding cooperativity may be derived from the tail-to-tail hydrophobic interactions.

1981; Wang, 1985). The process involves the following four steps: (i) binding of gyrase to DNA substrate to stabilize a positive DNA node, (ii) cleavage of DNA at four base pair staggered sites at the node and formation of covalent linkages between the tyrosine group on gyrase subunit A and the 5′-end of the DNA chain, (iii) passage of a remote DNA segment through the cleaved DNA gate, thus inverting the sign of the node, and (iv) resealing of the DNA break. The action of quinolone-type drugs such as oxolinic acid was shown to trap the intermediate by stabilizing the enzyme–DNA complex, thus preventing the enzyme's turnover. This mechanistic inference was based on the observation that a cleavage product, i.e., the linearized DNA with gyrase subunit A covalently attached to the 5′-end of the DNA, can be detected only in the presence of the drug upon addition of a protein denaturant. No definite explanation was given regarding exactly how the drug stops the enzyme reaction and what the structure of the stabilized enzyme–substrate complex is. Such a model is proposed here. It is evident that the stabilization effect results from the formation of a ternary complex in which the drug binds to a DNA site induced by the enzyme. The proposed model favors the concept that the drug binds where DNA gyrase binds on DNA and is consistent with the basic assumption adopted by many investigators to probe the gyrase binding sites on DNA or on bacterial chromosome with quinolone analogues (Morrison & Cozzarelli, 1979; Fisher et al., 1981, 1986; Kirkegaard & Wang, 1981; Morrison et al., 1980; O'Connor & Malamy, 1985; Lockshon & Morris, 1985; Snyder & Drlica, 1979; Drlica et al., 1980; Drlica & Franco, 1988).

Results from previous investigations on the mechanism of DNA supercoiling by gyrase have provided sufficient evidence

leading to the general belief that subunit A of the enzyme is the target of the drug. Genetic analysis of some high-level quinolone-resistant mutants also revealed that mutations were exclusively in *gyrA*, the structural gene of subunit A (Higgins et al., 1978; Sato et al., 1986). Enzyme reconstituted by combining subunit A purified from the resistant mutant and the wild-type subunit B was also resistant to the drug. The conclusion on the exclusive involvement of subunit A in quinolone inhibition, however, was overshadowed by the recent discovery of some lower level drug-resistant mutations which were found to occur in *gyrB*, the structural gene of subunit B (Yamagishi et al., 1981, 1986). The seemingly controversial observations may be explained by the proposed model. Our model in fact suggests that quinolone-resistant mutations can occur anywhere on the enzyme molecule and are not necessarily to be confined in *gyrA*. Any mutation which affects the configuration of the binding site on DNA is capable of conferring drug resistance. It is known that subunit B is the ATP-dependent energy-transduction component of the enzyme (Higgins et al., 1978; Mizuuchi et al., 1978). Binding of ATP results in a conformational transition that is essential to the supercoiling cycle (Sugino et al., 1978; Maxwell & Gellert, 1984; Rau et al., 1987). It is conceivable that a mutation in subunit B would also result in a change in the configuration of the drug binding site, thus reducing drug binding affinity.

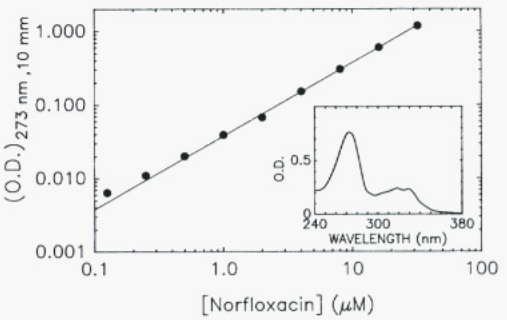

FIGURE 4: Double-logarithmic plot of UV absorption at 273 nm versus concentration of norfloxacin. The buffer used was 50 mM Hepes, pH 7.4/20 mM KCl/5 mM $MgCl_2$/1 mM EDTA. The line drawn through the data points is an arbitrary straight line with slope equal to 1 in the double-logarithmic scale. Insert: UV spectrum of norfloxacin in the same buffer.

More importantly, the proposed model provides an unprecedented and unique description of how a small drug with a simple structure acquires selectivity and high binding affinity to a site on DNA which is generally considered not to possess

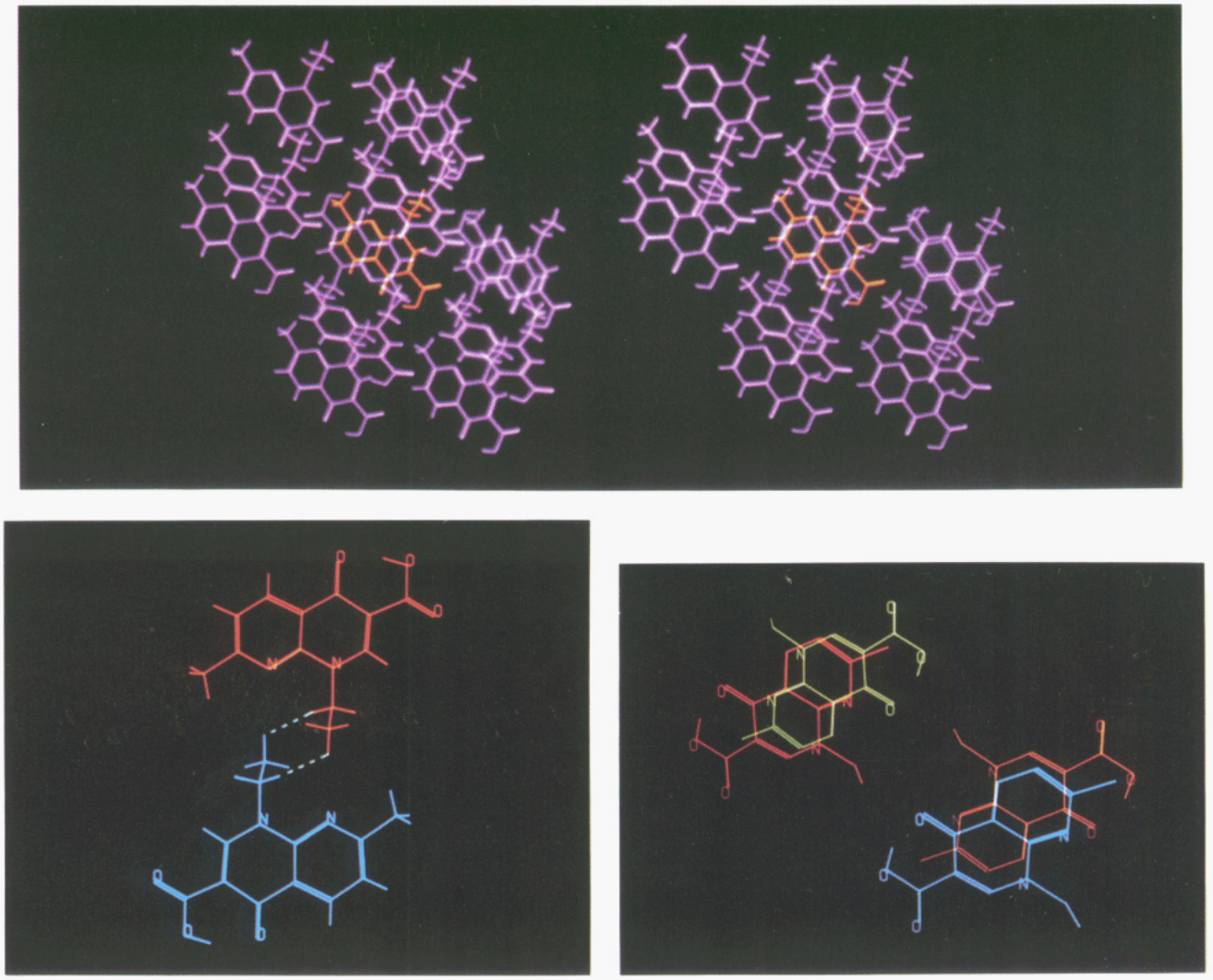

FIGURE 5: Crystal structure of nalidixic acid. (Top) Stereoview of the crystal structure of nalidixic acid. (Bottom left) A nalidixic acid molecular pair in the crystal showing the tail-to-tail hydrophobic interaction between the *N*-ethyl groups of the adjacent molecules. (Bottom right) Two pairs of nalidixic acid molecules in the crystal showing stacked naphthyridine rings. Coordinates for nalidixic acid crystal structure (Achari & Neidle, 1976; Huber et al., 1980) were retrieved from the Cambridge Crystallographic Data Bank. The asymmetric unit of this crystal contains one molecule of nalidixic acid. There are four symmetry-related molecules in the unit cell. In order to investigate the intermolecular interactions, symmetry-related molecules in neighboring unit cells were generated in order to reveal all molecules within 4 Å of a central reference molecule (in orange color).

a specific receptor configuration as an enzyme molecule may have. Quinolone drugs have a simple fused bicyclic ring structure which is unlikely to have strong base stacking capability as judged from the absence of a DNA intercalating effect at high drug concentration (Shen et al., 1988); the drug also has only a few hydrogen-bond acceptor groups (such as C=O and F) located on one side of the molecule, and these are of limited use because the hydrogen-bond donor groups on the DNA chain may be of limited number and/or not at the right position. But when the drug molecules aggregate as proposed, the self-association not only increases the number of total hydrogen-bond acceptor groups of the drugs as a whole, but most importantly, it allows the drug to search for additional available hydrogen-bond donors at specific distances from the binding site of the first bound drug molecule. The self-association of drug molecules through such hydrophobic interactions permits some flexibility in adjusting the positions of the drug molecules for optimal hydrogen-bonding configuration. The importance of the involvement of hydrogen bonding in protein–inhibitor interactions has been well depicted recently (Bartlett & Marlowe, 1987; Tronrud et al., 1987; Bash et al., 1987). It was shown that depletion of a single specific hydrogen bond would result in an 840-fold decrease in binding affinity of an inhibitor to the endopeptidase thermolysin. A normal hydrogen bond of the type involved in the proposed quinolone binding was shown to have an intrinsic binding energy of about 4 kcal/mol. If four drug molecules form a complex at the DNA site and each drug contributes only one hydrogen bond to the DNA, then the drug complex will bind with a net intrinsic binding energy of 20 kcal/mol, assuming that the strength of intermolecular hydrophobic interactions is infinitely high. This simply demonstrates that such a mode of drug self-association will greatly stabilize drug binding.

Quinolone inhibitors require the 3-carboxyl group for activity. This leads to a general belief that quinolones do not bind to DNA, owing to the similar charge properties of the ligand and the receptor molecules. The proposed model shows that the specific binding is taking place at a relatively disordered DNA site so that negatively charged phosphate groups no longer form a blockade for drug entry. The formation of the denatured binding pocket shown in Figure 1 requires a certain degree of unwinding of the cleaved DNA strands at the binding site. The unwinding may be accomplished by the conformational change of DNA gyrase upon ATP binding and/or by the intrinsic magnesium-dependent DNA unwinding effect of quinolones (Tornaletti & Pedrini, 1988).

It is known that there is no unique nucleotide sequence specificity for DNA cleavage by DNA gyrase. But for certain specific DNA substrates, there are prominent cleavage sites which may be demonstrated with a specific species of DNA gyrase. For example, the cleavage site on pBR322 DNA (a derivative of ColE1 DNA) has the nucleotide sequence of GGCC between the staggered cuts with *Escherichia coli* DNA gyrase (Fisher et al., 1981, 1986), whereas the sequence is TTAT when *Micrococcus luteus* DNA gyrase is used (Kirkegaard & Wang, 1981). The former is evidently more favorable for drug binding than the latter in terms of the number of available hydrogen-bond donors in the binding pocket. This may partially account for the overwhelmingly greater potency of the drug against *E. coli* DNA gyrase than *M. luteus* DNA gyrase (Zweerink & Edison, 1986; Fu et al., 1986; and see later in Table I). However, this argument needs further assessment with additional species of DNA gyrase.

Several additional pieces of supporting evidence and tests using the structure/property/activity relationship of quinolone derivatives are presented as follows:

Lack of Drug–Drug Interactions in Dilute Quinolone Solution. The proposed cooperative drug binding model is based on the assumption that the drug molecules in solution are monomers at the drug's inhibitory concentration (ca. 3 μM). Ultraviolet and fluorescence spectroscopic studies have therefore been used to investigate the possible self-association of quinolone drugs in solution. Figure 4 (insert) shows the UV spectrum of norfloxacin which has an absorption peak at 273 nm with a molar extinction coefficient of 37 500. As can be seen in Figure 4, the UV absorbance at the maximum does not show either a hyper- or hypochromatic effect as the concentration changes from 0.1 to 32 μM, the drug concentration range of interest. The fluorescence properties of norfloxacin in aqueous solution (10 mM Tris-HCl, pH 7.4) were also examined as a function of the drug concentration in the range of 1.6 nM to 20 μM. The steady-state emission spectrum has a constant shape, and the fluorescence intensity is simply proportional to the drug concentration (E. Matayoshi, personal communication). From the above observations, we may conclude that only one species is present over this concentration range. From the above results alone one cannot ascertain whether the single species is in fact a monomer rather than some specific oligomer. Gel filtration experiments using a Bio-Gel P-2 column for estimating the molecular form of norfloxacin in solution did not show formation of oligomers; instead, the elution profile of norfloxacin at two different initial concentrations gave an estimated molecular weight corresponding to that of the monomeric form (M_r = 319) of the drug (data not shown). Results suggest that the molecular species of norfloxacin observed at all concentrations is the monomer.

Crystal Structure of Nalidixic Acid. The proposed molecular assembly of the drug at the binding site (Figure 2) is supported by the packing of the drug molecules in the crystal (Achari & Neidle, 1976; Huber et al., 1980). Figure 5 (top) shows a stereoview of the crystal structure of nalidixic acid. It shows two major molecular interactions: stacking of the naphthyridine rings and tail-to-tail hydrophobic interactions between the ethyl groups attached to the N1 position. In fact, the closest intermolecular contact in the entire crystal is between a hydrogen atom on the *N*-ethyl side chain and a hydrogen atom on a neighboring *N*-ethyl side chain. This pair of H–H contacts is shown in Figure 5 (bottom left) as dashed lines connecting the hydrogen atoms at a distance of 2.44 Å. Figure 5 (bottom right) shows two pairs of stacked molecules in crystal packing.

The prevalence of ring stacking in the interaction of heterocyclic compounds in solution has been demonstrated by nucleosides in aqueous solution at millimolar concentration range (Solie & Schellman, 1968). As shown by techniques of thermal osmometry, equilibrium ultracentrifugation, and UV spectroscopy, deoxyadenosines formed aggregates only at concentrations greater than 10 mM, and this aggregation involved predominately the stacking of their constituent bases. The possibility of hydrogen-bonding formations was excluded. The absence of hydrogen bonding and the presence of intermolecular hydrophobic interactions and ring stackings in nalidixic acid crystal were consistent with these results. From the above observations, the chance is very slim that norfloxacin forms aggregates in solution at a concentration as low as 3 μM.

Model Favors Hydrophobic Group Substituents at the N1 Position. An important feature of the model is the tail-to-tail interactions between the N1 hydrophobic groups on the quinolone rings (see Figure 7 for ring numbering system).

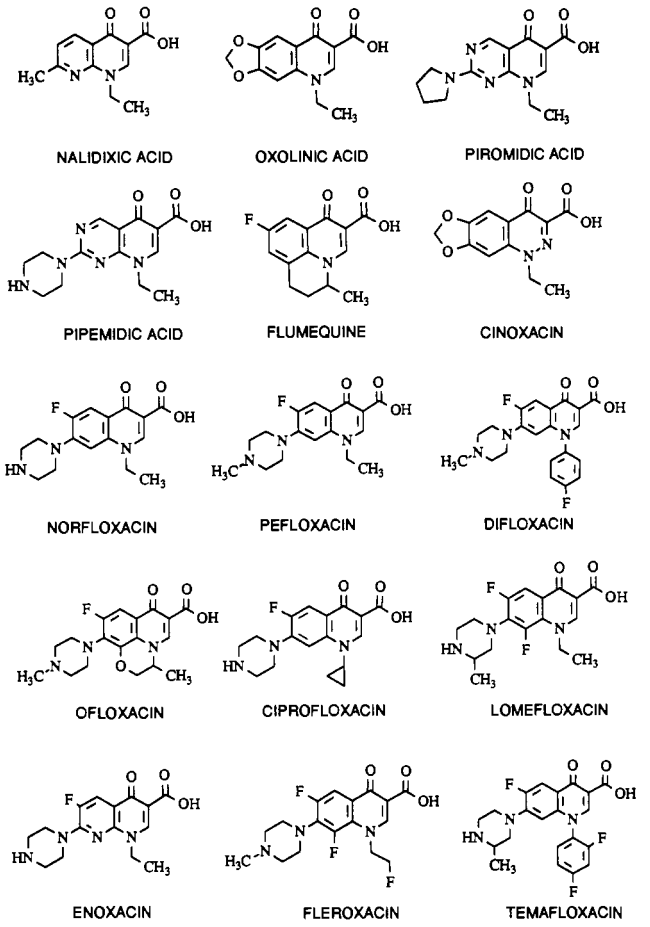

FIGURE 6: Structures of major quinolone antibacterial agents.

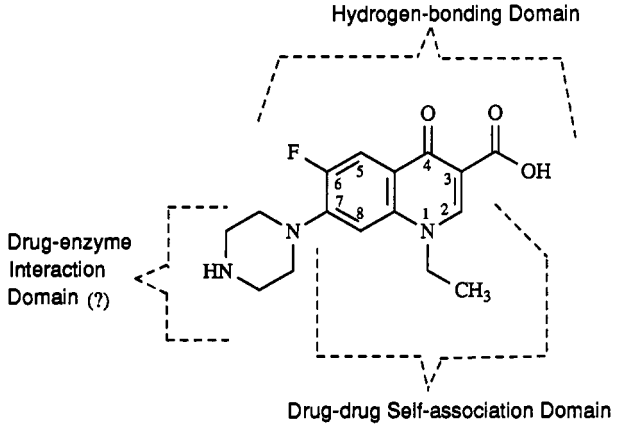

FIGURE 7: Proposed functional domains of a quinolone antibacterial agent (norfloxacin). The domain for the interaction with enzyme is hypothetical.

Though the strength of these interactions is not expected to be the sole determinant for the binding affinity, a minimum level of interaction between these groups is needed for optimal activity. Figure 6 shows the structure of some major quinolone drugs. It is evident that substituents at the N1 positions of these active drug molecules are indeed hydrophobic: ethyl, cyclopropyl, or phenyl groups.

Model Predicts That Bulky Group Substitutions at the C7 Position Are Allowed. As can be seen in Figure 7, there are three functional domains on the quinolone molecule as indicated by the model proposed. The only position on the drug molecule where substitutions of bulky functional groups are permitted is at the C7 ring position. Figure 8 shows a group of quinolones with the best K_i values. It is evident that large

COMPOUNDS	K_i (μg/ml)	SOLUBILITY (μg/ml)
A-62824	0.09	2
A-67107	0.16	5
A-57241	0.17	12
A-63210	0.18	4
A-63419	0.5	188
Ciprofloxacin	0.9	92
Norfloxacin	1.0	350
Ofloxacin	2.0	2,500
Difloxacin	2.9	94

FIGURE 8: Structure, anti-gyrase activity, and solubility of the five most active classes of new quinolones. The list is ranked according to the supercoiling inhibition constant (K_i) against *E. coli* DNA gyrase. Some selected old quinolones are also listed for comparison without showing the structures. For the determination of quinolone solubility, a measured but excess amount of quinolone compound was shaken with the buffer (50 mM sodium phosphate, pH 7.5) overnight. The excess drug was filtered, and the drug concentration in the filtrate was analyzed by HPLC.

substituents or substitutions of various functional groups on the rings at the C7 position are indeed allowed. It is possible that these groups further interact with the enzyme when the drug is bound.

Model Explains Why Some Highly Active Compounds Are Very Insoluble. We have observed that some very potent quinolone-type DNA gyrase inhibitors are very insoluble. Figure 8 shows chemical structures, K_i values, and solubilities of several of the most potent compounds listed in decreasing order of DNA supercoiling inhibitory potency. It is seen that more potent compounds generally have lower solubilities than the less potent ones. The rough inverse relationship between solubilities and potencies implies that the strength of intermolecular drug interactions may play an important role toward the inhibitory potency. This seems to be in agreement with our proposed model since a solid or crystal composed of molecules with higher intermolecular forces is more difficult to be solubilized as more energy must be supplied to overcome such forces (Morrison & Boyd, 1987).

Synthesis and Inhibitory Potencies of Norfloxacin Dimers. To provide a direct test of the model and the mode of drug binding proposed in Figures 1 and 2, three norfloxacin dimers with different lengths of methylene linkers between N1's were synthesized. The three compounds with three-, four-, and five-carbon linkers were designated as dimer-3, -4, and -5, respectively (Figure 9A). Figure 10 shows the *E. coli* gyrase IC_{50} values of these dimers plotted against number of carbons on the linker chain. It is clearly seen that dimer-4 is most active against *E. coli* DNA gyrase with IC_{50} roughly equal to that of the monomeric norfloxacin, while dimer-3 and di-

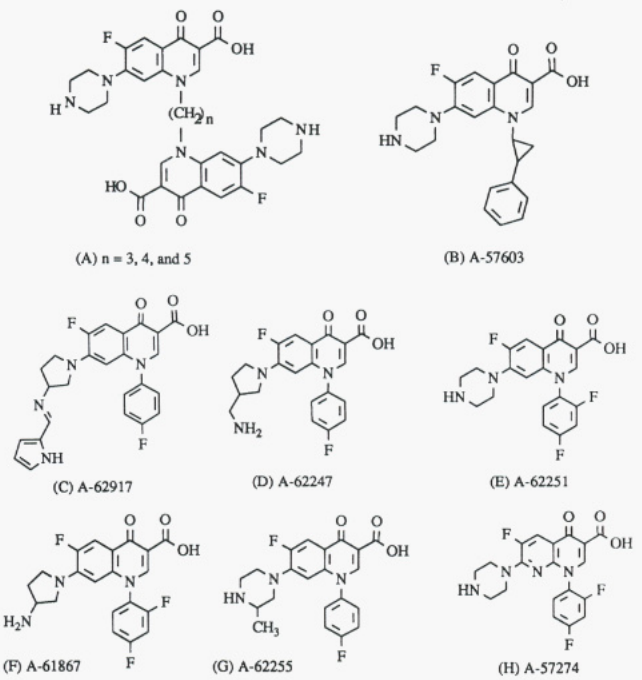

FIGURE 9: Structure of norfloxacin dimers (A) and some selected new quinolone analogues (B–H). Details of the procedures for the synthesis of norfloxacin dimers will be described elsewhere (Mitscher et al., unpublished results). The synthesis and inhibition potencies of A-57603 were described in Mitscher et al. (1986).

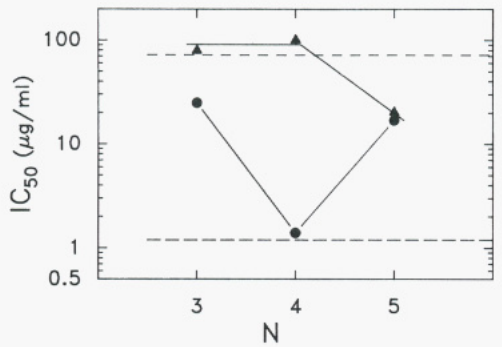

FIGURE 10: Activity profiles of norfloxacin dimers against *E. coli* and *M. luteus* DNA gyrase. Inhibition potency is represented by IC_{50} values against *E. coli* enzyme (●) and *M. luteus* enzyme (▲). *N* is the number of carbons of the methylene linker. Lower and upper horizontal dashed lines represent the IC_{50} values of norfloxacin monomer against enzymes from *E. coli* and *M. luteus*, respectively. Due to the limited solubility of these dimers in buffer at neutral pH, the inhibitory potencies of these compounds were tested in the presence of 2.5% DMSO which did not interfere with the quinolone inhibition test results. Sources of supercoiled ColE1 DNA and *E. coli* DNA gyrase were the same as described previously (Shen & Pernet, 1985).

mer-5 show inhibitory activities 25- and 17-fold lower, respectively. The result was predicted in advance of synthesis by considering the results illustrated in Figure 11, which shows a pair of interacting nalidixic acid molecules in the crystal. The ends of the *N*-ethyl groups are almost within their bonding distance of each other. When the dimer molecule with a four-carbon methylene linker is drawn with the nalidixic acid pair, there is a near correspondence of all important atoms. It is clearly seen that the distance between the N1 atoms of the interacting nalidixic acid molecules in the crystal is very close to the length of the four-carbon methylene linker. Therefore, dimer-4 was predicted to be the most active one among the three, and it was.

It has been well recognized that quinolones in general are poor inhibitors of *M. luteus* DNA gyrase (Zweerink & Edison, 1986; Fu et al., 1986). Table I lists the inhibition constants obtained in our laboratory of 25 quinolones which are either well-known or most active against either species of DNA gyrase. As the statistical mean of the ratio of the two K_i values shows, quinolones on the average are 79-fold more potent against *E. coli* DNA gyrase than *M. luteus* enzyme. We speculate that quinolones may bind poorly to the DNA site created by *M. luteus* DNA gyrase. Our results with dimers suggest that the DNA sites induced by different enzymes may indeed have different conformations so that quinolones with different structures or different ways to self-assemble through hydrophobic interactions have different binding affinities to these sites. This conclusion follows from the results also shown

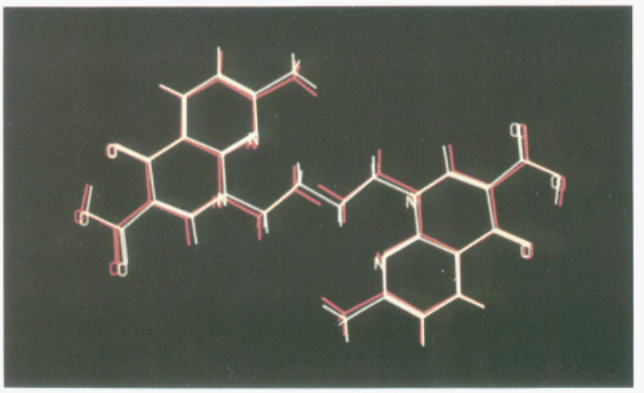

FIGURE 11: Illustration of nalidixic acid dimer drawn by connecting *N*-ethyl groups and its comparison with a pair of interacting nalidixic acid molecules in the crystal. The computer-drawn dimer molecule linked with a four-carbon chain between the N1 atoms gives a similar distance between the N1 atoms of the monomers in the crystal.

Table I: Supercoiling Inhibition Constants (K_i) of Some Selected Quinolones[a]

compd	K_i (μg/mL) *E. coli*	K_i (μg/mL) *M. luteus*	M/E[b]	structure ref
A-62824	0.09	17	189	*c*
A-67107	0.16	31	194	*c*
A-57241	0.17	22	129	*c*
A-63210	0.18	13	72	*c*
A-63419	0.50	12	24	*c*
A-62917	0.57	69	121	*e*
A-62247	0.60	59	98	*e*
rosoxacin	0.75	54	72	*f*
A-61867	0.90	47	52	*e*
A-62255	0.90	153	170	*e*
ciprofloxacin	0.90	38	42	*d*
norfloxacin	1.00	72	72	*d*
temafloxacin	1.10	110	100	*g*
CI-934	1.20	13	11	*g*
pefloxacin	1.34	162	121	*d*
lomefloxacin	1.60	105	66	*g*
A-62251	1.70	94	55	*e*
ofloxacin	2.00	45	23	*d*
A-57274	2.00	117	59	*e*
A-56620	2.00	43	22	*g*
A-61356	2.10	330	157	*h*
difloxacin	3.00	20	7	*d*
oxolinic acid	3.1	260	84	*d*
A-57603	12	9.6	0.8	*i*
nalidixic acid	26	650	25	*d*
average			78.6	

[a] Supercoiling inhibition constants were determined according to procedures previously described (Shen & Pernet, 1985). *M. luteus* DNA gyrase was purchased from Bethesda Research Laboratory (Gaithersburg, MD). [b] Ratio of supercoiling inhibition constants of *M. luteus* (M) versus *E. coli* (E). [c] Figure 8. [d] Figure 6. [e] Figure 9. [f] Hamanaka and Kellogg (1984). [g] Fernandes and Chu (1987). [h] Chu et al. (1987). [i] Mitscher et al. (1986).

in Figure 10 which demonstrate that the inhibition activity profile of the dimers against *M. luteus* DNA gyrase is indeed different from that displayed with *E. coli* DNA gyrase. With *M. luteus* DNA gyrase, dimer-5 shows optimal activity with IC_{50} equal to 21 μg/mL, which is a nearly 4-fold improvement in potency over monomeric norfloxacin as well as over dimer-3 and -4 which have an IC_{50} near 80 μg/mL.

It is intriguing when we examine the structure of the most potent quinolone inhibitor of *M. luteus* DNA gyrase in Table I. It is A-57603 (Mitscher et al., 1986), which is also the only compound on the list showing more potent activity against *M. luteus* DNA gyrase than *E. coli* DNA gyrase, i.e., M/E < 1. The compound is a ciprofloxacin derivative with a phenyl group attached to the cyclopropyl group (Figure 9B). It happens to be the quinolone compound which has the longest hydrophobic substitution at the N1 position known to date. This is consistent with the result shown in Figure 10 which demonstrates that there is an increase in activity against *M. luteus* enzyme with increasing chain length. It is not clear what the differences in binding site conformation created by different gyrase species may be. A simple but plausible interpretation is that the distance between the two separated DNA strands at the site created by *M. luteus* DNA gyrase is too great to be spanned by quinolones with normal N1 hydrophobic groups such as cyclopropyl, phenyl, or ethyl groups. For further probing of the binding site configuration, dimers with longer methylene linkers and molecules with various functional groups, positioned at opposite directions with proper distances, need to be synthesized and tested. Such structure–activity analysis based on the proposed model may shed light on designing more potent quinolones against DNA gyrase of different species or, more importantly, against the target enzyme of resistant mutants.

Acknowledgments

We gratefully acknowledge Prof. J. Hermans, Jr., for his critical readings of the manuscript. We thank J. Patel for quinolone solubility data, M. Bures and Y. Martin for their assistance in computer graphics, J. Baranowski for performing DNA gyrase supercoiling inhibition assays, and D. Weigl and W. E. Kohlbrenner for providing us with purified *E. coli* DNA gyrase.

Registry No. A-62824, 111279-87-9; A-67107, 100490-36-6; A-57241, 98106-49-1; A-63210, 118287-15-3; A-63419, 96568-21-7; A-62917, 100490-19-5; A-62247, 119530-20-0; A-61867, 102856-07-5; A-62255, 119530-21-1; A-62251, 108138-27-8; A-57274, 100490-19-5; A-56620, 98105-99-8; A-61356, 119618-89-2; A-57603, 103531-47-1; CI-934, 91188-00-0; dimer 3, 119530-22-2; dimer 4, 119530-23-3; dimer 5, 119530-24-4; rosoxacin, 40034-42-2; ciprofloxacin, 85721-33-1; norfloxacin, 70458-96-7; temafloxacin, 108319-06-8; pefloxacin, 70458-92-3; lomefloxacin, 98079-51-7; ofloxacin, 86784-41-0; difloxacin, 98106-17-3; oxolinic acid, 14698-29-4; nalidixic acid, 389-08-2.

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Mechanism of DNA Cleavage and Substrate Recognition by a Bovine Apurinic Endonuclease†

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Received November 3, 1988; Revised Manuscript Received January 23, 1989

ABSTRACT: The location of the phosphodiester bond cleaved by homogeneous Mg^{2+}-dependent apurinic endodeoxyribonuclease (EC 3.1.25.2; APE) of bovine calf thymus has been determined by using a 21-mer oligonucleotide containing a single central apurinic site as a substrate. A single product of cleavage consistent with cleavage of the oligonucleotide 5′ to the apurinic site, and leaving a 3′ hydroxyl group, was identified. This enzyme is, therefore, a class II apurinic endonuclease. The substrate specificities of this enzyme have been determined by using a variety of natural and synthetic DNAs or oligonucleotides containing base-free sites. Calf thymus APE has an absolute requirement for a double-stranded DNA and requires an abasic site as a substrate. The presence of a base fragment such as a urea residue, an alkoxyamine group attached to the C′-1 position of the abasic site, or reduction of the C′-1 aldehyde abolishes the APE activity of this enzyme. Synthetic abasic sites containing either ethylene glycol, propanediol, or tetrahydrofuran interphosphate linkages are excellent substrates for bovine APE. These results indicate that APE has no absolute requirement for either ring-opened or ring-closed deoxyribose moieties in its recognition of DNA-cleavage substrates. The enzyme may interact with the pocket in duplex DNA that results from the base loss or with the altered conformations of the phosphodiester backbone that result from the abasic site.

Apurinic and apyrimidinic sites, base-free sites in DNA, are mutagenic lesions for bacterial and mammalian cells (Schaaper & Loeb, 1981; Gentil et al., 1984; Kunkel, 1984; Loeb, 1985). Such sites are produced spontaneously (Lindahl, 1979), by the action of alkylating agents (Brooks & Lawley, 1963) and by the action of the DNA glycosylase repair enzymes (Friedberg, 1985). At AP[1] sites, adjacent phosphate residues are linked by a deoxyribose moiety. The action of X-rays or the antitumor antibiotic bleomycin also produces abasic sites in DNA. However, these base-free sites are linked by altered deoxyribose moieties (Hutterman, 1978; Sugiyama et al., 1988).

Both bacterial and mammalian cells contain enzymes that recognize and cleave DNA containing AP sites, the first step in the repair of such lesions. There are four potential sites for phosphodiesterase action adjacent to an AP site, and apurinic endonucleases (APEs) have been classified according to the phosphodiester bond cleaved (Linn et al., 1981). Class I and III APEs cleave 3′ to the base-free site but leave 3′ hydroxyl and phosphate end groups, respectively. Class II and IV APEs both cleave 5′ to the base-free site but leave 3′ hydroxyl and phosphate end groups, respectively. Thus, class II APEs cleave the phosphodiester bond most 5′ to the base-free site.

Recent studies of the *Escherichia coli* apurinic endonucleases reveal these enzymes to have distinctively different substrate specificities and mechanisms of action. For example, the apurinic endonuclease activity of exonuclease III (a class II apurinic endonuclease) recognizes and cleaves DNA at additional sites, such as urea residues (Kow & Wallace, 1985), and endonuclease III (a class I endonuclease) has been shown to cleave DNA by a novel β elimination reaction (Bailly & Verly, 1987; Kim & Linn, 1988).

A similar understanding of the mechanisms of action and the range of substrates recognized by the multiple forms of mammalian apurinic endonucleases should aid in the elucidation of the repair pathways in mammalian cells for base-free sites. This paper describes studies of the mechanism of action

† Research was supported by USPHS Grant CA 35767 and by a Leukemia Society of America Scholar Award to W.D.H. and by USPHS Grant CA 17395 and NIEHS Grant ES 04068 to A.P.G.
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[1] Abbreviations: AP, apurinic and/or apyrimidinic; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; APE, apurinic endonuclease; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethylamine.

0006-2960/89/0428-3894$01.50/0