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Proton NMR study of the [d(ACGTATACGT)]$_2$-2echinomycin complex: conformational changes between echinomycin binding sites

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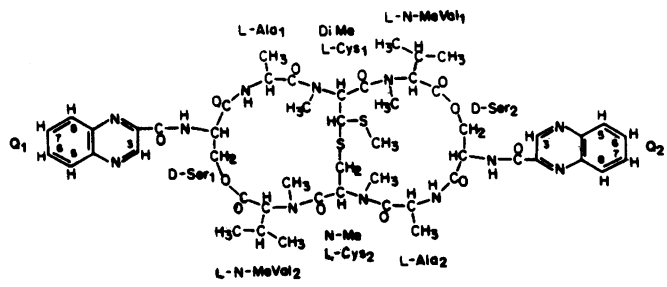
ABSTRACT

The interactions of echinomycin and the DNA decamer [d(ACGTATACGT)]$_2$ were studied by proton NMR. Echinomycin binds cooperatively as a bisintercalator at the CpG steps. The terminal A·T base pairs are Hoogsteen base paired, but none of the four central A·T base pairs are Hoogsteen base paired. However, binding of the drug induces unwinding of the DNA which is propagated to the central ApT step. All four central A·T base pairs are destabilized relative to those in the free DNA. Furthermore, based on these and other results from our laboratory, we conclude that the formation of stable Hoogsteen base pairs may not be the relevant structural change *in vivo*. The structural changes propagated between adjacent ACGT binding sites are the unwinding of the duplex and destabilization of the base pairing between binding sites.

INTRODUCTION

Echinomycin is a naturally occurring antibiotic comprised of an octadepsipeptide ring with a thioacetal cross bridge between opposing cysteines and quinoxaline rings attached via peptide linkages to the two D-serine residues (Chart I). It exhibits potent anti-viral and anti-tumor activity (1). Echinomycin is currently in phase two clinical trials as a chemotherapeutic agent (2).



Footprinting experiments established the preference for binding of echinomycin at CpG steps (3,4) and showed that echinomycin binding altered the structure of the DNA adjacent to the echinomycin binding sites, rendering it more reactive with DNase I and other DNA cleavage reagents (3, 5). This hyperreactivity to cleavage reagents is also observed several base pairs away from the nearest binding site. Mendel and Dervan (5) and Fox and Kentebe (6) showed that binding of echinomycin to DNA causes hyperreactivity to DNase I or DEPC up to 12 base pairs from the nearest binding site. The structural basis for the enhanced reactivity of DNA distal to the binding sites is not well understood. McClean and co-workers showed that DNA fragments in which the adenines were modified at the N7 position such that they could not Hoogsteen base pair remained hypersensitive to cleavage by OsO_4 (7). Based on these results, they proposed that this structural change was the unwinding of the DNA duplex induced by the intercalative binding of echinomycin rather than the formation of Hoogsteen base pairs (7).

None of the structural studies to date have addressed the issue of what structural changes are propagated between binding sites several base pairs apart. No structural information, either in solution or the crystalline state, has been obtained for DNA-echinomycin complexes in which two CpG binding sites are separated by more than two A·T base pairs. In order to address questions regarding the structure of the DNA between two echinomycin binding sites, the complex formed between echinomycin and [d(ACGTATACGT)]$_2$, in which the CpG binding sites are separated by four base pairs, was studied by ^{1}H NMR. The goals of this study were to determine: (1) if Hoogsteen base pairs form; (2) if so, are Hoogsteen base pairs propagated one base pair away from the binding site; and (3) if not, what other structural changes occur that account for the observed hyperreactivity to DNA cleavage reagents of DNA distal to the binding site.

Many of the results obtained in this study are consistent with those obtained on the [d(ACGTACGT)]$_2$-2echinomycin

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complex. Two drugs bind cooperatively per duplex, one at each of the CpG steps. The terminal A·T base pairs are Hoogsteen base paired. All four central A·T base pairs are destabilized relative to the free DNA. However, in contrast to the results obtained for the $[d(ACGTACGT)]_2$-2echinomycin complex, there is no direct evidence for the formation of Hoogsteen base pairs between the binding sites at any temperature. There is evidence that the helix is unwound through the four central A·T base pairs. Two structural changes, destabilization of base pairing and unwinding of the duplex, are propagated through all four A·T base pairs between the two echinomycin intercalation sites.

MATERIALS AND METHODS

Sample Preparation

The DNA decamer $d(A_1C_2G_3T_4A_5T_6A_7C_8G_9T_{10})$ was synthesized on the 10 μmole scale on an Applied Biosystems 381A DNA synthesizer using phosphoramidite chemistry. The decamer was purified by gel filtration as previously described (13). Two DNA samples were prepared by dissolving the DNA in 50 mM NaCl and adjusting the pH to 6.5 (uncorrected meter reading) with 0.1 M NaOH. No additional buffer was added. The samples were then transferred to NMR tubes and dried under a stream of N_2 gas and redissolved in 99.96%D D_2O several times. The samples were finally dissolved in 400 μl of 99.996%D D_2O (Cambridge Isotopes Laboratories, Cambridge, MA). The initial sample conditions were 1.9 mM duplex DNA, 50 mM NaCl, pH 6.5. Due to the presence of a DNA hairpin in equilibrium with the duplex, the NaCl concentration of the samples was raised to 104 mM by the addition of 1.27 mg of solid NaCl. For NMR experiments run in water the samples were dried in the NMR tube and redissolved in 400 μl 90% H_2O/10% D_2O.

Echinomycin was purchased from Fermical, Inc., Detroit, Michigan. The fully saturated complex was formed by adding two equivalents of echinomycin dissolved in methanol directly to the DNA solution in the NMR tube. The concentration of the echinomycin solution was calculated based on UV absorbance using an extinction coefficient of 11,500 $M^{-1}\,cm^{-1}$ at 325 nm (3). The methanol/water mixture was then evaporated under a stream of N_2 gas. Complex formation was assayed by 1- and 2-dimensional NMR. If the complex was not fully saturated, additional drug was added until no free DNA peaks were observed in the spectrum of the complex. The two partially saturated complexes were made from a second DNA sample by the addition of successive aliquots containing 0.5 equivalents of echinomycin in methanol.

The purine H8 protons in the complex were deuterated by heating the complex in D_2O at 65°C for approximately three days in the NMR tube (11, 14). Both GH8s and the A_1H8 were completely deuterated. The A_5 and A_7 H8s were approximately 80% deuterated.

NMR Spectroscopy

All NMR experiments were done on a General Electric GN500 (500.119 MHz, ^1H) spectrometer (GE NMR, Fremont, CA). Chemical shifts were referenced to the chemical shift of water, which had been previously calibrated relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Phase sensitive nuclear Overhauser effect (NOESY) spectra in D_2O were obtained using the method of States *et al.* (15) and the standard pulse sequence (16) with preirradiation of the solvent during the recycle delay. Phase sensitive NOESY spectra in H_2O were acquired by replacing the last pulse of the standard sequence with a 11 spin-echo pulse sequence and phase cycling appropriately to suppress the large solvent resonance (17). The carrier was set on the water resonance and the delay τ was adjusted so that the excitation maximum was centered at approximately 12 ppm (60 μsec). HOHAHA spectra were acquired using the MLEV 17 mixing scheme and two 2 msec trim pulses for the spin lock (18). COSY (19) and P.COSY (20) spectra were acquired with standard pulse sequences and phase cycling. All 2D NMR spectra were processed on a VAX 8820 (Digital Equipment Corporation) or Personal Iris 4D/25 (Silicon Graphics Incorporated) using the Fortran program FTNMR (Hare Research). Descriptions of the acquisition and processing parameters for each experiment are given in the figure captions.

RESULTS

Complex Formation

Figure 1 shows the aromatic region of the spectrum of $[d(ACGTATACGT)]_2$-echinomycin as a function of increasing drug:DNA ratio. Upon addition of 0.5 equivalents of drug, a second set of resonances appears. As the drug:DNA ratio is raised, the intensity of the second set of lines increases and the resonances from the free DNA decrease in intensity until, at a drug:DNA ratio of 2:1, only the resonances from the fully saturated complex are observed. The appearance of two sets of resonances indicates that the free and drug complexed DNA are

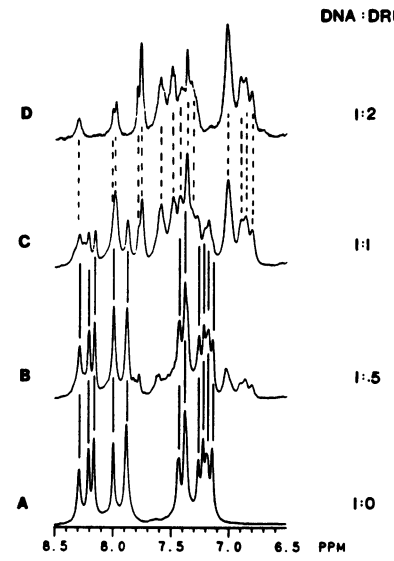
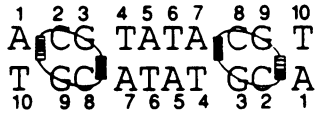


Figure 1. Proton NMR spectra of the aromatic region of $[d(ACGTATACGT)]_2$-echinomycin as a function of added drug concentration at 30°C in D_2O. DNA:echinomycin ratios of (A) 1:0, (B) 1:0.5, (C)1:1, and (D) 1:2 are shown. Solid lines connect the free DNA resonances to the free DNA resonances in the spectra of the 1:0 and 1:0.5 complexes; dashed lines connect the resonances of the fully saturated complex with the resonances of the fully saturated complex in the spectrum of the 1:1 complex. 0.5 equivalents of echinomycin in methanol were added to the DNA (1.9 mM duplex, 104 mM NaCl, pH 6.6) in the NMR tube. After each addition, the sample was dried under a stream of N_2 gas and redissolved in 99.996% D_2O. The spectra were acquired with a sweep width of 5000 Hz in 8K complex points. Data were Gaussian broadened by 3Hz prior to Fourier transformation.

in slow exchange on the NMR time scale. In NOESY spectra of the less than fully saturated complexes no exchange crosspeaks are observed between the free DNA resonances and the DNA resonances from the DNA-echinomycin complexes even at mixing times of 1 second (data not shown). This indicates that the drug binds essentially irreversibly to the DNA on the NMR time scale. The titration results are consistent with the 'all or none' binding characteristics seen in footprinting studies (3) and the cooperativity observed in the formation of the [d(ACGTACGT)]$_2$-2echinomycin complex (11). Chart 2 shows a schematic representation of the [d(ACGTATACGT)]$_2$-2echinomycin complex.



Imino Spectra as a Function of Temperature

The imino proton spectra of [d(ACGTATACGT)]$_2$ and the [d(ACGTATACGT)]$_2$-2echinomycin complex as a function of temperature are shown in Figure 2. The assignments of the imino resonances are indicated on both spectra. The T_4, T_{10}, G_3 and G_9 imino resonances shift upfield approximately 1 ppm when echinomycin binds. The T_6 imino resonances shifts only 0.2 ppm upfield. The large upfield shift of the imino resonances at the binding sites is characteristic of intercalative binding (21). The imino resonances of the free DNA were assigned based on imino-imino and imino-amino crosspeaks observed in NOESY spectra in H_2O (data not shown) using standard techniques (22). The assignments of the imino resonances in the [d(ACGTATACGT)]$_2$-2echinomycin complex are discussed below.

The imino resonances of the complex, except T_{10}, are much broader than those of the free DNA at all temperatures. The uniform broadening of all but the terminal resonances is, most likely, due to the increased viscosity of the solution caused by the drug binding (23). Additionally, both the T_4 and T_6 imino resonances of the complex begin to broaden and shift upfield due to exchange with solvent at lower temperature than those of the free DNA. The T_4 imino of the complex is broadened almost to baseline at 35°C. The T_{10} imino, however, is sharper in the complex than in the free DNA, indicating that echinomycin binding stabilizes this terminal base pair.

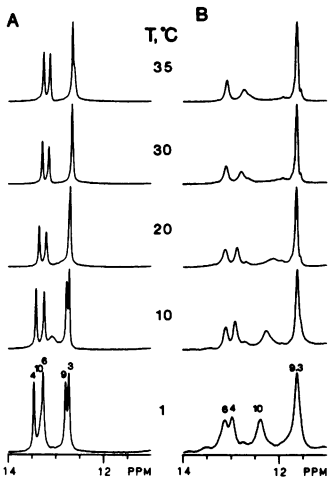


Figure 2. Imino proton spectra as a function of temperature of [d(ACGTATACGT)]$_2$, 1.9 mM duplex, 104 mM NaCl, pH 6.6 (A). [d(ACGTATACGT)]$_2$-2Echinomycin 1.9 mM duplex, 104 mM NaCl, pH 6.6 (B). Assignments of the imino resonances are indicated. Spectra were obtained with the $1\bar{1}$ spin echo pulse sequence (17) to suppress the H_2O resonance. The delay τ was adjusted to give maximum excitation at approximately 12 ppm, $\tau = 60\ \mu s$, $\Delta = 50\ \mu$sec. Spectra were acquired with a sweep width of 10,000 Hz in 8K complex points. All spectra were Gaussian broadened by 3 Hz prior to Fourier transformation.

NOESY Spectrum of [d(ACGTATACGT)]$_2$-2Echinomycin in D_2O

A short mixing time (50 msec) NOESY spectrum of the [d(ACGTATACGT)]$_2$-2echinomycin complex at 30°C is shown in Figure 3. There are no crosspeaks corresponding to free DNA resonances indicating that the complex is fully saturated. Crosspeaks that indicate changes in the DNA structure and identify the binding site are boxed and lettered in Figure 3. The significance of these crosspeaks will be discussed below.

Assignment of the Nonexchangeable Resonances in the DNA

Based on the difficulties we had assigning the DNA resonances in the spectrum of the [d(ACGTACGT)]$_2$-2echinomycin complex (11, 12), we anticipated difficulty in assigning the resonances in this complex. Several of the standard connectivities used in the sequential assignment of DNA fragments (24–26) are disrupted by the intercalation of the quinoxaline rings and the destabilization of the base pairs adjacent to the binding site.

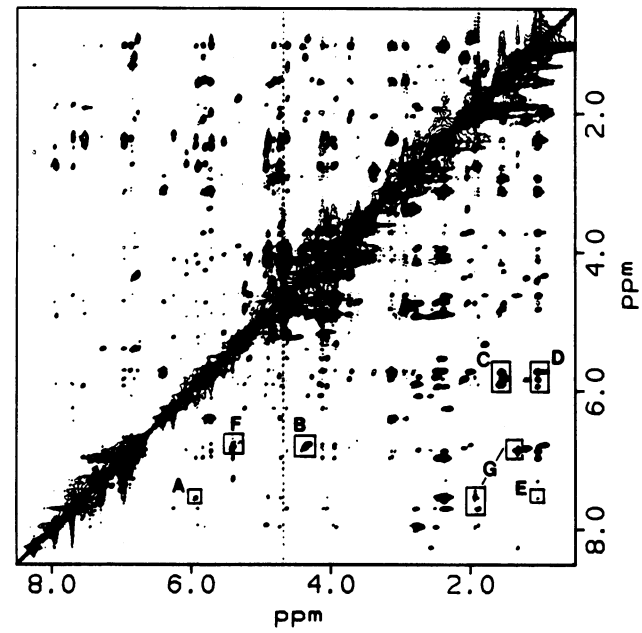


Figure 3. NOESY spectrum of d[(ACGTATACGT)]$_2$-2echinomycin 1.9 mM complex in 104 mM NaCl, pH 6.6 in D_2O, 30°C; $\tau_m = 50$ ms. Several important crosspeaks are boxed: the H8-H1′ crosspeak for A1 (A), the H6-H3′ crosspeaks for C_2 and C_8 (B), the H1′-AlaCH$_3$ crosspeaks for C_2, C_8 (C) ,G_3 and G_9 (D), the H8-ValCH$_3$ crosspeaks for A_1 (E). The spectrum was acquired with a sweep width of 5000 Hz in both dimensions, and a mixing time of 50 msec. The residual water was suppressed by irradiating the water during the 2 second recycle delay. 360 t_1 blocks of 2K points and 32 scans were acquired. The data in both dimension were apodized by a skewed sine-bell squared function (skew = 1.5) phase shifted by 60°. 360 points were apodized in both dimensions. Data in t_1 were zero-filled to 1K points prior to Fourier transformation.

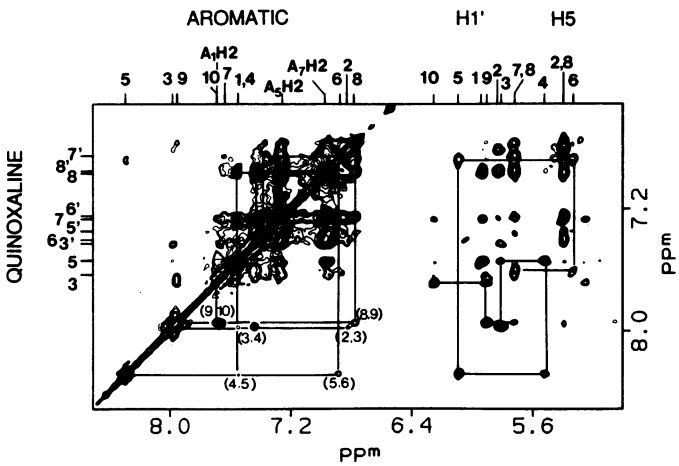


Figure 4. Expanded region of the NOESY spectrum of [d(ACGTATA-CGT)]$_2$-2echinomycin in D_2O, showing the aromatic resonances and their crosspeaks to the H1′ region at 20°C and τ_m=200 msec. The sample contained 1.9 mM complex in 104 mM NaCl, pH 6.6 in D_2O. The quinoxaline, aromatic DNA, H1′, and H5 resonance assignments are indicated. The sequential aromatic to aromatic connectivities are shown for bases C_2 through T_6, and C_8 through T_{10}. The crosspeaks are labeled. In the base-H1′ region the 3–7 and 8–10 sequential base-H1′ connectivities are drawn. The spectrum was acquired with a sweep width of 5000 Hz in both dimensions. 308 t_1 values of 32 scans and 1K complex points were collected. The data were apodized with a skewed sine-bell squared function (skew=1.5) phase shifted by 60°. 308 points in each dimension were apodized. Data in t_1 were zero-filled to 1K prior to transformation.

Fortunately, the strategy used to assign the [d(ACGTA-CGT)]$_2$-2echinomycin complex proved quite successful when applied to this complex. The DNA resonances were assigned essentially as was described (11, 12).

Figure 4 shows the aromatic and base-H1′ regions of the NOESY spectrum of the [d(ACGTATACGT)]$_2$-2echinomycin complex at 20°C (τ_m=200 msec). Base-base and base-H1′ connectivities can be traced in this region of the spectrum. Based on results obtained with the [d(ACGTACGT)]$_2$-2echinomycin complex, we expected the A_1 to be *syn*, and therefore have a large H8-H1′ crosspeak. There is one very large base-H1′ crosspeak which is assigned as A_1 (Figure 3, peak A). The resonance assigned as A_1H1′ has a crosspeak to a quinoxaline ring H7 proton which in turn has a crosspeak to a CH6 resonance tentatively assigned as C_2. This CH6 is the first CH6 in the sequence CGTAT for which base to base connectivities are observed. There is only one possible CGTAT sequence in the duplex. Therefore, the resonances could be assigned unambiguously and the assignments of the A_1H8 and H1′ resonances were confirmed.

Additionally, base-H1′ connectivities are observed from G_3 to A_7. The assignment of the A_7H8 resonance was initially ambiguous, because of the overlap of the A_7 and C_8 H1′ resonances, and the weakness of the T_6H6-H1′ and T_6H1′-A_7H8 crosspeaks . The assignment of the A_7H8 and the other H8 protons were confirmed by deuterating the sample, and comparing the spectra before and after deuteration. With the assignments of the A_7H8 and H1′ resonances confirmed, the remaining H8 and H1′ resonances were assigned based on base-H1′ connectivities observed between C_8, G_9 and T_{10}. The assignments of the non-exchangeable resonances at 20°C are listed in Table I.

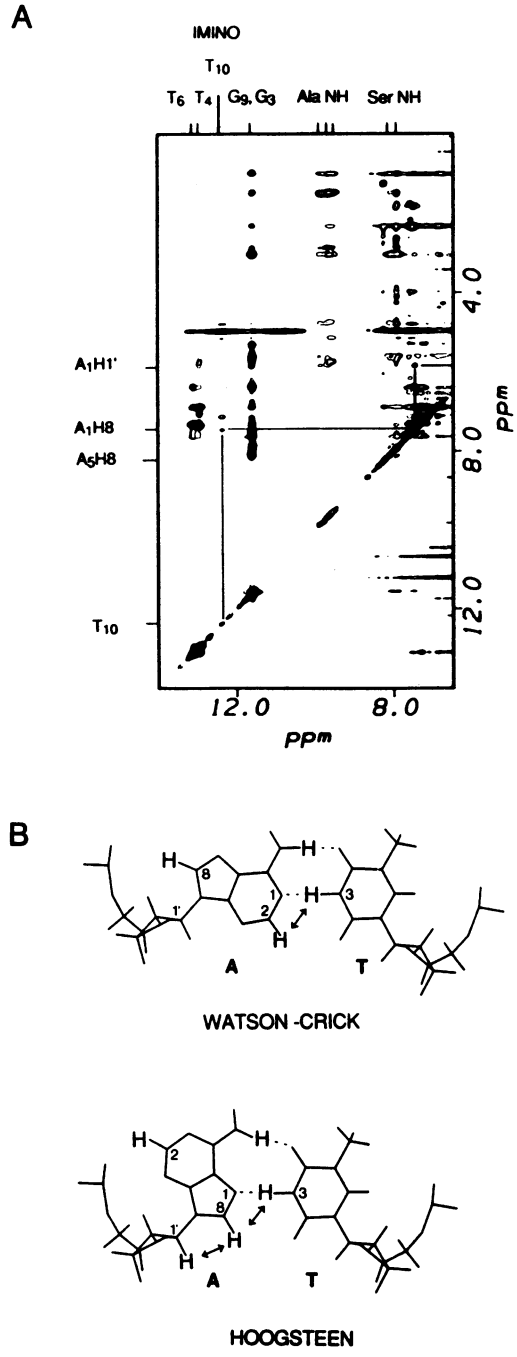


Figure 5. (A) Expanded region of the NOESY spectrum of d[(ACGTATA-CGT)]$_2$-2echinomycin, 1.9 mM complex in 104 mM NaCl, pH 6.6 in 90% H_2O/10% D_2O, 1°C, τ_m=50 msec. The region of the spectrum including the imino and aromatic resonances and their crosspeaks is shown. The T_{10} imino to A_1H8 and A_1H8 to A_1H1′ connectivities are traced. The position of the A_5H8 resonances is also indicated. The DNA imino and peptide amide protons are indicated. The spectrum was acquired with a sweep width of 10,000 Hz in each dimension. The data were acquired with 2K real points in t_2 and 180 points in t_1. The final pulse in the NOESY experiment was replaced with the $1\bar{1}$ spin echo pulse with τ=60 μsec, Δ=50 μsec. 96 scans were acquired for each t_1 experiment. Prior to Fourier transformation each FID was corrected using a Gaussian function with K=32 and M=16 (38) to remove the residual water signal. The data were apodized with a skewed sine-bell squared function (skew=1.5) phase shifted by 60°. 180 points were apodized in each dimension and data in t_1 were zero-filled to 2K. (B) Schematic representations of both Watson–Crick and Hoogsteen A·T base pairs. The short interproton distances for the two different base pairing schemes are indicated.

Table I: Assignments[a] of the DNA resonances in the complex [d(ACGTATACGT)]$_2$-2echinomycin at 20 °C

	H8,H6	H5,Me H2	H1′	H2′	H2″	H3′	H4′	H5′,5″	Amino[b]	Imino[b]
A_1	7.55	7.24	5.93	2.37	2.47	4.71	4.05	3.62		
C_2	6.81	5.39	5.83	1.19	2.11	4.36	3.95	4.12,3.74	7.46,6.38	
G_3	7.97		5.81	2.73	2.51	4.74	4.28	3.92	5.67	11.60
T_4	7.55	1.94	5.52	2.46	2.73	4.90	4.01	3.98,3.74		13.10
A_5	8.28	6.85	6.08	2.57	2.77	4.95	4.36	4.13,4.05	5.75	
T_6	6.88	1.32	5.34	1.39	1.81	4.63	3.92	4.16		12.95
A_7	7.61	7.34	5.71	2.39	2.54	4.90	4.09	3.92	6.37	
C_8	6.77	5.39	5.72	0.93	2.01	4.32	3.94	4.13,3.71	7.46,6.39	
G_9	7.95		5.90	2.40	2.77	4.91	4.18	3.97		11.60
T_{10}	7.69	1.88	6.25	2.29	2.39	4.64	4.18	4.10,4.05		12.36

a) Chemical shifts relative to DSS, b) Chemical shifts at 1°C

Table II: Chemical shifts[a] of the echinomycin resonances in the complex [d(ACGT-ATACGT)]$_2$-2echinomycin at 20°C

		α	β	β′	γ	NH	NCH_3	S-CH_3
Serine	1	5.19	4.62	4.13		7.92		
	1′	5.16	4.62	4.05		7.90		
	2	5.24	4.82	4.48		8.18		
	2′	5.26	4.79	4.48		8.21		
Valine	1	4.82	2.39		1.01,1.04		3.11	
	2	4.62	2.39		1.04,1.04		3.15	
Cysteine	1	6.38	4.72				2.96	2.05
	2	5.74	3.38	2.94			2.92	
Alanine	1	4.74	1.53			9.59,9.45		
	2	4.82	1.57			9.84,9.65		
Quinoxaline	1	7.66	7.40	7.26	6.97	6.97		
	2	7.43	7.36	7.25	6.94	6.86		

a) Chemical shift relative to DSS.

Assignment of the Exchangeable DNA Resonances in the [d(ACGTATACGT)]$_2$-2Echinomycin Complex

Figure 5 shows the NOESY spectrum of the [d(ACGTATA-CGT)]$_2$-2echinomycin complex in water at 1°C, τ_m=50. The region shown includes the imino and aromatic resonances and all of their crosspeaks. The T and G imino and the alanine and serine amide resonance positions are indicated.

Sequential imino-imino connectivities are disrupted by the intercalation of the quinoxaline rings, complicating the assignment of the imino resonances. The large peak at 12 ppm was assigned to both G imino resonances based on the strong crosspeaks to the C amino resonances and the CH5 resonances. The broad imino resonance at 12.36 ppm in the one dimensional spectrum was assigned as T_{10}. This assignment was confirmed by the presence of a crosspeak to the H8 of A_1. This crosspeak proves that the terminal base pair is Hoogsteen base paired. Figure 5b shows both Watson–Crick and Hoogsteen A·T base pairs with the short interproton distances characteristic of each base pair type indicated. The other T iminos were assigned at 20°C based on the presence of crosspeaks (due to spin diffusion) to the methyl protons. The T_6 imino has a crosspeak to its own methyl peak and the T_4 imino has crosspeaks to its own and to the T_6 methyl peak (data not shown). The AH2 resonances could not be assigned in the spectra in D_2O because of the overlap with the quinoxaline resonances. Therefore the AH2 resonances could not be used to assign the imino resonances. Crosspeaks between the imino resonances of G_3,T_4 and G_9 and the quinoxaline ring protons confirmed the assignment of the T_4 imino. T_6 should not have any crosspeaks to the quinoxaline ring protons. The A and G aminos are assigned at 1°C based on the broad crosspeaks to the T and G iminos. The assignments of the exchangeable DNA resonances at 1°C are listed in Table I.

Assignment of the Echinomycin Resonances in the [d(ACGTATACGT)]$_2$-2Echinomycin Complex

The amino acid spin resonances were assigned as previously described for the [d(ACGTACGT)]$_2$-2echinomycin complex (11, 12). Sequential assignment of the echinomycin resonances could be made on the basis of crosspeaks to the two distinct cysteine residues, between dimethylcys$_1$ and N-methylval$_1$, N-methylcys$_2$ and N-methylval$_2$, and N-methylcys$_2$ and ala$_2$. The amide resonances were assigned based on crosspeaks to the serine and alanine alpha protons in the NOESY in water shown in Figure 5. The doubling of several of the echinomycin resonances is discussed below. The assignments of the drug resonances at 20°C are listed in Table II.

DNA-Echinomycin Interactions in the Complex [d(ACGTATACGT)]$_2$-2Echinomycin

The mode of binding and the binding site are defined based on the intermolecular NOESY crosspeaks seen in the NOESY spectra shown in Figures 3,4 and 5. The DNA-echinomycin interactions are essentially the same as those seen in the complex [d(ACGTACGT)]$_2$-2echinomycin (12). The same patterns of

Table III: DNA-drug contacts in the complex [d(ACGTATACGT)]$_2$-2echinomycin

	H8/H6	ME/H5/H2	H1′	H2′,2″	H3′	H4′	H5′,5″	Amino	Imino
A1	Val_1CH_3(w) $Q_1$7,8(w)		$Q_1$7,8(m) Val_1CH_3(s)	$Q_1$7,8(s)	$Q_1$7,8(w)				
C2	$Q_1$7,8(w)	$Q_1$7,6(s)	$Q_1$7,8(w),Val_1CH_3(m) Ala_1CH_3(s),Val_1NCH_3(m) Ala_1NH(w)	Ala_1CH_3(m)		Val_1CH_3(w) Val_1NCH_3(w)	Ala_1CH_3(w)	$Q_1$6,7(w)	
G3	$Q_2$3(w)		Ala_1CH_3(s) Ala_1NH(s) Ser_1NH(m)						Ala_1CH_3(m),Val_1CH_3(m) Val_1NCH_3(s),Ala_1NH(w) Cys_1NCH_3(w),Ser_1NH(w) $Q_2$7,6(m)$Q_2$5(w)
T4			$Ser_1\alpha$(m) $Ser_1\beta$(w) Ser_1NH(m)						
A7	$Q_2$7,8(w)		Val_2NCH_3(w) Ser_1NH(w)						
C8	$Q_2$7,8(w)	$Q_2$7,6(s)	$Q_2$7,8(m),Val_2CH_3(m) Ala_2CH_3(s),Val_2NCH_3(m) Ala_2NH(m)	Ala_2CH_3(m)		$Q_2$7,6(w)	Ala_2CH_3(w)	$Q_2$7,6(w)	
G9	$Q_1$3(w)		Ala_2CH_3(s),$Ser_2\alpha$(w) Ala_2NH(s),Ser_2NH(w)	Ala_2CH_3(w)					Ala_2CH_3(m),Val_1CH_3(m) Val_1NCH_3(s),Ala_2NH(w) Cys_2NCH_3(w),Ser_2NH(w) $Q_2$7,6(m),$Q_2$5(w)
T10		$Q_1$3(w) $Ser_2\alpha$(w) Ala_2CH_3(m)							

note: All cross peaks between non-exchangeable resonances were taken from a NOESY spectrum at 30°C, τ_m= 50 msec. All contacts with exchangeable resonances were taken from a NOESY spectrum in H_2O, 20°C, τ_m=100 msec.

crosspeaks between the base and quinoxaline ring protons are observed, as well as similar interactions between the minor groove marker sugar protons and the peptide ring.

A list of the drug-DNA NOEs seen in the 50 ms mixing time NOESY spectrum is given in table III. A schematic representation of these drug-DNA interactions is shown in Figure 6. These interactions are discussed below.

NOESY Spectra of the Complex as a Function of Temperature

NOESY spectra in D_2O of the [d(ACGTATACGT)]$_2$-2echinomycin complex were obtained at 1, 10, 20, 30 and 45°C. Stacked plots of the base-H1′ region of spectra at 1 and 45°C are shown in Figure 7. In all the NOESY spectra the H8-H1′ crosspeak for A_1, labeled in the spectra, is much more intense than that for any other base in the complex. The intensities of all of the base-H1′ crosspeaks except A_1 are indicative of the *anti* glycosidic torsion angle expected for B-DNA, while that for A_1 is indicative of the short H8-H1′ interproton distance in the *syn* conformation. At 1°C the A_7H8-H1′ crosspeak is broader than the other base-H1′ crosspeaks. This crosspeak sharpens noticeably as the temperature is raised and its integrated intensity also increases.

DISCUSSION

Mode of Binding

A variety of experimental techniques, including viscometric studies (23), X-ray crystallography (8), and NMR studies in solution (9–12) have shown that echinomycin binds to DNA oligonucleotides as a bisintercalator. Echinomycin also binds to the two ACGT binding sites of [d(ACGTATACGT)]$_2$ with the quinoxaline rings intercalating at the ApC steps as illustrated in Chart 2 and the peptide binding in the minor groove. The 1 ppm upfield shift of the imino resonances adjacent to the binding site, characteristic of intercalative binding (21), seen in Figure 1, the large number of NOEs between the A and C base and sugar protons and the quinoxaline rings at both binding sites and the 0.5 ppm upfield complexation shift of the A_1 and A_7H8 and C_2 and C_8H6 protons confirm that the quinoxaline rings intercalate at the ApC steps.

The crosspeaks between the C and G sugar 1′ and 4′ protons and the peptide ring show that echinomycin binds in the minor groove at the CpG step. The crosspeaks between the alanine amides and the G imino and H1′ resonances and the 1 ppm down field shift of the alanine amide resonances are consistent with the pattern of hydrogen bonding proposed based on the crystal structure of the complex of echinomycin and [d(CGTACG)]$_2$ (8). In the crystal structure, hydrogen bonds were proposed to form between the alanine amide and GN3 at the CpG steps. An additional hydrogen bond was proposed to form between the carbonyl oxygen of alanine 2 and the amino group of the terminal G (8). These two hydrogen bonds were postulated to be the structural basis for the sequence specificity of the echinomycin analogue Triostin A (27).

The orientation of the two echinomycin molecules in the complex is shown in Chart II. The two nearly symmetrical halves of echinomycin can be distinguished from each other on the basis of crosspeaks between dimethylcys$_1$ and N-methylval$_1$, N-methylcys$_2$ and N-methylval$_2$, and N-methylcys$_2$ and ala$_2$. The val$_1$ methyl, ala$_1$ methyl, and quinoxaline ring 1 have crosspeaks to A_1, C_2, and G_9, while val$_2$ methyl, ala$_2$ methyl and quinoxaline ring 2 have crosspeaks to A_7, C_8, and G_3 (see Table III). If the drugs bound in either orientation, each alanine and valine methyl and quinoxaline ring spin system would have crosspeaks to both ends of the duplex, and this is not the case.

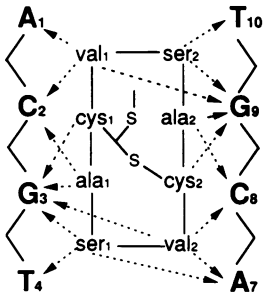


Figure 6. A schematic representation of the interactions between the peptide protons of echinomycin and one binding site of the DNA $[d(ACGTATACGT)]_2$. The arrows indicate NOESY crosspeaks seen in Figure 4, the short (50 ms) mixing time NOESY spectrum and discussed in the text. The length of the arrows is not correlated to the distances between the protons.

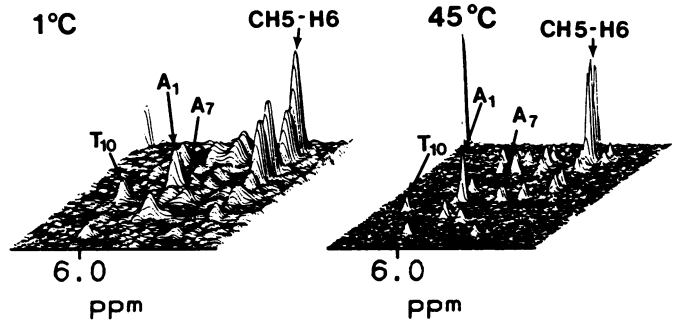


Figure 7. Stacked plots of NOESY spectra of $[d(ACGTATACGT)]_2$-2echinomycin, 1.9 mM complex in 104 mM NaCl, pH 6.6 in D_2O at (A) 1°C, $\tau_m = 50$ ms and (B) 45°C, $\tau_m = 150$ ms. The base-H1′ region of each spectrum is shown. Spectra were processed as follows: (A) 158 t_1 blocks of 1024 real points were collected. The data in both dimension were apodized by a skewed sine-bell squared function (skew = 1.5) phase shifted by 60°. 158 points were apodized in both dimensions. Data in t_1 were zero filled to 1k points prior to Fourier transformation. (B) 399 t_1 blocks of 1024 real points were collected. The data in both dimension were apodized by a skewed sine-bell squared function (skew = 1.5) phase shifted by 60°. 399 points were apodized both dimensions. Data in t_1 were zero filled to 1K points prior to Fourier transformation. The position of A_7H8 and H1′ resonances are indicated in each spectrum.

The representation in Chart II implies a two-fold symmetry axis at the A_5pT_6 step. However, in the complex, some of the echinomycin, although none of the DNA, resonances are doubled. There are four alanine amide resonances and four serine spins systems. Since echinomycin binds in only one orientation, this indicates that either the two halves of the complex are not completely symmetric or that the echinomycin can adopt two slightly different conformations on the DNA. In the crystal structure the $[d(CGTACG)]_2$-2echinomycin complex, the carbonyl of ala_2 but not ala_1 has a proposed hydrogen bond to the G amino group (8). A slight rearrangement of echinomycin on the DNA would bring the carbonyl of ala_1 rather than ala_2 into position to form a hydrogen bond with a G amino. This may be a possible explanation for the doubling of some of the echinomycin resonances.

Cooperative Binding

Spectra of complexes with 0.5 or 1 drug per $[d(ACGTACGT)]_2$ duplex show two sets of resonances corresponding to the free DNA and the saturated (2 drug/duplex) complex. There are no resonances that can be assigned to partially saturated (1 drug/duplex) complexes. In NOESY spectra of the 1:1 complex, all the crosspeaks observed can be assigned to either the free DNA or the fully saturated complex (data not shown). This apparent cooperativity is in agreement with the footprinting results that show 'all or none' binding over a large range of echinomycin concentrations (3). Our previous studies showed that 2 echinomycins bind cooperatively to $[d(ACGTACGT)]_2$ but independently to $[d(TCGATCGA)]_2$. Interestingly, the interaction between the binding sites which leads to cooperative binding for $[d(ACGTACGT)]_2$ is apparently propagated through the additional two A·T base pairs between the adjacent ACGT binding sites in $[d(ACGTATACGT)]_2$.

Structure of the DNA in the Complex [d(ACGTATACGT)]$_2$-2Echinomycin

The structure of the DNA in the complex differs from standard B-DNA in several ways. As was seen in the $[d(ACGTACGT)]_2$-2echinomycin complex, the terminal A·T base pairs are Hoogsteen base paired. In the short mixing time NOESY spectrum there is a very strong crosspeak between the A_1H8 and its own H1′ which confirms that A_1 is in the *syn* conformation (Figure 3, box A). In the NOESY in H_2O spectrum shown in Figure 5, the connectivities from the T_{10} imino to the A_1H8 and from the A_1H8 to A_1H1′ prove unambiguously that A_1 is *syn* and Hoogsteen base paired to T_{10}. This is the only complex reported so far for which the terminal T imino-AH8 crosspeak is observed, providing the first unambiguous proof that these base pairs are in fact Hoogsteen base paired in solution. In previous studies the Hoogsteen base pairing was inferred on the basis of the A_1 being in the *syn* conformation (9, 11). The crosspeak between the A_1H8 and the valine methyl protons is additional proof that the $A_1 \cdot T_{10}$ is a Hoogsteen base pair. The AH8 proton is in the minor groove only when the base pair is Hoogsteen base paired.

In contrast to the terminal A·T base pairs, there is no clear evidence that the central A·T base pairs are Hoogsteen base paired at any temperature. At 20°C there are no imino-H8 crosspeaks for either the $A_5 \cdot T_6$ or $A_7 \cdot T_4$ base pairs (not shown). Neither A_5 or A_7 has a strong H8-H1′ crosspeak at any temperature. In the NOESY in H_2O spectrum at 1°C (Figure 5) there is no T_6 imino to A_5H8 crosspeak. However, the structure of the $A_7 \cdot T_4$ base pair at low temperature is not clear. At 1°C many of the crosspeaks for A_7 are extremely broad and could not be unambiguously assigned. As the temperature is raised, the crosspeaks from A_7 protons sharpen (Figure 7). The intensity of the A_7H8-H1′ crosspeak is comparable to that of the other base-H1′ crosspeaks (except A_1), indicating that at 45°C the glycosidic torsion angle is *anti* and consistent with Watson–Crick pairing at this temperature. The differential line-broadening of the A_7 resonances at low temperature indicates that at low temperature this base is most likely exchanging between two conformations. These could be either a Watson–Crick base pair and an open state or a Watson–Crick and a Hoogsteen base pair (or all three). We are unable to distinguish between these possibilities.

Strong H6 to H3′ crosspeaks for both Cs (Figure 3, box B) indicate that the sugar pucker for these residues is N-type rather than the S-type sugar pucker observed in B-DNA. A comparison

of the fine structure of the H1'-H2',H2'' crosspeaks of C_2 and C_8 observed in the P.COSY (data not shown) with simulated COSY crosspeaks (28) indicates that the C sugars are predominantly N-type. The other sugars all appear to be primarily S-type. A C3'endo sugar pucker in the nucleotide on the 3'side of the intercalation site is observed in other DNA-intercalator complexes. We have observed a similar sugar pucker for the T_4 3' of the intercalation site in the complexes of [d(GGATATCC)]$_2$ and [d(GGTTAACC)]$_2$ and the related drug [N-MeCys^3, N-MeCys^7] TANDEM (29). In the [d(GCATGC)]$_2$-2nogalamycin complex the A sugar 3' of the intercalation site is also C3' endo (30). Theoretical calculations have shown that the C3' endo sugar pucker 3' of the intercalation site is energetically favorable (31).

There is NMR evidence for unwinding of the DNA duplex in the complex relative to the free DNA both in the C·G base pairs between the bis-intercalated quinoxaline rings and in the central A·T base pairs. Crosspeaks are observed between the CH6 and GH8 resonances. These crosspeaks are not observed in the NOESY spectra of the free DNA at comparable mixing times, and can most easily be explained by unwinding of the duplex. There is also a crosspeak between the A_5H8 and T_6H6 in the 50 msec mixing time NOESY spectrum. A comparison of the integrated intensities of this crosspeak with that of the CH5-CH6 yields a qualitative distance of 4 Å. Based on model building, unwinding the helix between bases 5 and 6 approximately 20 degrees brings these protons within 4 Å of each other.

Base-H1' connectivities are observed for G_3 through A_7. However, the crosspeaks from T_6H6 to T_6H1' and to A_7H8 and from A_7H8 to A_7H1' are much weaker than expected for B-DNA. In the NOESY spectrum with a 50 ms mixing time at 30°C the base-H1' crosspeaks observed at longer mixing times are no longer observed. The sequential assignments are only observed for bases 9 and 10. All the intraresidue base-H2',2'' crosspeaks are observed. This indicates that the structure of the central region of the DNA duplex from T_4 to A_7 differs from regular B DNA. This may be due to the destabilization of the $A_7 \cdot T_4$ base pairing discussed below as well as the observed unwinding.

Stability of Base Pairing Within the Duplex

The imino resonances of the two central Ts broaden to base line at a lower temperature in the complex than they do in the free DNA (see Figure 2). The increase in the rate of exchange of these protons with solvent indicates shorter base pair lifetimes for the central A·T base pairs. While it is true that base pair lifetimes can only be quantified from imino exchange rates in the presence of infinite catalyst concentration, the fact that the T_{10} imino is sharper in the complex indicates that the base pair lifetimes change for T_4 and T_6 rather than the drug acting as a catalyst for increased exchange. The conformational exchange observed for the A7·T4 base pair (discussed below) is consistent with a destabilization of base pairing for these base pairs. Recent results of Leroy *et al.* (32) confirm this conclusion. They report a comparison of the stability A·T base pairs adjacent to echinomycin binding sites in complexes containing single ACGT binding sites which shows that these base pairs are less stable in the complexes than in free DNA.

The crosspeaks between A_7H8 and A_7H1' and T_6H6 are extremely broad at 1°C. These crosspeaks sharpen as the temperature is raised. Figure 7 shows stacked plots of the base-H1' region of the NOESY spectra of [d(ACGTATACGT)]$_2$-2echinomycin at 1 and 45°C. At 1°C, the adenine appears to be in equilibrium between two conformations. At 45°C, the base-H1' crosspeaks for A_7 are similar in intensity and lineshape to those of the other residues in the duplex, indicating that at 45°C the base pairing is Watson–Crick. Thus, it appears that at low temperature the A_7 is exchanging between Watson–Crick pairing and some other conformation, either open or Hoogsteen paired, as discussed above.

Hoogsteen Base Pair Formation

In this complex, similar to the [d(ACGT)]$_2$-echinomycin complex (9) and the [d(ACGTACGT)]$_2$-2echinomycin complex (11), the terminal A·T base pairs are Hoogsteen base paired and remain so over the entire temperature range over which the duplex is stable. It has been postulated that the terminal 5' adenine adjacent to the CpG binding site will form Hoogsteen base pairs because the stacking interactions between the adenine in the *syn* conformation and the quinoxaline ring are stronger than those between the quinoxaline ring and adenine in the *anti* conformation (9, 11). Additionally, Hoogsteen base pairing may be stable at the ends of the duplex because there are fewer structural constraints to prevent the adenine from rotating around the glycosidic bond into the *syn* conformation.

Hoogsteen base pairs between adjacent echinomycin binding sites were observed in solution in the [d(ACGTACGT)]$_2$-2echinomycin complex (11,12) at low temperatures and in the crystalline state in the [d(CGTACG)]$_2$-2echinomycin (8). The formation of Hoogsteen base pairs between adjacent echinomycin binding sites in these molecules must be a result of favorable stacking interactions between the quinoxaline rings and the two Hoogsteen A·T base pairs. However, the Hoogsteen base pairs in the center of the complex in solution were considerably less stable than those on the ends and were not observed at higher temperatures (11,12). In addition, no Hoogsteen base pairs are formed in solution in [d(TCGA)]$_2$-echinomycin (9) and [d(TCGATCGA)]$_2$-2echinomycin (12) complexes.

There is no direct evidence for the formation of Hoogsteen base pairs at any of the four central A·T base pairs in the complex [d(ACGTATACGT)]$_2$-2echinomycin complex. The destabilization of the four central A·T base pairs in this complex is the only evidence that Hoogsteen base pairs may transiently form in the center of the [d(ACGTATACG)]$_2$ duplex when it is complexed with echinomycin. In the [d(TCGATCGA)]$_2$-2echinomycin complex, where no Hoogsteen base pairs are formed at any temperature, the central A·T base pairs are stabilized relative to free DNA (12), as is usually observed for complexes of intercalative drugs with DNA.

NMR studies of complexes of DNA and the echinomycin-like drug UK-65,662, in which the valines are replaced by methylcyclopropyl groups and the quinoxaline rings are replaced by 3-hydroxy-quinaldic acid, show that this drug binds similarly to echinomycin. Furthermore, UK-65,662 also induces formation of Hoogsteen base pairs at the ends of the duplex when complexed with [d(ACGT)]$_2$ but does not induce Hoogsteen base pair formation when complexed with [d(GACGTC)]$_2$ (33). The binding of [N-MeCys^3, N-MeCys^7] TANDEM, which binds at TpA sites, to the oligonucleotide [d(ATACGTAT)]$_2$ induces the formation of Hoogsteen base pairs at the terminal A·T base pairs, but not in the center (34).

Based on the results presented here, and previously (9–12), it appears that the formation of stable Hoogsteen base pairs occurs at terminal 5′ purines, and that Hoogsteen base pairs form within a DNA duplex only between immediately adjacent 3′ pyrimidine binding sites and then only under certain conditions. Therefore, the formation of Hoogsteen base pairs cannot account for the footprinting results (3,5–7), nor can it be the most relevant structural change *in-vivo*.

Propagation of Structural Changes Beyond the Binding Site

As discussed in the introduction, the results of footprinting experiments from several laboratories have shown that the hypersensitivity to DNA cleavage reagents is observed distal to the echinomycin binding sites (5, 6). Mendel and Dervan (5) probed a restriction fragment containing a run of 17 A·T base pairs between TCGA binding sites separated by 32 base pairs and showed that adenines up to 12 base pairs from the echinomycin binding sites were hyperreactive to DEPC. Fox and Kentebe showed that the ApT steps normally uncleaved by DNase I in the sequence $CG(AT)_nCG$ where n =5 or 17 were cleaved when echinomycin bound. One would expect that one or more of the structural changes observed in the complexes containing two adjacent echinomycin binding sites (11, 12) are propagated away from the binding site.

Based on the results presented here, Hoogsteen base pairing is not propagated away from echinomycin binding sites. The $A_5 \cdot T_6$ base pairs are not Hoogsteen base paired at any temperature studied. No imino-H8 crosspeak is observed for either the $A_5 \cdot T_6$ base pair or $A_7 \cdot T_4$ base pair. The presence of the extra A·T base pairs between the binding sites appears to prevent the formation of stable Hoogsteen base pairs immediately adjacent to the binding site.

We observe two major structural changes in the DNA duplex when echinomycin binds to $[d(ACGTATACGT)]_2$. The DNA duplex in the $[d(ACGTATACGT)]_2$-2echinomycin complex is underwound at the central ApT step. The presence of a crosspeak between the A_5H8 and T_6H6, as described above, indicates an unwinding of the duplex by approximately 20 degrees. Binding of echinomycin unwinds the duplex one base pair away from the ApC and GpT intercalation sites. In addition to the observed unwinding, all the internal A·T base pairs are less stable in the complex than in the free DNA. The destabilization of base pairs adjacent to the binding sites is also propagated one additional base pair away from the binding site. These changes are consistent with the footprinting results that have shown that the hyperreactivity of A·T rich regions adjacent to echinomycin binding sites is propagated a significant distance from the echinomycin binding site (5, 6). Both groups showed that the normally unreactive TpA steps become more reactive to DNase I and DEPC when echinomycin binds to the fragment.

Unwinding extended beyond the binding site can explain the footprinting results obtained by Mendel and Dervan and those of Fox and Kentebe. A·T rich regions of DNA have a narrower minor groove than mixed sequence DNA (35, 36). A·T rich regions have been shown to be less susceptible to cleavage by DNase I (37). Drew and Travers (37) also showed that unwinding the helix by the addition of 20%–40% DMSO to the solution substantially increased the reactivity of the A·T rich regions to DNase I. They proposed that unwinding of the duplex increases the width of the minor groove, thereby rendering the DNA more susceptible to cleavage by DNase I. It was also demonstrated that unwinding the helix also makes the N7 more accessible to DEPC (7).

SUMMARY AND CONCLUSIONS

The purpose of the studies presented here was to elucidate the structural changes induced in a longer DNA fragment when echinomycin binds. The sequence chosen, $[d(ACGTATACGT)]_2$, contains two strong echinomycin binding sites (ACGT) separated by two A·T base pairs. We have shown that two drugs bind per duplex and that the binding is cooperative. The terminal A·T base pairs are Hoogsteen base paired. In contrast to the results obtained with $[d(ACGTACGT)]_2$ (9,12), the central A·T base pairs adjacent to the quinoxaline ring are not observed to form Hoogsteen base pairs at any temperature, although we cannot completely rule out the possibility of Hoogsteen base pairs forming transiently at low temperature. All the central A·T base pairs are less stable in the complex than in the free DNA. Unusual crosspeaks between A_5 and T_6 indicate that the DNA is significantly unwound in the central region of the duplex. Therefore, based on the results presented here in combination with the footprinting results (5–7), we propose that the structural change responsible for the hypersensitivity to chemical footprinting reagents and nucleases of A·T base pairs distal to echinomycin binding sites is the unwinding induced by the drug binding.

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