An NMR Investigation of the Binding of the Anticancer Drug Actinomycin D to Oligodeoxyribonucleotides with Isolated 5′d(GC)3′ Binding Sites†

Robert L. Jones,‡ Elwood V. Scott,§ Gerald Zon,∥,⊥ Luigi G. Marzilli,*,§,# and W. David Wilson*,‡

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, Department of Chemistry, Emory University, Atlanta, Georgia 30322, and Molecular Pharmacology Laboratory, Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, Maryland 20892

Received January 7, 1988; Revised Manuscript Received April 11, 1988

ABSTRACT: Imino proton and ^{31}P NMR studies were conducted on the binding of actinomycin D (ActD) to self-complementary oligodeoxyribonucleotides with one GC binding site [d(ATATGCATAT) (**1**), d(ATACGCGTAT) (**2**), and d(ATATACGCGTATAT) (**3**)] and with two GC sites [d(ATGCATGCAT) (**4**)]. At $R = 1$ (molar ratio of ActD to oligomer duplex) ActD caused a doubling of the number of imino proton signals at, and adjacent to, the GC binding site of **1**. One of the G·C base pair signals shifted upfield while the other shifted downfield. Both of the signals for the A·T base pairs adjacent to the binding site shifted downfield. All imino proton signals of **2** and the longer sequence, **3**, shifted upfield on binding of ActD to the GC site, indicating a sequence-dependent change in base stacking on complex formation. For both **1** and **2** addition of ActD resulted in a similar pattern of three downfield ^{31}P NMR signals. The two most downfield signals have chemical shift and temperature dependence which are characteristic of phosphate groups at isolated intercalation sites. At $R = 1$ the ActD complex with **4** has very complex spectra with both upfield and downfield A·T and G·C imino signals. All these data were consistent with two 1:1 complexes with the unsymmetrical phenoxazone ring adopting both of the two possible orientations. Increasing R above 1 for **2** and **3** did not change the spectra further; but with **4**, which contains two GC binding sites off the C_2 center, at R ca. 2, all spectral studies (temperature dependence, ^{31}P NMR, 1D and 2D ^1H NMR) were consistent with the formation of at least two of the possible three 2:1 adducts. In contrast, **5** [d(ATGCGCAT)] and other oligomers with adjacent GCGC sites formed unique 2:1 complexes in a highly anticooperative manner [Wilson, W. D., Jones, R. L., Zon, G., Scott, E. V., Banville, D. L., & Marzilli, L. G. (1986) *J. Am. Chem. Soc. 108*, 7113–7114]. All three possible 2:1 complexes could be present if significant signal overlap occurs in the spectra of ActD with **4**. Obviously the two A·T base pairs between the GC sites in **4** significantly relax the intermolecular interactions which result in the unique ActD 2:1 complex with **5**. The variety of ActD adducts observed for these relatively simple sequences indicates that ActD binding to natural DNA must be much more complex than previously anticipated.

Actinomycin D[1] (Figure 1) plays a key role in elucidating DNA interactions as follows: (i) it serves as a small, well-defined model system for protein–DNA interactions (Sobell, 1973; Waring, 1981); (ii) it is useful in investigations of long-range effects on DNA conformation by bound molecules (Fox & Waring, 1984); (iii) this early model for base pair specific interactions may lead to the design of new sequence-specific DNA binding agents (Muller & Crothers, 1968; Sobell, 1973; Dervan, 1986); (iv) this antibiotic is the parent compound for a series of semisynthetic anticancer agents which bind to DNA (Remers, 1984); (v) it is an important molecule in the ongoing analysis of cooperative effects in DNA interactions (Walker et al., 1985).

We have initiated a series of studies designed to evaluate short- and long-range effects of ligand binding interactions on DNA conformation and cooperativity, base pair specificity, and binding site size in DNA interactions. We have used several duplexed oligomer sequences and three different types of DNA binding agents: porphyrins which intercalate selectively at CG sites (Marzilli et al., 1986), phenanthridines which are nonspecific intercalators (Wilson et al., 1986a), and ActD which intercalates selectivity at GC sites (Wilson et al., 1986b; Scott et al., 1988).

Our results have confirmed that ActD binds selectively at GC sites and that ActD can bind in *two* orientations at this site with the unsymmetric phenoxazone oriented in opposite directions. In the general model for the ActD–DNA complex, each of the large cyclic peptides, which are oriented in opposite directions from the intercalation site, lies along the DNA minor groove and covers two to three base pairs (Waring, 1981). This model might lead to the prediction that adjacent site binding is not possible. We have found, however, with oligomers such as **5** in Figure 1, that two ActD can bind at adjacent GC sites to form a unique symmetrical complex (2:1 complex), somewhat analogous to more complicated repressor protein–DNA interactions (Wilson et al., 1986b; Scott et al., 1988). Binding of the second ActD was highly anticooperative. To investigate factors influencing the formation of these novel ActD 2:1

† This work was supported by NSF Grant DBM-8603566 to W.D.W. and NIH Grant GM 29222 to L.G.M.

* Author to whom correspondence should be addressed.
‡ Georgia State University.
§ Emory University.
∥ Food and Drug Administration.
⊥ Present address: Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.
L.G.M. is also a member of the Winship Cancer Center.

[1] Abbreviations: ActD, actinomycin D; DNA, deoxyribonucleic acid; PIPES, piperazine *N*,*N*′-bis(2-ethanesulfonic acid); poly[d(G-C)], poly[d(G-C)·poly[d(G-C)]; EDTA, ethylenediaminetetraacetic acid; 2D, two dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate. Interchain base pairs are indicated with a dot, e.g., G·C, while intrachain sequences are indicated without a dot or dash, e.g., GC or TGCA; all sequences are written in the 5′–3′ direction.

0006-2960/88/0427-6021$01.50/0

OLIGODEOXYRIBONUCLEOTIDES

```
      1 2 3 4 5 6 7
1       A T A T G C A T A T
2       A T A C G C G T A T
3     A T A T A C G C G T A T A T
4       A T G C A T G C A T
5         A T G C G C A T
```

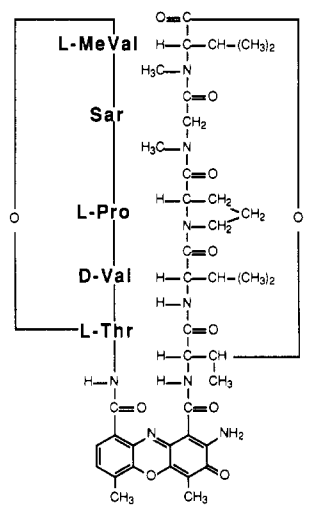


FIGURE 1: (Top) Self-complementary oligodeoxyribonucleotides, **1–4**, examined in this study and **5**, which has been previously reported (Scott et al., 1988). (Bottom) Structure of actinomycin D.

complexes, we have studied a self-complementary oligomer with two GC sites which are separated by two A·T base pairs (**4** in Figure 1). As model systems, we have examined the interaction of ActD with oligomers containing a single GC site in different sequences (oligomers **1–3** in Figure 1).

Materials and Methods

Materials. The ^{1}H spectrum of ActD (Sigma) agreed with literature results (Brown et al., 1984), and the compound was used without further purification. ActD stock solutions were made by dissolving ActD in water to a final concentration of approximately 1 mM. Final concentrations were determined by using an extinction coefficient of 24 500 M^{-1} cm^{-1} at 440 nm (Shafer et al., 1980). PIPES 10 buffer (10 mM PIPES, 1 mM EDTA, and 0.1 M NaCl, adjusted to pH 7.0 with NaOH) was used in all experiments. The oligonucleotides were prepared as previously described (Stec et al., 1985).

^{1}H NMR. Proton NMR spectra at 20 mM oligomer base concentration were obtained at either 270.15 MHz on a JEOL GX-270 or at 361.02 MHz on a Nicolet 360 NT FT spectrometer by using the Redfield 21412 pulse sequence to suppress the intense water signal (Redfield et al., 1975). At 270.15 MHz, spectra were obtained under the following conditions: 5000 scans; 0.4-s pulse repetition; 0.1-Hz line broadening; carrier frequency set at 13.4 ppm; 16K data points; TSP as the reference; 11 000-Hz spectral width; 0.6-mL sample volume in a 5-mm NMR tube. At 361.02 MHz, typical experimental conditions were as follows: 2000 scans; 0.3-s pulse repetition; 1-Hz line broadening; carrier frequency set at 14.4 ppm; 8K data points; TSP as the reference; 14 000-Hz spectral width; 0.6-mL sample volume in a 5-mm NMR tube.

A COSY experiment on the 2:1 complex of ActD with d(ATGCATGCAT) was conducted on the Nicolet 360 NT at 20 °C. Data were acquired with 1K data points in the t_2 dimension and 80 acquisitions in the t_1 dimension. Data in both the t_1 and t_2 dimensions were weighted with a combined Gaussian–sine apodization function prior to Fourier transformation.

^{31}P NMR. Phosphorus NMR spectra were obtained at 81.01 MHz on an IBM WP-200SY spectrometer under the following experimental conditions: 10 000 scans; 45° pulse angle; 2-s pulse repetition; broad-band proton decoupling; 2000-Hz spectral width; 3-Hz line broadening applied before Fourier transformation. Samples of 0.6 mL, contained in a 5-mm NMR tube, were inserted into a 10-mm tube containing PIPES 10/D_2O and trimethyl phosphate as a reference.

Table I: Imino ^{1}H Chemical Shifts and Assignments

oligomer	chemical shifts and assignments[a]
d(ATATGCATAT)	
free	13.49 (6), 13.44 (4), 13.39 (5), 12.95 (3), 12.50 (7)
complex	14.35 (6), 14.17 (6), 13.28 (4, 5), 13.21 (7), 12.78 (7)
d(ATACGCGTAT)	
free	13.61 (5), 13.42 (4), 13.0 (3), 12.87 (7), 12.80 (6)
complex	13.42 (5), 13.38 (5), 13.32 (4), 12.70 (6, 7), 12.37 (7)
d(ATATACGCGTATAT)	
free	13.41 (5, 2), 13.31 (4, 3), 13.0 (1), 12.80 (7), 12.75 (6)
complex	13.41,[b] 13.31,[b] 13.17,[b] 13.10,[b] 12.64 (6, 7), 12.31 (7)
d(ATGCATGCAT)	
free	13.72 (4), 13.51 (7), 13.07 (3), 12.69 (6), 12.56 (5)
complex	14.29,[b] 14.09,[b] 14.01,[b] 13.88,[b] 13.70,[b] 12.74,[c] 12.29[c]

[a] Chemical shifts (ppm) are listed for the free oligomer and for the complex at the maximum binding ratio studied. Assignments are given in parentheses with the numbering scheme shown in Figure 1. [b] Unassigned A·T peaks. [c] Unassigned G·C peaks.

Results

Oligomers with a Single GC Site. The oligomer d(ATATGCATAT) was used as a control here for longer range effects of ActD binding at the GC site. Such self-complementary oligomers have a C_2 symmetry axis located at the GC intercalation site and, thus, have relatively simple NMR spectra. Assignments for the imino protons of d(ATATGCATAT), when free and complexed with ActD, are shown in Table I and supplementary material Figure S1. The ^{1}H NMR assignments in this paper are based on chemical shift, NOE, and thermal melting studies as previously described (Scott et al., 1988). Addition of ActD to d(ATATGCATAT) results in a number of new signals which remain at the same positions at all R values (molar ratio of ActD/duplex). At R = 1.0, all of the new resonances have reached their maximum intensity with the total loss of the original peaks. The single signals for base pairs 7, 6, and 5 in the free duplex are replaced by two signals in the adduct due to binding of the unsymmetrical ActD phenoxazone ring at the GC site. The 1:1 complex, thus, does not have C_2 symmetry. Both new resonances for A·T base pair 6 are downfield of the original signal while both new signals for A·T base pair 5 are upfield. Compared to the G·C signal in the free duplex, one new G·C signal is upfield and one is downfield. This pattern can be observed clearly in the 0.5 ratio spectrum since the free and bound oligomers are in slow exchange. The chemical shift differences between the two related new peaks in the 1:1 complex decrease in the following order: 7 > 6 > 5 > 4.

The ^{31}P NMR spectra of d(ATATGCATAT) free and complexed to ActD are shown in Figure S2. Three new downfield peaks of equal intensity at −1.39, −2.50, and −2.67

ppm and a new upfield peak at −4.73 ppm are seen at R = 0.5 and greater. The most upfield of the three downfield peaks is the most sensitive to temperature changes (not shown). We have previously assigned the two most downfield peaks to phosphate groups at the intercalation site (Wilson et al, 1986b). The third downfield peak was assigned to the phosphate on the 3′ side of the benzenoid portion of ActD at a GC binding site (Wilson et al., 1986b).

The assignment and spectral changes are given in Table I and shown in Figure S3 for d(ATACGCGTAT) in the 9–15 ppm region of the proton spectrum. NOE experiments and peak areas have shown that the peak at 12.70 ppm is due to the two protons from base pair 6 and one of the protons from base pair 7 with the other base pair 7 proton resonance at 12.37 ppm. Except for slight upfield shifts and broadening, suggestive of some nonspecific stacking of ActD on the terminal base pair, no spectral changes were observed at $R > 1$. It is also possible that some weak binding of ActD at the GT site (Wells & Larson, 1970) occurs at the higher R values.

During temperature studies to assign the signals, we noted that at R = 1.2 and 50 °C all peaks are considerably broadened with the exception of the upfield peak at 12.3 ppm (top spectrum in Figure S3). The exchange rate for the G·C imino proton on one side is slower (narrow signal) than the rate for the G·C imino proton on the other side of the binding site (broad signal). The slower exchange rate is for the most upfield G·C signal which should be adjacent to the benzenoid side of the phenoxazone ring (Giessner-Prettre & Pullman, 1976). The reason for the difference in exchange rates is not clear.

^{31}P NMR spectra were monitored in the titration of d(ATACGCGTAT) with ActD (Figure S4). Three downfield peaks observed at −1.43, −2.59, and −2.87 ppm are characteristic of ActD intercalated at an isolated GC binding site.

The central sequence of the larger oligomer, d(ATATACGCGTATAT), is the same as d(ATACGCGTAT). Both ^{31}P and ^{1}H results for the titration of d(ATATACGCGTATAT) with ActD (not shown) are very similar to those obtained with the shorter oligomer. This finding indicates that the oligomer length does not have a significant effect on the ActD titration results with these sequences. Conclusions from these studies should, thus, also hold for similar sequences in high molecular weight DNA.

d(ATGCATGCAT): Nonadjacent GC Sites. The oligomer d(ATGCATGCAT) differs from the above oligonucleotides in that it contains two GC sites which are off the C_2 axis. The GC sites are flanked by A·T base pairs so that their local sequence environment is similar to the one GC site in d(ATATGCATAT). The addition of ActD to d(ATGCATGCAT) gives a number of new resonances in the 12–15 ppm region of the proton spectrum, and the chemical shift patterns depend on R (Figure 2). In the G·C region of the spectrum at R = 0.6, new peaks are observed at 12.32 and 12.73 ppm. These two peaks remain at the same positions but increase in intensity as R is increased. They are at almost identical positions as the peaks induced in the G·C region by ActD binding to d(ATATGCATAT). In the A·T region at R = 0.6, new signals are seen both upfield and downfield of the original A·T peaks. At R = 1.2, the parent signals and the new upfield A·T peaks have lost intensity relative to the signals in the 0.6 ratio spectrum. However, the new downfield A·T signals increase in intensity at R = 1.2. The new downfield peaks that are present in the 1.2 ratio spectrum again increase in intensity as R is increased to 1.8. There is no significant simplification of the spectrum as the ratio is increased from 0.6 to 1.8. At ratios above 1.8 no significant additional changes occur throughout the spectrum. We believe that these spectra can be understood, when R is in the range 0–1.8, if different mixtures of the free oligomer with 1:1 and 2:1 complexes coexist. Above the 1.8 ratio, only 2:1 complexes are present in significant amounts, simplifying the spectrum.

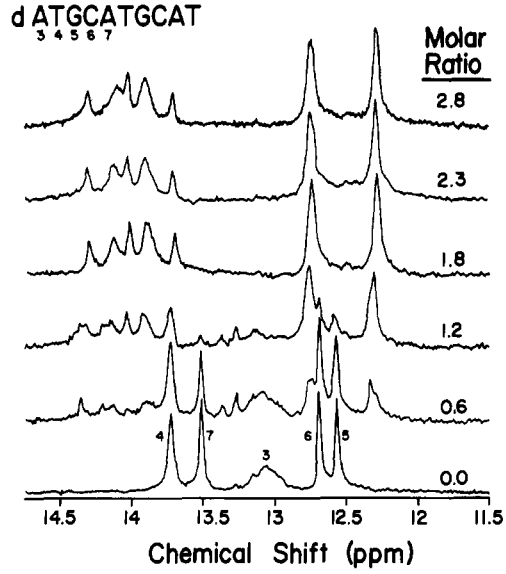
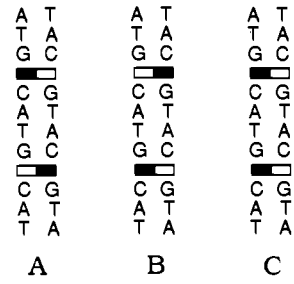


FIGURE 2: The 361.02-MHz imino proton spectra of d(ATGCATGCAT) at 5 °C as a function of the ratio of ActD to duplex. Peak assignments are given for the free oligonucleotide.

Scheme I



As shown in Scheme I, there are three possible ways of arranging ActD in the two GC binding sites of d(ATGCATGCAT). Both A and B have C_2 symmetry and could result in two G·C imino signals and two or three A·T imino signals depending on end fraying and peak separation. The unsymmetrical complex C could give twice as many signals as either A or B. Only two G·C signals are seen in Figure 2, as expected for either A or B, but there are more than three A·T signals. The sample at R = 2.8 was heated to increase the resolution (Figure S5). The signals sharpen and shift upfield with increasing temperature, and by 20 °C, the most downfield G·C signals have split into two peaks of approximately the same area, and six partially resolved peaks can be seen in the A·T proton region. The most upfield G·C signal contains twice the area of either of the downfield G·C signals, but it is not split into two peaks at any temperature. Signals in the A·T region are lost on increasing the temperature to 30 °C and above due to fraying of the terminal base pairs of the complex. These results clearly show that the spectra must contain at least four different G·C and six different A·T imino proton signals. This number of observed peaks is consistent with a mixture of A and B or with pure C (Scheme I). More complicated combinations are possible, however, and all three types of complex could be present if there is significant peak overlap.

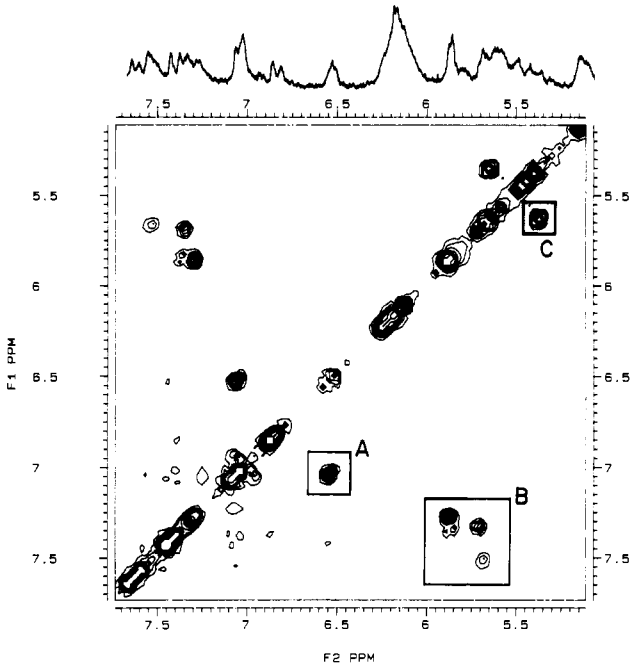


FIGURE 3: Low-field region of the 361.02-MHz COSY spectrum of d(ATGCATGCAT) at the ratio of two ActD per duplex. Box A shows the ActD H7–H8 connectivity. In box B the CH6 and CH5 connectivities for cytosine are shown. Box C contains a cross-peak for the Thr H_α–H_β protons (Brown et al., 1984).

In a COSY spectrum of the 2:1 complexes of ActD with d(ATGCATGCAT) either A or B (Scheme I) can give rise to two cytosine H5–H6 cross-peaks, depending on spectral resolution, while C can give up to four cross-peaks. The H5–H6 region of a COSY experiment for the ActD 2:1 complexes with d(ATGCATGCAT) is shown in Figure 3 (box B). There are four partially resolved H5–H6 cross-peaks, two at approximately 5.7 ppm and two overlapping peaks at approximately 5.9 ppm. As with the imino proton results, this finding is consistent with either a mixture of A and B or pure C (Scheme I), although all three complexes could be present if there is unresolved overlap of peaks in the 2D plot. Boxes A and C in Figure 3 contain cross-peaks for bound ActD. Box A contains the cross-peaks for the aromatic H7–H8 protons, and box C is for the peptide threonine H_α–H_β protons. Complexes A and B in Scheme I could each give one H7–H8 and one H_α–H_β cross-peak while the unsymmetrical complex C could give two cross-peaks in both boxes A and C. As can be seen in Figure 3, however, both box A and box C each contain only one cross-peak. Since the imino proton results and cytosine H5–H6 cross-peaks indicate that a minimum of A and B or pure C must be present, there must be significant overlap of ActD aromatic and peptide proton signals from the different complexes at the 2:1 ratio. The ActD cross-peaks, thus, do not provide additional information on the number of 2:1 complexes present with d(ATGCATGCAT).

On addition of ActD, new peaks are seen in the downfield spectral region of the ^{31}P NMR spectrum (Figure S6). The area of the downfield peaks increases up to a ratio of ca. 2. The ^{31}P results could also help resolve which of the A, B, or C combinations of Scheme I are present. A symmetrical complex such as A or B would be expected to give the pattern of three downfield ^{31}P signals observed with the simpler oligomers. The unsymmetrical complex C could give up to six downfield peaks depending on the spectral resolution. At R = 2.0 and above, five partially resolved downfield peaks can be seen with two signals overlapping at approximately −2.5 ppm; i.e., at least six downfield signals are present. Again, this number of peaks is consistent with either a mixture of A and B, pure C (Scheme I), or a more complex mixture with overlap of signals.

Increasing the temperature of an R = 2.8 sample (not shown) improves the resolution, and at 45 °C six downfield ^{31}P signals are seen. The downfield peaks shift with temperature according to the pattern observed for the ActD complex with d(ATATGCATAT).

Discussion

The first four oligomers of Figure 1 were designed to continue our analysis of ActD binding specificity, cooperativity, and long-range conformational effects when intercalated at GC sites. In agreement with earlier studies (Krugh & Nuss, 1979; Patel et al., 1981; Brown et al., 1984), imino proton shifts of d(ATATGCATAT) and d(ATACGCGTAT) indicate that ActD intercalates only at the GC site in the center of these oligomers. Adding ActD above a 1:1 ratio results in only small shifts at the terminal base pairs of these sequences. The three downfield ^{31}P signals induced by ActD binding to these two oligomers are similar and indicate that the backbone structural perturbations in the duplexes, caused by ActD intercalation, are similar for these sequences. This pattern of three ^{31}P peaks was also seen for the 1:1 complexes of ActD with oligomers containing a central GCGC sequence. Assignment of the peaks in the 1:1 ActD–d(TGCGCA) complex, with ^{17}O labeling, indicated that the two most downfield peaks of the three are for phosphate groups at the intercalation site (Wilson et al., 1986b). The third peak, which is shifted by a smaller amount due to binding of ActD and is more sensitive to temperature changes than the phosphate groups at the intercalation site, is for the phosphate on the 3′ side of the benzenoid portion of the ActD phenoxazone ring at the GC intercalation site. The ^{31}P spectra for 1:1 complexes of d(ATATGCATAT) and d(ATGCGCAT) are compared in Figure 4. The d(ATGCGCAT) complexes show twice as many peaks as for d(ATATGCATAT) because the GC binding sites are off the C_2 symmetry axis in d(ATGCGCAT), but the overall pattern of three downfield peaks for all 1:1 complexes is quite similar for these two oligomers.

A spectrum at the 1:1 ratio of ActD to d(ATGCATGCAT) is also shown in Figure 4. This spectrum again has the pattern of downfield ^{31}P signals expected for an unsymmetrical ActD complex. The difference in the number of peaks between the spectra for the oligomers containing two GC sites and the spectrum for the ActD complex with d(ATATGCATAT) is clear (Figure 4). The major conclusions from these ^{31}P results are that ActD at a 1:1 ratio can form two complexes at a single GC site, due to the two orientations of the phenoxazone ring system, and that these two complexes have similar ^{31}P chemical shifts regardless of the surrounding sequence.

Two downfield peaks are expected on ActD binding due to the two phosphate groups at the intercalation site. The presence of the third downfield signal indicates an asymmetrical conformational distortion of the double helix by bound ActD. The chemical shift of the third signal is more sensitive to temperature changes than the two signals for phosphate groups at the intercalation site, indicating that the long-range interactions of bound ActD are less strong or more flexible than those at the intercalation site.

Although the pattern of three downfield ^{31}P signals for oligomers with a central GC binding site (**1–3** in Figure 1) is similar, their imino proton shifts are significantly different. Interaction of the phenoxazone ring in either of the two possible orientations produces identical complexes with these oligomers since the GC binding site is on the oligomer C_2 axis.

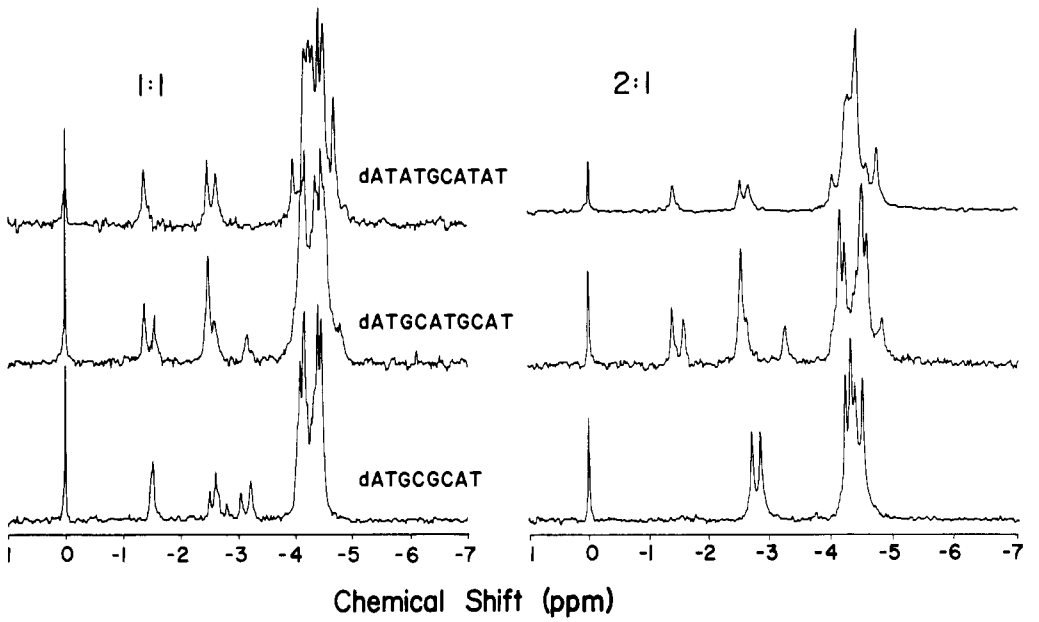
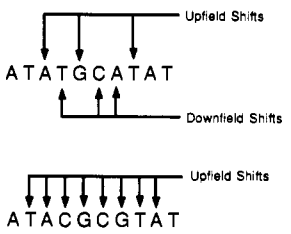


FIGURE 4: ^{31}P NMR spectra at the ratios of 1:1 (left) and 2:1 (right) (ActD/duplex) are shown to compare the features of d(ATGCATGCAT) (middle) to those of an oligomer that forms only a 1:1 complex [d(ATATGCATAT)] (top) and to those of an oligomer that forms a unique 2:1 complex [d(ATGCGCAT)] (bottom).

Scheme II



The direction of the shifts, relative to the free oligomers, is shown in Scheme II for the R = 1.0 complex with d(ATATGCATAT) and d(ATACGCGTAT). As can be seen, all shifts for the imino protons of d(ATACGCGTAT) on addition of ActD are upfield, whereas both upfield and downfield shifts are obtained with d(ATATGCATAT). This suggests that ActD produces long-range changes in base stacking which are dependent on the sequence flanking the GC binding site.

Although there are some basic similarities in the ^{31}P spectra for the 1:1 complexes, significant differences are evident in the comparison of the spectra at the 1:1 and 2:1 ratios in Figure 4. The downfield peaks of d(ATATGCATAT) do not change significantly when the amount of ActD is increased. The loss of resolution at R = 2.0 for the main peak is probably due to nonspecific end stacking of excess ActD as discussed above. However, the dramatic change in the ^{31}P spectrum in going from the 1:1 to the 2:1 ratio for d(ATGCGCAT) (Figure 4) provides strong evidence for a single symmetrical complex (Wilson et al., 1986b).

The spectral changes on going from the 1:1 to the 2:1 ratio are much smaller for d(ATGCATGCAT) than for d(ATGCGCAT). This complex 2:1 ^{31}P spectrum indicates that multiple orientations of ActD are still present with d(ATGCATGCAT). As discussed above, both mixtures of A and B or pure C could give this type of mixed spectrum (Scheme I). It is also possible that all three species are present but that the ^{1}H (both 1D and 2D) and ^{31}P NMR spectra appear less complicated than expected because of extensive signal overlap resulting from the similar nature of the complexes. It is quite clear that at both the 1:1 and 2:1 levels ActD can bind in both possible orientations at GC sites in d(ATGCATGCAT). Therefore, the dramatic structural constraints placed on ActD binding at adjacent GC sites are at least paritally relieved by the intervening A·T base pairs in d(ATGCATGCAT).

In conclusion, d(ATGCATGCAT) and d(ATGCGCAT), which have the same composition for the eight central base pairs, both form two 1:1 complexes with similar downfield ^{31}P signals. There are, however, significant differences with the G·C imino protons at the binding sites. All G·C signals are shifted upfield by ActD with d(ATGCGCAT), as with d(ATACGCGTAT), while both upfield and downfield shifts are obtained with d(ATGCATGCAT), as with d(ATATGCATAT). At the 2:1 level only a single orientation of ActD is observed in a unique complex with d(ATGCGCAT), whereas more than one orientation exists with d(ATGCATGCAT). These extremely sequence-specific binding situations could, at present, be resolved only by NMR investigations with oligonucleotides. The reported diversity of ActD interactions with natural DNA samples [cf. Waring (1981), Wells and Larson (1970), Walker et al. (1985), Wartell et al. (1975), and Friedman and Manning (1984)] is easily understood from the complex and quite different results obtained here with the oligomers containing two GC ActD binding sites.

Supplementary Material Available

Proton and ^{31}P NMR spectra of oligomers **1**, **2**, and **4** as a function of the molar ratio of ActD to oligomer duplex (7 pages). Ordering information is given on any current masthead page.

References

Brown, S. C., Mullis, K., Levenson, C., & Shafer, R. H. (1984) *Biochemistry 23*, 403–408.

Dervan, P. B. (1986) *Science (Washington, D.C.) 232*, 464–471.

Fox, K. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 9271–9285.

Friedman, R. A. G., & Manning, G. S. (1984) *Biopolymers 23*, 2671–2714.

Giessner-Prettre, C., & Pullman, B. (1976) *C. R. Seances Acad. Sci., Ser. D 283*, 675–676.
Krugh, T. R., & Nuss, M. E. (1979) in *Biological Applications of Magnetic Resonance* (Schulman, R. G., Ed.) Chapter 3, Academic, New York.
Marzilli, L. G., Banville, D. L., Zon, G., & Wilson, W. D. (1986) *J. Am. Chem. Soc. 108*, 4188–4192.
Muller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.
Patel, D. J. (1974) *Biochemistry 13*, 2396–2402.
Patel, D. J., Koglowski, S. A., Rice, J. A., Broka, C., & Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7281–7284.
Redfield, A. G., Kunz, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114–117.
Remers, W. (1984) in *Antineoplastic Agents* (Remers, W., Ed.) pp 167–178, Wiley, New York.
Scott, E. V., Jones, R. L., Banville, D. L., Zon, G., Marzilli, L. G., & Wilson, W. D. (1988) *Biochemistry 27*, 915–923.
Shafer, R. H., Burnette, R. R., & Mirau, P. A. (1980) *Nucleic Acids Res. 8*, 1121–1131.
Sobell, H. M. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 153–190.
Stec, W. J., Zon, G., Egan, W., Byrd, R. A., Phillips, L. R., & Gallo, K. A. (1985) *J. Org. Chem. 50*, 3908–3913.
Walker, G. T., Stone, M. P., & Krugh, T. R. (1985) *Biochemistry 24*, 7471–7479.
Waring, M. (1981) in *The Molecular Basis of Antibiotic Action* (Gale, F., et al., Eds.) pp 314–333, Wiley, London.
Wartell, R. M., Larson, J. E., & Wells, R. D. (1975) *J. Biol. Chem. 250*, 2698–2702.
Wells, R. D., & Larson, J. E. (1970) *J. Mol. Biol. 49*, 319–342.
Wilson, W. D., Jones, R. L., Zon, G., Banville, D. L., & Marzilli, L. G. (1986a) *Biopolymers 25*, 1997–2015.
Wilson, W. D., Jones, R. L., Zon, G., Scott, E. V., Banville, D. L., & Marzilli, L. G. (1986b) *J. Am. Chem. Soc. 108*, 7113–7119.

Determination of Equilibrium Binding Affinity of Distamycin and Netropsin to the Synthetic Deoxyoligonucleotide Sequence d(GGTATACC)$_2$ by Quantitative DNase I Footprinting†

Emily L. Fish,‡ Michael J. Lane,§ and John N. Vournakis*,‡

Molecular Therapeutics, Inc., 400 Morgan Lane, West Haven, Connecticut 06516, and Department of Medicine, SUNY—Health Science Center at Syracuse, Syracuse, New York 14850

Received February 18, 1988

ABSTRACT: A new method for determining the equilibrium binding constant of antitumor drugs to specific DNA sequences by quantitative DNase I footprinting is presented. The use of a short synthetic DNA oligomer to define a homogeneous population of DNA binding sites enables the calculation of the free drug concentration and the fraction of DNA sites complexed with drug in solution and is described for the first time. Since a 1:1 stoichiometry is observed for each drug–oligomer DNA complex, it becomes possible to calculate equilibrium binding constants in solution. By use of this technique, the binding affinities of the nonintercalating drugs netropsin and distamycin to the synthetic oligonucleotide d(GGTATACC)$_2$ are determined to be K_a(25 °C) = 1.0×10^5 and 2.0×10^5 M^{-1}, respectively. Quantitation of the temperature dependence associated with complex formation results in a determination of standard enthalpies of −3.75 and −8.48 kcal mol^{-1} for the binding of netropsin and distamycin, respectively. Calculation of other thermodynamic parameters are found to be in agreement with previous studies and indicate that the DNA binding process for these compounds is predominantly enthalpy driven. This method of quantitative DNase I footprinting is demonstrated to be a useful technique for the measurement of drug affinities to specific binding sites on DNA oligomers which are designed and synthesized expressly for this purpose. Applications of the technique to the determination of drug binding affinities at specific sites within native DNA sequences are discussed.

Studies of the interaction of antibiotic and antiviral compounds with DNA have become increasingly important in the development of a rational approach to design new chemotherapeutic agents which interact at the gene level. It is important, in attempting to elucidate the structure of complexes of such compounds with DNA, to have available an experimental technique for measuring binding affinities to specific DNA sequences. This has necessitated the development of a new method, described in this paper, for the direct measurement and comparison of binding constants at multiple sites in known DNA sequences.

Previously, studies of the affinities of DNA binding drugs utilized primarily synthetic homonucleotide and alternating dinucleotide sequence polynucleotides to determine binding

†This work was supported by NIH Grant GM31895, Molecular Therapeutics, Inc., and the Molecular Genetics Center at Dartmouth.
*Author to whom correspondence should be addressed.
‡Molecular Therapeutics, Inc.
§SUNY—Health Science Center at Syracuse.

0006-2960/88/0427-6026$01.50/0