Actinomycin D Binds Strongly to d(TGTCATTG), a Single-Stranded DNA Devoid of GpC Sites†

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ABSTRACT: Despite the absence of the GpC sequence and complete self-complementarity, d(CGTCGTCG) has recently been shown to bind strongly to actinomycin D (ACTD) with a binding density of about one drug molecule per strand. To further elucidate the nature of such a binding, studies are herein made with single-base G → A and C → T replacements in d(CGTCGTCG) to identify the DNA bases that play important roles in the strong ACTD binding of this oligomer. On the basis of these results, the octamer d(TGTCATTG) has been identified as a potentially strong ACTD binder. Indeed, binding titration confirms such an expectation and reveals an ACTD binding constant of about 1×10^7 M^{-1} and a binding density of roughly 0.8 drug molecule per DNA strand for this strong binding mode. Similar binding studies with single-base substitutions on d(TGTCATTG) further reveal the relative importance of the C and G bases on its ACTD binding, with the 3′-terminus G appearing to be the most crucial base. Further base substitutions lead to the conclusion that these C and G bases act in concert rather than individually in the ACTD binding of d(TGTCATTG). Spectral comparisons with the apparently single-stranded GpC-containing d(TGCTTTG) led to the proposal of a speculated monomeric hairpin binding model to account for the experimental observations. This model makes use of the notion that ACTD prefers to have the 3′-sides of both G bases stacking on the opposite faces of its planar phenoxazone chromophore, a principle akin to its classic preference for the GpC sequence in duplex form. The finding that ACTD can bind strongly to single-stranded DNA of special sequence motifs may have important implications.

Actinomycin D (ACTD)[1] is an antitumor antibiotic that contains a planar 2-aminophenoxazin-3-one chromophore and two bulky cyclic pentapeptide lactones. The biological effect of this drug is believed to be the consequence of its ability to bind to duplex DNA, which results in the inhibition of DNA-dependent RNA polymerase activities. Earlier studies with synthetic polynucleotides had indicated that this drug is G·C specific and exhibits negligible affinity for A and T bases (*1*, *2*). Detailed spectroscopic and hydrodynamic studies led to the conclusion that ACTD binds to DNA via intercalating its phenoxazone chromophore between the DNA base pairs and anchoring its pentapeptide rings on the minor groove (*3*, *4*). The binding is, however, quite sequence specific and had been shown to prefer greatly the GpC sequence. This base sequence specificity derives mainly from the formation of strong hydrogen bonds in the minor groove between the N-2 amino group and N-3 ring nitrogen of guanine residues and the carbonyl oxygen atom and amide groups of threonine residues of the cyclic pentapeptides, respectively (*5*, *6*). Additional stability derives from hydrophobic interactions between groups on the pentapeptides and sugar residues as well as from other specific weaker hydrogen bonds (*6*).

Binding of a single ACTD molecule spans about 4 base pairs of DNA (*6*). It is, thus, reasonable to suspect that the binding affinity of ACTD depends not only on the presence of the GpC sequence but also on the base pairs adjacent to this sequence. Indeed, systematic binding and kinetic studies with oligomers containing self- and non-self-complementary -XGCY- sequences have confirmed that the ACTD binding affinity and its dissociation kinetics are greatly affected by the nature of the adjacent bases (*7*, *8*). For example, ACTD binds stronger and dissociates considerably slower from the -TGCA- site than those of -AGCT- and −CGCG- sequences, and that ACTD binds weakly to and dissociates rapidly from sequences in which X = G and/or Y = C. Further studies with -XGCX- sequences that contain adjacent base pair mismatches, where X = any base, revealed that strong ACTD binding and slow dissociation are also exhibited by sequences containing adjacent T/T mismatches, and the pyrimidine/pyrimidine homo mismatches are more favorable than the corresponding purine/purine mismatches (*9*).

Although the GpC sequence specificity of ACTD has been well characterized, there have been recent reports to indicate that this drug may also bind strongly to some non-GpC-containing sequences (*10*−*12*) and even to some single strands (*13*−*17*). In particular, calorimetric studies by Synder et al. (*10*) have indicated that despite the absence of the GpC sequence ACTD can bind cooperatively to the octamer d(5′CGTCGACG3′) with a binding constant on the order of 10^7 M^{-1} and a 2:1 drug to duplex stoichiometry. Further optical studies led us to postulate that ACTD may in fact

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[1] Abbreviations: ACTD, actinomycin D; 7-AM-ACTD, 7-amino-actinomycin D; CD, circular dichroism; HEPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; AAEE, acryloylaminoethoxy-ethanol.

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stack on the G·C base pairs at both ends of this oligomeric duplex (*18*). This was based on the rationale that the classic ACTD preference of the G3′-p-5′C site implies the stacking preference of the drug on the 3′-side of the G base (*19*). Subsequent studies from this laboratory, however, led to the additional finding that, despite the lack of complete self-complementarity and the absence of the GpC sequence, ACTD also binds strongly to d(CGTCGTCG) and d(CGACGACG) (*20*). Although the experimental results appear not to contradict with the speculated binding model of ACTD stacking at the terminal G or G·C base pairs, the question remains as to whether it binds to a dimeric or a monomeric DNA. Indeed, the finding of significantly weaker ACTD affinity of the heteroduplex d(CGTCGTCG)·d(CGACGACG) as compared to its constituent oligomers and interesting capillary electrophoretic evidence seem to suggest that d(CGXCGXCG) binds to ACTD as a single-stranded or as a monomeric hairpin form (*20*).

To further delineate the nature of ACTD binding to d(CGXCGXCG), systematic binding studies were undertaken with related oligomers derived from progressive base replacements. These studies led to the finding that ACTD binds strongly to a sequence motif of d(TGTCATTG) of apparent single strand conformation. The results and their significance are reported herein, and a plausible binding model is presented to account for the experimental observations. Uncovering ligands that bind to single-stranded DNA with particular sequence preferences may be of importance in the regulation of processes that require single-stranded DNA as intermediates.

MATERIALS AND METHODS

Synthetic oligonucleotides were purchased from Research Genetics (Huntsville, AL) and used without further purification. These oligomers were purified by the vendor via reverse-phase oligonucleotide purification cartridges and exhibited single-band electrophoretic mobilities in denaturing polyacrylamide gel electrophoresis with stated purity of ≥95%. Concentrations of the DNA solutions (in nucleotide) were determined by measuring the absorbances at 260 nm after melting and calculating the concentrations by Beer's law. The extinction coefficients of DNA oligomers were obtained via nearest-neighbor approximation using mono- and dinucleotide values tabulated in Fasman (*21*). ACTD and 7-AM-ACTD were purchased from Serva. Concentrations of the drug solutions were determined by measuring the absorbances at 440 nm (for ACTD) and 528 nm (for 7-AM-ACTD), using extinction coefficients of 24 500 and 23 600 cm^{-1} M^{-1}, respectively. Stock solutions for oligonucleotides and drugs were prepared by dissolving in 10 mM Tris−borate buffer solution of pH 8 containing 0.1 M NaCl and 1 mM $MgCl_2$. Absorption spectra were measured with a Cary 1E spectrophotometric system. Data were collected from 600 to 350 nm. Absorption spectral titrations were carried out by starting with a 5 μM ACTD solution of 2 mL, followed by progressive additions of the oligomer stock at equal time intervals. Absorbance differences between 427 and 480 nm during absorption spectral titrations were used to obtain the binding isotherms and Scatchard plots. The binding parameters were deduced via least-squares fits on the linear portions of the Scatchard plots. The bound extinction coefficients were estimated through extrapolations to high DNA concentrations. Circular dichroic (CD) spectra were measured at room temperature with a Jasco J-500A recording spectropolarimeter using water-jacketed cylindrical cells of 2 cm path length. Data were collected from 350 to 230 nm with a scan speed of 50 nm/min. The CD difference spectra were obtained by subtracting out the spectra of 40 μM oligomers (per nucleotide) from those in the presence of 7 μM ACTD. Fluorescence spectra were obtained at 20 °C with a SLM 48000S system. Emission spectra were measured in the region of 550−750 nm with 525 nm excitation, whereas excitation spectra were taken in the region of 420−600 nm with 650 nm emission monitoring.

Table 1: Comparison of ACTD Binding and Melting Parameters of d(CGTCGTCG) and Its Single-Base Substituted Derivatives[a]

oligomer	K_b (μM^{-1})[b]	n (per strand)	T_m^0 (°C)	T_m (°C)
d(CGTCGTCG)	25 ± 2	1.1	<20	65
d(***T***GTCGTCG)	19 ± 1	1.0	<20	62
d(C***A***TCGTCG)	3.6 ± 0.1	1.1	<20	52
d(CGT***T***GTCG)	0.65 ± 0.01		<20	37
d(CGTC***A***TCG)	16 ± 1	1.0	<20	63
d(CGTCGT***T***G)	18 ± 1	0.8	<20	61
d(CGTCGTC***A***)	1.90 ± 0.01	0.9	<20	37

[a] T_m^0 and T_m designate the estimated (±0.5°C) melting temperatures of 40 μM DNA (nucleotide) in the absence and in the presence of 7 μM ACTD, respectively. Thermal denaturation experiments were carried out with 1 cm semimicro cells by monitoring absorbances at 275 nm, with a heating rate of 0.5 °C/min being maintained by the temperature controller accessory. [b] Equilibrium binding titration was carried out at 20 °C.

Capillary electrophoretic experiments were carried out with a Beckman P/ACE 5000 instrument. Capillary electrophoresis oligonucleotide run buffer and 75 μm i.d. × 375 μm o.d. capillaries internally coated with poly(AAEE) were purchased from Bio-Rad (Hercules, CA). Samples were loaded by pressure for 10 s and run at 15 kV and 25 °C with 254 nm absorbance detection. The sieving buffer was replenished before each run using a 5 min purge cycle at a pressure of 20 psi. Two water rinse cycles were used to remove residual buffer from the capillary and electrode surfaces to prevent buffer carryover into the sample vial.

RESULTS

Studies with Single-Base G → A or C → T Substitution in d(CGTCGTCG). In an effort to delineate the DNA bases which are responsible for the strong ACTD affinity of d(CGTCGTCG), studies were made with oligomers derived from single-base G → A or C → T substitutions on this octamer. Only the purine to purine or pyrimidine to pyrimidine replacement was made to ensure minimal perturbation on the structural integrity of the original sequence. Equilibrium binding parameters deduced via least-squares fits on the linear portions of the Scatchard plots along with their thermal melting characteristics are summarized in Table 1. The results indicate that the ACTD binding strengths of d(***T***GTCGTCG), d(CGTC***A***TCG), and d(CGTCGT***T***G) are basically unaltered, all exhibiting binding constants on the order of 2 × 10^7 M^{-1} with binding densities of near unity per strand. This suggests that the C and G bases at these locations are not essential for the strong ACTD binding of d(CGTCGTCG). On the other hand, significant reductions in ACTD affinity were observed for d(C***A***TCGTCG),

Table 2: Comparison of ACTD Binding and Melting Parameters for d(TGTCATTG) and Its Base-Substituted Derivatives[a]

oligomer	K_b (μM^{-1})[b]	n (per strand)	T_m^0 (°C)	T_m (°C)
d(TGTCATTG)	10.2 ± 0.2	0.8	26	62
d(T***A***TCATTG)	0.57 ± 0.01		<20	43
d(TGT***T***ATTG)	0.29 ± 0.02		<20	36
d(TGTCATT***A***)	<0.1		<20	33
d(T***A***T***T***ATTG)	≪0.1		<20	<20
d(T***A***TCATT***A***)	≪0.1		<20	<20
d(TGT***T***ATT***A***)	≪0.1		<20	<20
d(T***A***T***T***ATT***A***)	≪0.1		<20	<20

[a] T_m^0 and T_m designate the estimated (±0.5°C) melting temperatures of 40 μM DNA (nucleotide) in the absence and in the presence of 7 μM ACTD, respectively. Absorbance changes on melting were monitored at 275 nm. [b] Equilibrium binding titration was carried out at 20 °C.

d(CGT***T***GTCG), and d(CGTCGTC***A***), with binding constants of 3.7×10^6 M^{-1}, 0.65×10^6 M^{-1}, and 1.9×10^6 M^{-1}, respectively. The extents of melting temperature increases upon drug binding are qualitative measures of binding affinities and appear to generally conform to the titration results. All the strong-binding oligomers exhibit melting temperatures of around 62 °C for their drug complexes while those of the weaker binders are correspondingly lower.

The finding that d(CGT***T***GTCG) and d(CGTCGTC***A***) exhibit the weakest binding strengths strongly indicates the crucial roles played by C at the fourth position and the terminal G at the 3′-end. The less dramatic but still considerable reduction in the ACTD binding affinity for d(C***A***TCGTCG) suggests that the G at the second position also plays a significant role. Therefore, the C and G bases at these locations may have contributed individually or in concert to bind strongly to ACTD. On the basis of these results it seems reasonable that, by keeping intact those essential C and G bases, the octamer with the d(TGTCATTG) motif should be expected to exhibit a considerable ACTD binding affinity.

Actinomycin D Binds Strongly to d(TGTCATTG). Indeed, absorbance titrations revealed that d(TGTCATTG) binds strongly to ACTD with a binding strength of about 1×10^7 M^{-1}, comparable to those of the parent octamer d(CGTCGTCG) and its strong-binding single-base substituted derivatives. The strong ACTD binding of d(TGTCATTG) is further confirmed by the dramatic melting temperature increase upon its complex with the drug. The melting temperature for DNA alone in a buffer containing 0.1 M NaCl/1 mM $MgCl_2$ is below 20 °C whereas the melting temperature of its drug complex is found to be around 62 °C (see Table 2), nearly identical to that of d(CGTCGTCG) and its strong-binding single-base substituted derivatives. The absence of cooperative melting above 20 °C for d(TGTCATTG) in buffer suggests its single-stranded conformational dominance at room temperature, as to be expected from the non-self-complementary nature of the sequence. Additional supports for the strong ACTD binding of d(TGTCATTG) were provided by CD, fluorescence, and capillary electrophoretic measurements. Migration retardation upon ACTD binding was observed for d(TGTCATTG) in the gel-filled capillary. Considerable fluorescence spectral alteration was found with 525 nm excitation, exhibiting intensity reduction and enhancement in the 670 and 590 nm regions, respectively. Sizable CD intensities for ACTD were also induced upon binding to this oligomer in the 230−300 nm spectral region. Comparisons of these spectral characteristics with related oligomers and their significance will be presented in later sections.

The Interior C and the 3′-Terminus G in d(TGTCATTG) Are Critical for Its Strong ACTD Affinity. To elucidate the relative importance of C and G bases in the ACTD binding of d(TGTCATTG), single-base G → A or C → T substitutions on this octamer were made to form d(T***A***TCATTG), d(TGT***T***ATTG), and d(TGTCATT***A***), and their binding studies with ACTD were carried out. The results of binding and melting studies of these oligomers along with those of the parent octamer are shown in Table 2. The sequence d(TGTCATT***A***) exhibits the weakest ACTD affinity of less than 1×10^5 M^{-1}, which is followed by 3×10^5 M^{-1} and 6×10^5 M^{-1} for d(TGT***T***ATTG) and d(T***A***TCATTG), respectively. These results indicate that the G at the 3′-terminus plays a crucial role while the C and G bases of the GTC sequence are also of considerable importance. Binding isotherms for d(TGTCATTG) and its single-base substituted derivatives are compared in Figure 1A. The relative ACTD binding affinities for these oligomers can more easily be seen from the corresponding Scatchard plots and are shown in Figure 1B. It is worth pointing out the unusual feature of the Scatchard plot for d(TGTCATTG) in which the initial points of the titration (high r values) appear flat until around $r = 0.8$ whereupon a steep-rising curve sets in.

To further investigate whether the C and G bases at these positions are acting as individuals or in some concerted manners, studies were also made with oligomers with only one of these bases left unaltered. The results indicate that oligomers d(TGT***T***ATT***A***), d(T***A***TCATT***A***), and d(T***A***T***T***ATTG) all exhibit negligible ACTD affinity, similar to that of d(T***A***T***T***ATT***A***) in which all the C and G bases in the sequence have been replaced. These results imply that the C and G bases at these positions are acting in a concerted manner rather than individually to provide for strong ACTD binding.

Studies with Base Deletion from the 5′-End of d(TGTCATTG). In view of the finding that G at the 3′-terminus is essential for the strong ACTD binding of d(TGTCATTG), it is pertinent to ask what would be the minimal length of a sequence containing the 3′-terminus G that would still exhibit significant ACTD binding affinity. Studies were thus carried out with oligomers obtained via truncating bases from the 5′-end. The binding and melting results are included in Table 3. It was found that d(GTCATTG) still maintains a significant binding strength of 2.5×10^6 M^{-1}, albeit somewhat reduced. However, hexamer d(TCATTG) and pentamer d(CATTG) derived from removing the next one and two base(s) from the 5′-end exhibit very weak ACTD binding affinities. Thus, the shortest oligomer exhibiting significant ACTD binding appears to be d(GTCATTG). These results confirm the important role of the G base near the 5′-end and further support the notion that the strong ACTD binding of d(TGTCATTG) derives from the concerted action of the C and G bases in the sequence. The T base at the 5′-terminus appears to facilitate a somewhat tighter drug binding, likely via its interaction with one of the pentapeptide rings.

Studies with Oligomers Containing the GpC Sequence. To elucidate the possible role of GTC near the 5′-end in

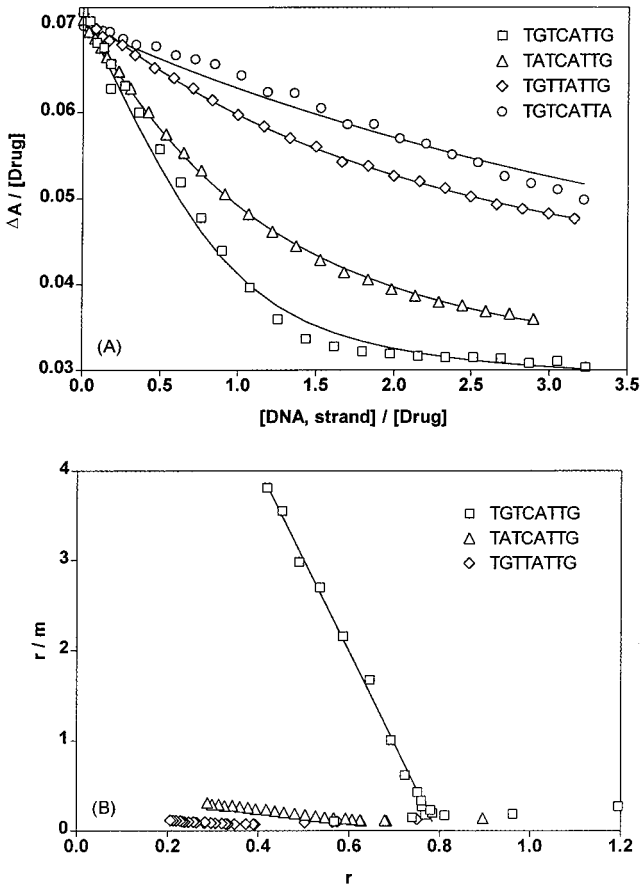

FIGURE 1: Comparison of binding isotherms (panel A) and Scatchard plots (panel B) of ACTD binding to d(TGTCATTG) and its single-base G → A or C → T substituted derivatives. The Scatchard plot for d(TGTCATTA) is nearly identical to that of d(TGTTATTG) and was not shown. Spectral titrations were carried out at 20 °C, and the absorbance differences between 427 and 480 nm were used to obtain the binding isotherms. [Bound drug]/[DNA, strand] is designated by *r*, and *m* represents the free drug concentration (in μM). Solid lines in panel B are linear least-squares fits on the linear portions of the data points. Solid lines in panel A are curve fits using a simple model of 1:1 drug to single strand binding. Binding constants of 1.29, 0.27, 0.081, and 0.017 μM^{-1} for d(TGTCATTG), d(TATCATTG), d(TGTTATTG), and d(TGTCATTA) were extracted from such fits.

d(TGTCATTG) on its ACTD binding and to see how it compares with the classic GpC site which differs only by the absence of a T base, studies were also made with d(TGCATTG) and related oligomers containing GpC sequences. These results along with those of melting experiments are also summarized in Table 3. Significant ACTD affinities were found for d(TGCATTG) and d(AGCTTTG) with binding constants of $1.3 \times 10^6\ M^{-1}$ and $3.4 \times 10^6\ M^{-1}$, respectively. These results appear to conform to the self-complementary nature of TGCA and AGCT sequences and the expectation of ACTD binding at the dimeric duplex form of these sites. Such a reasoning seems to be further supported by the much weaker ACTD affinity exhibited by d(AGCATTG) where AGCA is not self-complementary. Interestingly, however, despite the absence of self-complementary tetranucleotide sequence, strong ACTD binding was observed for d(TGCTTTG) with an affinity of $6.7 \times 10^6\ M^{-1}$, which is somewhat higher than those of d(TGCATTG) and d(AGCTTTG).

Table 3: Comparison of ACTD Binding and Melting Parameters of d(TGTCATTG) with Shorter Derivatives and Some GpC-Containing Oligomers[a]

oligomer	K_b (μM^{-1})[b]	*n* (per strand)	T_m^0 (°C)	T_m (°C)
d(TGTCATTG)	10.2 ± 0.2	0.8	26	62
d(GTCATTG)	2.46 ± 0.08	0.8	<20	58
d(TCATTG)	≪0.1		<20	<20
d(CATTG)	≪0.1		<20	<20
d(AGCATTG)	<0.1		<20	26
d(TGCATTG)	1.30 ± 0.03	1.0	<20	45
d(GCATTG)	0.85 ± 0.01	0.9	<20	31
d(AGCATTA)	≪0.1		<20	<20
d(TGCATTA)	<0.1		<20	27
d(AGCTTTG)	3.42 ± 0.07	0.64	<20	53
d(TGCTTTG)	6.7 ± 0.2	1.0	<20	50
d(GCTTTG)	1.15 ± 0.02		<20	41
d(AGCTTTA)	<0.1		<20	27
d(TGCTTTA)	≪0.1		<20	26

[a] T_m^0 and T_m designate the estimated (±0.5°C) melting temperatures of 40 μM DNA (nucleotide) in the absence and in the presence of 7 μM ACTD, respectively. Absorbance changes on melting were monitored at 275 nm. [b] Equilibrium binding titration was carried out at 20 °C.

To see if the G at the 3′-terminus, like that of d(TGTCATTG), plays a significant role in the ACTD binding of these GpC-containing oligomers, studies were then made with oligomers in which the G at the 3′-end of these heptamers was replaced by ***A***. If the binding were to occur mainly at the dimeric duplex sites of the self-complementary -XGCY- sequences, there would have been little difference between the ACTD binding affinities of d(XGCYTTG) and d(XGCYTTA). Interestingly, all oligomers including d(AGCTTT***A***), d(TGCATT***A***), and d(TGCTTT***A***) exhibit weak binding despite the fact that their corresponding oligomers containing the 3′-terminus G bind ACTD quite favorably. The much weaker binding of d(TGCATT***A***) and d(AGCTTT***A***) as compared to d(TGCATT***G***) and d(AGCTTT***G***) strongly implies that ACTD may not be dominated by intercalation at the *duplex* TGCA or AGCT site resulting from the dimeric duplex formation, but rather the 3′-terminus G is intimately involved in the ACTD binding. If it were a simple intercalation at the TGCA or AGCT *duplex* site, the ACTD binding affinities would have been nearly identical for d(TGCATT***G***) and d(TGCATT***A***) or for d(AGCTTT***G***) and d(AGCTTT***A***) since the bases near the 3′-end apparently are not involved in such a duplex formation. The important role of the 3′-terminus G is further manifested by the finding that d(TGCTTTG) binds to ACTD somewhat stronger than those of d(TGCATTG) and d(AGCTTTG). It is apparent that the ability to form a dimeric duplex is greatly reduced in d(TGCTTTG) as compared to d(TGCATTG) and d(AGCTTTG). It is also interesting to note that, in contrast to d(TGCATTG), d(AGCATTG) binds negligibly to ACTD, a dramatic illustration on how the bases adjacent to the GpC site can greatly affect its ACTD binding ability. The fact that d(TGCATTG) binds ACTD somewhat weaker than d(AGCTTTG) also argues against the simple intercalation at the *duplex* TGCA or AGCT site since our earlier ACTD binding studies with self-complementary -XGCY- sites have indicated a stronger ACTD binding at the -TGCA- than at the -AGCT- site (*7*). Further evidence against the notion of predominant ACTD binding at the -TGCA- or -AGCT- duplex site of these oligomers comes from the findings that

both d(GCATTG) and d(GCTTTG) exhibit binding constants of around 1×10^6 M^{-1}, only slightly lower than those of d(TGCATTG) and d(AGCTTTG), respectively. The absence of self-complementary tetranucleotide sequences for the above hexamers would have greatly reduced their ACTD affinities if the drug were to bind at the GpC duplex site. All of these results indicate that the G base at the 3′-terminus plays a crucial role in the ACTD binding even for these GpC-containing sequence motifs. It is interesting to note in passing that, despite the presence of GpC sequence, these strong-binding oligomers exhibit somewhat lower ACTD affinities than that of d(TGTCATTG).

CD and Fluorescence Spectral Characteristics. ACTD is an optically active compound and exhibits a weak intrinsic CD spectrum in the 230−350 nm region. Upon DNA binding, however, significant CD intensities can be induced. Inductions of a moderately strong positive CD intensity at 293 nm and a strong negative CD intensity in the DNA spectral region are characteristic features. Thus, comparison of the induced CD intensities can provide some qualitative measures as to the ACTD binding abilities of various DNA sequences. In addition, since CD spectral characteristics are intimately related to conformations, comparative studies with related oligomers could provide additional information as to the nature of the complexes formed.

Difference CD spectra (ACTD + DNA − DNA) for ACTD complexes with d(TGTCATTG) and the 5′-base-deleted derivatives are shown in Figure 2A. A positive intensity near 293 nm and a broad strong negative intensity (with double humps near 270 and 250 nm) are induced by the d(TGTCATTG) binding. Removing the T base at the 5′-end resulted in d(GTCATTG), which induces a reduction in the 270 nm intensity. Further deletions of the 5′-base resulted in d(TCATTG) and d(CATTG), which exhibit difference CD spectra very similar to that of the drug alone in buffer, confirming negligible ACTD affinities for these oligomers. It may be of interest to point out that in contrast to an induction of a negative CD intensity near 460 nm upon ACTD binding to a *duplex* GpC site, the binding to d(TGTCATTG) is similar to that of stacking to dGMP5′, which induces a weak positive CD intensity in this spectral region (*22*).

Difference CD spectra on ACTD binding to various GpC-containing oligomers are compared in Figure 2B. A strong negative CD intensity near 250 nm is induced by binding to d(TGCATTG) whereas no discernible enhancement is seen with d(AGCATTG) binding, in agreement with its much weaker ACTD affinity. Removal of the 5′-base, however, resulted in d(GCATTG), which exhibits nearly identical CD features as those of d(TGCATTG). It is interesting to note, however, that the induced CD caused by d(TGCTTTG) binding is nearly identical to that of d(TGTCATTG) with a double-humped feature, suggesting conformational similarity of these two drug complexes.

Difference fluorescence emission spectra (7-AM-ACTD + DNA − 7-AM-ACTD) for complexes of these oligomers are compared in Figure 3. As expected from the significantly different CD spectra exhibited by ACTD binding to d(TGTCATTG) and d(GTCATTG), their induced fluorescence characteristics are also distinctly different. The former induces an intensity depression near 670 nm with a concomitant larger intensity enhancement around 590 nm

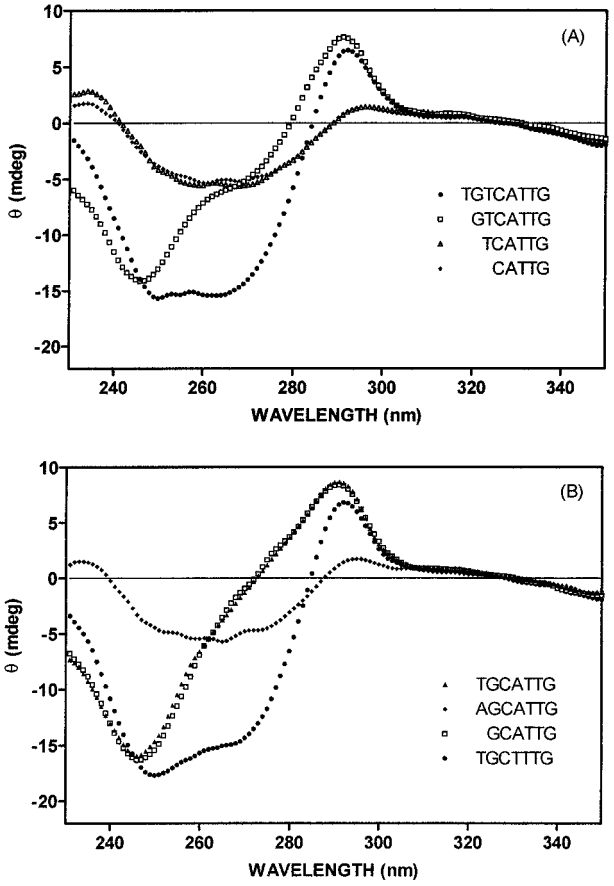

FIGURE 2: Comparison of difference CD spectra (ACTD + DNA − DNA) of d(TGTCATTG) and the 5′-base-deleted oligomers (panel A) and of some GpC-containing oligomers (panel B). CD spectra were measured at room temperature using a cylindrical cell of 2 cm path length with 40 μM (nucleotide) oligomer in the absence and in the presence of 5 μM ACTD.

whereas the latter exhibits only a strong intensity enhancement near 640 nm. Consistent with their weak ACTD affinities, d(TCATTG) and d(CATTG) exhibit only slight intensity reductions near 660 nm. In agreement with their near identical induced CD spectra, d(TGCATTG) and d(GCATTG) induce very similar fluorescence features with moderate intensity reduction near 660 nm upon binding to 7-AM-ACTD. And in line with the very similar induced CD spectral features to the d(TGTCATTG) complex, d(TGCTTTG) induces nearly identical fluorescence spectral characteristics except for a somewhat stronger effect upon binding to 7-AM-ACTD. In contrast, despite the presence of intensity reduction near 670 nm, the intensity enhancement around 590 nm is hardly evident for binding to d(TGCATTG). It is also noteworthy that both the difference CD and fluorescence spectra of d(GCATTG) do not differ greatly from those of d(TGCATTG), supporting the notion that binding at the -TGCA- duplex site may not be the dominant contributor to the ACTD binding of d(TGCATTG).

DISCUSSION

Single-base G → A and C → T replacements in d(CGTCGTCG) led us to the identification of the DNA bases which play critical roles in the strong ACTD binding of this oligomer. These results formed the basis for the choice of d(TGTCATTG) as a potentially strong ACTD binder. Indeed,

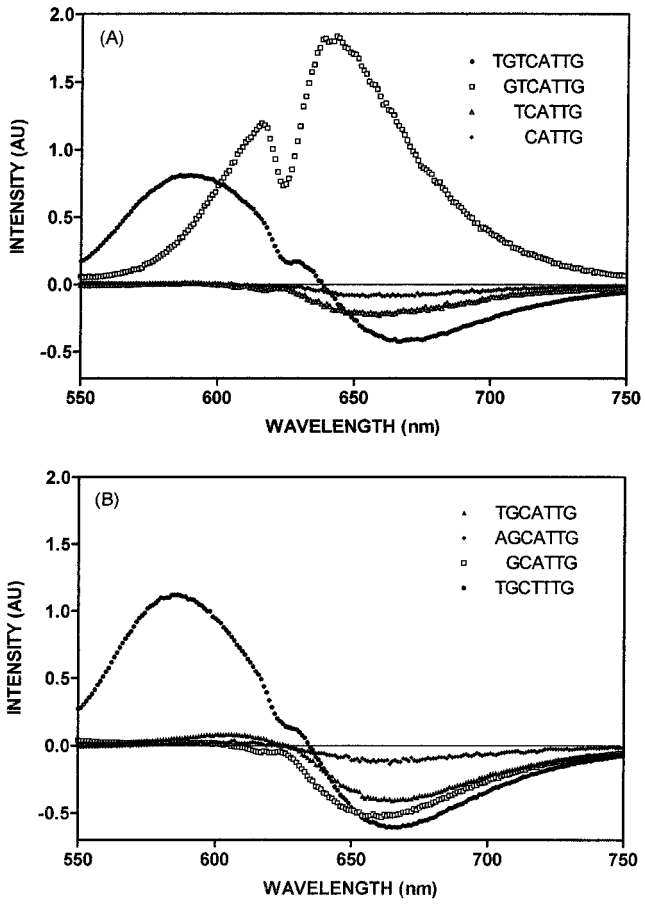

FIGURE 3: Comparison of fluorescence emission difference spectra (7-AM-ACTD + DNA − 7-AM-ACTD) of d(TGTCATTG) and its 5′-base-deleted derivatives (panel A) and of some GpC-containing oligomers (panel B). Fluorescence emission spectra (not corrected) were measured at 20 °C using 2 μM 7-AM-ACTD in the absence and in the presence of 40 μM (nucleotide) DNA with 525 nm excitation. The spectra were not corrected, and the dips near 630 nm are artifactual, likely the consequence of Wood's anomalies.

binding titrations confirmed such an expectation and revealed an ACTD binding constant of about 1×10^7 M^{-1} and a binding density of roughly 0.8 drug molecule per DNA strand for this strong binding mode. A similar binding study with single-base substitutions in d(TGTCATTG) further revealed the relative importance of these C and G bases on its ACTD binding, with the 3′-terminus G appearing to be most crucial. Additional base substitutions led to the conclusion that these C and G bases act in concert rather than individually in the ACTD binding of d(TGTCATTG). Thus, it appears that ACTD can bind strongly to single-stranded DNA of special sequence motifs.

Although the nature of such an ACTD + single strand binding cannot be deduced from our optical results, spectral comparisons with related oligomers provide some interesting insights. The induced CD spectrum in the 250−270 nm spectral region for the d(TGTCATTG) binding to ACTD is very similar to that of d(TGCTTTG) but is somewhat distinct from those of d(TGCATTG) and d(AGCTTTG). Analogous observations were also made with regard to the induced fluorescence spectra of 7-AM-ACTD upon binding to these oligomers. It is, thus, clear that the structure of ACTD complex with d(TGTCATTG) is likely very similar to that of d(TGCTTTG) but may be somewhat different from that of d(TGCATTG). One may be tempted to attribute the differences of d(TGCTTTG) with d(TGCATTG) and d(AGCTTTG) to be the consequence of dominant contributions of ACTD intercalation at the self-complementary duplex sites of TGCA and AGCT. However, the near identical spectral features for the ACTD complexes of d(TGCATTG) and d(GCATTG) as well as the weak binding of d(TGCATT***A***) strongly argue against such an assertion. It thus appears likely that these oligomers may be engaged mainly in a monomeric rather than dimeric binding mode, especially under the optical experimental conditions of dilute DNA concentrations. What then is the nature of such a single-stranded binding mode?

Speculated ACTD Binding Models for d(TGCTTTG) and d(TGTCATTG). An ACTD binding model for d(TGCTTTG) can be proposed on the basis of the following observations and rationales: (1) The classic GpC preference of ACTD implies the favorable stacking of the chromophore on the 3′-side of G. (2) The high ACTD affinity of the apparently single-stranded d(TGCTTTG) suggests a monomeric rather than a dimeric duplex binding. (3) A hairpin with a 3-base loop has been shown to form easily in DNA oligomers. (4) The critical requirement of the C base for ACTD binding in this sequence suggests a possible G·C base pair formation. On the basis of these considerations, a binding model for d(TGCTTTG) may be speculated: ACTD first stacks on the 3′-side of either of the G bases in the sequence, which then induces the other end of the oligomer to fold back for the interior C to form a base pair with the 3′-terminus G so that the 3′-sides of both G's are now stacked on the opposite faces of the drug chromophore. Alternatively, such a complex can be viewed as the ACTD chromophore intercalating at the GpC site with the TTTG folding back to form a G·C base pair and a TTT hairpin loop. Except for the absence of another C to form an additional G·C base pair, the model is very analogous to that of the classic intercalation at the duplex GpC site with both faces of the ACTD chromophore being stacked by G bases.

In view of the striking similarity of CD and fluorescence spectral characteristics between the drug complexes of d(TGCTTTG) and d(TGTCATTG), a similar binding model may also be speculated for d(TGTCATTG): ACTD first stacks on the 3′-side of either G in the sequence, which then induces the other end of the oligomer to fold back for the interior C to form a base pair with the 3′-terminus G so that the 3′-sides of both G's are now stacked on the opposite faces of the drug chromophore with the T of the GTC sequence being pushed out (see Figure 4a). One of the pentapeptide rings may rest and interact with the ATT loop of the hairpin while the other ring may interact with the T at the 5′-terminus. Such a monomeric hairpin binding model would account for the nearly 1:1 drug to DNA strand stoichiometry of the complex formation and the need for the presence of GTC sequence near the 5′-end for strong ACTD binding in d(TGTCATTG), as well as the binding enhancement due to the presence of T at the 5′-end. This binding model will likely be less favored in the presence of excess drug, as both G's will then be stacked with drugs so as to prevent the formation of the wrapped around complex.

An equilibrium scheme (see Figure 4b) can, thus, be proposed to account for the peculiar feature of the binding isotherm observed. The drug (D) may bind to the single-

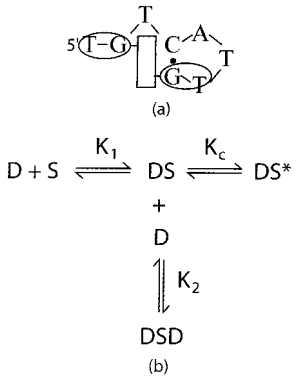

FIGURE 4: (a) Schematic drawing of the speculated ACTD binding to d(TGTCATTG) via a monomeric hairpin formation. The two oval shapes designate the two cyclic pentapeptide rings. (b) A proposed equilibrium binding scheme. D and S represent the drug and single-stranded DNA, respectively.

stranded DNA (S) at either of the two G sites to form single-drug-stacked complexes (DS) which can then bind another drug to form a double-drugged complex DSD in excess drug conditions. The double-drugged complex likely is that of single-stranded binding with two ACTD molecules stacking on the 3′-sides of the two G bases in d(TGTCATTG). Only the single-drug complex can fold back to form the proposed tight-binding complex DS*.

In our absorbance titration, DNA was progressively added to a drug solution. Thus at the initial stages of titration, [D] $\gg$ [S], and both G's in d(TGTCATTG) would have been stacked by the ACTD on the 3′-side of the base. Consequently, the equilibrium is expected to form mainly DSD that traps and hinders the formation of the tight-binding complex DS*. As the titration continues and more DNA are added, [S]/[D] becomes progressively larger and single-drug complexes are more prominent, which can then rearrange into tight-binding DS* complexes. Such a binding scheme may explain the peculiar feature of the Scatchard plot in which a weak-binding slope is followed by a steep-rising one of strong binding and that the binding isotherm does not fit well with a simple model of A + B = AB (see Figure 1A).

The finding of strong ACTD binding of single-stranded d(TGTCATTG) seems to support our recent speculation that the strong ACTD binding observed for d(CGTCGTCG) likely resulted from binding to single strands rather than to the dimeric duplex form (*20*). The proposed binding model for d(TGTCATTG) described in this report, if correct, should also be applicable to d(CGXCGXCG) and d(CGTCGACG), as all the crucial C and G bases are intact in these oligomers. The ability of some DNA sequences with C and G bases placed strategically to bind ACTD stronger for the single strands than the corresponding duplexes also gives credence to the notion that the previously observed cooperative-like binding of ACTD to the self-complementary sequence d(CGTCGACG) may not be the consequence of binding to the duplex but rather to that of the single-stranded form via drug-induced denaturation.

Despite the significant differences in the spectral characteristics of drug complexes between d(TGCTTTG) vs d(TGCATTG) and d(AGCTTTG) on one hand and d(TGTCATTG) vs d(GTCATTG) on the other, the proposed single-strand binding mode may be operative in all of these cases.

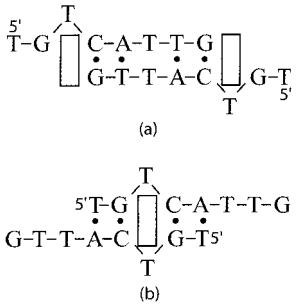

FIGURE 5: Speculated alternative binding modes for the off-registered dimeric duplex with 2 drugs:1 duplex (panel a) and 1 drug:1 duplex (panel b) stoichiometries.

The spectral differences likely originated from differences in the structural details resulting from interactions with the adjacent bases and/or some contributions from the binding at the duplex sites. A hairpin model for single-stranded binding has been suggested earlier but in quite distinct sequence contexts (*16*, *17*). For example, systematic binding studies of 7-AM-ACTD with single-stranded oligomers containing -TAGT- sequences led to the suggestion that the drug binds to metastable hairpins. The hairpins bound most tightly by ACTD contain non-Watson−Crick base pairs, including an A/G and two T/T mismatches (*17*).

Although the above results strongly implicate the important role played by the 3′-terminus G, the question remains as to whether the strong ACTD binding of d(TGTCATTG) is the consequence of the sequence motif or is due to the absolute requirement of a G base at the 3′-terminus. Studies were thus also made with oligomers derived from appending with one or two bases of A and/or T at the 3′-end of this sequence. The results indicate that oligomers of the form d(TGTCATTGX) and d(TGTCATTGXY), where X, Y = A or T, still exhibit considerably strong ACTD binding with binding constants ranging from 4 to 8.4 $\times$ 10^6 M^{-1}. These results indicate that the strong ACTD binding of the oligomers studied in this report is not simply the consequence of the presence of G at the 3′-end but rather is the result of the special sequence motif and the strategic placements of the C and G bases. Such a finding may have important implications on the ACTD binding to single-stranded DNA.

Despite the plausibility of our hairpin binding model for d(TGTCATTG) with 1 drug:1 DNA strand stoichiometry, there exists an alternative binding model with an off-registered dimeric binding with a 2 drugs:1 duplex (or 1:1 drug to strand) stoichiometry, utilizing the same binding principle of 2 G bases stacking on the opposite sides of the ACTD chromophore, as shown in Figure 5a. Such a contribution would be more prominent in a solution of higher DNA concentration. It is likely that both the monomeric and dimeric bindings coexist in solutions, with their relative contributions being dictated by the DNA concentration.

It should be noted that part of the sequence TGTCA in our oligomer may also form a duplex with 4 base pairs (same number of base pairs as the off-rigistered dimeric duplex mentioned earlier) and a T/T mismatch. An ACTD can, thus, bind to this site with bulged out T's to result in a 1 drug:1 duplex (or 2 strands) binding stoichiometry. However, the double-drugged complex will likely be favored because of the added stability resulting from the extra drug binding. The experimental binding density of 0.8 instead of 1 drug per

strand, however, may suggest some contributions from this single-drug to duplex binding mode.

Finally, attempts were made to clarify the relative contributions of dimeric vs momomeric binding via capillary electrophoretic measurements. In the absence of ACTD, d(TGTCATTG) (0.8 mM in nucleotide) exhibits a retention time of 8.05 min. In the presence of 0.1 mM ACTD (1:1 drug to strand ratio) in the DNA solution and 0.5 μM ACTD in the running buffer, the retention time slowed to 8.31 min. The observed slight mobility retardation appears more in line with the hairpin binding model. If it were to bind as a dimeric duplex with 2:1 drug to duplex stoichiometry, a considerably larger mobility retardation would have been observed due to both the dimeric duplex formation and the addition of two bulky drug molecules. The relatively small retardation upon drug binding may be due to the fact that, in the absence of drug, d(TGTCATTG) exists mainly as a single-stranded rather than hairpin conformation in the solution. In the presence of ACTD, however, the hairpin conformation is induced to accommodate complex formation. Thus, the observed small mobility retardation likely resulted from the partial compensation of mobility retardation due to the drug binding by a smaller mobility enhancement due to hairpin formation.

REFERENCES

1. Goldberg, I. H., Rabinowitz, M., and Reich, E. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 2094−2101.
2. Wells, R. D., and Larson, J. E. (1970) *J. Mol. Biol. 49*, 319−342.
3. Krugh, T. R., and Neely, J. W. (1973) *Biochemistry 12*, 4418−4425.
4. Waring, M. (1970) *J. Mol. Biol. 54*, 247−279.
5. Sobell, H. M., and Jain, S. C. (1972) *J. Mol. Biol. 68*, 21−34.
6. Kamitori, S., and Takusagawa, F. (1992) *J. Mol. Biol. 225*, 445−456.
7. Chen, F.-M. (1988) *Biochemstry 27*, 6393−6397.
8. Chen, F.-M. (1992) *Biochemstry 31*, 6223−6228.
9. Liu, C., and Chen, F.-M. (1996) *Biochemistry 35*, 16346−16353.
10. Snyder, J. G., Hartman, N. G., D'Estantoit, B. L., Kennard, O., Remeta, D. P., and Breslauer, K. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3968−3972.
11. Rill, R. L., Marsch, G. A., and Graves, D. E. (1989) *J. Biomol. Struct. Dyn. 7*, 591−604.
12. Bailey, S. A., Graves, D. E., and Rill, R. (1994) *Biochemstry 33*, 11493−11500.
13. Wadkins, R. M., and Jovin, T. M. (1991) *Biochemistry 30*, 9469−9478.
14. Hsieh, Y. L., Li, Y. T., and Henion, J. D. (1994) *Biol. Mass Spectrom. 116*, 272−276.
15. Wadkins, R. M., Jares-Erijman, E. A., Klement, R., Rudiger, A., and Jovin, T. M. (1996) *J. Mol. Biol. 262*, 53−68.
16. Rill, R. L., and Hecker, K. H. (1996) *Biochemistry 35*, 3525−3533.
17. Wadkins, R. M., Vladu, B., and Tung, C.-S. (1998) *Biochemistry 37*, 11915−11923.
18. Chen, F.-M., and Liu, C. D. (1996) *Biochemistry 35*, 7283−7291.
19. Krugh, T. R., and Neely, J. W. (1973) *Biochemistry 12*, 4418−4425.
20. Sha, F., and Chen, F.-M. (2000) *Biophys. J. 79*, 2095−2104.
21. Fasman, G. D., Ed. (1975) *CRC Handbook of Biochemistry and Molecular Biology*, 3rd ed., Vol. I, p 589, CRC Press, Cleveland, OH.
22. Hommer, R. B. (1969) *Arch. Biochem. Biophys. 129*, 405−407.

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