Is the Strong Actinomycin D Binding of d(5′CGTCGACG3′) the Consequence of End-Stacking?†

Fu-Ming Chen* and Chengdi Liu

Department of Chemistry, Tennessee State University, Nashville, Tennessee 37209-1561

Received December 11, 1995; Revised Manuscript Received March 21, 1996⊗

ABSTRACT: It has been reported that ACTD binds strongly and cooperatively to a non-GC containing self-complementary octamer d(CGTCGACG) with a 2:1 drug to duplex ratio (Synder et al., 1989). If one views the classic intercalative preference of ACTD for the 5′GpC3′ sequence to be the drug favoring the 3′-side of dG, the possibility exists that the drug molecules may in fact stack on the G·C base pairs at both ends of this oligomeric duplex. To investigate this possibility, d(CGTCGACG) and several related oligomers resulting from replacing the terminal base(s) or appending with dT and/or dA are used in a comparative study employing equilibrium titration, thermal denaturation, kinetic, and various spectral measurements. Absorbance titrations at 20 °C confirm the strong and highly cooperative nature of ACTD binding to this octamer. The stoichiometric association constants for the binding of the first and second drugs were found to be 1×10^5 and 3.2×10^7 M^{-1}, respectively. The base replacements of dG and dC at the respective ends resulted in a much weaker ACTD binding affinity, the loss of binding cooperativity, and much faster association and dissociation kinetics. These are consistent with the inability of the drug to stack on the 3′-side of dG due to base replacements. Appending the end(s) with dA and/or dT resulted in some diminution of binding affinity and cooperativity, appearance of slower association kinetic components, and unusually strong 7-amino-ACTD fluorescence enhancement for oligomers with dA or dT attached to dG at the 3′-terminal. To further support our postulate, studies were also made with d(CGACGTCG), which is related to the parent octamer by inverting the A·T pairs. It was found that, despite the altered internal sequence, this oligomer exhibits cooperative ACTD binding and kinetic characteristics very similar to those of the parent octamer, consistent with its ability to end-stack on the 3′-side of dG.

Actinomycin D (ACTD) is a chromopeptide antibiotic which consists of a 2-aminophenoxazin-3-one chromophore and two identical cyclic pentapeptide side chains. It has been well established that this drug prefers duplex DNA and binds via intercalation of the planar chromophore (Muller & Crothers, 1968; Waring, 1970; Sobell & Jain, 1972), preferably at the GpC sequence, with the two pentapeptide rings resting on the minor groove. X-ray studies (Sobell et al., 1971; Kamitori & Takusagawa, 1992) have clearly shown that its duplex and GpC sequence preference is the consequence of the ability of the carbonyl oxygens and the NH groups of the L-threonine residues to form hydrogen bonds with the 2-amino group and the N(3) of guanines on both sides of the intercalated drug. However, there have been recent reports to indicate that ACTD can also bind strongly to some non-GC sequences (Synder, et al., 1989; Rill et al., 1989; Bailey et al., 1994) as well as to some single-stranded DNA (Wadkins & Jovin; 1991). In particular, calorimetric studies by Synder et al. (1989) had led to the conclusion that ACTD binds cooperatively to the octamer d(5′CGTCGACG3′) with a binding constant higher than 10^7 M^{-1} and a 2:1 drug to duplex ratio. It is important to understand the nature of such a strong binding and to delineate the origin of its high cooperativity. Since it has been suggested by Snyder et al. (1989) that the mode of ACTD binding to d(CGTCGACG) is distinct from its classic mode of binding to GpC sequence, it will be of value to see if this is in fact the case. The possibility that the cooperative and strong ACTD binding of this oligomer is a consequence of the drug molecules stacking at the ends of the DNA duplex is hereby investigated. The basis for such a speculation stems from the fact that if one is to view the classic preferred ACTD intercalative site as G3′p5′C, then it follows that the drug favors stacking and hydrogen bonding at a G·C base pair on the 3′-side of dG. To elucidate the possibility of ACTD stacking at the duplex ends of d(CGTCGACG), comparative binding, melting, and kinetic studies were carried out with oligomers of the form d(XGTCGACY), in which X = A, T, G, or C, and d(X-CGTCGACG), d(CGTCGACG-Y), and d(X-CGTCGACG-Y) in which X is A or T, with Y being complementary to X. It is reasoned that if end-stacking of ACTD on the 3′-side of dG is the culprit, the replacement of the terminal dG by another base and a concomitant complementary replacement at the other end will lead to a considerable reduction in the ACTD binding affinity. In contrast, minimal alteration in binding characteristics should result if it is due to the internal sequence binding. Such a rationale should be particularly valid for the base-added oligomers, where the entire 8 base-paired duplex is now intact with only a dangling base or a base pair being appended to each end. On the other hand, significant effects on the binding behaviors will be expected for these oligomers

† Research Supported by USPHS Grant CA-42682 and Army Medical Research Grant DAMD17-94-J-4474.

* Corresponding author, Dr. Fu-Ming Chen at the Department of Chemistry, Tennessee State University, Nashville, TN 37209-1561. Tel: (615) 963-5325; FAX: (615) 963-5434; E-Mail: chenf@acad.tnstate.edu.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0006-2960(95)02907-2 CCC: $12.00

if the end-stacking mechanism is operative. Additional studies were also made with d(CGACGTCG), which is related to the parent octamer by inverting the A·T base pairs without altering the terminal bases and should thus retain its end-stacking abilities. The results of these experiments and their significance are presented and discussed in this report.

MATERIALS AND METHODS

Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, IA and used without further purification. Concentrations of these oligomers (per nucleotide) were determined by measuring the absorbances at 260 nm after melting, with use of extinction coefficients obtained via nearest-neighbor approximation using mono- and dinucleotide values tabulated by Fasman (1975). The extinction coefficients used for drug concentration determination are 24 500 M^{-1} cm^{-1} at 440 nm for ACTD and 23 600 M^{-1} cm^{-1} at 528 nm for 7-amino-ACTD. All experiments were carried out in 10 mM HEPPS (N-(2-hydroxyethyl)piperazine-*N*′-propanesulfonic acid) buffer solutions of pH 8 containing 0.1 M NaCl. Absorption spectra were measured with a Cary 1E spectrophotometric system. Absorbance changes at 427 and 480 nm were used to obtain Scatchard plots. Thermal denaturation experiments were carried out with 1-cm semimicro cells by monitoring absorbances at 275 or 427 nm. A heating rate of 0.5 °C/min was maintained by the temperature controller accessory. Melting temperatures were determined via differential melting plots.

Circular dichroic (CD) spectra were measured at room temperature with a Jasco J-500A recording spectropolarimeter using water-jacketed cylindrical cells of 1-cm path length. Fluorescence measurements were made with an SLM48000S system. Stopped-flow kinetic measurements were made with an Olis RSM-1000 rapid scan spectrophotometer. Nonlinear least-squares fits on the kinetic data and binding isotherms were carried out with the MINSQ program of Micromath (Salt Lake City, UT).

RESULTS

Equilibrium Binding Titrations. (A) Qualitative Binding Order. Spectral measurements indicate that the binding of DNA to ACTD results in an absorbance increase and decrease at the 480 and 427 nm spectral regions of the drug, respectively. Thus, a comparison of absorbance difference spectra at a given P/D ratio, where P and D are nucleotide and drug concentrations, respectively, can provide qualitative ACTD binding orders for these oligomers. Representative absorbance difference spectra at P/D = 10 are compared in Figure 1 for these oligomers. It is apparent that the qualitative ACTD binding order for the d(XGTCGACY) series (panel A) is as follows: d(CGTCGACG) > d(GGTCGACC) > d(TGTCGACA) ≥ d(AGTCGACT). On the other hand, the order for the oligomers containing terminally-added base(s) of dA and/or dT (panel B) is found to be the following: d(CGTCGACG) > d(CGTCGACG-T) ≥ d(A-CGTCGACG) > d(CGTCGACG-A) ≥ d(T-CGTCGACG) ≅ d(A-CGTCGACG-T) > d(T-CGTCGACG-A). It is apparent that the replacement and blocking of the 5′-end of dC and/or 3′-end of dG had considerably diminished the

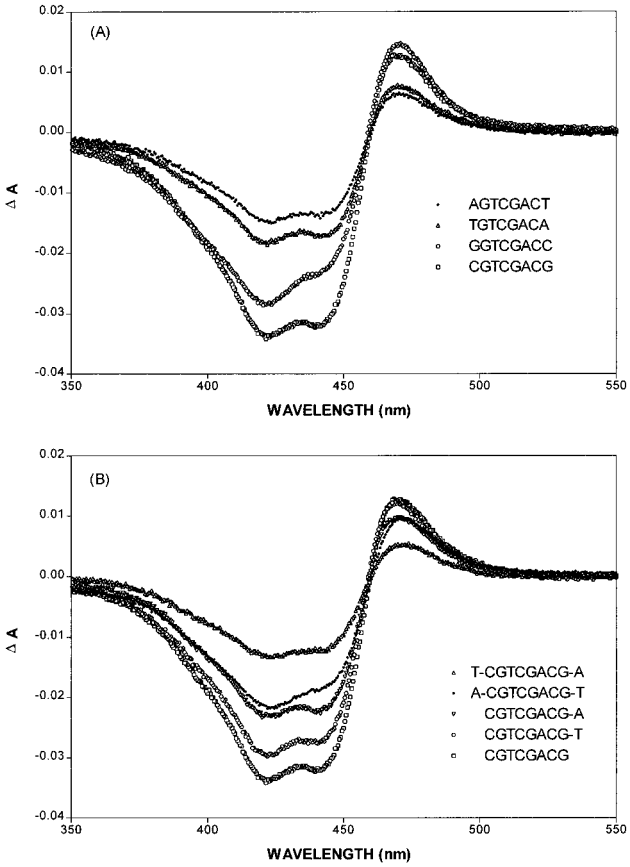

FIGURE 1: Absorbance difference spectra (ACTD/DNA − ACTD) for P/D = 10 at 20 °C. (A) Comparison for octamers of the form d(XGTCGACY). (B) Representative plots for oligomers formed by adding dA and/or dT to the terminal(s) of d(CGTCGACG). The difference spectra for oligomers with dA and dT attached to the dC of the 5′-terminal are somewhat smaller than the corresponding oligomers with dT and dA attached to the dG of the 3′-terminal, respectively, and are thus not shown.

oligomer's ability to bind ACTD. In addition, the binding affinities are strongly dependent on the nature and the location of the replacing/blocking base(s). The moderate ACTD binding to d(GGTCGACC) most likely is the consequence of binding to the GG/CC sequence.

(B) Scatchard Plots. To obtain more quantitative binding parameters, results of spectral titrations were converted to binding isotherms. Scatchard plots were constructed using absorbance differences between 427 and 480 nm, and the representative plots are shown in Figure 2. It is apparent that the plot for d(CGTCGACG) is decidedly curved and its binding parameters cannot be obtained via linear least-squares fits. It is also clear that the oligomers with the terminal base replacements had significantly diminished binding affinities, as evidenced by the much reduced slopes (panel A). Their binding constants were estimated from linear least-squares fits, and their values are included in Table 1. Effects due to the terminal addition with A and/or T base(s) can be seen in panel B, where representative plots are compared with the parent octamer. Although the weaker binding plots appear to be linear, the stronger binding ones are decidedly curved. Thus, a more straightforward approach was taken by directly fitting the experimental binding isotherms with a binding model.

(C) Fitting the Binding Isotherms with a Binding Model. If ACTD were to bind via end-stacking, two binding sites

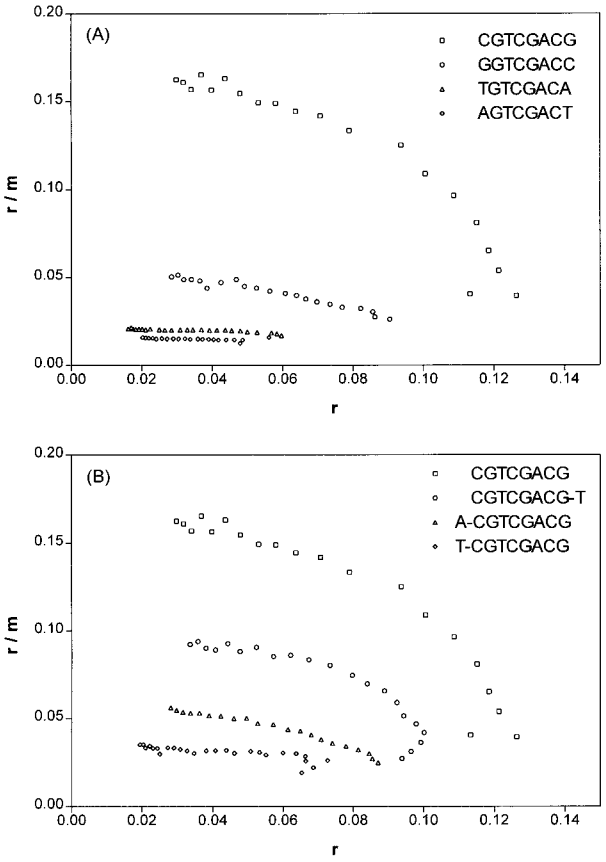

FIGURE 2: Scatchard plots derived from absorbance titrations at 20 °C. (A) Comparison of the d(XGTCGACY) oligomers. (B) Representative plots for oligomers with dA and/or dT added to either or both ends of the parent octamer d(CGTCGACG). Absorbance difference between 427 and 480 nm has been used to construct the plots. [Bound drug]/[DNA, nucleotide] is designated by *r*, and *m* represents the free drug concentration.

are available. Thus, the following binding model was assumed:

$$S + L_2 \overset{K_1}{\rightleftharpoons} SL_2 \qquad S + SL_2 \overset{K_2}{\rightleftharpoons} S_2L_2$$

where S and L_2 represent the drug and oligomeric duplex, respectively. With use of mass balance equations of the DNA and drug concentrations, the following equations can be derived:

$$K_1K_2S^3 + [K_1K_2(L_t - S_t) + K_1]S^2 + [K_1(0.5L_t - S_t) + 1]S - S_t = 0 \quad (1)$$

$$L_2 = (L_t - S_t + S)/(2 + K_1S) \quad (2)$$

$$\Delta A = [\epsilon_S + \epsilon_1K_1L_2(1 + 2K_2S)]S \quad (3)$$

where ΔA is the observed absorbance difference between 427 and 480 nm. ϵ_S and ϵ_1 are extinction coefficients of the free and bound drugs, respectively. L_t and S_t are the respective total DNA oligomeric (in strand) and drug concentrations. These equations were used to extract binding parameters via nonlinear least-squares fits on the experimentally observed data. As can be seen in Figure 3, excellent fits are obtained for most of the binding isotherms, and the extracted binding parameters from these fits are included in Table 1 for comparison. Of particular interest are the values

Table 1: Summary of Binding and Melting Parameters[a]

DNA Oligomer	K_a (μM^{-1})	K_1 (μM^{-1})	K_2 (μM^{-1})	$T_m°$ (°C)	ΔT_m (°C)
CGACGTCG	-	0.08	35.6	42	21
AGTCGACT	< 0.1	-	-	36	6
TGTCGACA	< 0.1	-	-	34	9
GGTCGACC	0.38	-	-	40	6
CGTCGACG	-	0.11	31.5	45	20
A-CGTCGACG	-	0.87	0.80	49	14
CGTCGACG-T	-	0.70	4.16	45	18
A-CGTCGACG-T	-	0.56	0.15	52	14
T-CGTCGACG	-	0.22	0.94	47	13
CGTCGACG-A	-	0.03	7.96	45	16
T-CGTCGACG-A	-	0.03	0.96	50	11

[a] K_a is the binding constant estimated via linear least-squares fit of the Scatchard plot. K_1 and K_2 are stoichiometric association constants for binding one and two molecules, respectively. The values are extracted via nonlinear least-squares fit using eqs 1−3 in the text. $T_m°$ is the melting temperature of 40 μM (nucleotide) oligomeric solution, and ΔT_m is the melting temperature increase in the presence of 7 μM ACTD.

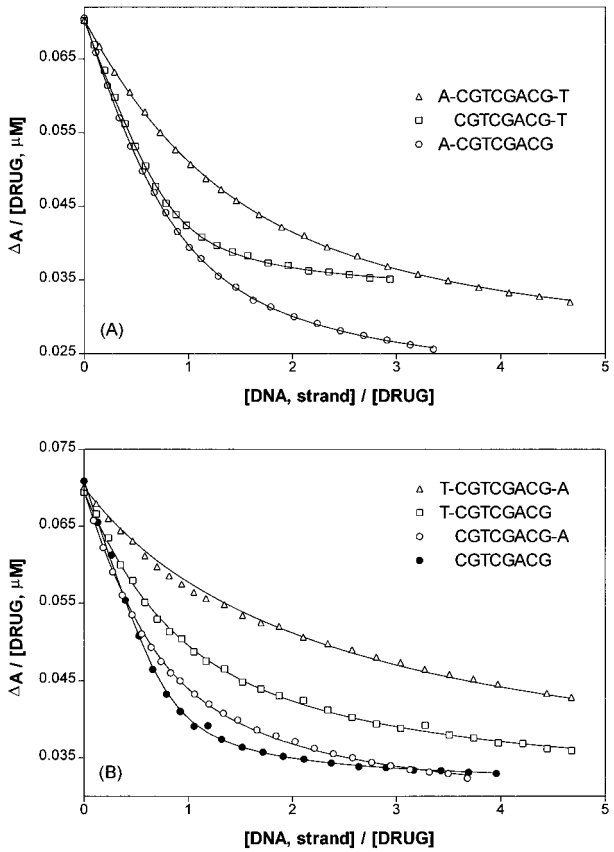

FIGURE 3: Comparison of the experimental binding isotherms at 20 °C and the theoretically fitted curves (connected lines) using the binding model and eqs 1−3 as described in the text. Panel A: Plots for oligomers of the form (A)-CGTCGACG-(T). Panel B: For oligomers of the form (T)-CGTCGACG-(A) and the parent octamer.

found for d(CGTCGACG) of 1×10^5 and 3.2×10^7 M^{-1} for the binding constants K_1 and K_2, respectively. The highly cooperative nature of ACTD binding to this octamer is confirmed by $K_2 \gg K_1$ in which binding of the second drug

is much stronger than that of the first. Although a good nonlinear least-squares fit does not guarantee the correctness of the model, the finding of a highly cooperative binding for the parent octamer and the considerably reduced binding affinities and cooperativities for the terminally replaced or base-added oligomers suggest the plausibility of the assumed model. It is interesting to note that significant binding affinity and cooperativity are retained in d(CGTCGACG-T) and d(CGTCGACG-A) (see Table 1).

It should be pointed out that K_1 and K_2 in the above model are stoichiometric rather than microscopic binding constants. They are, however, related and in the special case of two identical binding sites can be shown (Connors, 1987) to be:

$$K_1 = 2k_{11} \quad \text{and} \quad K_2 = wK_1/4$$

where k_{11} is the microscopic binding constant for the formation of 1:1 drug to duplex complex and w is the interaction (or cooperativity) parameter which measures the extent of interaction between the two sites in a 2:1 complex formation.

Thermal Denaturation Measurements. The extent of melting temperature increases upon drug binding can also provide information on the drug binding affinity of a DNA. Melting temperatures of the oligomers and the increases upon ACTD binding are also included in Table 1 for comparison. It is apparent that the pattern of melting temperature increases is in general agreement with that of binding affinities. For example, the ACTD-induced melting temperature increase is about 20 °C for d(CGTCGACG), whereas it is less than 10 °C for any of the terminally replaced oligomer, in agreement with their much reduced binding affinities. In addition, the base-added oligomers exhibit somewhat smaller melting temperature increases than that of the parent octamer. Consistent with the retention of significant binding affinity and cooperativity, a dT or dA addition to the 3′-end of dG resulted in the largest drug-induced duplex stability among the derived oligomers.

Stopped-Flow Kinetic Measurements via Absorbance Monitoring. (A) Association Kinetics. Association kinetic measurements were made by mixing equal volumes of 8 μM ACTD and 100 μM (nucleotide) DNA in a stopped-flow rapid-scanning instrument. Representative kinetic profiles with 428-nm absorbance monitoring are shown in Figure 4, and the results of 1- or 2-exponential fit along with their total absorbance changes are compared in Table 2. As can be seen from Figure 4A, the two oligomers with terminal A·T base pair replacement exhibit small absorbance changes and fast association kinetics with characteristic times of around 0.25 s at 20 °C. Despite an observed significant total absorbance change, the oligomer d(GGTCGACC) exhibits even faster association kinetics, with a measured slowest association characteristic time of around 0.04 s. In contrast, nearly 50% of the absorbance changes were measurable by the stopped-flow technique for d(CGTCGACG), with the bulk of the measured changes exhibiting the slow characteristic association time of around 14 s. Interestingly, oligomers with bases added to both ends exhibit significantly smaller total absorbance changes and slower association kinetics than those of oligomers with a dangling base, which in turn are slower than the parent octamer. It should also be noted that the two oligomers with respective dangling dT and dA added to the 3′-end of dG exhibit the largest

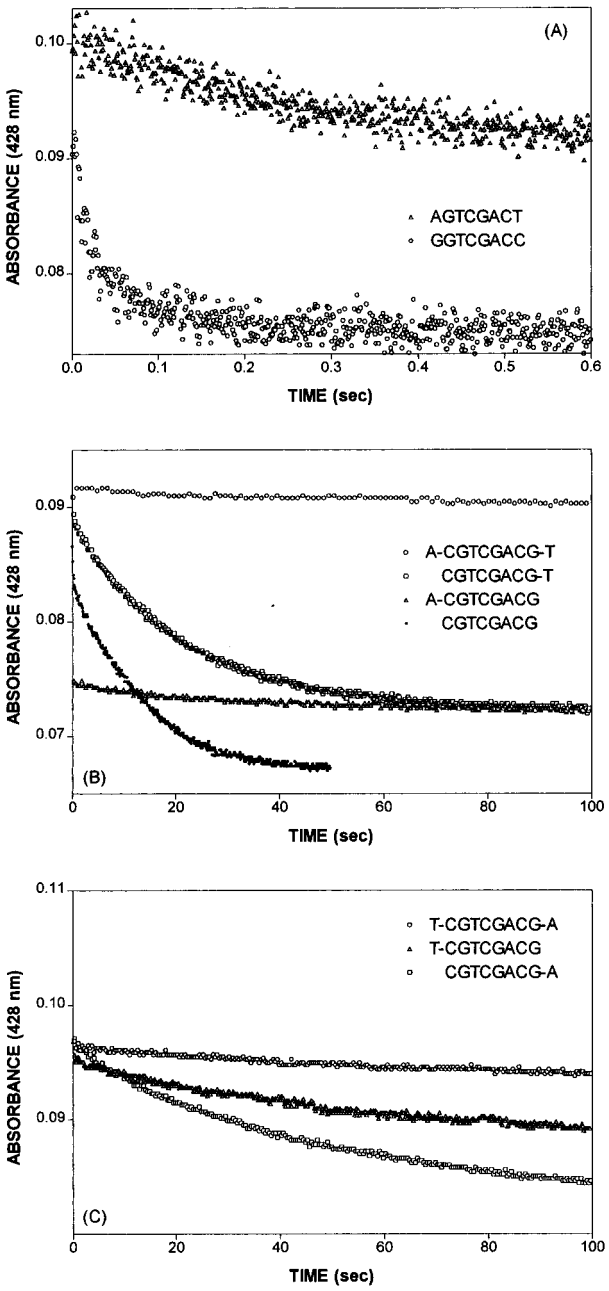

FIGURE 4: Typical ACTD association kinetic profiles with 428-nm absorbance monitoring at 20 °C. Panel A: GGTCGACC (○) and TGTCGACA ≅ AGTCGACT (△). Panel B: Comparison of CGTCGACG and oligomers of the form (A)-CGTCGACG-(T). Panel C: Oligomers of the form (T)-CGTCGACG-(A).

absorbance changes for the slow association component (see panels B and C) and the magnitudes of the total absorbance changes ΔA_t (see Table 2) are in general agreement with the qualitative binding orders established in the earlier section.

(B) Dissociation Kinetics. SDS-induced ACTD dissociation kinetics were also measured at 20 °C. The results indicate that, in the d(XGTCGACY) series, only the parent octamer d(CGTCGACG) exhibits a slow enough dissociation kinetics to be measured by the stopped-flow technique to yield a characteristic dissociation time of 0.8 s. Except for d(CGTCGACG-T), the considerably smaller binding-induced total absorbance changes of the base-added oligomers prevented us from obtaining meaningful dissociation kinetic profiles. Nevertheless, a dissociation time of about 1.3 s is

Table 2: Summary of Association Kinetic Parameters of ACTD at 20 °C[a]

DNA Oligomer	τ_f (s)	% (fast)	τ_s (s)	% (slow)	ΔA_t
CGACGTCG	0.22 ± 0.03	6.1	10.3 ± 0.2	36.5	0.0386
AGTCGACT	0.26 ± 0.02	62.8			0.0156
TGTCGACA	0.24 ± 0.01	69.4			0.0170
GGTCGACC	0.04 ± 0.003	42.1			0.0335
CGTCGACG	0.14 ± 0.02	4.8	14.1 ± 0.4	42.4	0.0397
A-CGTCGACG	0.17 ± 0.03	8.2	52.6 ± 5.5	7.0	0.0348
CGTCGACG-T	0.15 ± 0.01	6.7	34.5 ± 1.2	50.8	0.0341
A-CGTCGACG-T	0.15 ± 0.03	21.1	222 ± 30	14.9	0.0178
T-CGTCGACG	0.20 ± 0.02	20.3	55.5 ± 3.1	37.7	0.0175
CGTCGACG-A	0.16 ± 0.01	17.3	50.0 ± 2.5	55.9	0.0238
T-CGTCGACG-A	0.15 ± 0.01	34.8	109 ± 2	24.9	0.0140

[a] τ_f and τ_s are the fast and slow components of the 428-nm association kinetic trace as extracted via 1- or 2-exponential fit. ΔA_t is the total absorbance change.

obtained for the base-added oligomer d(CGTCGACG-T), which is slower than that of the parent octamer.

(C) Circular Dichroic Spectral Characteristics. Binding of ACTD to DNA induces a characteristic positive and negative CD maxima near 293 and 270 nm, respectively. Thus, the extent of induced CD intensity at these wavelengths can be used to provide qualitative binding information. Representative CD difference spectra (drug/DNA − DNA) are compared in Figure 5. Consistent with the absorbance results, d(CGTCGACG) exhibits the largest CD intensity enhancement at 293 nm. Much weaker CD intensities were induced at this wavelength for the end-base-replaced oligomers (panel A). The distinctly different induced CD spectral characteristics can also clearly be seen. The progressive reduction of the 293-nm CD intensity on the base-added oligomers are also apparent (panels B and C). Thus, the CD measurements are in general agreement with the binding order established earlier via absorbance titrations. CD spectral measurements in the 520−320 nm region were also made to indicate weak broad positive maxima near 460 nm (not shown), in agreement with the work of Snyder et al. (1989).

Fluorescence Spectral Enhancement of 7-Amino-ACTD. (A) Emission Spectral Characteristics. In contrast to ACTD, its 7-amino derivative is highly fluorescent. Despite the presence of an amino group at the 7 position, its DNA binding mode has been shown to be similar to that of ACTD (Chiao et al., 1979). Thus, this compound was used for the fluorescence binding studies. Binding of 7-AM-ACTD to an oligomeric duplex containing a GpC sequence usually results in a strongly enhanced fluorescence emission spectrum exhibiting a maximum at 650 nm and a shoulder near 610 nm. Fluorescence intensity enhancement patterns for 7-AM-ACTD upon binding to d(CGTCGACG) and the related oligomers are compared in Figure 6 as difference spectra (drug/DNA − drug). It is immediately apparent that,

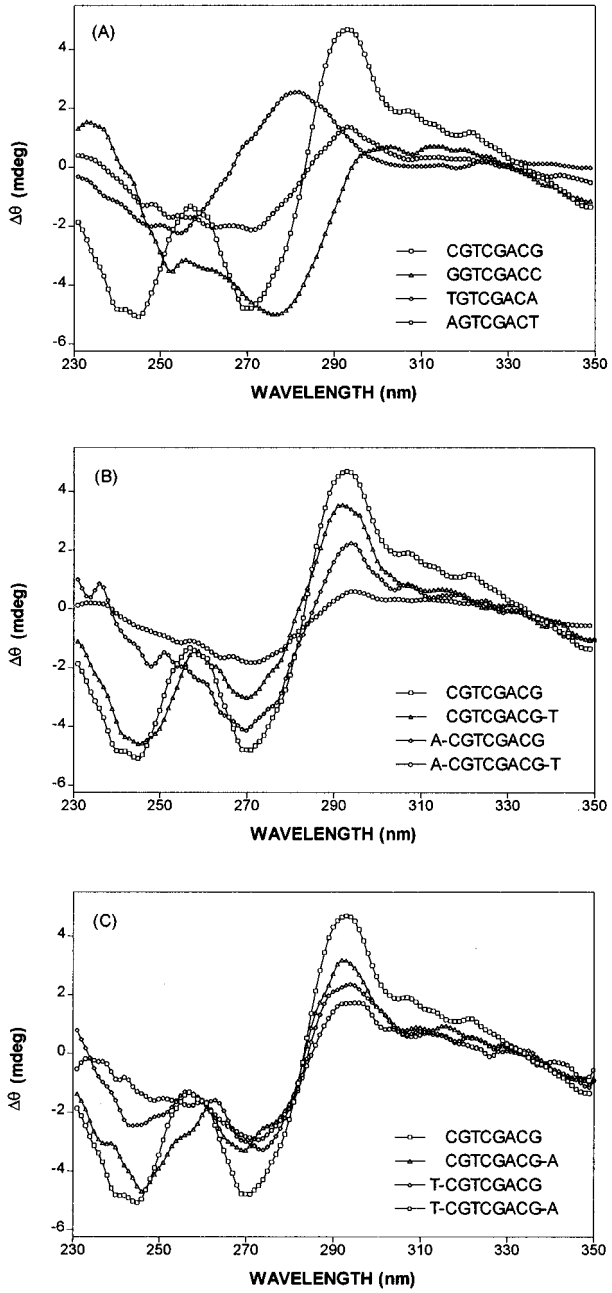

FIGURE 5: Comparison of difference CD spectra at room temperature for 5 μM ACTD in 40 μM/base of oligonucleotide solutions with the contributions due to DNA subtracted. Panel A: Octamers of the form XGTCGACY. Panel B: Oligomers of the form (A)-CGTCGACG-(T). Panel C: Oligomers of the form (T)-CGTCGACG-(A).

in contrast to the GpC containing oligomers, d(CGTCGACG) and the base-added oligomers induced a much stronger fluorescence enhancement at 610 nm than at 650 nm. This results in a double-humped spectral pattern, with the intensity of the former now being larger than that of the latter. Panel A compares the effect of adding dA to the 5′-terminal and/or dT to the 3′-terminal of the parent octamer on the fluorescence spectral patterns of 7-AM-ACTD. Consistent with its weaker ACTD binding, d(A-CGTCGACG) induces a smaller fluorescence intensity enhancement than that of d(CGTCGACG). In contrast, a dramatic intensity enhancement much more than the parent octamer is induced by d(CGTCGACG-T), and despite its considerably weaker binding affinity, d(A-CGTCGACG-T) induces nearly identi-

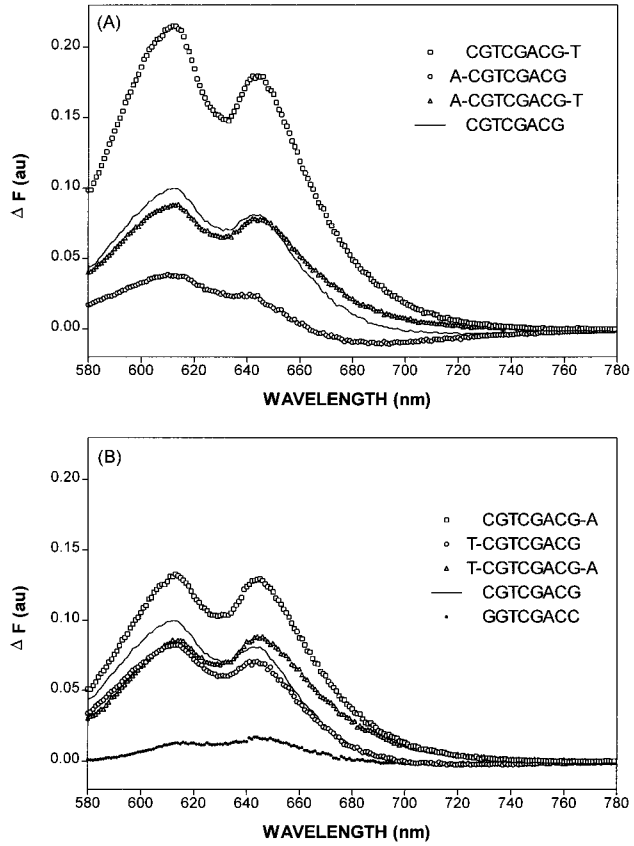

FIGURE 6: DNA-induced fluorescence emission spectral characteristics of 2 μM 7-amino-ACTD at 20 °C. Panel A: Comparison of the parent octamer and oligomers of the form (A)-CGTCGACG-(T). Panel B: Comparison with oligomers of the form (T)-CGTCGACG-(A) and GGTCGACC.

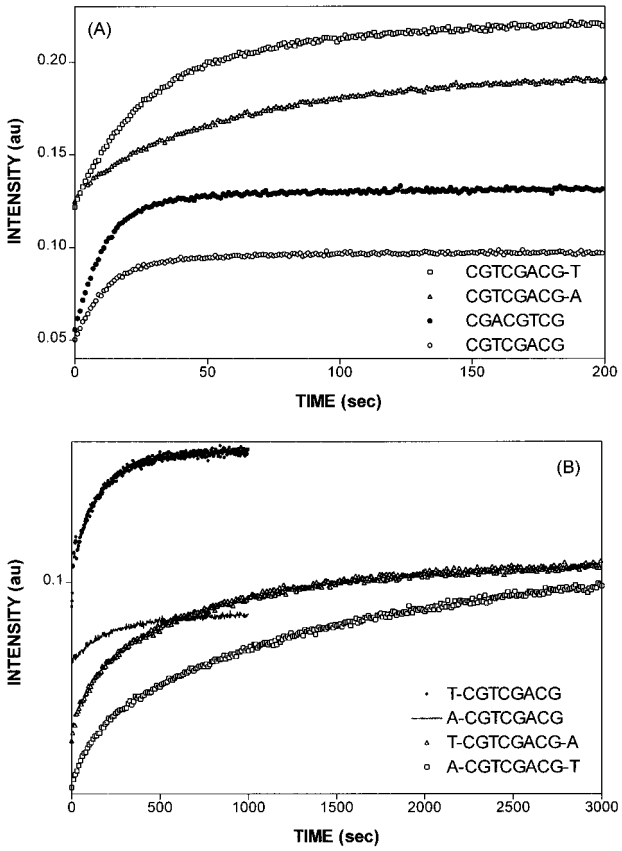

FIGURE 7: Association kinetic profiles of 7-AM-ACTD at 20 °C via fluorescence monitoring at 610 nm. Excitation wavelength is at 550 nm. Panel A: Comparison of d(CGTCGACG) and d(CGACGTCG) vs d(CGTCGACG-X), where X = A or T. Panel B: Comparison of d(X-CGTCGACG) and d(X-CGTCGACG-Y), where X = A or T and Y is complementary to X.

cal intensity enhancement as that of the parent octamer (panel A). Similarly, d(CGTCGACG-A) induces a stronger fluorescence intensity enhancement than its parent octamer, but the effect is not as dramatic as that of dT attachment (see panel B). Also, despite their weaker binding affinities, d(T-CGTCGACG) and d(T-CGTCGACG-A) induced nearly identical fluorescence enhancements as that of the parent octamer. As for the end-base-replaced octamers, significantly weaker fluorescence enhancements than the parent octamer were observed (see also panel B).

(B) Fluorescence Kinetic Measurements. In contrast to the small absorbance changes for some of the base-added oligomers, those of the corresponding fluorescence changes are quite considerable even for the oligomers with bases added to both ends. Thus, the slow component of the association kinetics, which was barely discernible via absorbance monitoring, can now be seen clearly in the fluorescence monitoring. The association kinetic profiles at 20 °C for the base-added oligomers are shown in Figure 7. It is immediately apparent that the association kinetics for the base-added oligomers are considerably slower than those of the parent octamer. In particular, the decamers with terminal A·T base pairs exhibit more than an order of magnitude slower kinetics than the parent octamer. The nonlinear least-squares fitted kinetic parameters with 1- or 2-exponential equation are summarized in Table 3.

SDS-induced dissociation kinetics were also measured. These non-stopped-flow measurable dissociation kinetic profiles can be adequately fitted with single exponential decays, and the extracted parameters were also included in Table 3 for comparison. It is apparent that the rates of dissociation for the base-added oligomers are considerably slower than that of the parent octamer. Also, the rates of dissociation show strong dependence on the nature and the location of the attached base(s).

Studies with d(CGACGTCG). To further support our thesis on end-stacking, studies were also made with d(CGACGTCG) which is related to the parent octamer by inverting the A·T base pairs without altering the terminal bases. This oligomer is expected to bind well to ACTD if end-stacking is the culprit. Indeed, the following are found: (1) that ACTD binds strongly to this oligomer with high cooperativity, as indicated by the considerable curvature of its Scatchard plot (not shown) and the respective values of 1×10^5 and 3.6×10^7 M^{-1} for K_1 and K_2 (see Table 1) obtained via nonlinear least-squares fit on its binding isotherm using the described binding model; (2) that the ACTD-induced CD exhibits a sizable positive intensity near 293 nm as well as 460 nm; (3) that the melting temperature increase upon drug binding is around 21 °C; and (4) that the characteristic association and SDS-induced dissociation times were estimated to be 10 and 0.6 s, respectively. Furthermore, this oligomer induces strong fluorescence intensity enhancement, exhibiting a 600-nm maximum (not shown). All these characteristics are very similar to those of the parent octamer d(CGTCGACG).

Table 3: Summary of Fluorescence Kinetic Parameters of 7-AM-ACTD at 20 °C[a]

DNA Oligomer	τ_1 (s)	% (1)	τ_2 (s)	% (2)	τ_d (s)
CGACGTCG	12.7 ± 0.2	62			~ 1
AGTCGACT					
TGTCGACA					
GGTCGACC					
CGTCGACG	13.7 ± 0.2	57			~ 1
A-CGTCGACG	232 ± 5	~ 100			15.5 ± 1.4
CGTCGACG-T	32.8 ± 0.4	67			3.3 ± 0.1
A-CGTCGACG-T	169 ± 6	18	1640 ± 19	51	7.1 ± 0.5
T-CGTCGACG	150 ± 2	65			19.1 ± 1.2
CGTCGACG-A	59.2 ± 0.4	58			25.6 ± 1.3
T-CGTCGACG-A	119 ± 6	20	813 ± 13	42	16.9 ± 1.2

[a] τ_1 and τ_2 are characteristic association times, and τ_d is the characteristic SDS-induced dissociation time.

DISCUSSION

Consistent with previous calorimetric studies of Synder et al. (1989), our equilibrium binding titrations indicate that d(CGTCGACG) binds strongly to ACTD and exhibits very high cooperativity to result in a 2:1 drug to DNA complex. Nonlinear least-squares fits of the experimental binding isotherms with a binding model yielded stoichiometric binding constants of 1×10^5 and 3.2×10^7 M^{-1} for the 1- and 2-drug binding processes, respectively. In support of our postulate that the strong ACTD binding of this octamer is the consequence of ACTD stacking on the 3′-side of dG at the terminal G·C base pairs, the replacement of G at the 3′-terminal by A, T, or C and the complementary base at the other end resulted in more than an order of magnitude reduction in the binding affinities, the loss of binding cooperativity, the considerably smaller ACTD-induced melting temperature increases, and the much faster drug−DNA association as well as dissociation kinetics. The weak binding of d(AGTCGACT), d(TGTCGACA), and even d(GGTCGACC) which contains as many G·C base pairs as in the parent octamer may partly be due to the decreased duplex stability, as indicated by their lower melting temperatures (see Table 1). However, the most likely reason for these observations is the absence of dG at the 3′-terminal in these oligomers for the ACTD end-stacking.

To further support our thesis on end-stacking, studies were also made with oligomers by appending dA or dT to the 5′- and/or 3′-ends, the rationale being that, by adding a dT or dA to the terminal(s) without altering the 8-base self-complementary internal sequence, minimal effect on the binding affinity will be expected if binding occurs at the internal sequences. On the other hand, significant alteration on the binding characteristics should occur if end-stacking to the G·C base pairs is the culprit. The results indicate that, in contrast to their parent octamer, these oligomers exhibit significantly weaker ACTD binding affinities with considerably reduced cooperativity as more bases are added. In addition, association kinetic measurements indicate that each end-base-added oligomer exhibits a slow association component which is significantly slower than that of the parent octamer. In particular, the oligomeric duplexes with A·T base pairs at both ends exhibit considerably weaker binding and slower association kinetics than the corresponding duplexes with a dangling A or T at the end. These results are consistent with the drug molecules stacking at the duplex ends. Interestingly, both the binding and kinetic characteristics are strongly dependent on the nature and the location of the added bases. For example, the (A)-CGTCGACG-(T) series of oligomers exhibit somewhat higher binding affinities than the corresponding (T)-CGTCGACG-(A) counterparts. Significant ACTD binding affinity and cooperativity are retained when dA or dT is added to the 3′-end of dG, whereas weaker binding and cooperativity are apparent for oligomers with dA or dT attached to the 5′-end of dC.

Despite the fact that the extent of fluorescence enhancements of 7-amino-ACTD do not exactly correlate well with the binding order of oligomers studied, the fluorescence results appear to provide the most convincing evidence that the binding of ACTD to d(CGTCGACG) occurs at the duplex termini rather than at the internal sequences. If the binding were to occur at the internal sequences, a dangling base at either end of a duplex is not expected to have a significant influence on the fluorescence spectral characteristics of the bound drug. Yet, our results clearly indicate that when the dangling dA or dT is attached to the dG of the 3′-terminal, dramatic fluorescence enhancement is observed. In contrast, weak or moderate enhancement is seen when the dangling base is attached to dC at the 5′-terminal.

The large fluorescence intensity enhancements for the base-appended oligomers provided us with the opportunity to study the SDS-induced dissociation kinetic behaviors of the drug, which was not possible with the absorbance monitoring. In addition to confirming the expected slower dissociation rates for the base-added oligomers than their parent octamer, these kinetic results provided us with a powerful argument for the end-stacking mechanism. For example, characteristic times for the dissociation of 7-AM-ACTD from d(CGTCGACG), d(CGTCGACG-T), and d(CGTCGACG-A) are about 1, 3.3, and 26 s, respectively. One would be hard pressed to explain how a dangling A base can induce a 30-fold decrease in the drug's off rate, if the binding were at the internal sequences rather than at the ends. Also, how can a change in the dangling base from A to T result in a rate change of nearly 10-fold? These results can, however, be more easily explained in terms of the drug interacting at the terminal G·C base pairs. The strong fluorescence enhancement is likely the consequence of the drug's experiencing a more hydrophobic environment via wrapping around of the dG-attached dangling A or T base to stack on the benzenoid portion of the phenoxazone ring of ACTD. Such an interpretation is consistent with the observation and interpretation on the splitting of the H7 and H8 NMR proton signals during the ACTD titrations with dinucleotide pdG-dT or pdG-dA (Krugh & Neely, 1973). It is further supported by the earlier fluorescence studies of 7-AM-ACTD (Modest & Sengupta, 1974; Chiao, et al., 1979) indicating that binding of dAMP enhances the fluorescence intensity near 600 nm. The extent of such interactions should be dependent on the nature of the base, with base A expected

to provide stronger stacking interactions than base T.

Another piece of evidence implicating stacking at dG comes from the positive sign of the CD band at 460 nm. Binding of ACTD to DNA usually results in a negative intensity in this region except for binding to the mononucleotide pdG (Homer, 1969; Brown & Shafer, 1987). The unusual feature of the positive CD intensity at this wavelength observed for ACTD binding to d(CGTCGACG) was pointed out earlier by Synder et al. (1989). It is also interesting to note that the long wavelength CD band of 7-AM-ACTD reverts from negative with intercalative binding of dinucleotide pdG-dC to positive with stacking interactions of pdC-dG (Chiao et al., 1979). Thus, the observation of a positive 460-nm CD band is consistent with ACTD stacking to dG at the duplex ends.

Additional corroborating evidence supporting the notion of ACTD stacking on the 3′-side of dG at the terminal G·C base pairs comes from the study with the octamer d(CGACGTCG). This octamer is related to the parent octamer by inverting the A·T pairs without disturbing the C and G at the respective 5′- and 3′-ends. It was found that this octamer exhibits a strong ACTD binding affinity with high cooperativity, a large ACTD-induced melting temperature increase, a significant fluorescence intensity enhancement of 7-AM-ACTD near 600 nm, a positive CD band around 460 nm for the bound ACTD, and relatively slow association as well as dissociation kinetics. These characteristics are very similar to those of the parent d(CGTCGACG) and are consistent with its ability to accommodate ACTD at the duplex ends.

Our results suggest that the binding constant (K_1) for the initial ACTD stacking at one of the termini is around 1×10^5 M^{-1}. This value is more than an order of magnitude higher than binding to the mononucleotide pdG (Krugh & Neely, 1973), but about an order of magnitude lower than intercalation at the GpC sequence of a duplex DNA. The stronger affinity compared to the mononucleotide is most certainly due to the ability of the oligomer to interact with one of the drug's pentapeptide rings. The weaker binding, compared to the intercalation at the duplex GpC site, is likely the consequence of the facts that (1) end-stacking only results in the formation of half the number of hydrogen bonds as that of intercalation at the duplex GpC site, (2) only one of the two pentapeptide rings can be anchored at the minor groove, and (3) the fraying of the duplex ends may somewhat hamper the initial ACTD binding. No ready explanation, however, is at hand to account for the origin of the observed highly cooperative binding of the second drug ($K_2 = 3.2 \times 10^7$ M^{-1}). Somehow the binding of the first drug results in more favorable stacking, hydrogen-bonding, groove−pentapeptide ring interactions for the second drug.

It should be noted that Snyder et al. (1989) have also studied d(CGTACG) and d(CATCGATG). They found the binding constants to be 3.3×10^5 and 7.1×10^5 M^{-1}, respectively. Weaker binding affinities were also found for d(CTAGATCTAG) and d(CGTTAACG), with corresponding binding constants of $<7.4 \times 10^4$ and $<3.1 \times 10^4$ M^{-1}, respectively. Since end-stackings can occur with these oligomers, their weaker ACTD bindings thus suggest strong sequence as well as length dependence on the proposed cooperative binding. To further investigate the length-dependent binding characteristics, studies were also made with d(CGTXCGX′ACG), d(CGTCGXX′CGACG), and d(CGXCGYCGY′CGX′CG), where X or Y is either A or T while X′ and Y′ are complementary to X and Y, respectively. The results indicate that these oligomers with longer lengths exhibit ACTD binding constants on the order of 10^5 M^{-1} with no evidence of cooperativity.

The above two-step process of binding may also account for the observed much slower association kinetics exhibited by d(CGTCGACG) and d(CGACGTCG) than the other XGTCGACY octamers studied. The weak bindings of the other XGTCGACY octamers most likely occur at the internal sequences. The slower dissociation kinetics exhibited by the parent octamer may be attributed to hydrogen bonding with the stacked guanines and minor groove interactions with one of the two pentapeptide rings. These interactions are similar to those observed for intercalative binding at the GpC site. The observation of an association or dissociation process slower than that of the parent octamer for each of the (X)-CGTCGACG-(Y) series of oligomers is consistent with the varying degrees of interference by the dangling bases or A·T base pairs.

An alternative model which seems plausible in light of our data is that binding occurs to the termini of the oligomers in a hairpin, rather than duplex, conformation. If the hairpin conformation is preferred by ACTD, then the drug will drive the equilibrium to the hairpin with an associated energy cost. Such a scenario could explain positive cooperativity, since the cost of shifting the equilibrium (melting the duplex) is paid only when ACTD binds to the first oligomer of the associated duplex. Binding to the second oligomer is "free". The difference between K_2 and K_1 would then be a measure of the relative free energies of the duplex and hairpin. This free energy difference would be sequence-dependent, which might explain why some oligomers with 3′-G residues exhibit cooperative binding, and others do not. To test the possible strong ACTD binding to the hairpin termini, spectral titrations were made with d(CGGTTCCG) and d(CGTTTTCG) which favor hairpin conformations. The results, however, indicate only moderate binding strengths ($\sim 10^5$ M^{-1}) for these oligomers. Comparison of the gel electrophoretic patterns of these oligomers as well as d(CGTCGACG) in the absence and in the presence of ACTD also failed to show evidence of gel retardation due to drug binding to hairpin (results not shown).

If ACTD were indeed stacking at the termini of dimeric duplex ends, there exist the possibilities that a drug-induced self-association of oligonucleotides can occur to form higher aggregates via ACTD dimerization or via ACTD sandwiching between two stacked duplexes (Lancelot & Thuong, 1986) to simulate a preferred classic GC site. Contribution from the former scenario probably will not be significant since the dimerization constant of ACTD in aqueous solution is roughly 10^3 M^{-1} (Crothers et al., 1968). Although attempts were made to observe such possible aggregate formation, no evidence of ladder-like gel patterns with progressively slower moving bands was observed in our gel experiments.

ACKNOWLEDGMENT

We thank Q. Johnson for her careful reading of the manuscript.

REFERENCES

Bailey, S. A., Graves, D. E., & Rill, R. (1994) *Biochemistry 33*, 11493−11500.

Chiao, Y.-C. C., Rao, G., Hook, J. W., III, & Krugh, T. R. (1979) *Biopolymers 18*, 1749−1762.

Connors, K. A. (1987) *Binding Constants*, pp 50−51, John Wiley & Sons, New York, NY.

Crothers, D. M., Sabol, S. L., Ratner, D. I., & Muller, W. (1968) *Biochemistry 7*, 1817−1823.

Fasman, G. D., Ed. (1975) *CRC Handbook of Biochemistry and Molecular Biology*, 3rd ed., Vol. I, p 589, CRC Press, Cleveland, OH.

Homer, R. B. (1969) *Arch. Biochem. Biophys. 129*, 405−407.

Kamitori, S., & Takusagawa, F. (1992) *J. Mol. Biol. 225*, 445−456.

Krugh, T. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1911−1914.

Krugh, T. R., & Neely, J. W. (1973) *Biochemistry 12*, 4418−4425.

Lancelot, G., & Thuong, N. T. (1986) *Biochemistry 25*, 5357−5363.

Modest, E. J., & Sengupta, S. K. (1974) *Cancer Chemother. Rep. 58*, 35−48.

Muller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251−290.

Rill, R. L., Marsch, G. A., & Graves, D. E. (1989) *J. Biomol. Struct. Dyn. 7*, 591−604.

Snyder, J. G., Hartman, N. G., D'Estantoit, B. L., Kennard, O., Remeta, D. P., & Breslauer, K. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3968−3972.

Sobell, H. M., & Jain, S. C. (1972) *J. Mol. Biol. 68*, 21−34.

Wadkins, R. M., & Jovin, T. M. (1991) *Biochemistry 30*, 9469−9478.

Waring, M. (1970) *J. Mol. Biol. 54*, 247−279.

BI952907T