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Kinetic and Equilibrium Binding Studies of Actinomycin D with Some d(TGCA)-Containing Dodecamers†

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ABSTRACT: Comparative kinetic, melting, and equilibrium binding studies of actinomycin D (ACTD) with d(ATATACGTATAT), four d(TGCA)-containing dodecamers, and poly(dG-dC)·poly(dG-dC) revealed that (1) the affinity of ACTD for the dC-dG sequence is much less than for the dG-dC sequence; (2) ACTD forms 1:1 and 2:1 drug–duplex complexes with d(TATATGCATATA) and d(TATGCATGCATA), respectively, and their SDS driven dissociations exhibit single-exponential characteristics with rates ($\sim 5 \times 10^{-4}$ s^{-1} at 20 °C) slightly slower than that of poly(dG-dC)·poly(dG-dC); (3) although the melting temperature of d(CATGCATGCATG) is 8–9 deg higher than that of d(TATGCATGCATA), the rates of ACTD dissociation from these two oligomers are not greatly different and binding constants of (1–5) $\times 10^7$ M^{-1} have been estimated for both; (4) a 3:1 stoichiometry is exhibited by ACTD binding to duplex d(TGCATGCATGCA) and the complex dissociates with two characteristic times, the fast component ($1/k = \sim 100$ s) comprising $^2/_3$ of the contribution and the slow process (~ 2000 s) contributing the other $^1/_3$; and (5) the slow dissociation kinetics of an oligomer appears to be correlated to the higher percentage of slow association kinetics detectable by non-stop-flow techniques. These results indicate that the d(TGCA) sequence is a stronger binding and a slower dissociation site than the d(CGCG) sequence and suggest that base pairs flanking the dG-dC intercalative site may modulate interactions of the pentapeptide rings of ACTD with the DNA minor groove. The fast ACTD dissociation from the near-end dG-dC sites in d(TGCATGCATGCA) is most likely due to the inability of one of the pentapeptide rings to anchor securely, a consequence of end-fraying effects.

Actinomycin D (ACTD) is an antitumor antibiotic that contains a 2-amino-phenoxazin-3-one chromophore and two cyclic pentapeptide lactones. The biological activity of ACTD is believed to be the consequence of its ability to bind to duplex DNA, which results in the inhibition of DNA-dependent RNA polymerase. Earlier binding studies with synthetic polynucleotides (Goldberg et al., 1962; Wells & Larson, 1970) had established the guanine specificity of this drug. Detailed spectroscopic and hydrodynamic studies led Muller and Crothers (1968) and Waring (1970) to conclude that ACTD binds to DNA via insertion of its phenoxazone chromophore between the DNA base pairs. On the basis of their X-ray diffraction results of a 2:1 complex of deoxyguanosine with ACTD, Sobell and Jain (1972) subsequently proposed a binding model with intercalation at the dG-dC sequence and specific hydrogen bonding between the 2-amino group of guanine and the carbonyl oxygen of the threonine of the peptide rings. NMR studies (Patel, 1974; Krugh et al., 1977; Brown et al., 1984) using oligonucleotides containing the dG-dC sequence had generally agreed with such a binding model. Recent DNase I footprinting experiments (Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984a) have also confirmed the dG-dC binding specificity of ACTD. These observations are further supported by the finding that the most prominent RNA elongation inhibition sites are encoded by a consensus tetranucleotide sequence XGCY, where X is any nucleotide but G and Y is any nucleotide but C (Aivasashvilli & Beabealashvilli, 1983), suggesting possible effects of neighboring base pairs on the ACTD binding.

The combination of a planar intercalating phenoxazone chromophore and the two pentapeptide rings appears to be responsible for some of the unusual kinetic properties observed for the binding of ACTD to DNA. For example, the association of ACTD to natural DNA is characterized by five

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separate rate constants with the three slow processes appearing to be unimolecular (Bittman & Blau, 1975). The sodium dodecyl sulfate (SDS) induced drug dissociation from DNA also exhibits multiexponential decay, with the slowest of the order of 1000 s at room temperature. These kinetic results led Muller and Crothers (1968) to suggest that the complicated kinetics are the result of a series of conformational changes in the peptide backbones of ACTD. Krugh et al. (1980), however, found that the contribution from this slow component increases as the G:C content of the DNA increases and the dissociation of ACTD from poly(dG-dC)·poly(dG-dC) exhibits a single exponential decay. These observations led them to suggest that the multiexponential nature of ACTD dissociation from native DNA is the consequence of site heterogeneity. The slow dissociation of ACTD from DNA is of significance, as it apparently correlates with the pharmacological activities of this drug (Muller & Crothers, 1968).

Although the intercalative binding preference of ACTD to the dG-dC sequence has been well established through optical and NMR studies using various dinucleotides (Krugh, 1972; Krugh & Neely, 1973; Krugh & Chen, 1975; Chiao & Krugh, 1977; Krugh et al., 1977), first neighbor frequency circular dichroism (CD) analysis of ACTD + DNA systems had led others (Allen et al., 1977; Allen & Gray, 1984) to suggest that this drug binds to the dC-dG sequence as strongly as it binds to dG-dC. It is, thus, of some interest to study oligonucleotide systems which can demonstrate directly the possible binding differences of these two sequences and to establish conclusively that the slow dissociation site is also dG-dC. It is also of value to investigate the possible effects of neighboring base pairs on the binding and kinetic characteristics of the dG-dC site. To this end, binding and kinetic studies of ACTD have been carried out with the self-complimentary dodecamers d(ATATACGTATAT), d(TATATGCATATA), d(TATGCATGCATA), d(CATGCATGCATG), and d(TGCATGCATGCA).

Comparative binding studies of ACTD with d(TATATGCATATA) and d(ATATACGTATAT) are designed to give straightforward answers to the binding preference of dG-dC vs dC-dG sequences, since ACTD binds negligibly to the AT or TA sequence and binding at the central sequence should preclude the binding at the neighboring guanine-containing sequences. Comparative binding and kinetic studies of the d(TGCA)-containing dodecamers d(TATATGCATATA), d(TATGCATGCATA), and d(TGCATGCATGCA) are intended to investigate the role of neighboring A-T base pairs on the ACTD intercalation at the dG-dC sequence and possible effects resulting from multiple binding sites, such as binding cooperativity and kinetic differentials of nonidentical binding sites. The replacement of the terminal A-T base pairs in d(TATGCATGCATA) to result in d(CATGCATGCATG) will increase the melting temperature, and thus, comparative studies with these two oligomers should yield the effect of duplex stability on the association and dissociation kinetics.

Materials and Methods

Deoxyoligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer using β-cyanoethyl phosphoramidite chemistry (Sinha et al., 1984). The detritylated products were subsequently purified by anion-exchange and C-18 reverse-phase high-performance liquid chromatography (HPLC). The lyophilized oligomers were then dissolved in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer of pH 8 containing 0.1 M NaCl. All experiments were carried out in this buffer. Concentrations of these oligonucleotides (per nucleotide unless stated otherwise) were determined from absorbances at 260 nm after melting, with the following extinction coefficients:

oligonucleotide	extinction coeff (per base) (M^{-1} cm^{-1})
d(ATATACGTATAT)	10700
d(TATATGCATATA)	10600
d(TATGCATGCATA)	10000
d(TGCATGCATGCA)	9900
d(CATGCATGCATG)	8900

These extinction coefficients were obtained through nearest-neighbor approximation by use of mono- and dinucleotide values tabulated in the *CRC Handbook of Biochemistry and Molecular Biology* (1975). Poly(dG-dC)·poly(dG-dC) was purchased from P-L Biochemicals, and an extinction coefficient of 8400 M^{-1} cm^{-1} at 255 nm had been used for its concentration determination. Sodium dodecyl sulfate was obtained from Pierce Chemical Co. Actinomycin D was purchased from Sigma and used without further purification. The concentration of ACTD was determined with an extinction coefficient of 24 500 M^{-1} cm^{-1} at 440 nm.

Absorption spectra were measured with a Cary 210 spectrophotometric system. Melting experiments were carried out by monitoring absorbances and collecting data every 15 s with an Apple II microcomputer. A heating rate of 0.5 deg/min was maintained by a Neslab RTE-8 refrigerated circulating bath and an EPT-4RC temperature programmer. Differential thermal melting curves were obtained from these collected data. CD spectra were measured by a JASCO J-500A recording spectropolarimeter at appropriate temperatures with water-jacketed cells.

Kinetic measurements by means of absorbance monitoring were carried out at 20 °C with a Cary 210 using the stirrer accessory. Time-dependent absorbance changes were monitored at 440 or 427 nm for the association and 452 nm for the 1% SDS-induced dissociation experiments. Data were collected with an Apple II microcomputer, and the first data point was taken 5 s after the sample application in a typical association reaction. Kinetic studies with CD were carried out by monitoring the ellipticity changes at 293 nm with a chart recorder and rigorous manual shaking for mixing (requires about 10 s).

Results

Absorption Spectral Titrations. In the absence of DNA, actinomycin D exhibits a broad absorption maximum around 440 nm. Addition of DNA results in the depression of the 440-nm band with a concomitant absorbance enhancement in the 480-nm region (spectra not shown). An isosbestic point is evident at 460 nm, suggesting a two-state equilibrium. Comparative spectral titrations with d(ATATACGTATAT) and d(TATATGCATATA) reveal that the dC-dG-containing oligomer exhibits much less of a spectral alteration (results not shown), suggesting that the binding of ACTD to the dC-dG or dA-dC sequence is much weaker than to dG-dC.

The spectral features resulting from DNA binding are more clearly seen with difference spectra in which the free drug contribution has been subtracted. Typical difference spectra (not shown) suggest that the greatest absorbance increase and decrease are seen at 475 and 427 nm, respectively. A somewhat more quantitative result of DNA binding can be achieved if the apparent molar drug absorptivity at a given wavelength is plotted against the DNA/drug concentration ratio. In Figure 1A the apparent molar absorptivity at 440 nm is plotted against [DNA, duplex]/[ACTD] to indicate drug to duplex

Table I: Comparison of Dissociation and Association Kinetics at 20 °C for Some Dodecamers and Poly(dG-dC)·Poly(dG-dC)

oligonucleotide	[DNA] (μM)	[ACTD] (μM)	k(dissoc) (s^{-1})	k(assoc) (s^{-1})[a]	% A(slow)[b]
poly(dG-dC)	10.0	1.42	7.8×10^{-4}	2.7×10^{-2}	84
	29.0	1.40	1.1×10^{-3}	5.5×10^{-2}	58
d(TATGCATGCATA)	16.9	1.41	4.4×10^{-4}	2.7×10^{-2}	~100
d(CATGCATGCATA)	17.3	1.40	4.3×10^{-4}	2.8×10^{-2}	93
d(TGCATGCATGCA)	15.4	1.41	1.7×10^{-2}	3.2×10^{-2}	36
			6.3×10^{-4}		

[a] k(assoc) is approximated by fitting the data of the first 3 min with a single exponential. [b] % A(slow) is obtained by comparing the detectable absorbance change at 440 nm during the association run with the total absorbance change measured from the spectra at this wavelength.

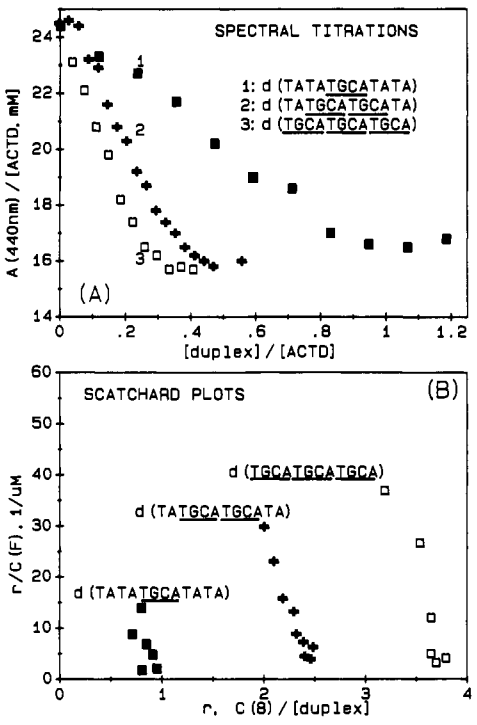

FIGURE 1: Absorption spectral titrations of actinomycin D with some dodecamers in 10 mM Tris-HCl pH 8 buffer containing 0.1 M NaCl. (A) Plots of apparent extinction coefficients at 440 nm vs [DNA, duplex]/[ACTD] for titrations with d(TATATGCATATA), d(TATGCATGCATA), and d(TGCATGCATGCA). Except for d(TATATGCATATA) at 10 °C, all spectral titrations have been carried out at 20 °C. (B) Scatchard plots using the actinomycin D absorbances at 440 nm for the three dodecamers. C(F) and C(B) are free and bound ACTD concentrations in μM, respectively, and r is the ratio of bound ACTD to oligomer concentration in duplex.

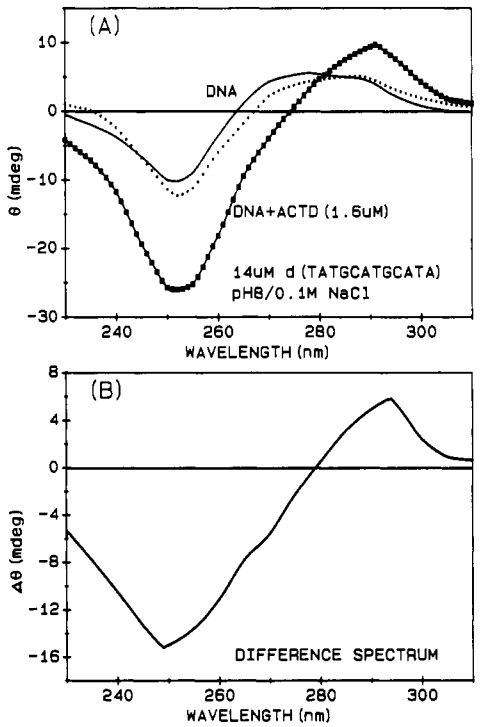

FIGURE 2: Effects of ACTD binding on the circular dichroism. (A) CD spectrum of 14 μM d(TATGCATGCATA) (solid line) using a 5-cm water-jacketed cell; spectral sum of 14 μM d(TATGCATGCATA) and 1.6 μM ACTD in buffer, no physical interactions (dotted line); CD spectrum of 14 μM d(TATGCATGCATA) solution containing 1.6 μM ACTD (connected squares). (B) Difference CD spectrum obtained by subtraction of the DNA and ACTD spectral sum from the DNA + ACTD mixture (connected squares – dotted curve) in (A). Molar ellipticity can be obtained by $[\theta] = 100\theta/(lC)$, where θ is the ellipticity in millideg, l is the path length in cm, and C is the concentration (drug or DNA) in mM.

binding stoichiometries of roughly 1, 2, and 3 for d(TATATGCATATA), d(TATGCATGCATA), and d(TGCATGCATGCA), respectively.

Scatchard plots for the ACTD titrations with these three dodecamers are shown in Figure 1B. It is apparent that the plots are roughly linear and the slopes are not greatly different. Binding constants of $(1-5) \times 10^7$ M^{-1} and r intercepts of 1, 2.3, and 3.7 have been estimated from these plots. It is noteworthy that the binding strengths of ACTD to these d(TGCA)-containing oligomers are higher than that to poly(dG-dC)·poly(dG-dC) (Wells & Larson, 1970).

CD Spectral Characteristics. Free actinomycin D exhibits only weak optical activity. Binding to DNA, however, results in an enhancement of circular dichroism in the drug as well as in the DNA spectral regions. These features are illustrated in Figure 2A with d(TATGCATGCATA). It is apparent that the interaction of ACTD with DNA results in the enhancement of Cotton effects at 293- and 250-nm regions. This can be seen more clearly by the difference spectrum (Figure 2B), in which the CD contributions of free drug and DNA have been subtracted. Although the CD intensities are severalfold smaller in the drug spectral region, enhancements of negative ellipticities at 465 and 380 nm are also evident from the difference spectra at long wavelengths (not shown). Although spectral alterations upon drug binding to d(TATATGCATATA) and d(TGCATGCATGCA) are similar to those of d(TATGCATGCATA), only slight changes in the CD spectrum are observed for d(ATATACGTATAT) upon the addition of ACTD (not shown). This is consistent with the much lower binding ability of the dC-dG sequence as observed in the absorbance titrations.

SDS-Induced Dissociation Kinetics. Although it is well-known that dissociation of ACTD from poly(dG-dC)·poly(dG-dC) is quite slow, it was, nonetheless, surprising to find that the dissociation rates of ACTD from some d(TGCA)-containing oligonucleotides are of the same order or even slower. Time-dependent absorbance changes at 452 nm after the introduction of 1% SDS (by adding appropriate amounts of 20% SDS) are shown in Figure 3A for the ACTD–d(TATATGCATATA) and ACTD–d(TATGCATGCATA) solutions. The corresponding semilog plots are presented in Figure 3B. Single exponential decays are apparent with characteristic times of 1800 and 2200 s for ACTD dissociations from d-

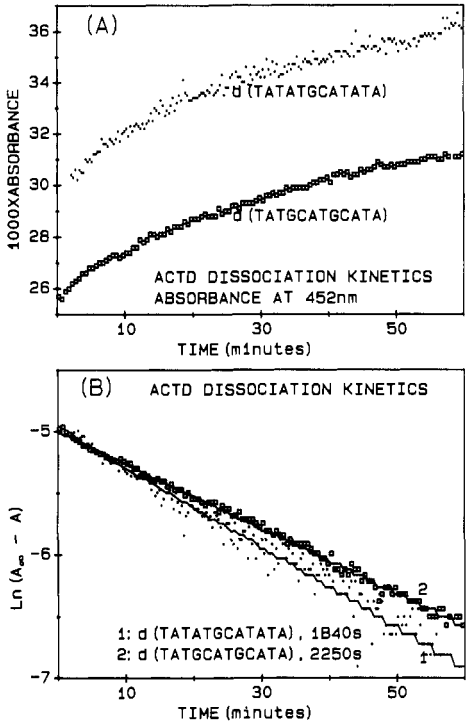

FIGURE 3: Kinetics of SDS- (1%) induced ACTD dissociation from some dG-dC containing oligonucleotides at 20 °C. (A) Time-dependent absorbance change at 452 nm after the introduction of 1% SDS for the 60 μM d(TATATGCATATA) + 1.41 μM ACTD mixture (dots) and the 33.7 μM d(TATGCATGCATA) + 1.41 μM ACTD mixture (squares). (B) Semilog plots of the dissociation curves of (A). The connected lines are the linear least squares fits which yield the time constants indicated.

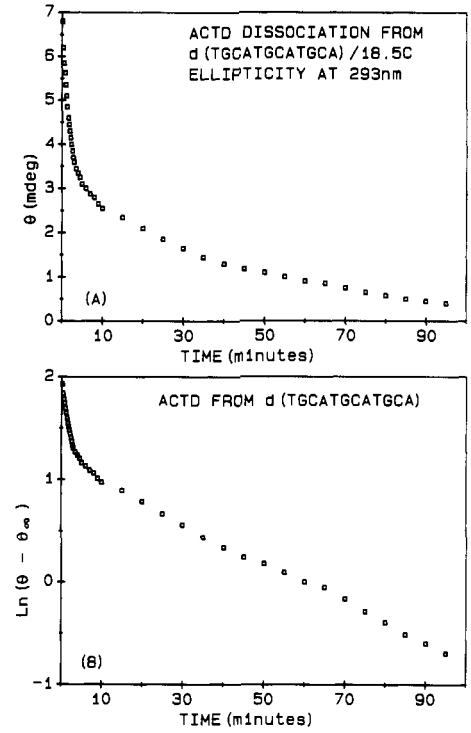

FIGURE 4: (A) Time-dependent ellipticity change at 293 nm after the introduction of 1% SDS for the 30.7 μM d(TGCATGCATGCA) + 1.4 μM ACTD mixture. (B) Semilog plot of the data in (A). CD kinetic measurements were carried out at 19 °C with a 5-cm water-jacketed cylindrical cell.

(TATATGCATATA) and d(TATGCATGCATA), respectively. A dissociation time of 1300 s is obtained for poly(dG-dC)·poly(dG-dC) under similar conditions (see Table I).

To study the reaction kinetics, advantage can also be taken of the fact that binding of ACTD to DNA results in a strong CD enhancement at 293 nm (see Figure 2B). The rate parameters obtained by both absorbance and ellipticity changes are in general agreement. The kinetic measurements of ACTD dissociation from d(TGCATGCATGCA) through such ellipticity monitoring are shown in Figure 4. It is apparent that the decay is not a single exponential. A faster time constant of around 100 s contributes a total ellipticity change of $^2/_3$, whereas a much slower process of 2500 s constitutes only about $^1/_3$ of the total ellipticity change. These observations are, thus, consistent with the notion that the slow component is derived from ACTD dissociating from the central dG-dC sequence whereas the near-end dG-dC sequences give rise to the faster dissociation.

Kinetics of Drug–DNA Association. To investigate the possible correlation between the kinetics of dissociation and association, rates of ACTD binding to various oligomers were also measured. Consistent with earlier association kinetic measurements (Bittman & Blau, 1975; Fox & Waring, 1984b; Brown & Shafer, 1987), ACTD complexation with our oligonucleotides containing identical binding sites, in contrast to the observed single-exponential dissociation kinetics, exhibits multiple rate processes. These earlier association kinetic studies have indicated that the two slowest association components contribute no more than 25% of the total absorbance changes. To our surprise, however, the slow association components for some oligonucleotides contribute more than 50%, and several even contribute nearly 100% at 20 °C. There appears to be an apparent correlation between the slow dissociation and the percentage of detectable (non-stop-flow) slow association as can be seen in Table I. For d(TATGCATGCATA) and d(CATGCATGCATG), where single-exponential slow dissociation kinetics are observed, more than 90% of association kinetics can be detected. On the other hand, only 36% of the absorbance change during the association kinetics has been detected by our technique for the ACTD + d(TGCATGCATGCA) system in which the SDS-driven dissociation exhibits a fast ($^2/_3$ contribution) and a slow ($^1/_3$ contribution) kinetics.

Although the detectable slow association kinetics are not single exponential, the contribution to the slowest component (of the order of 10^{-3} s^{-1}) is only a few percent. Thus, qualitative rate comparisons can be made by attempting to fit the absorbance decay curve with a single exponential. By use of the data for the first 3 min, the fitted first-order rate constants are also included in Table I. The slow-dissociating oligomers do indeed exhibit slower association rates.

The ability to detect large portions of slow-association components, to be sure, is partially due to the low oligomer and drug concentrations used in our studies which resulted in significant slowing down of the bimolecular events which then greatly overlap with the slow unimolecular kinetics. Indeed, kinetic studies with 10 and 29 μM poly(dG-dC)·poly(dG-dC) with 1.4 μM ACTD revealed that with a 3-fold increase in DNA concentration the slow-association components observable by our non-stop-flow technique decreased from 84 to 58% and the association rate increased roughly 2-fold (see Table I). It is also interesting to note in passing that the dissociation rate increased somewhat, consistent with the P/D dependence observed by Krugh et al. (1980) for the poly(dG-dC)·poly(dG-dC) system. Another possible contributing factor in the oligonucleotide association kinetics, however, is that at these low DNA concentrations a large fraction of these oligomers exists in the single-stranded form. Thus, the observed slow-association rates may partially reflect processes of initial

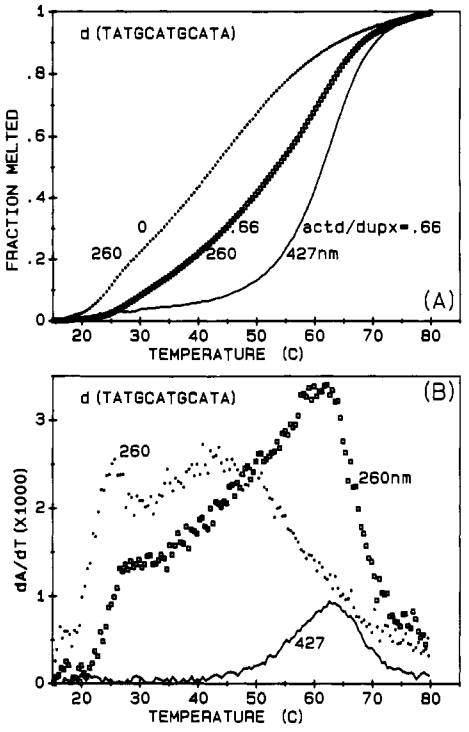

FIGURE 5: (A) Comparison of thermal denaturation profiles of 61 μM d(TATGCATGCATA) (dots) and in the presence of 1.7 μM ACTD monitored at 260 nm (squares) and 427 nm (solid curve). (B) Differential melting curves of 61 μM d(TATGCATGCATA) (dots) and in the presence of 1.7 μM ACTD with 260-nm (squares) and 427-nm (solid curve) monitoring.

ACTD–single strand interactions and subsequent duplex formation resulting in intercalated complexes. Indeed, an association experiment with d(TATGCATGCATA) carried out at 44.7 °C (melting temperatures of this oligomer and its ACTD complex are 43.5 and 65.0 °C, respectively) revealed a similar slow kinetic pattern with a rate constant estimated from the first 3 min to be around 3.2×10^{-2} s^{-1}, not significantly different from that at 20 °C.

Thermal Denaturation Experiments. To investigate the effect of drug binding on the duplex stability, thermal denaturation measurements were carried out both in the presence and in the absence of ACTD. Melting profiles are represented by d(TATGCATGCATA) at 5.2 μM strand concentration in the absence and in the presence of nonsaturating amounts of ACTD (1.7 μM) (Figure 5A). In the absence of ACTD the oligomer exhibits a rather broad melting curve with a melting temperature of roughly 44 °C. Binding of ACTD results in significant enhancements in both duplex stability (t_m = 64 °C) and melting cooperativity. In contrast to solutions with saturating amounts of drug (not shown), the melting profiles resulting from monitoring the drug and DNA spectral regions are substantially different as can be seen in Figure 5A. Such differences are to be expected since at an [ACTD]/[strand] ratio of 1/3 some oligomers will be devoid of drug and consequently melt at lower temperatures.

Some melting features can be seen more clearly with differential plots as shown in Figure 5B. A biphasic melting is readily apparent in such a plot for d(TATGCATGCATA) in the absence of ACTD. The lower temperature transition (~26 °C) is most likely due to the premelting of the TAT regions at both ends of the oligomer. This is supported by the appearance of a premelt transition at around the same temperature even in the presence of the drug, as ACTD is not expected to bind at these regions. The higher cooperative transition resulting from the drug binding is indicated by a more sharply defined differential plot. The correlation of drug release with duplex disruption is clearly indicated by the near identical melting temperatures obtained, regardless of whether the absorbance is monitored at the drug or at the DNA spectral region.

A biphasic melting for d(CATGCATGCATG) is also apparent from the differential plot and melting temperatures of 31.0 and 52.8 °C obtained for the two respective transitions (not shown). The 5 deg higher first-phase transition, in comparison to d(TATGCATGCATA), can be attributed to the presence of G-C base pairs at both terminals which most likely reduces the fraying effects. Binding of saturating amounts of ACTD results in the duplex melting at 68 °C (not shown).

The much weaker binding of ACTD at the dC-dG site is also supported by a mere 2 deg increase in the melting temperature of 18 μM d(ATATACGTATAT) when 1.7 μM ACTD is added. In contrast, the melting temperature of d(TATATGCATATA) increases roughly by 18 deg under similar conditions (results not shown).

Discussion

Comparative spectral titrations and melting experiments with d(ATATACGTATAT) and d(TATATGCATATA) clearly demonstrate that actinomycin D strongly prefers dG-dC but only binds very weakly, if at all, to the dC-dG sequence. The strong binding at the dG-dC sequence is further supported by the ability of d(TATGCATGCATA) and d-(TGCATGCATGCA) to respectively bind two and three drugs for each duplex and suggests that the ACTD binding site size is equal to or less than four base pairs. These conclusions are in conformance with the ones reached by Wilson et al. (1986) in which NMR studies were carried out with a series of oligomers containing d(CGCG) and d(GCGC) sequences. Their results in fact suggest that ACTD can bind to contiguous dG-dC sequences. Our preliminary studies (not shown) with oligomers having one or no base pair separating dG-dC sequences appear to support such a conclusion.

The observation that ACTD dissociates from d(TATATG-CATATA), d(TATGCATGCATA), or d-(CATGCATGCATG) single exponentially but slower than from poly(dG-dC)·poly(dG-dC) appears to suggest that the slow dissociation of ACTD from DNA may partly be the consequence of the pentapeptide ring–DNA interactions at the minor groove which are modulated by the neighboring base pairs flanking the dG-dC binding site, with the A-T base pair imparting more favorable interactions than the G-C base pairs. The faster dissociation of ACTDs bound to the near-end dG-dC sequences in d(TGCATGCATGCA) is most likely due to the inability of one of the pentapeptide rings to anchor securely to DNA, a consequence of end-fraying effects. Duplex stability appears to also dominate the dissociation of ACTD from d(ATGCAT) as suggested by a 1-min characteristic time (Krugh and Chen, unpublished results), where the melting temperature of this hexanucleotide under the experimental conditions is around room temperature. However, the fact that the rates of dissociation of ACTD from d(TATGCATGCA-TA) and d(CATGCATGCATG) are not greatly different, even though there is an 8–9-deg difference in their melting temperatures while poly(dG-dC)·poly(dG-dC) melts roughly 50 deg higher than these two oligomers and exhibits faster ACTD dissociation rates, suggests that the groove–ring interactions dictate the rapidity of the ACTD dissociation from DNA as long as the duplex is reasonably stable.

Our finding that d(TGCA) is a stronger binding and slower dissociation site than d(CGCG) is consistent with the site-

heterogeneity dissociation model of Krugh et al. (1980) for the natural DNA and further extends such a heterogeneity concept beyond the 10 distinct dinucleotide intercalation sites. The observation of multiexponential association kinetics for oligomers with equivalent binding sites, however, appears to also support the multistep model of complex formation as suggested by Muller and Crothers (1968). An observed single-exponential dissociation kinetics for the oligomers with equivalent binding sites may not necessarily be in conflict with the multistep association model, if the dissociation is governed by a rate-limiting step. It is interesting to note that the site heterogeneity beyond dinucleotide intercalation sites may be the consequences of interactions involving the pentapeptide rings.

Phillips and Crothers (1986) recently developed an in vitro transcriptional assay to detect the DNA sequence specificity of the drug binding sites and the kinetics of dissociation of drugs from those sites. They found an ACTD-induced blockage to the elongation of RNA transcript around a d(AGCT) site, and the ACTD dissociation from this site is significantly slower than the rate constants for dissociation from the whole promoter fragment as measured by the detergent sequestration method. The major difference between these two techniques, as they correctly pointed out, is that an averaged dissociation from all binding sites is detected by the SDS sequestering process whereas in the transcriptional process a discrete site is measured. Their results are, thus, consistent with the interpretation that d(AGCT) is the slowest dissociation site in the fragment and support our notion that the A-T base pairs adjacent to the dG-dC intercalative site favor interactions with the pentapeptide rings.

It is also worth noting that recently Duffy and Lindell (1985) carried out ACTD binding studies at low drug concentrations (<80 nM) which are relevant to biological use. They studied rat liver DNA and identified a strong ACTD binding site(s). This site (1 out of every 330 base pairs) has an apparent binding constant of 7.6×10^7 M^{-1} and dissociates twice as slowly as that bound at higher drug concentrations. Our estimated binding constants of $(1-5) \times 10^7$ M^{-1} and the slow dissociation rates of the d(TGCA)-containing oligomers are, thus, consistent with their observations and may further suggest that one such site is most likely related to the d(TGCA) or d(AGCT) sequence in DNA.

One can only speculate on the reasons for the adjacent A-T base pair preference over G-C on the pentapeptide ring–DNA interactions. It may be that the hydrophobic character of the inner surface of the pentapeptide ring (Takusagawa, 1985) prefers the minor groove region in which no 2-amino group of guanine is exposed. Indeed, NMR studies with ACTD–d(ATGCAT) complex have detected close contacts between N-CH_3 of L-methylvaline and the adenosine (internal) H2 protons (Brown et al., 1984). The adjacent base pair effect may also arise from sequence-dependent conformational differences around the binding site which manifest in differential stacking and ring–groove interactions. The observed slow unimolecular components in the association kinetics may very well be due to such ring–groove interactions, although it does not appear to be the sole contributor since slow components have also been found in the ACTD–dG reactions (Brown & Shafer, 1987). Fox and Waring (1984) have attributed the slow association kinetics in native DNA to the "shuffling" of drugs to stronger binding sites. Such a model, however, is difficult to reconcile with the large fraction of slow-association components observed in our d(TGCA)-containing oligomers.

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