# Binding Specificities of Actinomycin D to Self-Complementary Tetranucleotide Sequences -XGCY-†

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ABSTRACT: Binding of actinomycin D (ACTD) to self-complementary decamers d(ATA-XGCY-TAT), where XGCY = TGCA, AGCT, CGCG, and GGCC, has been investigated by equilibrium, kinetic, and thermal denaturation studies. The results indicate that despite the presence of a GC dinucleotide sequence, -GGCC- exhibits a much weaker binding affinity toward ACTD than the other three tetranucleotide sequences. Binding constants estimated from Scatchard plots indicate that binding to the -GGCC- site is at least an order of magnitude weaker than binding to -CGCG- and -AGCT-, which in turn is only slightly weaker than binding to the -TGCA- sequence. At 18.5 °C and 1% SDS, ACTD dissociates from d(ATA-TGCA-TAT) with a slow characteristic time of 3300 s, roughly 4 times slower than dissociation from those containing -CGCG- and -AGCT- sequences and more than 2 orders of magnitude slower than that from -GGCC-. An 18.2 °C increase in the melting temperature is observed for the -TGCA-containing decamer upon binding of the ACTD, whereas increases of 10.3, 6.7, and 2.0 °C are observed for the -CGCG-, -AGCT-, and -GGCC-containing decamers, respectively. The effects observed by changing the adjacent base pair (sequence) may occur as a result of differential stacking and/or peptide ring–DNA groove interactions. Base sequence alterations adjacent to the ACTD binding site may result in differences in the minor groove environment and/or subtle conformational alterations at the intercalation site. The greatly reduced binding at the -GGCC- sequence is likely a consequence of a local non-B-genus, possibly an A-genus, conformation around the binding site. Our results from the SDS-driven dissociation kinetics support the site-heterogeneity model of drug dissociation from native DNA and further extend the heterogeneity concept beyond the dinucleotide sequence level.

Actinomycin D (ACTD) is an antitumor antibiotic that contains a 2-aminophenoxazin-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 of 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 a 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 further confirmed the dG-dC binding specificity of ACTD. Most recently, comparative studies with oligonucleotides of specific lengths and sequences (Wilson et al., 1986; Chen, 1988) have conclusively demonstrated that the binding of ACTD to the dC-dG sequence is much weaker than that to dG-dC. Thus, at the dinucleotide level, the binding preference of ACTD at the dG-dC sequence appears to be firmly established.

In 1983 Aivasashvilli and Beabealashvilli determined that the most prominent RNA elongation inhibition sites are encoded by a consensus tetranucleotide sequence XGCY, where X can be any nucleotide except G and Y can be any nucleotide except C. Although their findings are supportive of the fact that dG-dC is the strong binding site, the observation that ACTD exhibits negligible binding to the GGCC sequence strongly suggests that the base pairs adjacent to the G-C sequence may exert a profound influence on the ability of ACTD to bind. 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. Their results with ACTD appear to also support the notion that base sequences adjacent to the dG-dC intercalation site influence ACTD–DNA kinetics. Studies at low drug concentrations (<80 nM) that are relevant to biological use led Duffy and Lindell (1985) to the identification of a strong binding site. This site (one of every 330 base pairs) has an apparent binding constant of $7.6 \times 10^7$ $M^{-1}$ and exhibits a dissociation rate twice as slow as that of bound drugs at higher concentrations. Since there are several dG-dC sites within the stretch of 330 base pairs, this result again suggests that the binding ability of a dG-dC sequence must be strongly dependent on the adjacent base pairs or sequences.

The purpose of this research is to elucidate the base sequence specificity of ACTD binding beyond the dG-dC dinucleotide level through the use of selected synthetic oligonucleotides of defined length and sequence. Earlier, this laboratory embarked on the oligonucleotide–ACTD studies by synthesizing several -TGCA-containing dodecamers (Chen, 1988). The binding and kinetic measurements revealed ACTD to bind more strongly and to dissociate more slowly from the -TGCA- ol-

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igomers than from poly(dG-dC)·poly(dG-dC). Although these results appear to suggest that the d(TGCA) sequence provides a stronger binding and slower dissociation site than the d(CGCG) sequence, the roughly 50 °C higher melting of poly(dG-dC)·poly(dG-dC) may obscure some of the conclusions. To draw more definitive conclusions and to extend to other tetranucleotide sequences, a more systematic investigation on the relative ACTD binding abilities and kinetic behaviors of a series of decadeoxyoligonucleotides d(ATA-XGCY-TAT), each containing a unique tetranucleotide binding sequence at the center and nonbinding sequences at the terminals, has been carried out. In this paper, attention will be focused on the ACTD binding and kinetic characteristics of the self-complementary tetranucleotide binding sequences -TGCA-, -AGCT-, -CGCG-, and -GGCC-.

The rationale for choosing these oligomers relies on the fact that ACTD binds negligibly to the A-T and T-A sequences. Furthermore, the binding of ACTD at the central G-C sequence should result in the inhibition of ACTD binding at adjacent guanine containing weaker binding sequences, thus assuring a single binding site at the center of each oligomer. Oligomers containing single binding sites should provide model binding systems that will eliminate some of the complications arising from multiple-site processes. Other reasons for the choice of these oligomers are that, with the presence of at least two G-C base pairs, they are long enough to form duplexes at room temperature and their melting temperatures are not much higher than ambient, so that thermal denaturation experiments in the presence and in the absence of ACTD can be conveniently carried out for obtaining thermodynamic parameters of drug binding. Altering only 2 of the 10 base pairs is not expected to alter the global conformations of these four oligomers and, thus, ensures some measure of conformational integrity and rational comparison.

Elucidations of strong binding and slow dissociation tetranucleotide sequence(s) will greatly facilitate a detailed understanding of ACTD–DNA binding, which in turn may assist in the rational design of effective anticancer drugs. The slow dissociation of ACTD from DNA is of significance, as it apparently correlates with the pharmacological activities of this drug (Muller & Crothers, 1968).

## Materials and Methods

Oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer using the β-cyanoethyl phosphoramidite chemistry (Sinha et al., 1984). The crude oligomer was purified by a strong anion exchange (SAX) HPLC followed by reverse-phase HPLC chromatography. The SAX method consisted of a linear gradient of 100% A/0% B to 50% A/50% B, where buffer A refers to 20% acetonitrile and 3.4 mM potassium phosphate and buffer B refers to 20% acetonitrile and 0.68 M potassium phosphate, both at pH 6.8. A C-18 column was used in the reverse-phase chromatography with a mobile phase consisting of a linear gradient from 0 to 50% acetonitrile in the presence of 50 mM ammonium acetate.

The purified and lyophilized oligomers were dissolved in 10 mM Tris-HCl buffer at pH 8 containing 0.1 M NaCl. All experiments were carried out in this buffer. Concentrations of these oligomers (per nucleotide) were determined by measuring the absorbance at 260 nm after melting. Extinction coefficients (as follows) were obtained through nearest-neighbor approximation using mono- and dinucleotide values tabulated in the *CRC Handbook of Biochemistry and Molecular Biology*: 10 500 $cm^{-1}$ $M^{-1}$ for d(ATA**TGCA**TAT); 10 300 $cm^{-1}$ $M^{-1}$ for d(ATAA**GCTT**AT); 10 100 $cm^{-1}$ $M^{-1}$ for d(ATA**CGCG**TAT); and 10 000 $cm^{-1}$ $M^{-1}$ for d(ATA**GGCC**TAT).

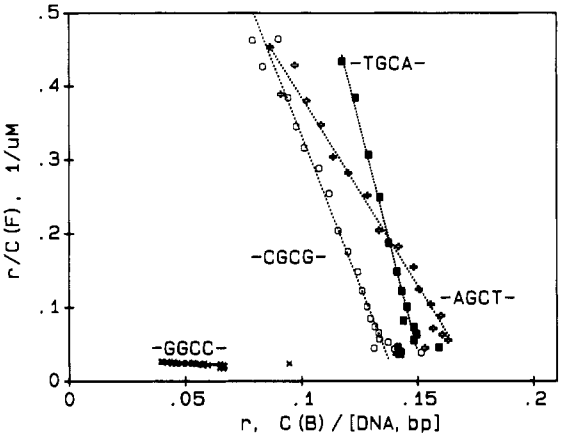


FIGURE 1: Scatchard plots for equilibrium titrations of ACTD with d(ATA-TGCA-TAT) (solid squares), d(ATA-AGCT-TAT) (+), d(ATA-CGCG-TAT) (open circles), and d(ATA-GGCC-TAT) (×). Absorbances of 440 nm after isosbestic correction at 462.5 nm have been used for obtaining the binding isotherms. The bound extinction coefficient was obtained through a plot of $1/(\epsilon-\epsilon_f)$ vs $1/([DNA,bp] - [ACTD])$ for d(ATA-AGCT-TAT) and an extrapolation of the last few points to infinite DNA concentration. The same $\epsilon_b$ was used for all four Scatchard plots. Straight lines are those of least-squares fits excluding the discordant points.

Absorption spectra were measured with a Cary 210 spectrophotometric system. CD spectra were measured by a Jasco J-500A recording spectropolarimeter at appropriate temperatures using water-jacketed cells. Spectral titrations have been carried out at 18.5 °C by starting with an ACTD solution and a progressive addition of the oligomer stock. Absorbance changes at 427 or 440 nm were used to obtain Scatchard plots. Kinetic meaurements by means of absorbance monitoring were carried out with a Cary 210 spectrophotometric system using the stirrer accessory. Time-dependent absorbance changes were monitored at 440 or 427 nm for the association and at 452 nm for the 1% sodium dodecyl sulfate (SDS) induced dissociation experiments. Data were collected with an Apple II microcomputer, and in a typical association reaction the first data point taken was about 5 s after the sample application. Kinetic studies with CD were carried out by monitoring the ellipticity changes at 293 nm by use of a chart recorder and rigorous manual shaking for mixing (requires about 10 s).

Thermal melting profiles of each oligomer (60 μM in nucleotide) and its drug–DNA mixture (containing 4.3 μM ACTD) were carried out with 1-cm semimicro cells by monitoring absorbances at 275 and 427 nm, respectively, and collecting data at 15-s intervals with an Apple II microcomputer. A heating rate of 0.5 °C/min was maintained by a Neslab RTE-8 refrigerated circulating bath and an EPT-4RC temperature programmer. Numerical differentiations were performed to obtain differential melting profiles from which melting temperatures and half-widths were deduced.

## Results

*The -GGCC- Sequence Exhibits a Much Weaker ACTD Binding Affinity.* The representative Scatchard plots resulting from absorbance measurements at 440 nm are shown in Figure 1. The slopes of these plots revealed that the -GGCC- containing decamer exhibits a binding constant of less than 0.2 × $10^6$ $M^{-1}$, at least an order of magnitude weaker than the -AGCT- (5 × $10^6$ $M^{-1}$), -CGCG- (8 × $10^6$ $M^{-1}$), and -TGCA- (12 × $10^6$ $M^{-1}$) containing oligomers. The highest ACTD binding affinity exhibited by the -TGCA- sequence is the more

Table I: Comparison of Equilibrium, Kinetic, and Thermal Denaturation Results[a]

| oligonucleotide | $T_m$ (°C) | $\Delta T_m$ (°C) | $K$ ($1/\mu M$) | $n^b$ | $k^{-1}$ (dissoc) (s) |
|---|---|---|---|---|---|
| d(ATATGCATAT) | 22.6 | 18.2 | 12 | 0.15 | 3340 |
| d(ATACGCGTAT) | 41.5 | 10.3 | 8 | 0.14 | 850 |
| d(ATAAGCTTAT) | 28.0 | 6.7 | 5 | 0.18 | 795 |
| d(ATAGGCCTAT) | 37.6 | 2.0 | 0.2 | 0.20 | |

[a] The experimental data shown for the kinetics and melting are averages of duplicate experiments. [b] Binding sites per base pair.

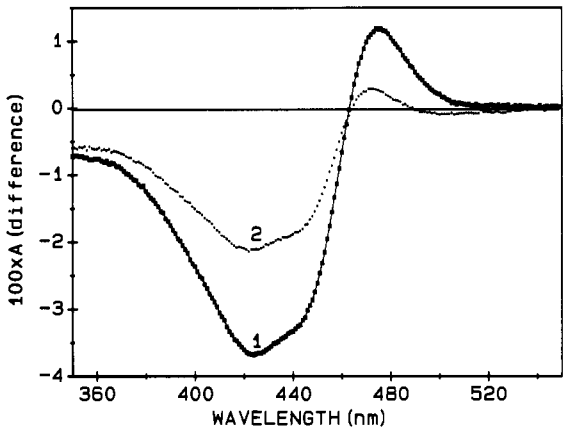


FIGURE 2: Comparison of absorption difference spectra of decamers containing -AGCT- vs -GGCC-. The difference spectra were obtained by subtracting those of initial ACTD solutions at the start of the equilibrium titration with appropriate dilution corrections from those of final mixtures of 3.38 $\mu$M ACTD + 71 $\mu$M d(ATA-AGCT-TAT) (1) and 3.18 $\mu$M ACTD + 83 $\mu$M d(ATA-GGCC-TAT) (2). Measurements were made at 18.5 °C by using cuvettes of 1-cm path length.

remarkable in view of the fact that our equilibrium titrations were carried out at 18.5 °C, a mere 4 °C lower than the melting temperature of d(ATA-TGCA-TAT) (see Table I). Due to the humidity problem in our laboratory, no titration had been attempted at lower temperatures.

*Additional Spectral Evidence for the Weak Binding of the -GGCC- Sequence.* The much reduced ACTD binding to the -GGCC- site can also be seen by comparisons of the absorption or CD difference spectra. Difference absorption spectra between drug-DNA complexes and free ACTD are shown in Figure 2. From this figure, it is apparent that ACTD binding to the d(ATA-AGCT-TAT) results in an absorbance increase at 475 nm and an absorbance decrease at 427 nm. Spectral alteration on binding to the -TGCA- and -CGCG-containing oligomers are of similar magnitudes and thus are not shown. The weaker binding of the -GGCC- containing decamer is indicated by the much reduced spectral change. Difference CD spectra at shorter wavelengths, obtained by subtracting the DNA contributions from the ACTD–oligomer complexes, are shown in Figure 3 for the four oligomers studied. The -TGCA-, -AGCT-, and -CGCG-containing oligomers all exhibit strong ellipticity increases at the 293-nm region and decreases at the 255-nm region. The considerably weaker binding of the -GGCC-containing oligomer is again indicated by the much reduced CD spectral alterations.

*The -TGCA- Sequence Exhibits the Slowest ACTD Dissociation Kinetics.* The SDS–induced ACTD dissociation kinetics have been measured at 18.5 °C by monitoring the rate of ellipticity change at 293 nm or absorbance change at 452 nm. Semilog plots of ellipticity change for the ACTD dissociation from the decamers containing -TGCA-, -AGCT-, and -CGCG- are shown in Figure 4. Single-exponential kinetics

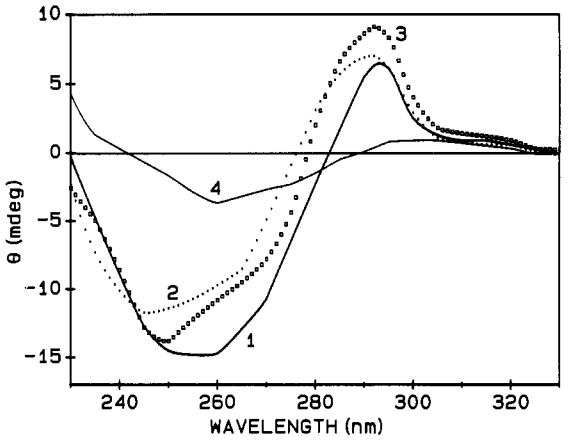


FIGURE 3: Comparison of difference CD spectra of 1.6 $\mu$M of ACTD in 40 $\mu$M oligonucleotide solutions with the oligomer contributions subtracted: (1) d(ATA-TGCA-TAT); (2) d(ATA-AGCT-TAT); (3) d(ATA-CGCG-TAT); (4) d(ATA-GGCC-TAT). Measurements were made at 18.5 °C by using 5-cm water-jacketed cylindrical cells.

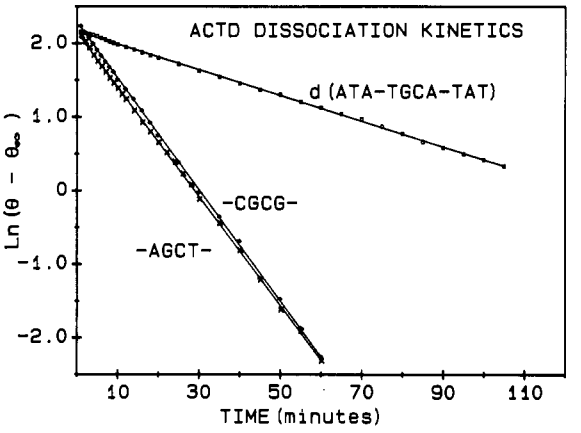


FIGURE 4: Semilog plots of 1% SDS-driven ACTD dissociation kinetics for the three strong binding decamers. Straight lines are those of linear least-squares fits. Dissociation of ACTD from d(ATA-GGCC-TAT) is too fast to be measurable with our non-stopped-flow technique and is not shown. Measurements were made at 18.5 °C with 293-nm ellipticity monitoring.

are exhibited by all three oligomers, as evidenced by straight lines obtained in such a plot. Least-squares fits to these data points yielded characteristic dissociation times of 3340, 850, and 800 s for the -TGCA-, -CGCG-, and -AGCT-containing decamers, respectively, and the zero time extrapolated amplitudes suggest that these decays account for the total ellipticity changes. The ACTD dissociation rate from the -GGCC-containing oligomer, however, is too fast to be measurable by our non-stopped-flow techniques.

The kinetics of ACTD association to these decamers have also been investigated. Except for the much faster kinetics of the -GGCC-containing decamer that binds ACTD weakly, the bulk of the association kinetics can be approximated by single exponentials with time constants in the order of 100 s (not shown). The bimolecular origin of this process is indicated by a rate increase at higher DNA concentration. The resolution of our experimental data is not sensitive enough to warrant multiexponential analyses for extracting the small contributions due to the slower components (presumably unimolecular).

*Thermal Denaturation Experiments.* Although the ACTD binding results in a duplex stabilization of each decamer studied, the extent of such a stabilization depends greatly on the tetranucleotide binding sequence. Melting temperature increases of 18.2, 10.3, and 6.7 °C have been observed for the

decamers containing -TGCA-, -CGCG-, and -AGCT-, respectively. Consistent with the weak ACTD binding to the -GGCC-containing decamer, a mere 2 °C increase in melting temperature is observed. The observation of a largest melting temperature increase for d(ATA-TGCA-TAT) appears to also be consistent with the observed slowest ACTD dissociation from this oligomer. Comparison of the melting temperature increase, equilibrium binding constant, and characteristic dissociation time of ACTD for each of these oligomers can be found in Table I.

It is interesting to note that the extent of melting temperature increase agrees qualitatively with the general trend in binding constants. To see how well they agree quantitatively with the theoretical expectation, comparisons were made with the predictions of Crothers (1971) and McGhee (1976). According to the theoretical model, the melting temperatures in the absence ($T_m^0$) and in the presence ($T_m$) of drug are related to the binding constant ($K$) and the free ligand activity ($L$) by the equation (assuming only duplex binds to the drug)

$$1/T_m^0 - 1/T_m = R \ln (1 + KL)^n/\Delta H$$

where $\Delta H$ is the enthalpy change for the melting of a base pair and $n$ is the number of binding sites per base pair. Approximating $L$ with 2.8 $\mu$M under our experimental conditions and $\Delta H$ as one-tenth of the decamer enthalpy value calculated according to the method of Breslauer et al. (1986), $1/T_m^0 - 1/T_m$ values of 1.6, 1.2, 1.3, and 0.22 $\times 10^{-4}$ 1/K° are predicted for the -TGCA-, -CGCG-, -AGCT-, and -GGCC-containing decamers, respectively. These values are to be compared with the respective experimental results of 1.96, 1.01, 0.72, and 0.21 $\times 10^{-4}$. Except for the -AGCT-containing oligomer, the agreement between theory and experiment appears to be quite satisfactory (in view of the fact that the cited equation was derived for an infinitely long homogeneous polynucleotide).

## Discussion

Our decamer studies presented above revealed that of the four self-complementary tetranucleotide sequences -TGCA- exhibits the strongest binding and the slowest dissociation characteristics. In a 1% SDS solution at 18.5 °C, ACTD dissociates from d(ATA-TGCA-TAT) with a surprisingly long characteristic time of 3300 s, about 4 times slower than from the corresponding decamers containing -AGCT- and -CGCG- sequences. Despite the presence of a GC dinucleotide sequence in -GGCC-, this tetranucleotide sequence is found to have a much weaker ACTD binding affinity. These results are further supported by an 18.2 °C increase in the melting temperature of d(ATA-TGCA-TAT) upon ACTD binding, whereas increases of 10.3, 6.7, and 2.0 °C are observed for the -CGCG-, -AGCT-, and -GGCC-containing decamers, respectively.

These results clearly indicate that base pairs (sequences) flanking the dG-dC sequence can have a dramatic influence on the ACTD binding to and dissociation from this site. Although the reasons for such effects are not clear, they may be due to differential stacking and peptide ring–DNA groove interactions, possibly the consequence of changes on the minor groove environment and/or subtle sequence-dependent conformational alterations at the intercalation site.

The observed weak binding of the -GGCC- sequence is consistent with the results of Aivasashvilli and Beabealashvilli (1983) on the RNA elongation inhibition studies. This most likely is the consequence of a local non-B-genus, possibly an A-genus, conformation around the site. This assertion appears to be supported by the X-ray crystal results of d(GGCCGGCC) (Wang et al., 1982), d(GGTATACC) (Shakked et al., 1983), and d(GGGGCCCC) (McCall et al., 1985) in which all three oligomers contain the GG (CC) sequence and all exhibit A conformations. It must be cautioned, however, that crystal and solution structures do not always agree. The existence of a possible conformational heterogeneity in such a short oligomer and the feasibility of using ACTD for such a probe may be of significance.

As mentioned in the introduction, Duffy and Lindell (1985) found a strong ACTD binding site in rat liver DNA that dissociates twice as slowly as the others at higher drug concentration. It may very well be that this site is the -TGCA- sequence which has been found to be the strongest binding and the slowest dissociating self-complementary tetranucleotide sequence. The strong binding at the -TGCA- site is consistent with the earlier spectroscopic study of Brown and Schafer (1987) and the X-ray crystal study of Takusagawa et al. (1984) on the complexation of ACTD with hexamer d(ATGCAT).

The combination of a planar intercalating phenoxazone chromophore and the two pentapeptide rings most likely is 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 separate rate constants, with the three slow processes appearing to be unimolecular (Bittman & Blau, 1975). The SDS-induced ACTD dissociation from natural DNA had earlier been shown to exhibit multiexponential decay, with the slowest component of the order of 1000 s at room temperature (Muller & Crothers, 1968). 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. Our dissociation kinetic results with oligonucleotides containing single binding sites, all of which exhibit single-exponential decays but with significant rate differences, are consistent with the latter model and further extend the site heterogeneity concept beyond the dinucleotide sequence level.

Although the slowest ACTD dissociation rate from d(ATA-TGCA-TAT) is consistent with the strongest binding and the largest melting temperature increase of this decamer, it must be pointed out that the -CGCG- and -AGCT-containing oligomers exhibit nearly identical dissociation kinetics despite a somewhat stronger binding and a larger melting temperature increase for the former. This may be due to the fact that although the strength of intercalative binding is mainly determined by the stacking interactions between the base pairs and the phenoxazone chromophore, the interactions between the pentapeptide rings and the DNA groove likely dictate the slow drug dissociation. Such an interpretation appears to be plausible in view of the fact that actinomine, an actinomycin without the bulky pentapeptide rings, binds to DNA as strongly as ACTD but dissociates several orders of magnitude faster (Muller & Crothers, 1968), whereas chromomycin $A_3$, which is known to bind nonintercalatively at the minor groove, dissociates slowly from DNA (Behr et al., 1969).

By comparing the data of -TGCA- vs -CGCG- (pyrimidine–purine alternating) and -AGCT- vs -GGCC-, it is

tempting to suggest that a G-C site flanked by A-T base pairs dissociates ACTD slower than the site having flanking G-C base pairs. This may possibly be attributed to the presence of the 2-amino group of the adjacent guanine at the minor groove, which can render interactions with the pentapeptide rings less favorable. This may also help explain why the -CGCG- and -AGCT-containing decamers exhibit nearly identical dissociation kinetics despite the somewhat weaker binding strength and the less favorable duplex stabilization upon ACTD binding for the latter and despite the fact that d(ATA-CGCG-TAT) is purine–pyrimidine alternating, whereas d(ATA-AGCT-TAT) contains a 3-purine stretch that likely destabilizes B conformation.

Recently, Fox and Waring (1984b) have attributed the slow ACTD association kinetics in native DNA to the "shuffling" of drugs from weaker to stronger binding sites. Brown and Shafer (1987), however, have found that these slow processes are also present in the oligomer–ACTD interactions, casting doubt on such a shuffling model. Our association kinetic results carried out at 18.5 °C do not shed light on this point, as they can be approximated by single exponentials on the order of 100 s of apparent bimolecular origin, the consequence of low oligomer concentrations used in our experiment. The basis for the presence of slow unimolecular association kinetics, however, is currently under active investigation.

## Acknowledgments

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**Registry No.** ACTD, 50-76-0; d(ATATGCATAT), 81065-64-7; d(ATAAGCTTAT), 115364-12-0; d(ATACGCGTAT), 84744-83-2; d(ATAGGCCTAT), 115364-14-2; d(ATATGCATAT)·ACTD, 115290-14-7; d(ATAAGCTTAT)·ACTD, 115364-13-1; d(ATACGCGCTAT)·ACTD, 115290-15-8; d(ATAGGCCTAT)·ACTD, 115364-15-3.

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