Observation of an Anomalously Slow Association Kinetics in the Binding of Actinomycin D to d(CATGGCCATG)†

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ABSTRACT: An unusually slow association process which accounts for the bulk of its dichroic changes at 293 nm is observed for d(CAT-GGCC-ATG) when it reacts with actinomycin D (ACTD). This is in contrast to an order of magnitude faster association rates exhibited by oligomers containing a self-complementary tetranucleotide ACTD binding sequence (-TGCA-, -AGCT-, or -CGCG-). The number of drug molecules bound and the melting temperature increase upon ACTD binding are significantly higher for d(CAT-GGCC-ATG) than for other decamers studied. Temperature-dependent spectral measurements of this oligomer in the presence of ACTD suggest additional drug binding prior to denaturation. This particular decamer sequence may be unique, as other decamers containing central -GGCC- sequence and even those differing only by the terminal bases such as d(TAT-GGCC-ATA) and d(GAT-GGCC-ATC) are only weakly binding and do not exhibit such anomalously slow ACTD association kinetics, whereas the dodecamer d(CCAT-GGCC-ATGG) does. CD evidence indicates that, in contrast to the other -GGCC- containing oligomers, both d(CCAT-GGCC-ATGG) and its parent decamer exhibit nonstandard B conformations. The observed slow association kinetics and its interesting D/P dependence are rationalized in terms of a model in which the ACTD molecules initially end-stack and distort the oligomer duplex to a favorable ACTD-binding conformation so that intercalation at the central G-C sequence can occur via DNA breathing. Such a mechanism may be partly responsible for the earlier observed presence of minor contributions due to slow unimolecular rate processes in the association reaction between ACTD and native DNA, likely via induced conversions of some nonbonding regions of DNA to binding conformations.

Actinomycin D (ACTD) is an antitumor antibiotic consisting of a 2-aminophenoxazin-3-one chromophore and two cyclic pentapeptide lactones. The intercalative binding specificity of this drug at the dG-dC sequence has by now been firmly established [Wilson et al., 1986; Chen (1988) and references cited therein]. ACTD bindings to the other guanine-containing dinucleotide sequences are at least an order of magnitude weaker and are negligible at the dinucleotide sequences devoid of guanine. 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. Kinetic studies on the ACTD–DNA interactions are, thus, of some interest as they are relevant to the pharmacological activities of the drug (Muller & Crothers, 1968).

It is generally believed that the combination of a planar intercalating phenoxazone chromophore and the two bulky pentapeptide rings is responsible for some of the unusual kinetic properties observed in 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 also been shown to exhibit multiexponential decay, with the slowest component on 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.

Recently, our laboratory has embarked on studies aimed at elucidating the base sequence specificity of ACTD–DNA binding beyond the dG-dC dinucleotide level by means of systematic comparative studies using synthetic oligonucleotides of defined length and sequence (Chen, 1988a,b). An investigation had recently been made (Chen, 1988b) on the relative ACTD binding abilities and dissociation kinetic behaviors for a series of decadeoxyoligonucleotides of the form d(ATA-XGCY-TAT), each containing a unique tetranucleotide binding sequence at the center and nonbinding sequences at the terminals. These studies revealed that, of the four dG-dC-containing 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 single exponential characteristic time of 3300 s, 4–5 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 and its dissociation rate is too fast to be measurable by the non-stopped-flow techniques. 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. Our dissociation kinetic results of these oligonucleotides containing single binding sites, all of which exhibit single exponential decays but with significant rate differences, are consistent with the site heterogeneity dissociation model and further extend

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the site heterogeneity concept beyond the dinucleotide sequence level.

Although the dissociation kinetics have thus far been greatly clarified by these oligonucleotide studies, the same cannot be said of the association kinetics. Although site heterogeneity, to be sure, is also partially responsible for the multiexponential nature of the association kinetics, the origin of the observed very slow unimolecular rate processes of minor contributions is not yet clear. Fox and Waring (1984) have attributed the slow association kinetics in native DNA to the "shuffling" of drugs from weaker to stronger binding sites. Brown and Shafer (1987), however, have observed slow unimolecular association rate processes in oligonucleotides, thus casting doubts on the "shuffling" model.

In our effort to elucidate the effect of nonbinding flanking sequences on the binding characteristics of the self-complementary tetranucleotide binding sequences, a rather surprising association kinetic result was uncovered which shed considerable light on the origin of slow unimolecular association rate processes observed in native DNA. Some interesting findings are detailed in this report.

Materials and Methods

Oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. The crude oligomer was purified by a strong anion-exchange (SAX) HPLC and repurified by reverse-phase HPLC chromatography as detailed earlier (Chen, 1988a,b). The purified and lyophilized oligomers were 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 oligomers (per nucleotide) were determined with absorbances at 260 nm after melting, with extinction coefficients obtained through nearest-neighbor approximation using mono- and dinucleotide values tabulated in the *Handbook of Biochemistry and Molecular Biology* (Fasman, 1975).

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 cylindrical cells. Spectral titrations were carried out at 18.5 °C by starting with an ACTD solution (~5 μM) and a progressive addition of the oligomer stock. Due to its slow rate of association, a 30-min waiting period was allowed after each addition. ACTD and 7-amino-ACTD were purchased from Sigma and Calbiochem, respectively, and actinomine is a kind gift from Professor Krugh. The extinction coefficients used for drug concentration determination are 24500 M^{-1} cm^{-1} at 440 nm for ACTD, 23600 at 528 nm for 7-amino-ACTD, and 22500 at 445 nm for actinomine, respectively. Kinetic measurements via 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 452 nm for the 1% 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, using a chart recorder and rigorous manual shaking for mixing (required about 10 s).

Thermal melting profiles of oligomers and their drug–DNA mixtures were carried out by monitoring absorbances at 275 and 427 nm, respectively, and collecting data every 15 s 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 were deduced.

Results

Unexpected Significant ACTD Binding to d(CAT-GGCC-ATG). As mentioned earlier, our previous studies suggest that d(ATA-GGCC-TAT) exhibits little affinity for ACTD and its association as well as dissociation rates are too fast to be measurable by the non-stopped-flow techniques. To investigate the effect of flanking sequences on the binding and kinetic behaviors of this "nonbinding" -GGCC- tetranucleotide sequence, decamers d(GTA-GGCC-TAC), d(TAT-GGCC-ATA), d(GAT-GGCC-ATC), and d(CAT-GGCC-ATG) were synthesized. Our binding results indicate that the replacement of the terminal A and T by the corresponding G and C in d(ATA-GGCC-TAT) does not improve the ACTD binding ability of the oligomer. And as expected, d(TAT-GGCC-ATA) and d(GAT-GGCC-ATC) are also found to have little affinity for ACTD. These results reinforce our earlier conclusion that although -GGCC- contains a dG-dC site, this tetranucleotide sequence exhibits weak affinity for ACTD.

Surprisingly, however, d(CAT-GGCC-ATG) exhibits a significant ACTD binding affinity, as shown by the equilibrium binding titrations. In fact, its binding strength is only slightly lower than binding decamers differing only in base pairs adjacent to the central dG-dC sequence. A binding constant of about 1.6 μM^{-1} is extracted for this decamer, as compared to 2.5 μM^{-1} for d(CAT-TGCA-ATG) and 2.6 μM^{-1} for d(CAT-AGCT-ATG). These binding parameters have been obtained from linear least-squares fits of the data points to the simple Scatchard equation $r/m = K(n - r)$, where r is the ratio of bound drug to the DNA base pair concentrations, n is the number of binding sites per base pair, and K is the apparent binding constant. No attempt was made to fit the isotherms using the McGhee–von Hippel analysis (1974), as no significant curvatures were apparent. It is interesting to note that the saturating binding density for d(CAT-GGCC-ATG) is close to 3 drug molecules per duplex, suggesting that binding at sites other than dG-dC may be important.

Observation of Anomalously Slow Association Kinetics for d(CAT-GGCC-ATG) and Comparisons with Other Decamers of the Form d(CAT-XGCY-ATG). The most surprising finding, however, is the observation that in its binding to ACTD the association kinetics exhibited by d(CAT-GGCC-ATG) is being dominated by an anomalously slow process. In fact, its association rate is almost an order of magnitude slower than other oligomers containing strong binding sites, and this slow component constitutes the bulk of its CD spectral changes at 293 nm, even though it contributes only about 25–40% absorbance changes at 427 nm. Comparisons of the ACTD association kinetics for decamers of the form d(CAT-XGCY-ATG) are shown in Figure 1 as semilog plots of ellipticity changes at 293 nm. The much slower association rate for d(CAT-GGCC-ATG) ($1/k \sim 1000$ s at 18.5 °C) is readily apparent when compared to decamers containing tetranucleotide binding sites, which exhibit nearly identical kinetics of an order of magnitude faster (~100 s). It is seen that the bulk of the association kinetics measured by our non-stopped-flow techniques can be approximated by single exponential decays, as indicated by the apparent good linear least-squares fits. The decamer results are summarized in Table I.

ACTD Dissociation Kinetics from d(CAT-GGCC-ATG) Are Rapid. Even though d(CAT-GGCC-ATG) exhibits an unusually slow association rate, its SDS-induced dissociation

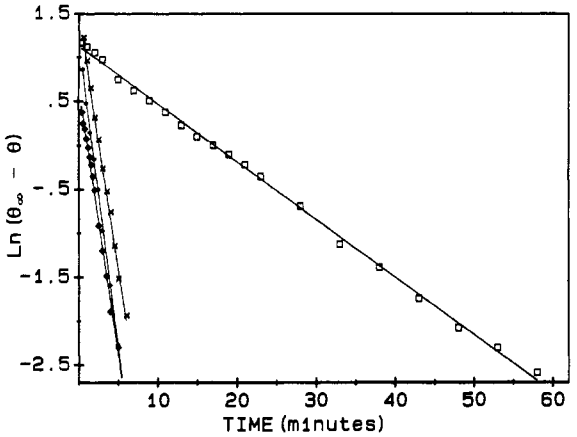

FIGURE 1: Association kinetics at 18.5 °C for the binding of ACTD (1.7 μM) to oligomers of the form d(CAT-XGCY-ATG) (20 μM), shown as a semilog plot of ellipticity changes at 293 nm. Measurements were made with a water-jacketed cylindrical cell of 5-cm path length. -GGCC- (open squares), -CGCG- (×), -AGCT- (diamonds), and -TGCA- (+). Solid lines are linear least-squares fits with a single exponential decay kinetics.

Table I: Comparison of ACTD Association and Dissociation Kinetics of Some Oligonucleotides at 18.5 °C[a]

oligomer	t_m (°C)	Δt_m (°C)	$1/k_{assoc}$ (s)	$1/k_{dissoc}$ (s)
d(ATA-GGCC-TAT)	38	2	*	*
d(GTA-GGCC-TAC)	42	4	*	*
d(TCA-GGCC-TGA)	44	3	*	*
d(TAT-GGCC-ATA)	41	2	*	*
d(GAT-GGCC-ATC)	43	6	*	*
d(CAT-GGCC-ATG)	45	21	960	*
d(CAT-TGCA-ATG)	36	10	95	640
d(CAT-AGCT-ATG)	35	10	95	290
d(CAT-CGCG-ATG)	49	5	98	440

[a] Entries with (*) markings refer to the values too fast to be measurable by our non-stopped-flow techniques. Melting temperatures (monitored at 275 nm) were measured for 50 μM oligomer concentration (nucleotide) in the absence and in the presence of 3.3 μM ACTD. Kinetic measurements were made with solution mixtures containaing 20 μM nucleotides and 1.7 μM ACTD in a 5-cm water-jacketed cylindrical CD cell.

kinetics is too fast to be measured by the non-stopped-flow techniques. This is in contrast to the other d(CAT-XGCY-ATG) oligomers which exhibit relatively slow dissociation kinetics, as can be seen in Table I. It is noteworthy, however, that ACTD dissociates from the d(CAT-XGCY-ATG) oligomers significantly faster than the corresponding d(ATA-XGCY-TAT) counterparts studied earlier (Chen, 1988b), despite the presence of two terminal G·C base pairs to enhance the duplex stability of the former. In fact, the rates of ACTD dissociation from d(CAT-XGCY-ATG) are 5-, 3-, and 2-fold faster than the corresponding d(ATA-XGCY-TAT) for the -TGCA-, -AGCT-, and -CGCG- containing oligomers, respectively. These results clearly suggest that the nonbinding flanking sequences can exert significant influence on the dissociation rate of a given tetranucleotide binding sequence, a topic of considerable interest but not the focus of this report.

ACTD Binding Greatly Enhances the Duplex Stability of d(CAT-GGCC-ATG). Another unexpected finding is the fact that ACTD binding results in the largest melting temperature increase (~20 °C) for d(CAT-GGCC-ATG) as compared to the other closely related decamers (see Table I). This is unexpected, in view of its fast ACTD dissociation and a mere 10 °C melting temperature increase for the corresponding oligomer containing the strongest binding sequence -TGCA-. The much smaller increases in the melting temperature for the other -GGCC- containing decamers, however, are consistent with their weak ACTD binding.

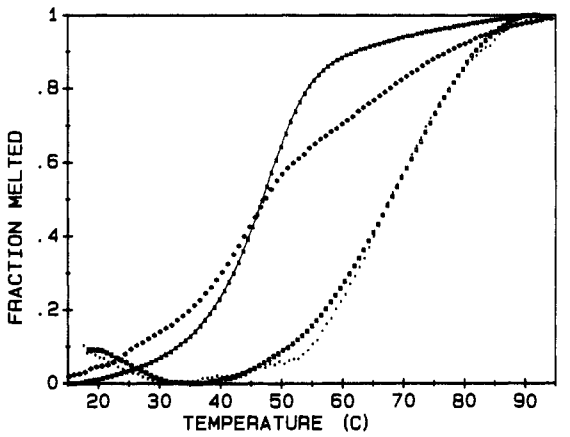

FIGURE 2: Thermal denaturation profiles of d(CAT-GGCC-ATG) in the absence and in the presence of ACTD. Absorbance monitored at 275 nm for oligomer of 130 μM (×) in 10 mM Tris-HCl buffer of pH 8 containing 0.1 M NaCl and a 50 μM oligomer solution containing 3.6 μM ACTD (+), D/P = 0.072. Absorbance monitored at 427 nm for 50 μM oligonucleotide solutions containing 1.8 μM (dots), D/P = 0.036, and 11.4 μM ACTD (squares), D/P = 0.23, respectively.

Thermal denaturation for an ACTD + d(CAT-GGCC-ATG) mixture was monitored at both DNA and ACTD spectral regions, and the results are shown in Figure 2. The biphasic nature of the 275-nm melting for an unsaturated solution is readily apparent, yielding melting temperatures of 45 and 66 °C, respectively. The lower temperature transition corresponds to the denaturation of the weakly bound and/or nonbound DNA regions (as suggested by its agreement with the melting of the oligomer itself) while the higher temperature corresponds to the disruption of the stronger complexes. In addition to a melting temperature of around 66 °C, however, the 427-nm monitoring reveals a minimum around 37 °C. Such a hypochromic effect near 427 nm is characteristic of the ACTD binding to DNA. These results, thus, suggest that if one starts below room temperature, additional ACTD binding will occur as the temperature is increased and will reach a maximal binding around 30–40 °C; further temperature increase results in the melting of the nonbound or weakly bound oligomers around 45 °C and subsequent melting of strong drug–oligomer complexes around 66 °C.

Melting experiments further revealed that the drugs are released at about the same temperature (~66 °C) even at saturation. Denaturation profile via monitoring drug release for an ACTD-saturated solution is also included in Figure 2 and is seen not to be very different from the melting profile of a low D/P counterpart. This suggests that even at low D/P, the additional drug binding observed around 30 °C most likely corresponds to ACTD binding to the oligomers which already have drugs bound.

CD Evidence Supports Thermally Induced ACTD Binding. To further investigate the basis for the enhanced ACTD binding at the 30 °C region, temperature-dependent CD spectral measurements were also made. ACTD binding results in the appearance of an additional CD maximum at 293 nm and a concomitant intensity reduction and enhancement at 266 and 248 nm, respectively (see Figure 3). Interestingly, as the temperature is raised (e.g., from 15 to 30 °C), the intensity at 293 nm is increased (rather than decreased) and is accompanied by an intensity reduction at the 266-nm region with no discernible change at the 248-nm intensity. These CD results are consistent with the enhanced ACTD binding with moderate temperature increase, as indicated by the appearance

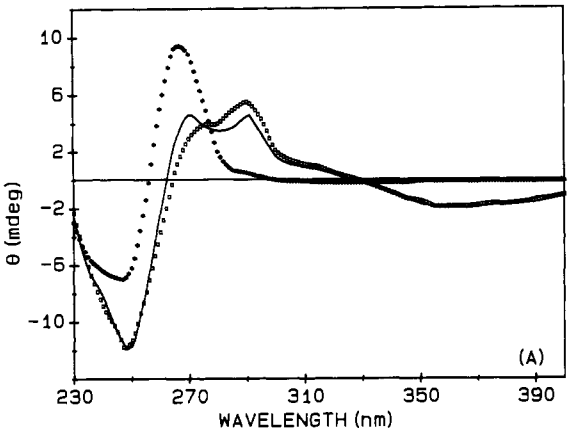

FIGURE 3: CD spectra of a 15 μM d(CAT-GGCC-ATG) solution containing 1.5 μM ACTD at 15 (connected curve) and 30 °C (open squares). For comparison, the spectrum of oligomer in the absence of ACTD at 30 °C (+) is also shown. Measurements were made in a water-jacketed cylindrical cell of 5-cm path length.

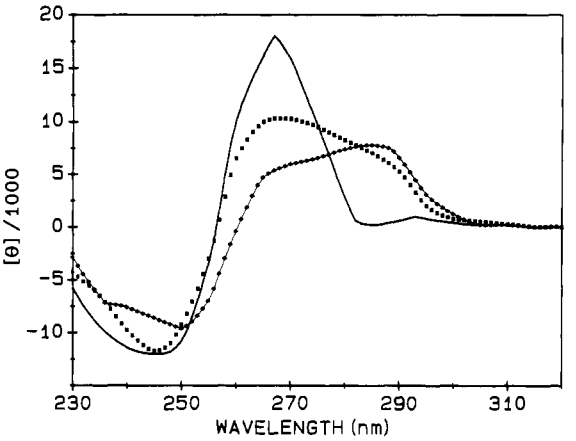

FIGURE 4: Comparison of CD spectra for d(CAT-GGCC-ATG) (connected curve) and d(GAT-GGCC-ATC) (squares) at 18.5 °C in pH 8 Tris-HCl buffer containing 0.1 M NaCl. Measurements were made with a 2-cm cell and solutions of 40 μM nucleotide concentration. The spectrum for d(TGT-GGCC-ACA) (+) is also included for additional comparison.

of a hypochromic effect around 30 °C in the absorbance melting profile monitored at 427 nm. Temperature-dependent CD spectra of d(CAT-GGCC-ATG) alone were also measured to see if such an ability to bind additional ACTD may be the consequence of premelting conformational alterations. Indeed, increasing the temperature from 15 to 30 °C results in an intensity increase at 285 nm and a decrease at 266 nm with negligible changes observed at 248 nm (not shown), suggesting subtle conformational alterations without the duplex disruption. Duplex disruptions at higher temperatures are characterized by CD intensity reductions at 248 nm, in addition to spectral changes at the 285- and 266-nm regions.

CD Spectral Comparison of d(CAT-GGCC-ATG) and d(GAT-GGCC-ATC). Since d(GAT-GGCC-ATC) binds ACTD weakly and does not exhibit slow association kinetics, it is of interest to see if its conformation is significantly different from that of d(CAT-GGCC-ATG). CD spectra of these two decamers are compared in Figure 4, and indeed, they differ greatly from each other. The CD characteristics of d(GAT-GGCC-ATC) are typical of a B-DNA with a negative maximum at 245 nm, a broad positive maximum around 265 nm, and a shoulder at 285 nm. The spectrum of d(CAT-GGCC-ATG), on the other hand, exhibits an enhanced 267-nm maximum but a slight *negative* dip around 285 nm. Although these features resemble those of A form, the conformation most likely is a variant of B (Wolk et al., 1989). In view of the fact that these two oligomers differ only in the terminal base pairs, the observed spectral differences are rather surprising and may partly be the basis for their differing ACTD binding characteristics.

Kinetic and Binding Comparisons with 7-Amino-ACTD and Actinomine. To investigate the roles played by intercalation and cyclic pentapeptide lactones on the binding and kinetic characteristics, studies were also made with 7-amino-ACTD, in which the amino group at the 7-position imparts a slight hindrance to intercalation, and actinomine, an actinomycin without the bulky pentapeptide rings. Comparisons of binding isotherms for d(CAT-GGCC-ATG) with the three compounds indicate that the presence of an amino group at the 7-position affects the binding characteristics only slightly, whereas the absence of bulky pentapeptide rings in actinomine results in a significant reduction in the binding strength and a considerable increase in the saturation binding density. The extracted n value of 0.44 for actinomine corresponds to nearly one drug molecule per every two base pairs of the neighbor-exclusion limit.

It is worthy to note that no slow association kinetics were observed for actinomine, implicating the important role played by the pentapeptide rings in these processes. Consistent with some steric hindrance for intercalation, a factor of 2 slower association kinetics is observed for the 7-amino-ACTD when compared to ACTD.

Interesting D/P Dependence of Association Rates. Interesting concentration-dependent association kinetics were also observed. As expected, the association rate at a constant drug concentration increases as the DNA concentration is increased. At a constant d(CAT-GGCC-ATG) concentration, however, the association rate is progressively reduced as the ACTD or 7-amino-ACTD concentration is increased. This peculiar concentration dependence is in direct contrast to what is expected of a simple bimolecular mechanism. The extracted rate constants when plotted against 1/[drug added] exhibit linearity in the region of high drug concentration for both ACTD and 7-amino-ACTD.

ACTD Binding to the Dodecamer d(CCAT-GGCC-ATGG). To further investigate the peculiar behaviors of CAT-GGCC-ATG sequence, ACTD binding studies were also carried out with dodecamer d(CCAT-GGCC-ATGG). As expected, the additional terminal G·C base pairs enhance the duplex stability of the oligomer which now melts around 54 °C, a roughly 10 °C increase from the parent decamer. Measurements at 18.5 °C revealed only a weak induced CD around 293 nm upon the ACTD addition. Consistent with the results of the parent decamer, however, this CD intensity is significantly enhanced upon moderate temperature increase (Figure 5A), suggesting additional drug binding. It should also be noted in passing that in the absence of ACTD the CD features of the dodecamer, in particular the presence of a negative dip at 285 nm, are almost identical with those of its parent decamer (see Figure 4).

The thermally enhanced drug binding is also evidenced by the hypochromic effect of the drug absorbance which is manifested by the appearance of a minimum around 50 °C in the absorbance melting profile with 427-nm monitoring (Figure 5B). Similar to the decamer, the additional drug binding occurs in the premelting region of the dodecamer. In the presence of 10 μM ACTD, a concentration sufficient to saturate the DNA, a 256-nm absorbance monitoring of the 40 μM d(CCAT-GGCC-ATGG) solution revealed a biphasic behavior with a prominent low-temperature transition around 37 °C and a high-temperature transition around 74 °C [a 20

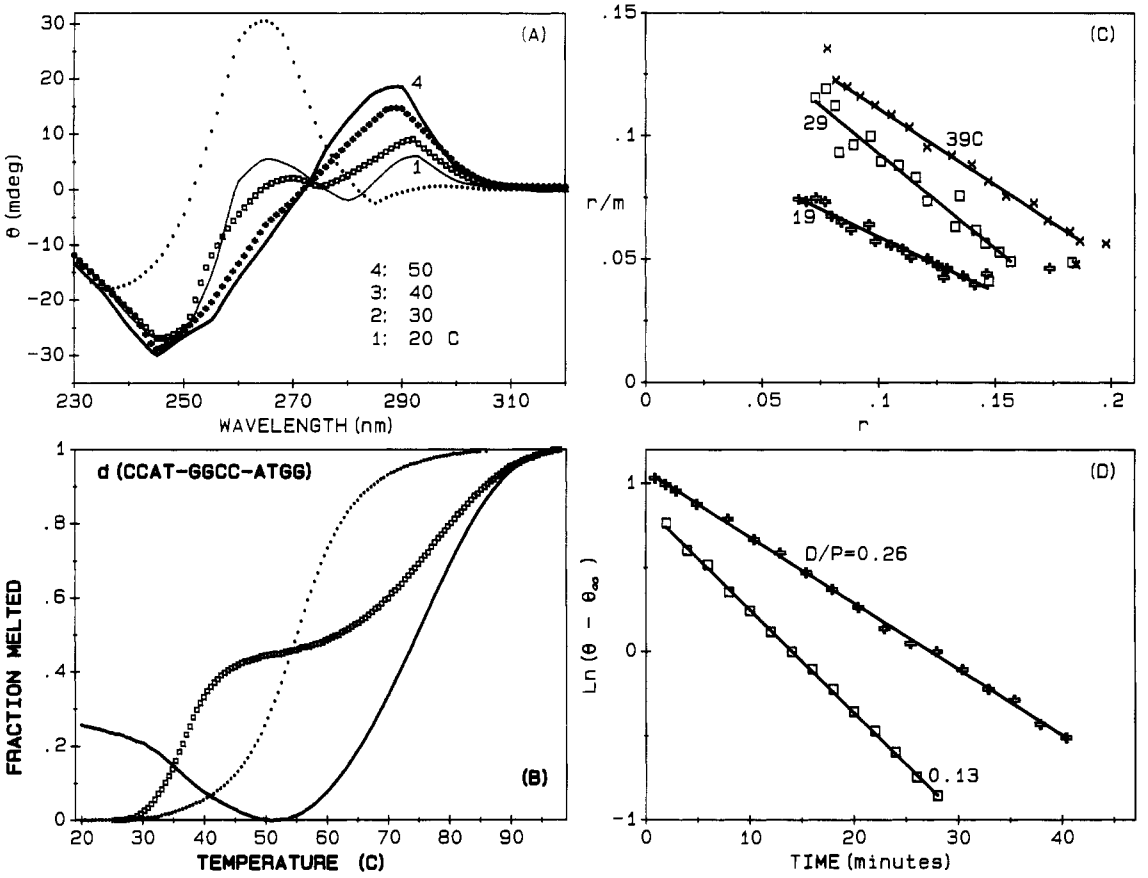

FIGURE 5: (A) Temperature-dependent CD spectra of a solution containing 10 μM ACTD and 84 μM (nucleotide) d(CCAT-GGCC-ATGG). The CD spectrum of the oligomer alone (dotted curve) is also shown for comparison. (B) Thermal melting profiles of 80 μM d(CCAT-GGCC-ATGG) in the absence (dotted curve, monitored at 275 nm) and in the presence of 10 μM ACTD, monitored at 256 nm (squares) and 427 nm (connected curve). (C) Scatchard plots of ACTD + d(CCAT-GGCC-ATGG) titrations at three different temperatures. (D) Temperature jump kinetics (from 20 to 29 °C) for the ACTD + d(CCAT-GGCC-ATGG) solutions at two different D/P. The DNA concentrations were kept at 40 μM.

°C increase from the naked DNA and about 10 °C higher than the ACTD + d(CAT-GGCC-ATG) complex melting]. The low-temperature transition occurs in the region of hypochromic effects exhibited by the 427-nm monitoring. It is also of interest to note that in the presence of an ACTD concentration below saturation the drug release is also not greatly different from 74 °C (not shown).

Scatchard plots of ACTD titrations with d(CCAT-GGCC-ATGG) at three temperatures are shown in Figure 5C. Contrary to the usual behavior of binding strength decrease with increasing temperature, a significant increase in the binding constant, as judged from the slopes, is seen as the temperature is increased from 18.5 to 28.5 °C. Even though the binding strength at 38.5 °C appears to be slightly weaker than at 28.5 °C, it is still stronger than that at 18.5 °C. Interestingly, the saturation binding density at 38.5 °C is the highest, as can be seen from the *r* axis intercept, and amounts to about 3 drug molecules per duplex. These results are consistent with the notion of additional drug binding at moderately high temperatures, as suggested by the CD and absorption spectral evidence.

The anomalously slow ACTD association kinetics seen in the parent decamer is also observed for this dodecamer except for a significantly slower rate at the same temperature. For example, the dodecamer exhibits a slow ACTD association kinetics with a characteristic time in the order of 1000 s at 28.5 °C as compared to that of its parent decamer at 18.5 °C. It is interesting to note the corresponding 10 °C increase in the melting temperature observed for the dodecamer, which seems to suggest a possible correlation of the slow association kinetics with the base-pair opening. The trend of D/P dependence on the association kinetics observed in the decamer (i.e., the rate decrease as the ACTD concentration is increased) is also evident in the dodecamer, as seen in Figure 5D. Due to the small ellipticity change and much slower association kinetics at 18.5 °C, kinetic measurements were carried out at 29 °C for the dodecamer. It should be noted that the results of relaxation measurements are at variance with the mechanism of direct bimolecular drug binding which would predict the relaxation rate to be linearly dependent on both the drug and DNA concentrations. Despite its slow association rate, the SDS-induced dissociation process is too fast to be measurable by the non-stopped-flow techniques, again consistent with results of the decamer.

Discussion

The observation of an unusually slow ACTD association kinetics in d(CAT-GGCC-ATG) which accounts for the bulk of its CD spectral changes at 293 nm is unexpected, in view of the fact that other -GGCC- containing decamers exhibit little affinity for ACTD and their kinetics are too fast to be measurable by the non-stopped-flow techniques. This association rate is nearly an order of magnitude slower than decamers containing strong tetranucleotide binding sequences (-TGCA-, -AGCT-, or -CGCG-) under similar conditions. Although the ACTD binding to d(CAT-GGCC-ATG), which contains the "nonbinding" -GGCC- sequence, is only slightly weaker than to decamers containing other tetranucleotide binding sequences, its saturation binding density is considerably higher (nearly 3 drug molecules per duplex) and the melting

temperature increase upon drug binding is much more dramatic (>20 °C increase). Despite the fact that the duplex structure of this oligomer is greatly stabilized by ACTD binding, its SDS-induced drug dissociation rate is too fast to be measurable by the non-stopped-flow techniques. Temperature-dependent spectral measurements of this oligomer in the presence of ACTD further suggest additional drug binding during duplex premelting. CD evidence indicates that the conformation of this oligomer is distinctly different from those of other -GGCC- containing decamers which exhibit characteristic B features. These as well as other kinetic results are further confirmed by experiments with the dodecamer d(CCAT-GGCC-ATGG) which contains this particular decamer sequence.

The fact that the melting temperature obtained from monitoring the drug release is almost identical for a solution of low D/P ratio when compared to a solution containing a saturating amount of drug further suggests that the thermally induced additional ACTD binding likely occurs at the drug-bound rather than the naked oligomers. The thermally induced ACTD binding results in a sizable positive CD at 293 nm, which suggests that the mode of additional ACTD binding most likely is intercalation at the central dG-dC site, as decamers containing binding tetranucleotide sequences -XGCY- all exhibit significant induced positive CD at this wavelength. The good correlation between the melting temperature increase and the slowing of association kinetics as one proceeds from decamer d(CAT-GGCC-ATG) to dodecamer d(CCAT-GGCC-ATGG) further suggests that a locally denatured (possibly base-pair-opened) conformation may be involved in intercalative binding at the dG-dC site.

Contrary to the prediction of a simple bimolecular mechanism, the observed slow association rate decreases upon increasing ACTD concentration. A kinetic model that allows for such a behavior as well as the aforementioned observations likely consists of a ACTD-binding duplex conformation (P) dynamically converting into a locally distorted conformation (P*) prior to intercalatively accommodating the sizable phenoxazone chromophore. The scheme is summarized as

$$P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P^* \qquad P^* + D \underset{k_d}{\overset{k_b}{\rightleftharpoons}} C$$

It can be shown that the solution of the coupled differential rate equations results in a fast and a slow rate constant which under the condition $k_b[D] \gg k_1 + k_{-1}$ can be approximated by

$$k_{fast} \cong k_b[D] + k_d \quad (1)$$

and

$$k_{slow} = k_1 + (k_{-1}K_d)/([D] + K_d) \quad (2)$$

where $K_d = k_d/k_b$. Thus, under the condition in which $[D] \gg K_d$, the slow association rate becomes inversely proportional to the drug concentration (see eq 2), in agreement with experimental observations at high [ACTD]. As the lowering of drug concentration will result in a rate decrease for the fast process but at the same time a rate increase for the slow process, as can bee seen from eqs 1 and 2, considerable kinetic overlap can occur. This may account for the significantly faster association rate observed at the lowest drug concentration measured, as the fast process makes a relatively more important contribution in this case. Thus, the observed slow ACTD association kinetics is seen to be reasonably accounted for by such a mechanism. It should be noted that the proposed mechanism applies equally well to the intercalative binding at the strong binding -XGCY- sequence if one identifies the binding conformation (P) to be the B form of the oligomers.

It is likely that, in contrast to the other tetranucleotide sequences -XGCY-, the minor groove of the -GGCC- sequence in a standard B conformation does not permit very favorable hydrophobic interactions with the pentapeptide rings of ACTD. The decamer d(CAT-GGCC-ATG), on the other hand, exhibits a peculiar conformation and the ACTD molecules may initially bind at both ends of the duplex to create a favorable ACTD binding conformation (P) at the central region. The much slower rate observed in d(CAT-GGCC-ATG), as compared to other -XGCY- containing binding decamers, may thus be attributed in part to the greatly enhanced duplex stability of this oligomer upon initial drug bindings which considerably slowed the base-pair opening around dG-dC for further ACTD intercalation.

The initial end binding for d(CAT-GGCC-ATG) may either be intercalation at the dC-dA (dT-dG) sequence or end stacking at the G·C base pairs. Krugh et al. (1977), however, had observed that ACTD does not form an intercalative complex with the dinucleotide miniduplex of d(CA)·d(TG). Indeed, our measurements with d(TCA-GGCC-TGA), which also contains CA (TG) sequence, revealed only weak binding and the absence of slow association kinetics. One is, thus, left with the alternative of ACTD stacking with the terminal G·C base pairs. The ability for the end-stacking interactions of ACTD to alter the conformation of the central region most likely arises from requirements of specific hydrogen bondings to the guanine base as well as hydrophobic interactions at the minor groove (Sobell et al., 1971). Even though terminal G·C base pairs are also present in d(GTA-GGCC-TAC) and d-(GAT-GGCC-ATC), the terminal guanines are at the 5′-end of these oligomers. It had been shown (Sobell et al., 1972; Krugh, 1972; Krugh & Neely, 1973) that the phenoxazone chromophore much prefers stacking at the 3′ side of guanosine at both the benzenoid and quinoid rings with the orientations permitting specific hydrogen bonding with guanine. The consequence of this is the well-known intercalative specificity of ACTD to the dG-dC sequence and the observation of ACTD end stacking to the dinucleotide dC-dG. The B conformations as well as their inability to terminally stack with ACTD may be the reasons why these two decamers are weak ACTD binders.

Our proposed binding model can thus be summarized: The conformation of the -GGCC- sequence in d(CAT-GGCC-ATG) exists predominantly in an unusual conformation, possibly a variant of B form. Thus, the ACTD molecules initially bind to the decamer via end stacking with the terminal G·C base pairs. Their hydrogen-bonding and minor groove interaction requirements with one of the pentapeptide rings result in significant conformational alterations at the central -GGCC- sequence so that intercalative ACTD binding at the dG-dC site now becomes favorable. To intercalatively accommodate the sizable phenoxazone ring requires a local denaturation (likely via breathing) of the duplex at the central region. The initial binding of drugs, however, has considerably stabilized the oligomer so that the rate of base-pair opening becomes significantly slower than decamers containing a strong binding -XGCY- sequence and hence the observed order of magnitude slower ACTD association rate.

Such a model can nicely explain several curious experimental observations. For example, even though d(CAT-GGCC-ATG) exhibits anomalously slow association with ACTD, the SDS-induced ACTD dissociation rate is very rapid. This can be attributed to the instantaneous removal of the end-stacked ACTD by SDS with the consequence of rapid ejection of the drug molecule from the now unfavorable in-

tercalative environment at the central -GGCC- site. The low-temperature transition around 37 °C of a biphasic 256-nm melting profile of d(CCAT-GGCC-ATGG) saturated with ACTD (see Figure 5B) may likely correspond to the local melting of the central regions of the drug-end-stacked oligomers with concomitant ACTD intercalations at the dG-dC sites, as suggested by hypochromic effects observed in the 427-nm monitoring. The model also appears to be in line with the observation that although the slow association kinetics account for the bulk of the ellipticity changes at 293 nm, they contribute only about 25–40% when monitoring absorbance changes at 427 nm. This may be attributed to the fact that although conformational changes of the oligomer itself do not directly affect the absorbance at 427 nm, they contribute significantly to the 293-nm ellipticity alterations. The more than 60% contribution to the fast absorbance changes at 427 nm is consistent with the initial ACTD end stacking.

Since the presence of the amino group slightly hinders the intercalative binding mode, the observation of a slower association kinetics for 7-amino-ACTD is also consistent with the proposed model. Studies with actinomine, an actinomycin without the bulky pentapeptide rings, however, revealed no presence of slow association kinetics in d(CAT-GGCC-ATG) but exhibited higher binding density. This can be rationalized in terms of the absence of stringent requirements for hydrogen bonding with guanine and hydrophobic interactions between the pentapeptide lactones and the minor groove. It must be cautioned, however, that the presence of two positive charges in actinomine may render its mode of binding to be different from other neutral actinomycins.

As has already been mentioned in the introduction, various models have been proposed to account for the existence of minor contributions in native DNA of very slow ACTD association kinetics of unimolecular character. Muller and Crothers (1968) have suggested the role of pentapeptide conformational rearrangement to account for these slow rates. NMR studies of the ACTD + d(ATGCAT) complex, however, revealed no significant conformational alterations of the pentapeptide lactones upon DNA binding (Brown et al., 1984). Fox and Waring (1984), on the other hand, have reasoned that the observed slow association kinetics are the consequence of ACTD shuffling from weak to stronger sites. The existence of the slow unimolecular components in poly(dG-dC)·poly(dG-dC) and short oligonucleotides, however, is difficult to reconcile with such a model (Brown & Shaefer, 1987). Our observation of an association kinetics in d(CAT-GGCC-ATG) being dominated by a slow process, thus, offers an alternative model in which the presence of slow unimolecular association kinetics may be partly due to the ACTD-induced conformational alteration in some regions of DNA and the subsequent further ACTD binding.

Although the dissociation kinetics is not the focus of this report, it is worth noting that the rate of ACTD dissociation from a given tetranucleotide binding site -XGCY- can be greatly affected by the nonbinding flanking sequences. One of the more telling examples is the observation that ACTD dissociates from d(ATA-TGCA-TAT) about 5-fold slower than from d(CAT-TGCA-ATG). This probably is the consequence of conformational fine tuning of the central binding site from the nonbinding flanking sequences. The significant conformational differences, as evidenced by the CD spectra of two decamers d(CAT-GGCC-ATG) and d(GAT-GGCC-ATC) differing only at the terminal bases, underscore the extreme conformational sensitivity of a not-so-short stretch of DNA on the flanking bases (or sequences).

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Supplementary Material Available

Binding and kinetic comparison of ACTD, 7-amino-ACTD, and actinomine (Table I), association kinetics of ACTD in d(CCAT-GGCC-ATGG) solutions at 29 °C (Table II), Scatchard plots for the ACTD binding to three decamers of the form d(CAT-XGCY-ATG) (Figure 1), Scatchard plots of d(CAT-GGCC-ATG) binding to ACTD, 7-amino-ACTD, and actinomine (Figure 2), and D/P dependence of association kinetics (Figures 3 and 4) (6 pages). Ordering information is given on any current masthead page.

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