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

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Received February 4, 1992; Revised Manuscript Received April 22, 1992

ABSTRACT: Studies on the binding specificity of actinomycin D (ACTD) to tetranucleotide sequences of the form -XGCY- have been extended to include the non-self-complementary sequences. ACTD binding characteristics are investigated by equilibrium, kinetic, and thermal denaturation for decameric duplexes d(ATA-XGCY-ATA)-d(TAT-Y′GCX′-TAT), where X and Y are complementary to X′ and Y′, respectively, but not to each other. The results indicate that when X = G or Y = C, the oligomers exhibit significantly weaker ACTD binding affinities, smaller melting temperature increases upon drug binding, and faster SDS-induced ACTD dissociation rates than the other sequences. Estimated binding constants at 18.5 °C for decameric duplexes containing -AGCA-/-TGCT-, -AGCG-/-CGCT-, or -CGCA-/-TGCG- are in the range of 4–9 μM^{-1}, whereas for the ones containing -GGCT-/-AGCC-, -GGCA-/-TGCC-, or -GGCG-/-CGCC- they range from 0.6 to 2 μM^{-1}. In contrast to the characteristic SDS-induced ACTD dissociation times of 600–1000 s for the stronger binding sites, the sequences containing X = G or Y = C exhibit at least an order of magnitude faster dissociation kinetics. These observations are further supported by the induced CD results and fluorescence measurements with 7-amino-ACTD. The findings from these non-self-complementary -XGCY- tetranucleotide sequences are consistent with those found earlier for the self-complementary counterparts, and they together clearly demonstrate that a base sequence alteration adjacent to the GC site can have a profound effect on the ACTD binding as well as dissociation characteristics, likely a consequence of subtle conformational alterations near the binding site. Our results on 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 activities. 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 with specific hydrogen bonding between the 2-amino group of guanine and the carbonyl oxygen of threonine in 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. DNase I footprinting experiments (Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984a) have further confirmed the dG-dC binding specificity of ACTD. Comparative studies with oligonucleotides of specific lengths and sequences (Wilson et al., 1986; Chen, 1988a) 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.

Aside from being supportive of the fact that dG-dC is the strong binding site, Aivasashvilli and Beabealashvilli (1983)

†Supported by USPHS Grant CA-42682 and by a subproject of MBRS Grant S06GM0892.

0006-2960/92/0431-6223$03.00/0

found 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. Their finding strongly suggests that the base pairs adjacent to the G-C sequence may exert a profound influence on its ability to bind ACTD. Phillips and Crothers (1986) developed an in vitro transcriptional assay to detect the DNA sequence specificity of the drug binding sites and the kinetics of drug dissociation from those sites. Their results appear to also support the notion that base sequences adjacent to the dG-dC intercalation site influence the ACTD dissociation kinetics. Studies at low drug concentrations led Duffy and Lindell (1985) to the identification of a strong binding site having an apparent binding constant of 7.6×10^7 M^{-1} and exhibiting a dissociation rate which is twice as slow as that of bound drugs at higher concentrations. This result again suggests that the binding ability of a dG-dC sequence may be strongly dependent on the adjacent base pairs or sequences.

In an effort to elucidate the base sequence specificity of ACTD binding beyond the dG-dC dinucleotide level, our laboratory recently embarked on investigations using selected synthetic oligonucleotides of defined length and sequence. A systematic study on the relative ACTD binding abilities and kinetic behavior of a series of self-complementary decadeoxyoligonucleotides, d(ATA-XGCY-TAT), each containing a unique tetranucleotide binding sequence at the center and nonbinding sequences at the terminals, had been carried out (Chen, 1988b). It was found 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 3700 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 and its ACTD dissociation kinetics are too fast to be measured by non-stopped-flow techniques. These results confirm that base pairs (sequences) flanking the dG-dC sequence can have a dramatic influence on ACTD binding to and dissociation from this site.

In order to complete the binding specificity studies at the tetranucleotide level, non-self-complementary -XGCY- sequences are hereby included. Binding of actinomycin D (ACTD) to non-self-complementary decameric duplexes of the form d(ATA-XGCY-ATA)–d(TAT-Y′GCX′-TAT), where X and Y are complementary to X′ and Y′, respectively, but not to each other, has been investigated by equilibrium, kinetic, and thermal denaturation studies. The results are detailed in this report.

Materials and Methods

Oligonucleotides were synthesized with a Biosearch 8600 DNA synthesizer with use of β-cyanoethyl phosphoramidite chemistry. The crude oligomer was purified by a strong anion-exchange (SAX) HPLC followed by reverse-phase HPLC chromatography as detailed earlier (Chen, 1988a). The purified and lyophilized oligomers were dissolved in 10 mM Tris-HCl buffer of pH 7.3 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, with use of extinction coefficients obtained via nearest-neighbor approximation using mono- and dinucleotide values tabulated in Fasman (1975). Complementary oligomers were annealed by heating the equal molar mixtures to 90 °C and slowly cooled back to ambient

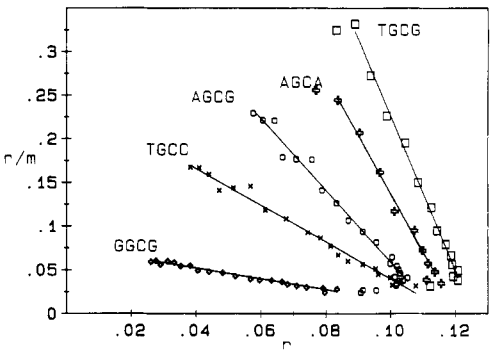

FIGURE 1: Scatchard plots for equilibrium titrations of ACTD at 18.5 °C with d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT) (squares), d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) (+), d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT) (circles), d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT) (×), and d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT) (diamonds). The plot for d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT) is nearly identical to that of -GGCG- and is not shown. The absorbance difference between 427 and 480 nm has been used to obtain the binding isotherms. [Bound drug]/[DNA, bp] is designated by r, and m represents the free drug concentration.

temperature. The extinction coefficients used for drug concentration determination are 24 500 M^{-1} cm^{-1} at 440 nm for ACTD and 23 600 M^{-1} cm^{-1} at 528 nm for 7-amino-ACTD.

Absorption spectra were measured with a Cary 210 spectrophotometric system. CD spectra were obtained by a JASCO J-500A recording spectropolarimeter at appropriate temperatures using water-jacketed cells. Fluorescence measurements were made with an SLM48000S system. Spectral titrations were carried out at 18.5 °C by starting with an ACTD solution and a progressive addition of the oligomer stock. Absorbance changes at 427 and 480 nm were used to obtain Scatchard plots. Kinetic measurements 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 452 nm for the 1% sodium dodecyl sulfate (SDS)-induced dissociation experiments. 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 (requires about 10 s). Scatchard plots via fluorescence titrations of 7-amino-ACTD were made by integrating emission spectral intensities from 580 to 780 nm while exciting the sample at 560 nm. Fluorescence kinetic measurements were carried out by monitoring emission intensity changes at 650 nm using a stirrer accessory.

Thermal melting profiles of each oligomer and its drug–DNA mixture were carried out with 1-cm semimicro cells by monitoring absorbances at 275 nm 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 were deduced.

Results

Significantly Lower ACTD Binding Affinities Are Exhibited by -XGCY- Sequences with X = G or Y = C. The representative Scatchard plots resulting from absorbance titrations are shown in Figure 1. It is immediately apparent that these oligomers exhibit varied ACTD binding affinities. Although considerable variation in slope is clearly evident, all curves appear to converge near $r = 0.12$, suggestive of a saturation binding density of slightly greater than 1 drug molecule per

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

oligonucleotide	T_m (°C)	ΔT_m (°C)	K (μM^{-1})	k^{-1}(dissoc) (s)	K (μM^{-1})[b]	k^{-1}(dissoc)[b] (s)
d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT)	25.1	13.3	6.7	730	8.3	4100
d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT)	32.3	11.3	4.1	600	4.9	2900
d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT)	33.8	11.7	8.8	1000	5.8	5860
d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT)	32.1	4.0	0.63	~60	1.4	180
d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT)	36.6	5.4	0.62	~60	0.85	160
d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT)	32.7	6.9	2.1	~60	2.5	300

[a] Binding and kinetic experiments for ACTD were carried out at 18.5 °C. Melting measurements were made with 40 μM/bp DNA in the absence and in the presence of 7.5 μM ACTD by monitoring the absorbance at 275 nm in solutions containing 0.1 M NaCl. [b] 7-Amino-ACTD with fluorescence monitoring at 20 °C.

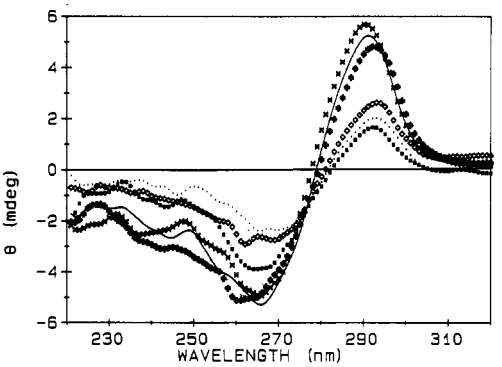

FIGURE 2: Comparison of difference CD spectra of 7.5 μM ACTD in 40 μM/bp of oligonucleotide solutions with the oligomer contributions subtracted: d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT) (×), d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT) (solid curve), d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) (+), d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT) (diamonds), d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT) (dotted curve), and d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT) (squares). Measurements were made at 18.5 °C using a 2-cm water-jacketed cylindrical cell.

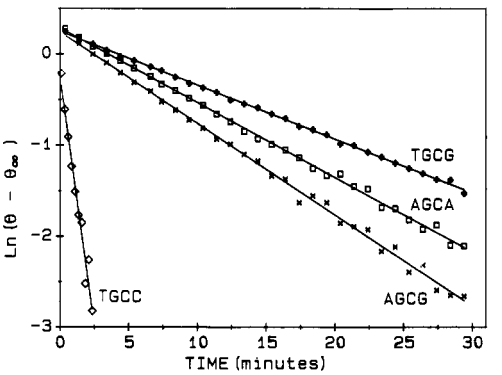

FIGURE 3: Representative semilog plots of 1% SDS-driven ACTD dissociation kinetics for the decameric duplexes d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT) (+), d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) (open squares), d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT) (×), and d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT) (diamonds). Those of d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT) and d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT) are nearly identical to that of -TGCC- and not shown. Measurements were made at 18.5 °C with 293-nm ellipticity monitoring. Straight lines are those of linear least-squares fits.

duplex, as to be expected of these single-site systems. As the data points do not deviate greatly from linearity, binding parameters were estimated via least-squares fits to the linear portions. The deduced ACTD binding constants at 18.5 °C for decameric duplexes containing these non-self-complementary -XGCY- sequences are summarized as part of Table I. Of particular interest is the observation that the binding constants for sequences with X = G or Y = C range from 0.6 × 10^6 to 2 × 10^6 M^{-1}, considerably lower than those of the other three which range from 4 × 10^6 to 9 × 10^6 M^{-1}. These values are in general agreement with those found earlier for the self-complementary tetranucleotide sequences of (5–12) × 10^6 M^{-1} for -TGCA-, -AGCT-, and -CGCG- and 0.2 × 10^6 M^{-1} for the weak binding -GGCC- sequence.

CD Evidence Supports the Observed Differential Binding Affinities. Binding of ACTD induces positive and negative CD intensities at 293 and 265 nm, respectively. Relative binding order can, thus, be estimated by comparing the net CD intensities induced at these wavelengths. Difference spectra obtained by subtracting the oligomer from the drug mixture (40 μM/bp DNA + 7.5 μM ACTD) are compared in Figure 2. It is readily apparent, especially at the 293-nm region, that the three sequences containing X = G or Y = C exhibit considerably smaller induced CD intensities than the other three, in agreement with the relative binding affinities deduced via absorbance titrations. Advantage has also been taken of the CD intensity increase at 293 nm to investigate the kinetic behavior.

ACTD Dissociation Kinetics Are Considerably Faster for -XGCY- Sequences with X = G or Y = C. The SDS-induced ACTD dissociation kinetics were measured at 18.5 °C. Semi-log plots on the rate of ellipticity changes at 293 nm are shown in Figure 3 for ACTD dissociations from decameric duplexes containing -TGCG-/-CGCA-, -AGCA-/-TGCT-, -AGCG-/-CGCT-, and -TGCC-/-GGCA-. Those of -GGCG-/-CGCC- and -AGCC-/-GGCT- are nearly identical to that of -TGCC-/-GGCA- and, thus, not shown. Single-exponential kinetics are exhibited by all oligomers, as evidenced by straight lines obtained in such a plot. The most striking feature is the much faster dissociation rates exhibited by the GG/CC oligomers as represented by the -TGCC-/-GGCA- sequence. Characteristic times of about 60 s as opposed to 600–1000 s for the other three non X = G or non Y = C sequences are obtained via linear least-squares fits and are included in Table I. These results are again comparable to those found earlier for oligomers containing self-complementary sequences in which -GGCC- exhibits more than an order of magnitude faster ACTD dissociation kinetics than those of -TGCA-, -AGCT-, and -CGCG- (Chen, 1988b).

Evidence from Thermal Denaturation Experiments. Although ACTD binding results in duplex stabilization for each decameric duplex studied, the extent of such a stabilization depends significantly on the tetranucleotide binding sequence. Melting temperature increases of 11.3, 11.7, and 13.3 °C have been observed for the decameric duplexes containing -AGCG-/-CGCT-, -TGCG-/-CGCA-, and -AGCA-/-TGCT-, respectively. Consistent with the weaker ACTD binding abilities of X = G or Y = C oligomers, 4.0, 5.4, and 6.9 °C increases in melting temperatures are observed for -AGCC-/-GGCT-, -GGCG-/-CGCC-, and -TGCC-/-GGCA-, respectively. It is also interesting to note in passing that the self-complementary decamer d(ATA-GGCC-TAT) studied earlier in which X = G *and* Y = C exhibits a weaker binding and a mere 2 °C increase in the melting temperature (Chen, 1988b).

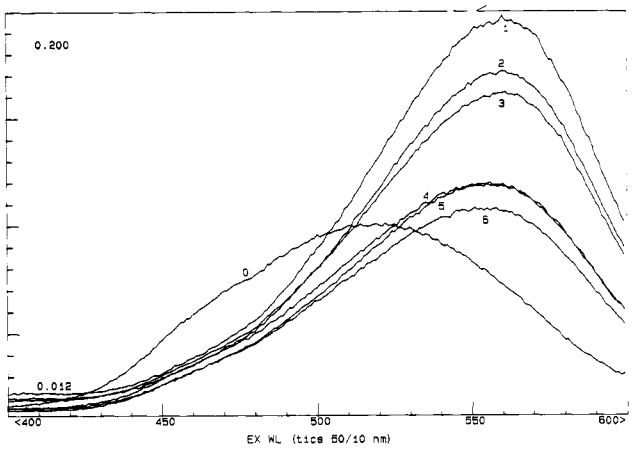

FIGURE 4: Comparison of 2 μM 7-amino-ACTD fluorescence excitation spectra in the absence (0) and in the presence of 40 μM/bp of d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) (1), d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT) (2), d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT) (3), d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT) (4), d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT) (5), and d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT) (6). Measurements were made at 20 °C with emission monitored at 660 nm.

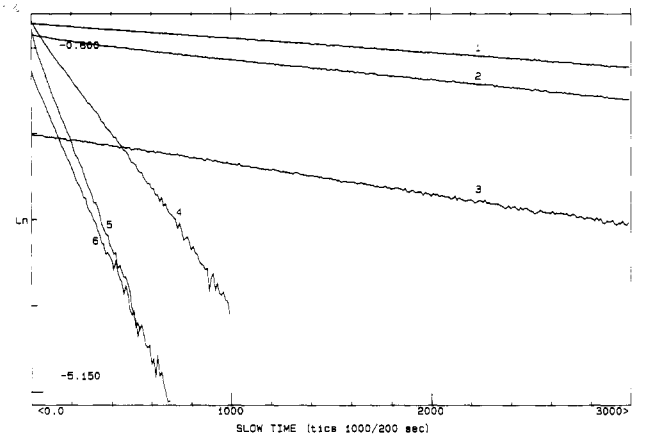

FIGURE 5: Comparison of 1% SDS-induced 7-amino-ACTD (4 μM) dissociation kinetics at 20 °C from 40 μM/bp decameric duplexes of d(ATA-TGCG-ATA)–d(TAT-CGCA-TAT) (1), d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) (2), d(ATA-AGCG-ATA)–d(TAT-CGCT-TAT) (3), d(ATA-TGCC-ATA)–d(TAT-GGCA-TAT) (4), d(ATA-GGCG-ATA)–d(TAT-CGCC-TAT) (5), and d(ATA-AGCC-ATA)–d(TAT-GGCT-TAT) (6). Measurements were made with a stirrer accessory, and semilog plots of fluorescence intensity changes at 650 nm (with 560-nm excitation) were shown.

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:

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

where ΔH is the enthalpy change for the melting of a duplex and n is the binding sites per duplex. Approximating L with 5.5 μM under our experimental conditions and ΔH calculated from that of Breslauer et al. (1986), calculated (observed value in parentheses) $1/T_m^0 - 1/T_m$ values of 1.00 (1.43) $\times 10^{-4}$ K^{-1} for -AGCA-/-TGCT-, 0.84 (1.17) $\times 10^{-4}$ K^{-1} for -AGCG-/-CGCT-, 0.40 (0.42) $\times 10^{-4}$ K^{-1} for -AGCC-/-GGCT-, 1.07 (1.20) $\times 10^{-4}$ K^{-1} for -TGCG-/-CGCA-, 0.70 (0.72) $\times 10^{-4}$ K^{-1} for -TGCC-/-GGCA-, and 0.39 (0.55) $\times 10^{-4}$ K^{-1} for -GGCG-/-CGCC- are predicted. In view of the fact that the equation was derived for long polymers, the general qualitative agreement between the theory and experiments is quite gratifying.

Fluorescence Measurements of 7-Amino-ACTD Support the Observed Differential Binding and Kinetic Results. Although actinomycin D is only weakly fluorescent, the presence of an amino group at the 7-position significantly enhances its luminescence characteristics. The fluorescence technique is, thus, also employed in the study of binding and kinetic behaviors of this compound. In the absence of DNA, 7-amino-ACTD exhibits an excitation spectrum with a maximum at 510 nm. Upon binding to DNA, this maximum is red-shifted to 560 nm with concomitant fluorescence intensity enhancement. Consequently, the relative fluorescence intensity enhancement at this wavelength can be used as a qualitative gauge for the affinity of an oligomer for this drug. Fluorescence excitation spectra of 2 μM 7-amino-ACTD in the absence and in the presence of each of the six decameric duplexes of 40 μM/bp concentration are compared in Figure 4. Indeed, fluorescence intensity enhancements in the presence of oligomers containing -AGCA-/-TGCT-, -TGCG-/-CGCA-, or -AGCG-/-CGCT- are significantly greater than those containing the -XGCY- sequence with X = G or Y = C, in agreement with our absorbance and CD results. Fluorescence titrations have also been carried out to obtain more quantitative results, and the estimated binding constants via Scatchard plots are also included in Table I for comparison. SDS-induced drug dissociation kinetics have also been carried out, the results are shown in Figure 5, and the values estimated from linear least-squares fits are also included in Table I. The striking differences in the dissociation kinetics between the GG/CC and non-GG/CC sequences are evident from the <300-s vs >2900-s characteristic times. The slower dissociation rates of 7-amino-ACTD as compared to ACTD are the consequence of additional intercalative hindrance imposed by the presence of the 7-amino group.

Discussion

Binding of actinomycin D (ACTD) to non-self-complementary decameric duplexes of the form d(ATA-XGCY-ATA)–d(TAT-Y′GCX′-TAT), where X and Y are complementary to X′ and Y′, respectively, but not to each other, has been investigated by equilibrium, kinetic, and thermal denaturation studies. The results indicate that when X = G or Y = C, the oligomers exhibit significantly weaker binding affinities toward ACTD, smaller melting temperature increases upon drug binding, and faster SDS-induced ACTD dissociation rates than the other sequences. Estimated binding constants at 18.5 °C for decameric duplexes containing -AGCA-/-TGCT-, -AGCG-/-CGCT-, and -CGCA-/-TGCG- are in the range of 4–9 μM^{-1}, whereas for the ones containing -GGCT-/-AGCC-, -GGCA-/-TGCC-, or -GGCG-/-CGCC- they range from 0.6 to 2 μM^{-1}. Furthermore, the three weaker binding sequences exhibit rapid SDS-induced ACTD dissociations with characteristic times of about 60 s, in contrast to an order of magnitude slower rates for those of stronger binding sequences. These observations are further supported by fluorescence studies on 7-amino-ACTD. The findings are consistent with those found earlier on self-complementary -XGCY- sequences (Chen, 1988b) and clearly indicate that base pairs (sequences) flanking the dG-dC sequence can have a dramatic influence on ACTD binding to and dissociation from this site.

The observed weaker ACTD binding to and much faster dissociation from the -XGCY- sequence with X = G or Y =

C is consistent with the results of Aivasashvilli and Beabealashvilli (1983) on the RNA elongation inhibition studies, our earlier results on the self-complementary -GGCC- sequence (Chen, 1988b), and some of the more recent results (Rill et al., 1989; Rehfuss et al., 1990). Using DNA sequencing techniques and 7-azidoactinomycin D as a photoaffinity probe, Rill et al. (1989) found that GC doublets were strongly preferred only if the 5′-flanking base was a pyrimidine and the 3′-flanking base was not cytosine. Quantitative footprinting methods were employed by Rehfuss et al. (1990) to measure site-specific binding constants for ACTD on a restriction fragment from pBR322 DNA. It was found that sites with the highest binding constants possess the GC sequence which is flanked on the 5′ side by either T or C, but not G. Although the basis for the weak ACTD binding and fast dissociation with X = G or Y = C is not clear, it most likely is the consequence of a local peculiar conformation deriving from the GG/CC sequence and/or the interfering presence of the 2-amino group of the adjacent guanine at the minor groove to render less favorable interactions with the pentapeptide rings. Although crystal and solution structures do not necessarily agree, it is interesting to note that 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, all exhibit A conformations.

Despite the fact that there are 12 possible oligomers of the form d(ATA-XGCY-ATA) with non-self-complementary -XGCY- and the same number of complementary d(TAT-Y′GCX′-TAT), only 6 of these 12 pairs have been synthesized and studied in this report. As these six oligomeric pairs made possible the comparison of the ACTD binding characteristics of all six non-self-complementary tetranucleotide duplex pairs, -AGCA-/-TGCT-, -AGCG-/-CGCT-, -AGCC-/-GGCT-, -TGCG-/-CGCA-, -TGCC-/-GGCA, and -GGCG-/-CGCC-, the other six oligomeric pairs were not synthesized. The general validity of our results on the relative binding abilities of these six sites, however, rests on the implicit assumption that the ACTD binding characteristics are little affected by the nonbinding A/T flanking sequences. For example, the binding characteristics of ACTD to d(ATA-AGCA-ATA)–d(TAT-TGCT-TAT) are not expected to differ greatly from d(ATA-TGCT-ATA)–d(TAT-AGCA-TAT), as they both contain the -AGCA-/-TGCT- binding site. Since the DNA conformation is known to be sequence-dependent, the validity of such an assumption may require further investigations.

Although this report as well as our earlier investigations has focused on the effects of adjacent bases (sequences) on the binding to the dG–dC sites, it should be pointed out that strong ACTD binding to sequences not containing this dinucleotide has recently been reported (Synder et al., 1989; Rill et al., 1989, Rehfuss et al., 1990). Also noteworthy is the demonstration via fluorescence spectral measurements of strong binding of 7-amino-ACTD to single strands of certain specific sequences (Wadkins & Jovin, 1991). All these serve to illustrate that the mechanism of ACTD binding to DNA may be more complex than generally believed.

The SDS-induced ACTD dissociation from natural DNA had earlier been shown to exhibit multiexponential decay, with the slowest component in 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.

Acknowledgments

I thank E. Miller for her technical assistance.

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Probing Structural Factors Stabilizing Antisense Oligonucleotide Duplexes: NMR Studies of a DNA·DNA Duplex Containing a Formacetal Linkage

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Received November 27, 1991; Revised Manuscript Received April 21, 1992

ABSTRACT: The duplex formed by annealing the formacetal backbone modified dodecamer d-(CGCGTT$_{OCH_2O}$TTGCGC) to its complementary strand, d(GCGCAAAACGCG) (duplex I), has been studied by NMR techniques and analyzed with reference to its unmodified counterpart (duplex II). Comparison of parameters such as 2D cross-peak intensities, coupling constants, and spectral patterns indicates that structural perturbations caused by the incorporation of the formacetal linkage are minimal and localized to the central T_4·A_4 block. Duplex I adopts a B-type helical conformation with regular Watson–Crick base pairing and normal minor groove width. The methylene group is accommodated along the phosphate backbone in a conformation similar to that of the PO_2 group found in the B-form DNA family. The central T6-T7 base pairs of duplex I melt simultaneously with the duplex, indicating a cooperative transition to single strands. Although the formacetal linkage affects global melting, as evidenced by a 3 °C reduction in T_m for duplex I with respect to duplex II, the present study indicates that this is not the result of localized premelting at the formacetal site of duplex I but rather reflects the subtle interplay of several structural and energy factors which need to be further explored.

The idea of preventing gene transcription and/or gene translation at the DNA/mRNA level is an attractive one for many reasons. Classical approaches to drug discovery involve the design and identification of compounds directed against enzymes, receptors, or ion channels, the structure and mode of action of which are usually complex and often poorly understood. Conversely, the potential for therapeutic intervention at the nucleic acid level follows a well-ordered, generalizable strategy which is targeted at the initiating events of an amplifying cascade: transcription of a gene gives rise to many copies of mRNA which on translation affords an even greater number of protein molecules. Inhibition of gene expression ought, therefore, to be more efficient than inhibition of the gene product.

The potential of oligonucleotides (ODNs) to serve as code-blocking therapeutic principles was first recognized by Stephenson and Zamecnik (1978). Stimulated by their findings, interest in ODNs as sequence-specific code blockers has grown steadily over recent years [reviewed by Uhlmann and Peyman (1990) and Miller (1991)]. Whether the intended use is as an antisense or triplex agent, the requirement is for ODNs which are stable to both extracellular and intracellular enzymes and capable of being transported to their site of action. In chemical terms this means that some or all of the usual phosphodiester linkages must be replaced with nuclease-stable and preferably nonpolar surrogates which still allow for hybridization. Not surprisingly, initial attention focused on close phosphodiester analogues and attempted to preserve the central role of phosphorus. Methylphosphonates, alkyl phosphotriesters, phosphorothioates, and phosphoramidates typify the first-generation analogues [reviewed by Uhlmann and Peyman (1990)]. Although such modifications impart ODN stabilization and are claimed by some to show promise in enhancing cellular permeability, they often result in reduced binding affinity as judged by T_m depression when compared with phosphodiester counterparts and introduce an additional complication in that substitution of one phosphate oxygen atom in a phosphodiester linkage generates a new center of chirality (Pramanik & Kan, 1987; Froehler & Matteucci, 1988; Lin et al., 1989; Kibler-Herzog et al., 1991; Dagle et al., 1991; Piotto et al., 1991; Kibler et al., 1991). This latter issue is problematical, since biological systems are known to discriminate between enantiomers, and becomes acute in the context of a multiply modified oligomer where each stereoisomer is likely to possess a unique biological profile. An elegant solution to this problem is to only target achiral

0006-2960/92/0431-6228$03.00/0