Actinomycin D induced DNase I cleavage enhancement caused by sequence specific propagation of an altered DNA structure

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ABSTRACT

Two DNA hexadecamers containing one central 5'-GC-3' base step have been examined by footprinting methodology in the presence and absence of actinomycin D. The results of these studies, coupled with imino proton NMR measurements indicate that the antitumor drug causes a change in DNA conformation at a distance from the actinomycin intercalation site in a molecule of sequence d[ATATATAGCTATATAT] that does not occur in d[AAAAAAGCTTTTTT]. The experiments demonstrate that DNase I rate enhancements associated with actinomycin D binding are caused by ligand alteration of equilibrium DNA structure.

INTRODUCTION

DNA can adopt a variety of helical forms that depend upon local sequence and environmental factors such as ionic strength and superhelix density. Deoxyribonuclease I (DNase I) cleavage rate has been shown to respond to changes in DNA minor groove width as well as helical twist at a given base step (1-3). We (4,5), and others (6,7), have postulated that drug associated DNase I rate enhancements are caused by induced changes in DNA structure. This manuscript presents enzymatic evidence that d(AT)_n : d(AT)_n stretches change their structure in response to the underwinding stress provided by an intercalated actinomycin D molecule bound at a nearby d(GC) site, whereas similarly placed (dA)_n : (dT)_n sequences resist a similar conformational change. This evidence supports the hypothesis of "telestability" advanced by Wells and co-workers (8-10) that a ligand bound at one sequence can influence DNA structure at some distance from the binding site. This distal response will be modulated by

the receptivity of a sequence to conformational change.

A dramatic example of ligand induced structural change is cooperative conversion of Z DNA to a right-handed helical configuration by intercalating compounds (11-14). Cooperative DNA binding of the non-intercalative antibiotics distamycin and netropsin (15,16) as well as intercalative binding of actinomycin D to poly d(GC) (17), have also been attributed to a conformational switch of the DNA substrate to one more favorable for binding these ligands. Single crystal examinations of DNA and DNA:ligand complexes have shown conformations consistent with ligand evoked changes being able to propagate through the DNA helix (18-21). However, the interpretation that DNase I enhancements are due to structural changes propagating through DNA from a ligand binding site is not straightforward. Two other plausible explanations may be advanced to account for such rate enhancements having nothing to do with DNA structural response to ligand binding. First, it is possible that such DNase I rate increases are due to a ternary complex between the DNA substrate, the bound ligand, and the DNase I molecule. If a ligand bound to a DNA site has an affinity for DNase I, more cleavage should occur near the bound ligand since the local enzyme concentration will increase. Second, as DNA molecules become bound by a ligand, the concentration of available DNase I substrate will decrease. As a consequence enzyme rate might increase at all ligand-free base pairs. This type of kinetic effect has recently been suggested as a source of DNase I rate enhancements (22).

In order to ascertain which of these mechanisms gives rise to drug associated DNase I enhancements we have studied the interaction of actinomycin D with two synthetic hexadecamer DNA duplexes. The sequences of the two 16-mers are shown below with the drug intercalation sites indicated by vertical bars. Both duplexes have identical base content but differ in sequence motif in the regions of the DNA duplexes flanking the actinomycin D binding site, 5'- AGCT- 3'. Actinomycin D was chosen as the ligand in these

MOLECULE 1 **MOLECULE 2**

studies because its interactions with DNA have been the focus of a great deal of attention (reviewed in 23,24). The evidence accumulated to date indicates that this drug binds DNA with a strong preference for 5'- GC - 3' base pairs, in an intercalative, or pseudo-intercalative mode, with the two pentapeptide rings lying in the DNA minor groove (25-27). The intercalated phenoxazone chromophore of the drug causes unwinding of the neighboring GC base pairs and the interaction is stabilized by two hydrogen bonds formed between the N2 amino group of the guanine residues and the carbonyl oxygen of the L-threonine residues of the cyclic pentapeptide segment of the drug. The drug is efficacious against some cancers, a powerful inhibitor of RNA transcription, and exhibits complex DNA binding kinetics.

MATERIALS AND METHODS**Oligonucleotide Synthesis and Manipulations.**

Oligonucleotides were synthesized on a Biosearch (Model 8600) DNA synthesizer in 1.0 or 15.0 uMole quantities. Hexadecamers were purified by electrophoresis through a 20%, 7.0 M urea polyacrylamide gel, visualized by backshadowing against a

fluorescent TLC plate and the gel slice containing the purified oligomer excised from the gel with a razor blade. The gel slice was ground up and suspended in 1.0 ml of a 10 mM Tris/ 1.0 mM Na₂EDTA (pH 7.5) solution and incubated overnight at 4°C. Subsequently, oligonucleotide was separated from the gel by passage of the slurry through silanized glass wool and then precipitated from a 0.3 M sodium acetate/ 70% ethanol solution. 15.0 uMole products were purified by HPLC on a DEAE 60-7 (Nucleogen) column. Optical density at 260 nm was used to determine concentrations. Labeling and purification of the labeled oligonucleotides has been described (5). Labeled samples were heated to 90°C and slow cooled (1°C/min.) to avoid snap-back of the molecules to themselves. Native acrylamide gels showed only duplex product with no contaminating hairpin structures evident (not shown).

DNase I Digestions. Procedures used were essentially identical to those described previously (5). Briefly, DNase I digests were performed in 8.0 ul (buffer was composed of 10 mM Tris (pH 7.5)/ 8.0 mM MgCl₂/ 2.0 mM CaCl₂); 2.0 ul of a 40 uM 'carrier' d[ATATAGCTATAT] duplex, 2.0 ul labeled duplex and 2.0 ul drug (or buffer) were combined and then incubated at 25°C for thirty minutes, then 2.0 ul DNase I was added to initiate the reaction. The reaction was allowed to proceed for 1.0 min. and then terminated by addition of 10.0 ul of a solution composed of 10 M urea/ 100 mM NaOH/ 50 mM Na₂EDTA/ 0.1% bromophenol blue/ 0.1% xylene cyanol. All reactions were performed under conditions determined to give 80 (+/-3)% full length oligonucleotide remaining (in the drug free controls) after the reactions were terminated.

Autoradiography/ Densitometry. X-Omat AR film was used to visualize bands after electrophoresis for periods of up to one week in the absence of intensifying screens. Densitometry was performed on a Jarrel-Ash model 23-100 densitometer coupled to an NEC microcomputer. Band intensities were obtained as integrated absorbance. This equipment has been described previously (28).

NMR Spectroscopy. Imino proton spectra were obtained on a General Electric GN-500 instrument. Samples consisted of 2 mM (single strand) DNA oligomer in the DNase I buffer described above plus 20% D₂O. A jump-return method was used to suppress the water signal (29). Spectra were referenced to DSS through the water peak. Actinomycin D was added to the NMR samples as a solid and allowed to equilibrate with the oligonucleotide for at least 30 minutes before spectra were recorded. Assignment of imino protons was accomplished by examining the temperature dependence of the protons as described (5).

Actinomycin D Handling. Actinomycin D was purchased from Sigma (product # A 1410) and used without further purification. An extinction coefficient of 24450 (17) was employed for drug concentration determinations.

RESULTS

Figure 1a shows the electrophoresis pattern generated by resolution of the partial DNase I cleavage products from the d[ATATATAGCTATATAT] hexadecamer in the presence of various stoichiometric ratios of added actinomycin D. The pattern is drastically altered at all visible DNase I cleavage sites as actinomycin D is titrated onto the duplex. Reduced cleavage, signifying intercalative binding of the actinomycin D phenoxazone moiety between the 5'- GC- 3' base step (5), becomes increasingly evident as the ratio of total drug to total duplex increases. Additionally, two bonds (both ApT base steps) located between base positions 5-6 and 13-14 show dramatically increased rates of DNase I cleavage as more drug becomes bound to the duplex. The extent of the rate changes at these two positions is more apparent in the densitometric scans shown in Figure 1b.

DNase I partial digestion patterns of the duplex hexadecamer d[AAAAAAGCTTTTTT] in the presence of actinomycin D concentrations identical to those used for the d[ATATATAGCTATATAT] duplex are shown in figure 2a and the

Figure 1. DNase I partial digests of the d[ATATATAGCTATATAT] hexadecamer.

a) Autoradiogram of a 15% denaturing gel showing alteration in DNase I cleavage pattern as a function of actinomycin D concentration. Enzyme concentration was fixed such that only one cleavage was sustained per duplex during the course of the digestion. Sequence position is indicated on the right side of the autoradiogram. Lanes are 1: Alu I partial digest of the 16 mer - note that Alu I (recognition site AGCT) cleaves the molecule between bases 8-9; Lane 2: No enzyme; Lanes 3 and 9 DNase I partial digests in the absence of actinomycin D; Lanes 4- 8: DNase I partial digests in the presence of 3.4, 1.8, 1.2, 0.8, an 0.5 equivalents of actinomycin D added per duplex, respectively.

b) Densitometric scans obtained from the autoradiogram shown in a. X-axis is sequence position, Y-axis is band intensity and Z- axis is drug:duplex ratio. Note that in addition to the expected footprinting pattern surrounding the actinomycin D binding site (5'-AGCT-3'), bands on either side of the drug binding site, at positions 5-6 and 13-14 (arrows), show increased susceptibility to DNase I as the duplex becomes bound by drug.

corresponding densitometric traces in figure 2b. It is clear that 1) a similar inhibition of DNase I rate occurs in the central domains of the two duplexes, and 2) no rate enhancements are observed outside of the drug binding site.

Figure 2. DNase I partial digests of the duplex hexadecamer d[AAAAAAGCTTTTTT].

a) Autoradiogram of a 15% denaturing gel showing alteration of DNase I cleavage intensity as a function of actinomycin D binding to the d[AAAAAAGCTTTTTT] 16 mer. DNase I concentration necessary for obtaining appropriate degree of digestion was twice that used for d[ATATATAGCTATATAT]. Lane 1: Alu I; Lanes 2 and 8: DNase I partial digests in the absence of added actinomycin D; Lanes 3-7: DNase I partial digests in the presence of 3.4, 1.8, 1.2, 0.8, and 0.5 mole equivalents of actinomycin D per duplex.

b) Densitometric scans obtained from the autoradiograph shown in a. Axes are identical to those in figure 1b. No change in cleavage intensity is seen at positions 5-6 or 13-14 (arrows) in this duplex as more actinomycin D is bound to this duplex.

Specifically, the 5-6 and 13-14 phosphodiester bonds which exhibit enhanced cleavage in molecule 1 exhibit roughly equivalent susceptibility to DNase I attack both in the absence and presence of complexed actinomycin D on this DNA molecule.

In order to view the actinomycin induced changes in cleavage rate on a more quantitative basis, the measurements

Figure 3. Performance of DNase I cleaved phosphodiester bonds as a function of actinomycin D concentration for $d[ATATATAGCTATATAT]$ and $d[AAAAAAGCTTTTTTT]$. The ratio of band intensity for each cleaved site in both molecules in the presence of drug (I) was divided by the intensity of the same band in the minus drug lanes (I_0). This value was obtained in triplicate experiments and the standard error of this ratio is indicated by error bars. This value was then plotted versus the actinomycin D: DNA duplex ratio. Phosphodiester bonds are delineated in a base-phosphate-base numbering scheme (see text for base numbering) where C9pT10 signifies cleavage at the internucleotide linkage between cytosine 9 and Thymine 10. The scale on the plots in the two vertical columns is from -0.10 to 1.25. Scales for positions 5-6 (A5pT6) and 13-14 (A13pT14) in the $d[ATATATAGCTATATAT]$ duplex are from -0.10 to the indicated values. The 5-6 and 13-14 phosphodiester linkages in the $d[AAAAAAGCTTTTTTT]$ show no response to the presence of actinomycin D. Base position 6-7 shows footprinting behaviour in $d[AAAAAAGCTTTTTTT]$ but remains available for DNase I cleavage in $d[ATATATAGCTATATAT]$ (see text).

Figure 4. Proton NMR spectra of the imino protons of the dodecamer d[AAAAAGCTTTT].

a) Temperature dependence of the imino proton spectra was used to assign the protons of this dodecamer. Peak assignments are indicated above the 5°C spectrum. The single GC imino proton falls on the right side of the spectra.

b) Changes in the imino proton spectra as a function of increasing ratio of actinomycin D to duplex at 15°C. The resonances labeled C are GC imino protons from the drug-bound duplex.

were repeated in triplicate and the average ratio of cleavage rate in the presence of drug versus the no drug control for all cleaved phosphodiester linkages, in both DNA molecules, are plotted in Fig. 3. In this representation,

phosphodiester bonds inaccessible to DNase I, indicating steric blockage of the enzyme by the drug, appear as inhibition curves (ratio drops below 1.0). Conversely, phosphodiester bonds displaying drug associated enhanced cleavage appear as enhancement curves (ratio greater than 1.0). Bonds inaccessible to the enzyme are not shown but we note that the inability of the enzyme to cleave base steps at the 5' end of a duplex has been demonstrated to be a function of the mechanism by which DNase I binds to DNA (30,31).

Examination of the plots in Fig. 3 shows that common to both DNA duplexes is inhibition of cleavage by the drug at phosphodiester bonds between bases 7-8, 8-9, 9-10, 10-11, 11-12 and 12-13. These phosphate positions clearly are protected from DNase I as was shown in figures 1 and 2. The phosphodiester linkages between bases 5-6 and 13-14 do not show any change within the error of our measurements in the d[AAAAAAGCTTTTTT] duplex. Cleavage rates of the same bonds in d[ATATATAGCTATATAT] are increased by six-fold and three-fold, respectively (note y axis scale change in these plots). The bond between bases 6-7 behaves anomalously. In the d[AAAAAAGCTTTTTT] duplex clear inhibition at this bond is observed while its congener in the d[ATATATAGCTATATAT] duplex showed no response to drug binding. This raises an alternative hypothesis to the three outlined above, namely, that the binding geometry of the actinomycin D molecule to the two duplexes is different. If this were the case then differing drug binding geometries could explain the different responses of the two DNA molecules to binding of the drug. In order to test this possibility a dodecamer of sequence 5' - AAAAAGCTTTTT -3' was synthesized and the imino proton NMR spectrum assigned by correlating the disappearance of proton signals with increasing temperature (Fig. 4a). The effect of actinomycin D on the imino proton resonances was then observed. As shown in figure 4b, binding of actinomycin D to this molecule results in a decrease in the ratio of the unbound GC imino proton peak area relative to the actinomycin bound GC proton peak areas; these are

split due to the asymmetry of the drug (peaks C of Fig. 4b). The GC imino protons in this and the dodecamer duplex, d[ATATAGCTATAT] (5), have virtually identical chemical shifts, in both the free and drug-bound forms. The AT imino protons in the two duplexes have different chemical shifts because they have different nearest-neighbors, however, actinomycin binding causes little, if any, change in their chemical shifts and only slight broadening in each duplex. This indicates that the 5'-AGCT-3' site responds very similarly to actinomycin D binding in both cases. Therefore, it appears that neither the DNase I enhancements nor the anomalous response of the 6-7 phosphodiester bond are caused by different binding modes of the drug to the two hexadecamers. We have shown previously that the DNase I detected structural change is not reflected in a large change in the imino-proton chemical shifts of the (AT)_n sequence (5).

DISCUSSION

Given that the drug assumes a similar intercalation geometry with the GC core of these molecules, it is most likely that any affinity of the enzyme molecule for the bound drug will be similar in both cases. If that is so then the ternary complex hypothesis, which would predict that the same positions on both duplexes will show enzymatic enhancements, can be discounted as the source of the drug associated rate enhancements in d[ATATATAGCTATATAT], because no enhancements are observed in d[AAAAAAGCTTTTTT]. Such a mechanism is also inconsistent with previous observations of actinomycin D complexed to (AT)_nAGCT(AT)_n duplexes (5). The second mechanism for generating enhanced DNase I cleavage, a kinetic effect caused by a decrease in effective substrate to enzyme ratio (22), can also be dismissed. An effect of this type can explain neither the sequence specificity nor the extent of the enhancements observed. The combined observations indicate that the DNase I rate increases are due to sequence specific, actinomycin D induced, alteration

in DNA structure. A possibility we cannot exclude is that actinomycin D induces a structural change in the portion of the $d(A)_n:d(T)_n$ duplex accessible to DNase I that does not lead to altered cleavage by the enzyme. This possibility seems remote based on the DNA structure dependence of DNase I cleavage rate as the rate of cleavage of a phosphodiester bond is not dependent on sequence, per se, but rather sequence dependent structure (1-3).

Why does a flanking $d(AT)_n : d(AT)_n$ sequence motif display the ability to change conformation on binding of actinomycin D while a $d(A)_n:d(T)_n$ flanking sequence does not? A definitive answer to this question must await resolution of the structures of the hexadecamers in both actinomycin D bound and unbound states. We are currently pursuing this problem via 2-D NMR techniques. However, even in the absence of the specific structural details governing these complexes some inferences can be drawn. Actinomycin D, and intercalaters in general, cause a flattening of the propeller twist and an unwinding of the helix at the insertion site (for a recent review see 32). In our view this disruption is then propagated through the helix in the case of a flanking $d(AT)_n : d(AT)_n$ sequence, whereas a $d(A)_n : d(T)_n$ sequence has sufficient rigidity to prevent transmission of this structural change. Measurements of helix repeat length suggest that a $d(A)_n:d(T)_n$ helix has a repeat length of 10.0 base-pairs per turn either in the absence or presence of torsional strain provided by supercoiling, while $d(AT)_n : d(AT)_n$ sequences adopt a 10.5-10.6 base-pair helical repeat in the absence and an 11.7 base-pair helical repeat in the presence of supercoiling strain (33-35). This suggests that $d(AT)_n : d(AT)_n$ sequences are susceptible to underwinding and lends support to the possibility that local unwinding caused by the intercalated phenoxazone chromophore might be propagated to neighboring residues. An underwound $d(AT)_n : d(AT)_n$ helix geometry might also explain the differing 6-7 phosphodiester response mentioned earlier. Additionally, the ability of actinomycin D

to alter DNA structure at a distance may lead to the complex association and dissociation kinetics observed for the binding of this drug to various duplex DNA substrates (36-42).

CONCLUSION

We have established that a structural change can be propagated through a DNA helix by a structurally disruptive DNA binding ligand, such as an intercalater, and that such an effect can be monitored with DNase I. We have suggested that unwinding induced by the drug can be transmitted to the surrounding DNA if the flanking sequence motif is receptive to unwinding. However recent crystallographic analysis of DNase I bound to DNA (43) demonstrates a large DNase I induced bend in the DNA substrate. This implicates flexibility of the double helix as important to cleavage by the enzyme molecule. As the sequence dependence of overwinding (44,45) has been related to DNA flexibility (46) it is possible that it is not underwinding which directly causes enhanced DNase I cleavage but rather an increase in flexibility of the helix caused by underwinding.

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