Actinomycin D induced DNase I hypersensitivity and asymmetric structure transmission in a DNA hexadecamer

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ABSTRACT

DNase I cleavage rates and nmr chemical shifts are shown to change for DNA sequences distal to an intercalated actinomycin D molecule in a duplex hexadecamer upon drug binding. Both sets of observations suggest that the source of these changes is a DNA-mediated structural response. The nmr results imply the response is transmitted preferentially in a 5'-to-3' direction from the drug binding site. An inequivalent response of the two strands to a ligand-induced conformational change immediately suggests a mechanism for distinguishing the sense and antisense strands of DNA.

INTRODUCTION

Flanking DNA sequences adjacent to active genes can exhibit unusual increases in sensitivity to nucleases (1,2). In some cases, this 'hypersensitivity' has been related to local alterations in DNA structure associated with underwinding stress (reviewed in 3). Although radical changes in structure have been proposed for such sequences it is also possible that less drastic structural alterations can switch a control sequence between active and inactive forms; such alterations might occur as a response to the binding of proteins or other ligands (4). We have previously shown that actinomycin D binding increases DNase I sensitivity at sites distal to the binding domain of the drug (5,6), and that the appearance of enhanced cleavage is a function of sequence (7). In this communication, DNase I cleavage kinetics and 1H-nmr are used to show that actinomycin D binding increases DNase I sensitivity at sites distal to the binding domain of the drug (5,6), and that the appearance of enhanced cleavage is a function of sequence (7).

The vertical bars represent the intercalation site of the drug while the (*) symbols indicate phosphodiester bonds that are cleaved at 4 to 10-fold faster rates in the presence of a saturating amount of ActD. Previous studies demonstrated the 1:1 stoichiometry of complex formation and intercalation at the G-C in the central dodecamers of I, II and III (6,7). The alternating (A-T)n motif in I is permissive to drug induced enhancements while the (A)n:(T)n sequence in II is not (7). Sequence III is shown in this communication to have enhancements identical in location and similar in degree to I. Significant enhancements also occur in IV but the positions are shifted an additional base outward from the drug intercalation site relative to I and III (Y.-Q.H., K.D.B., & M.J.L., unpublished). The present study examines whether there is a structural basis for these DNase I enhancements. Duplex III was chosen because its two-dimensional (2D) nmr spectrum has fewer overlapping crosspeaks than I, II or IV.

MATERIALS AND METHODS

DNA Synthesis

The DNA was synthesized on a Biosearch 8600 DNA Synthesizer using the solid state phosphoramidite method at 15μmole scale. Purification of the crude DNA was performed by ion exchange HPLC over a DEAE Sephadex column with a LiCl gradient in 20% acetonitrile and 0.05M sodium acetate at pH 6.1. Desalting and salt exchange with NaCl of the purified DNA was accomplished by elution on a G-10 Sephadex column.
DNase I Reactions
The endonuclease reaction was performed under single-cleavage conditions in 8 μl: 2 μl of carrier DNA, [d(AT)₂GCT(CT)₂], 2 μl ³²P labelled duplex, 2 μl ActD (or buffer) and 2 μl DNase I. DNA concentration was 10μM in carrier duplex, and DNase I concentration was identical in all reactions. The drug:DNA mixture was incubated for at least 30 minutes at 25°C prior to addition of enzyme. DNase I digests were performed at 25°C and terminated after one minute by addition of 10 μl of a 10M urea/100mM NaOH/50mM Na₂EDTA/0.1% bromophenol blue/0.1% xylene cyanol solution. Samples were heated to 90°C for two minutes and quickly cooled on ice prior to loading onto a 20% polyacrylamide/7.0M urea sequencing gel. (More details are given in refs. 6,7). DNase I patterns were visualized by autoradiography at -80°C using Kodak X-omat AR film.

NMR Sample Preparation and Analysis
The NMR samples of sequence III were prepared by drying 250 A₂₆₀ OD units of the sodium salt of the DNA and adding an appropriate amount of a stock buffer. The final concentration of the buffer in the NMR tube contained 8mM MgCl₂, 2mM CaCl₂, 10mM phosphate and 0.01% NaN₃ at pH=7.1. The H₂O in the sample was exchanged several times with D₂O (Aldrich, 99.8%); brought up to 0.5ml with 99.96% D₂O and transferred to a 5mm NMR tube (Wilmad 528pp) in an argon atmosphere. The 1:1 16-mer/ActD complex was formed by adding 1.5mg of solid ActD directly to the 16-mer sample. The NMR data were acquired at 500 MHz on a GN500 NMR spectrometer. Both spectra were acquired in hypercomplex mode (8) with 1K real points in t₂ and 512 points in t₁, and zero-filled twice in t₂ and three times in t₁ to 4K×4K; spectral width was 4000 Hz for the free duplex and 4404 Hz for the complex. Sequential assignments (9-11) were made using the 2DNOE spectrum at 90ms mixing time. HOHAHA/TOCSY (12,13) spectra were acquired on both samples, which together with the 2DNOE spectra allowed assignment of most of the protons in the drug-free 16-mer and its complex with ActD.

RESULTS AND DISCUSSION
Figure 2 shows the result of partial digestion of III with DNase I in the presence and absence of bound ActD. When ActD binds, steric blockage of the nuclease occurs in the central portion of the duplex, while enzymatic enhancements (*) are observed outside the drug binding site. Thus in III, the drug ‘footprint’ is found between phosphodiester positions 7-8 through 12-13 while sites for enhanced cleavage induced by the drug are located at phosphodiester positions 5—6 and 13 — 14, an identical response to that reported for I (7). This data is summarized in Fig. 1b.

In considering the nature of the enhancements, it is necessary to examine the relative accessibility of the phosphodiester to DNase I cleavage. Inspection of x-ray diffraction (14,15) and nmr (16—20) studies of ActD binding to short oligonucleotides predicts that the drug physically contacts only the central AGC-T tetranucleotide, with the pentapeptide lactones oriented in the minor groove. Models derived from the x-ray and nmr studies agree that the helix twist angle is much smaller at the intercalation site than elsewhere. A model derived from x-ray diffraction analysis of a cocrystal of DNase I and an oligonucleotide duplex shows that the enzyme contacts seven phosphates, representing a four-to-five base distance in the minor groove (21), so most of the linkages away from the drug site should be accessible to the enzyme. Duplex IV is especially interesting in regard to enzyme accessibility, since the 6—7 linkage is cleaved at a high...
rate in the presence or absence of added drug, even though it is very close (A6-T7-G8-ActD) to the intercalation site (Y.-Q.H., K.D.B. & M.J.L., unpublished). There are numerous other examples of phosphates in these four duplexes that have equal cleavage rates whether or not ActD is bound at the AGCT tetramer. These observations lend support to the idea that the enhancements arise from a rearrangement in structure distal to the binding site.

We turned to high-resolution nmr analysis in order to learn more about the structural basis for the observed DNase I enhancements. We used the sequential assignment scheme (9-11) for 2DNOE (nuclear Overhauser enhancement) spectra to assign the H8/H6/H2 (base protons) and H1'/H3'/H4' (sugar protons; K.D.B., M.J.L. & P.N.B. in preparation). Drug-DNA NOE contacts were used to aid in the assignment of the base and sugar protons. The free duplex was classified as a member of the B DNA family by the inter and intra nucleotide NOE intensities. The strands are equivalent in the free duplex; however, drug binding causes the two strands to be magnetically inequivalent. This is due to the asymmetry of the actinomine chromophore, where G and C bases along one strand overlap the quinoid ring, while those on the other strand overlap the benzenoid ring (16,17). The resonances from the two strands become indistinguishable for the nucleotides nearest the ends of the duplex. This makes it difficult to evaluate NOE intensities and produce a well-defined conformational model from interproton distances. However, the chemical shifts are easily evaluated to within ±0.003 ppm, so the changes in chemical shift upon drug binding can be used as a monitor of structural change.

The chemical shift changes (Δδ) of the H8/H6, H1' and H3' protons are summarized in Fig. 3, where Δδ = δbound−δfree (by convention chemical shifts are taken as positive numbers, e.g., δH8 > δH1'). The Δδ values depend on changes in the local electronic environment of the individual nuclei. The electronic distribution changes as a complicated function of the conformational equilibrium, so a large alteration in structure may actually result in Δδ being small or null. On the other hand, a non-zero Δδ can occur only if there is a change in average conformation. Also, when the Δδ values for two protons correlate strongly for a single residue, it may be expected that the changes arise from a common structural origin. Finally, when the Δδ values are nearly zero for several protons on the same residue, we may infer that there is little or no structural change.

Figure 3 presents the chemical shift changes as a function of the sequence, which is arrayed along the bottom of each panel; the solid and cross-hatched bars distinguish the Δδ measurements.
for the quinoid and benzenoid strands, respectively. The greatest shielding changes occur near the GC intercalation site (Fig. 3a-d), as expected. There are also significant $\Delta\delta$ values in the flanking A/T regions that are best illustrated by the vertically expanded plots at the top of each panel of Fig. 3. These measurements affirm the prediction of the DNase I experiments that ActD binding alters the average conformation in the distal regions.

The most surprising feature of our results on sequence III is that the distal effect propagates more strongly through the regions 3' to the binding site compared to the 5'-flanking DNA. This is most obvious for the H3' and H4' (Fig. 3a,b) where comparison of the five residues nearest each end shows that the average $|\Delta\delta|$ is larger for the 3'-distal protons by more than a factor of 3. The magnitude of the 3'-distal effect is also larger for the H8/H6 and H1' (almost 2-fold, see Fig. 3c,d). The H3' and H4' profiles also correlate highly with each other (see Fig. 3a,b) a scatter plot of the $\Delta\delta$ values for H3' vs. H4' has a linear least squares coefficient, $r=0.85,$ for the 12 residues flanking the central AGCT tetramer. This indicates that a defined structural change occurs in the distal deoxyribose moieties in response to ActD binding.

The $\Delta\delta$ values for H8/H6 and H1' protons on the same residue (see Fig. 3c,d) are also highly correlated ($r=0.90$ for all 16 residues). This correlation argues that the shielding changes, although small, are not random fluctuations in the measurements; they are on the order of previously reported premelting changes (22). As both the H8/H6 and H1' chemical shifts respond to changes in base orientation, the correlation suggests that distal alterations in base stacking occur in response to drug binding. The largest $|\Delta\delta|$ values in the distal residues for the H8/H6 and H1' are about 0.05 ppm. This is much smaller than the ~0.5 ppm changes that are seen for some of the H8/H6 and H1' protons in comparisons of B and A (23) or B and Z (24) forms of DNA. Thus, it is likely that there are no radical deviations in distal structure between the drug-bound and free duplexes. Interestingly, the local structural changes in the bases do not appear to be directly correlated with the changes in the deoxyribose (cf. Fig. 3a,b with 3c,d).

It is valuable to compare the $\Delta\delta$ values observed for duplex III (the present study) with those in a control duplex where no DNase I enhancements are observed. Such a control is offered by II; however, its 2D-nmr spectrum is much more difficult to interpret. First, a relatively subtle change in the equilibrium

Our experiments suggest that the structural disturbance induced by the drug in III propagates most strongly in the 3'-direction from the binding site, producing what Arnott and colleagues (25) have termed a 'heteronomous' structure. For clarity, our interpretation of the response of the duplex to actinomycin D binding is shown below:

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5'-A A A T A T A G | C T A T A T T T-3'
3'-T T T A T A T C | G A T A T A A A S-5'
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We are unaware of any other direct demonstration of the structural consequences of ligand binding on sequences half a helical turn from the binding site. However, the potential for such transmission effects has been recognized for some time having been previously referred to as 'telestability' by Wells and coworkers (26).

Our chemical shift results do not point toward a specific structural change as the source of the increase in hydrolysis rate. In retrospect, this can be rationalized by considering the magnitude of the changes in rate. The increases in the rate constants are 4 to 10-fold (vide supra), which would indicate a decrease of 0.8 to 1.4 kcal in activation free energy ($\Delta G^\circ$) for the reaction at 25°C. While we are aware of the dangers in mixing kinetic and thermodynamic arguments, it is useful to note that ActD binding lowers the free energy of the duplex by more than this amount (27). In any reasonable mechanical model, most of the energy from the binding event would be expected to alter the structure near the binding site, but some of the energy could be used to alter distal regions of the DNA strands. The distal energy increment could then lower the barrier to cleavage at the enhancement sites by an alteration in the average (or transient) structure toward a form more susceptible to cleavage. These structural changes do not need to be large, since the $\leq 1.4$ kcal required to account for the rate changes is much less than that required to open an internal base-pair [3.8 kcal in d(A):d(T) vs 2.4 kcal in d(AT):d(AT) (28)], and the effects are evidently spread over several base-pairs (see Fig. 3).

Perhaps the ease with which structural information can be transmitted through a particular sequence is simply related to the free energy of base-pair opening. This is the most obvious correlation from the d(A):d(T) vs d(AT):d(T) comparison just noted. Others have reported that the d(AT)$_n$ sequence is much more susceptible to underwinding than d(A)$_n$d(T)$_n$ (29,30). Our DNase I and nmr results also suggest that d(AT)$_n$ sequences are especially permisive to the propagation of structural change (7, vide supra). From the results presented here and our previous observations (5–7), it is our opinion that ligand-induced structural alterations are sequence dependent and such effects will propagate most efficiently through sequences that are relatively unstable to helix melting, such as d(AT)$_n$ (6,28). We also note that a correlation has been drawn between DNA flexibility variation and DNase I cleavage rate (31). This is significant because the values used to calculate flexibility variation (32) correlate closely with the well-known order of base pair stability (28).

The evidence presented here leads to two important conclusions. First, a relatively subtle change in the equilibrium
structure of DNA caused by a bound ligand clearly affects the subsequent binding and activity of a second ligand. Second, the structural disturbance in d(AT) sequences is transmitted in an asymmetric fashion at least five nucleotides from the ActD binding site. Therefore, DNase I hypersensitivity in active chromatin may be due to the transmission of relatively minor structural perturbations caused by proteins bound at some distance. This does not rule out instances of dramatic structural changes (cruciforms, left-handed forms, etc.) that may require local supercoiling as their driving force. The response of DNA to protein binding could propagate further since the free energy of binding is usually much larger than for ActD. The asymmetry of transmission is especially interesting in the context of the regulation of genes by bound proteins. A structural disturbance caused by binding at upstream regulatory elements may also propagate most strongly in the 3'-direction, thus providing a natural mechanism to distinguish the sense strand of a gene.

REFERENCES

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