The Compatibility of Netropsin and Actinomycin Binding to Natural Deoxyribonucleic Acid*

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The simultaneous binding of netropsin and actinomycin to four natural DNAs was studied to determine the influence of one ligand on the binding of the other. Actinomycin binds specifically to GC sites, whereas netropsin binds specifically to AT sites. Spectral titrations, thermal denaturation, and analytical buoyant density centrifugation were employed to measure the binding interference of these drugs.

The binding of actinomycin to DNA was decreased by the presence of netropsin. Increasing the GC content of the DNA resulted in a decreased effect of netropsin on actinomycin binding. Quantitative analysis of the binding parameters indicated that netropsin and actinomycin can bind in close proximity along the DNA chain. Supercoiled DNA gave the same result as linear DNA. These results imply that DNA can absorb alterations in conformation within a short distance.

Prior studies have provided composite evaluations of the conformation of natural DNA. Studies such as x-ray diffraction (1–3), thermal denaturation (Tm), and buoyant density have demonstrated the influence of environmental factors (such as cation or humidity) on the over-all characteristics, changes along the entire length, of DNA. However, prior studies from this laboratory (4, 5) have demonstrated that the properties and structure of a DNA are dictated by the nucleotide sequence. This influence was demonstrated by a variety of physical, chemical, and enzymatic measurements on 14 helical biosynthetic DNAs with defined repeating nucleotide sequences. Hence, the over-all conformation of natural DNA is probably a composite of many types of DNA structures.

Our present goal is to determine the proximity of binding (total neighbor exclusion lengths) of actinomycin and netropsin in order to provide information on the range of influence of one ligand on the other. Also, these studies should give an indication of the conformational flexibility of DNA over the length of a few base pairs, as opposed to the more composite measurements. These results may be relevant to the problem of packaging DNA in a phage head as well as to the conformational distortions created by DNA-binding proteins. Drugs with complementary specificities were employed in order to obviate direct competition for identical sites on the DNA.

Netropsin specifically binds to GC sites on duplex DNA (6–9) whereas netropsin specifically binds to AT sites (10).

Actinomycin D intercalates into DNA whereas netropsin binds by a non-intercalating mechanism.

Experimental Procedure

DNAs—Escherichia coli, Clostridium perfringens, and Micrococcus luteus DNA were prepared as previously described (10). Analytical zone sedimentation studies were performed on the DNAs in 0.9 M NaCl-0.1 M sodium hydroxide solution (11). smw values ranged from 8.3 to 79. Supercoiled φX174 RF1 DNA was the gift of J. B. Dodgson (this laboratory). The degree of superhelicity was checked by titrating the sedimentation velocity of φX174 RF1 DNA as a function of actinomycin D concentration (10, 12). A value of −15.0 superhelical turns was obtained. Linear φX174 RF DNA was generated by sonicating the supercoiled DNA. Zone sedimentation analysis (13) in 3 M CaCl2-0.2 M NaOH-0.001 M EDTA solution indicated that all of the supercoiled DNA was converted to linear DNA (s = 5.2). DNAs were normally dialyzed into SP1 buffer (0.1 M NaCl-0.001 M sodium phosphate, pH 6.5). Unless stated otherwise, this buffer was used in all studies.

Spectral Titrations—A Cary 15 spectrophotometer was used to monitor the actinomycin-DNA interaction. A DNA solution at 8 to 11 μM in base pairs was filtered through a sintered glass funnel to remove dust and pipetted into 10-cm light path cells. The actinomycin D titration was carried out by adding 4- to 8-μl aliquots of actinomycin D (6.5 × 10−4 M) and measuring the absorbance at 260 nm. After adding the actinomycin D, the absorbance was measured after a 15- to 30-min interval. The absorbance of free actinomycin D was measured by carrying out a similar titration with the SP1 solvent replacing the DNA solution. The concentration of bound actinomycin D (Db) was calculated from

\[ D_b = \Delta A_{260/4} / (c_{260} - c_{425})10 \]  

Where \( \Delta A_{260/4} \) is the absorbance difference between the solvent and the DNA solution with the same amount of total actinomycin D. \( c_{260} \) and \( c_{425} \) were the molar extinction coefficients of free and bound actinomycin. Values of \( c_{260} = 23,500 \) (7) and \( c_{425} = 12,410 \) were employed. \( c_{260} \) was determined by adding actinomycin (to 3 μM) to 1-cm path length cells.
containing DNA solutions at 0.5 mg/ml. At this high ratio of DNA to actinomycin D, it was assumed that all the drug was bound.

Measurement of actinomycin binding to DNA in the presence of netropsin was carried out in a manner similar to the above. Netropsin was added to the DNA solution before the actinomycin D titration began. Studies showed that netropsin does not absorb in the 400- to 500-nm region and has no effect on the free actinomycin spectra. Also, the circular dichroism spectra of actinomycin D was not changed by netropsin. In addition, netropsin did not have any detectable effect on the spectral titration of actinomycin D with (dG-dC)₆ or (dG-dC)₉. Hence, it was concluded that the interference of netropsin with actinomycin D binding to DNA (see under “Results”) was effected through netropsin-DNA binding.

The equilibrium binding data was plotted as r/D, versus r, where r was the moles of bound actinomycin D divided by the DNA concentration in base pairs, and D, was the concentration of free actinomycin D. From this plot, binding parameters were obtained from the intercepts of the tangent to the binding curve in the limit of r → 0 with the horizontal and vertical axes (see Fig. 1).

\[
\lim_{r \to 0} \left( \frac{r}{D_0} \right) = KB
\]

\[
\lim_{r \to 0} \left( \frac{\ln \delta (r/D_0)}{\delta r} \right) = B_{ap}
\]

K is the binding constant and B the number of potential binding sites per base pair. Gurskii et al. have shown that B, can be related to binding parameters of ligand-DNA interaction (Equations 36 and 37 of Ref. 14). By itself, however, B, cannot be used as a measure of the number of binding sites per DNA base pair. In the results which follow we will only employ Equation 2a.

The number of potential binding sites, B, for actinomycin binding to DNA depends on the actinomycin binding site. Müller and Coulter have illustrated several cases where actinomycin intercalates and has specific orientations with respect to GC pairs (8). For the case where actinomycin D can attach to only one side of the guanine residue B equals C,C, the GC content. This is the model we will assume. Work by Sobell (15) and Krugh (16) indicate actinomycin D binds to the 3' side of guanine.

Drugs—The netropsin and actinomycin used in this study were described previously (9, 10).

Other Procedures—Helix-coil transitions of DNA with varying amounts of netropsin or actinomycin D, or both, were performed with a Gilford spectrophotometer as described previously (4). SP1 buffer was used throughout. Analytical cesium sulfate density gradient centrifugation experiments were similar to those previously described (9). (dI)_n was used as a marker to measure the binding of both actinomycin D and netropsin to DNA. Previous studies (9, 10) have shown that neither netropsin nor actinomycin D bind to (dI)_n; also the isocencentration method was used for some determinations.

RESULTS

Effect of Netropsin on Actinomycin-DNA Binding Parameters—The binding of actinomycin D to three natural DNAs was examined in the presence and absence of netropsin. Fig. 1 shows the effect of two different netropsin concentrations on the binding curve of actinomycin D with C. perfringens DNA. At a ratio of 0.07 mol of netropsin/DNA base pairs, both KB and B, decreased. A further decrease in the actinomycin-DNA binding parameters was found at 0.18 mol of netropsin/DNA base pair. Table I lists the binding parameters of actinomycin D for C. perfringens, E. coli, and M. luteus DNAs in the absence and presence of saturating netropsin concentrations. Saturation values were determined from previous binding data of netropsin with each of these DNAs (10). Increasing the GC content of DNA resulted in a decreased effect of netropsin on actinomycin D binding. This result was consistent with inability of netropsin to bind GC paired sequences. For M. luteus DNA (72% GC), there was no significant difference in the actinomycin D binding curves with or without netropsin.

Quantitative Analysis of Binding Data—To interpret the data of Table I, the neighbor exclusion model of ligand DNA binding was employed (8, 14). In the neighbor exclusion model, bound netropsin and actinomycin D have exclusion lengths lₙ and lₐ, respectively. l is the number of base pairs per ligand which are covered or structurally affected such that other ligands cannot bind. The length of DNA physically covered by netropsin has been estimated as three base pairs (10), and actinomycin D appears to cover five to six base pairs (8, 15). All intercalative sites with the 3' side of guanine available for binding were considered as potential actinomycin D sites. Denoting sites by the base pair composition surrounding them, actinomycin D sites include three GC, GC sites and two each of AT, GC and GC, AT sites. One of the GC, GC sites has a 2-fold degeneracy and is counted twice. Previous studies (10) have indicated that the affinity of netropsin for three contiguous AT pairs was considerably greater than its affinity for one or two AT pairs. Therefore, all AT pairs were regarded as potential netropsin sites, but only those sites with two adjacent ATs interfered with actinomycin D-DNA complex formation.

The goal of this analysis was to estimate the total neighbor exclusion lengths of actinomycin D and strongly bound netropsin. To correctly analyze the actinomycin D binding parameters with netropsin present, one must obtain equations from

![Fig. 1. Scatchard plot of the binding of actinomycin D to Clostridium perfringens DNA at three concentrations of netropsin. No netropsin (○), 0.07 mol netropsin/DNA base pair (O), and 0.18 mol netropsin/DNA base pair (A). r is the moles of bound actinomycin/ base pair and D, the concentration of free actinomycin.](http://www.jbc.org/)

**Table 1**

| DNA     | GC | \(C_{mb}/C_{dm} \) | \(K_B \times 10^{-4} \) M⁻¹ sites/base pair | \(B_{ap} \) sites/base pair |
|---------|----|-------------------|---------------------------------------------|---------------------------|
| C. perfringens | % | 0.18 | 2.2 | 0.052 |
|          | 30 | 0.18 | 1.4 | 0.022 |
| E. coli  | 50 | 0   | 9.4 | 0.080 |
|          |   | 0.20 | 4.4 | 0.066 |
| M. luteus| 72 | 0   | 4.1 | 0.065 |
|          |   | 0.12 | 4.1 | 0.088 |

*Netropsin.
the statistical sum of a system consisting of a random sequence DNA with saturating netropsin and very little actinomycin D. Since equations for this system have not been solved to date, we have employed a simplified analysis.

Assuming a random sequence of AT and GC pairs, Equation 2a was used to describe actinomycin D-DNA binding with no netropsin. When saturating netropsin was added, changes in KB were interpreted as changes in the number of potential actinomycin D sites. It was assumed that the only effect of bound netropsin was to decrease the potential actinomycin D sites by neighbor exclusion. Thus by taking ratios of KB with and without netropsin (Table I), the number of potential actinomycin D sites blocked by netropsin was estimated. From Equation 2a

$$\frac{KB(-\text{netropsin})}{KB(+\text{netropsin})} = \frac{C_{GC}}{B(+\text{netropsin})}$$

(3a)

$$N_B = C_{GC} - B(+\text{netropsin})$$

(3b)

where B(+ netropsin) was the fraction of base pairs which are potential actinomycin D sites with netropsin present, and N_B the number of actinomycin D sites per base pair removed because of netropsin binding. Table II lists values of N_B, calculated from KB ratios, for the three DNAs.

A theoretical estimate of N_B also was made to estimate l_A + l_N from the data. For each netropsin bound to three contiguous ATs, the average number of actinomycin D sites removed was estimated.

We assume netropsin binds to one end of three AT pairs and excludes l_A base pairs. Actinomycin D is assumed to centrally bind to the sites previously described and exclude l_A sites (l_A - 1 base pairs are covered). Fig. 2 illustrates an adjacent netropsin and actinomycin D for l_A = 3 and l_A = 5. The dots represent intercalative sites.

By examining several values of l_A and l_N it can be shown that for a random sequence DNA the average number of actinomycin D intercalative sites blocked for each netropsin bound to three contiguous ATs is (l_N + l_A - 5)C_{GC} for l_A ≥ 3, l_N ≥ 3.

Thus

$$N_B = R_{max}(l_N + l_A - 5)C_{GC}$$

(4)

where R_{max} is the saturating density of bound netropsin with three AT pairs per site. R_{max} is C_{AT} times the saturating density of bound netropsin for all AT sites given in Reference 14, or

$$R_{max} = C_{AT}/(1 + (l_n - 1)C_{AT})$$

(5)

Table II lists values of N_B for two values of l_A + l_N. l_N was kept constant since it was assumed that R_{max} for netropsin does not change upon titrating with actinomycin D. This assumption appears justified since the netropsin concentration was saturating, and netropsin's binding constant was greater than or of the same magnitude as for actinomycin D (see under "Discussion").

As observed from Table II, the theoretical values of N_B with l_N = 3, l_A = 5 compared reasonably well with the experimental values for C. perfringens DNA and M. luteus DNA. For l_N + l_A = 12 theoretical values of N_B were generally greater than the experimental values and outside the error limits. Except for the discrepancy with E. coli DNA, which is not understood, the results indicate l_N + l_A ≈ 8. This was also true for linear φX174 RF DNA (next section).

Although this analysis was theoretically nonrigorous, it indicated that netropsin and actinomycin D can bind DNA within a short distance from each other. The values of l_A = 5 and l_N = 3 correspond roughly to the van der Waals radii of the adsorbed molecules. It is clear that l_A and l_N were not substantially larger than the value expected from studies of each drug separately (8, 10).

Thus linear DNA can accommodate actinomycin D and netropsin, which have different binding modes, within a few base pairs of each other. The DNA conformations of the actinomycin D and netropsin complexes can exist in close proximity. An examination of the assumptions of the above analysis and their effect on the results will be made in the "Discussion."

**Two-drug Binding to Supercoiled DNA**—The binding of actinomycin to supercoiled φX174 RF1 DNA was examined in the presence and absence of netropsin. The purpose of these experiments was to determine if employing a supercoiled DNA affects the above results. For a linear DNA, conformational changes induced by ligand binding can be propagated to the free ends. Similar changes in a supercoiled DNA can change the tertiary structure. Since actinomycin D unwinds DNA and netropsin does not, the binding of actinomycin D to supercoiled DNA should initially be greater than for linear DNA. Fig. 3 shows the binding of actinomycin D to RF1 φX174 DNA in the presence and absence of saturating netropsin. Employing Equation 3 the number of potential actinomycin D sites blocked was N_B = 0.10 from KB ratios. Equation 4 yielded a
theoretical prediction of $N_a = 0.12$ for $l_N + l_A = 8$. Experiments performed with linear φX174 DNA gave results similar to that shown in Fig. 3. These experiments indicated that covalent closure of the free ends of a linear DNA did not measurably affect the minimum distance between actinomycin and netropsin.

The effect of binding a saturating quantity of actinomycin to φX174 DNA on the netropsin-DNA binding parameters also was examined. This approach was technically more difficult than the one previously described because the absorbance change per bound netropsin was smaller than for actinomycin (10). Methods previously described (10) were employed to obtain netropsin binding parameters. Supercoiled and linear φX174 DNA gave similar results. The $KB$ values decreased about 16% when saturating actinomycin was added (from $7.4 \times 10^4$ to $6.2 \times 10^4$), and $B_{ap}$ decreased by 10% from a value of approximately 0.068 to 0.061.

**DNA Denaturation**—The effect of netropsin and actinomycin D on the thermal denaturation of DNA was examined in order to have an independent measure of the binding interference. The midpoint of the thermal denaturation curve, $T_m$, was measured as a function of drug concentration for each drug separately. A netropsin titration was then performed on DNA saturated with actinomycin D. Fig. 4 shows the $T_m$ change versus drug concentration for C. perfringens DNA. Titrating actinomycin to saturation increased the $T_m$ by 5.0°. Netropsin yielded a $T_m$ increase of 14.6° at saturation. When netropsin was titrated in the presence of saturating amounts of actinomycin D, the increase in $T_m$ was always less than the additive effect expected from the independent binding of each drug. At 0.2 mol both actinomycin D and netropsin/DNA nucleotide, $\Delta T_m = 15.7°$, whereas $\Delta T_m$ expected from independent binding was 19.6°. Analytical CsSO$_4$ density gradient titrations of C. perfringens DNA with netropsin or actinomycin D, or both, were performed in the manner previously described (10). The nonadditivity of the density decrements (results not shown) were in complete agreement with the conclusion drawn from $T_m$ studies.

A similar result was obtained with E. coli DNA (results not shown). When this DNA was complexed with both saturating netropsin and actinomycin D, it showed a $T_m$ increase of 10°. The expected elevation of $T_m$ from titrating both drugs separately was 12.5°. The results indicated that netropsin and actinomycin D binding interfere with each other. The larger interference for C. perfringens DNA was in agreement with the spectral titration results.

**DISCUSSION**

The objective of this study was to determine the proximity of binding (total neighbor exclusion lengths) of actinomycin D and netropsin in order to provide information on the range of influence of one ligand on the other. Actinomycin D specifically binds to GC sites (6-9) whereas netropsin specifically binds to AT sites (10). Also, an indication of the conformational flexibility of DNA might derive from this data. The spectral titration results yielded an estimate of eight base pairs for the shortest outside end to outside end distance between bound netropsin and actinomycin. The van der Waals radius of bound netropsin has been estimated as three base pairs (10), and actinomycin D appears to cover five to six base pairs (8, 15). This indicated that DNA can accommodate both of these ligands within a short distance despite the distortions in DNA structure created by these ligands.

Since the evaluation of $l_N + l_A$ critically depends on the assumptions underlying Equations 3, 3, and 4, we will review them here. It was assumed that all intercalative sites with a 3'-guanine were equivalent actinomycin D-binding sites. Although there is evidence that the base pairs adjacent to these sites influence actinomycin D binding (9), this effect appears to be small for natural DNAs. The $l_N$ values listed by Müller and Crothers (8) for DNAs with varying GC content all give the same approximate value of six. This would not be the case (10) if actinomycin D sites were strongly influenced by neighboring base pairs.

In the analysis, it was assumed that only the netropsin molecules which were bound to three contiguous AT pairs could eliminate actinomycin D sites. Previous studies (10) have indicated that netropsin can also bind to a site with only one or two consecutive AT pairs. The binding affinities, however, were weaker than for three AT pairs. Thus $R_{max}$ was, if anything, underestimated by this assumption. If the $R_{max}$ values were larger, then the theoretical estimate of $l_N + l_A$ would be smaller (see Equation 4). A third assumption was that titrating the DNA with actinomycin D did not lower $R_{max}$. This depends on the binding constants of the ligands for their respective sites, and their concentrations. For (dG⋅dC)$_n$ the actinomycin D binding constant (9) was $K = 5.4 \times 10^8$ M$^{-1}$ in 0.01 M Na$^+$ (normalized to moles of base pairs). Netropsin has a binding constant of $4 \times 10^5$ M$^{-1}$ for (dA⋅dT)$_n$ and (dA⋅dT)$_n$ in 0.1 M Na$^+$ (10). Increasing the salt concentration from 0.01 M to 0.1 M decreases actinomycin binding to DNA (8). Therefore, it was unlikely that $R_{max}$ was substantially altered by adding small amounts of actinomycin D to DNA saturated with netropsin.

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