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Determination of Equilibrium Binding Affinity of Distamycin and Netropsin to the Synthetic Deoxyoligonucleotide Sequence d(GGTATACC)$_2$ by Quantitative DNase I Footprinting†

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ABSTRACT: A new method for determining the equilibrium binding constant of antitumor drugs to specific DNA sequences by quantitative DNase I footprinting is presented. The use of a short synthetic DNA oligomer to define a homogeneous population of DNA binding sites enables the calculation of the free drug concentration and the fraction of DNA sites complexed with drug in solution and is described for the first time. Since a 1:1 stoichiometry is observed for each drug–oligomer DNA complex, it becomes possible to calculate equilibrium binding constants in solution. By use of this technique, the binding affinities of the nonintercalating drugs netropsin and distamycin to the synthetic oligonucleotide d(GGTATACC)$_2$ are determined to be K_a(25 °C) = 1.0×10^5 and 2.0×10^5 M^{-1}, respectively. Quantitation of the temperature dependence associated with complex formation results in a determination of standard enthalpies of −3.75 and −8.48 kcal mol^{-1} for the binding of netropsin and distamycin, respectively. Calculation of other thermodynamic parameters are found to be in agreement with previous studies and indicate that the DNA binding process for these compounds is predominantly enthalpy driven. This method of quantitative DNase I footprinting is demonstrated to be a useful technique for the measurement of drug affinities to specific binding sites on DNA oligomers which are designed and synthesized expressly for this purpose. Applications of the technique to the determination of drug binding affinities at specific sites within native DNA sequences are discussed.

Studies of the interaction of antibiotic and antiviral compounds with DNA have become increasingly important in the development of a rational approach to design new chemotherapeutic agents which interact at the gene level. It is important, in attempting to elucidate the structure of complexes of such compounds with DNA, to have available an experimental technique for measuring binding affinities to specific DNA sequences. This has necessitated the development of a new method, described in this paper, for the direct measurement and comparison of binding constants at multiple sites in known DNA sequences.

Previously, studies of the affinities of DNA binding drugs utilized primarily synthetic homonucleotide and alternating dinucleotide sequence polynucleotides to determine binding

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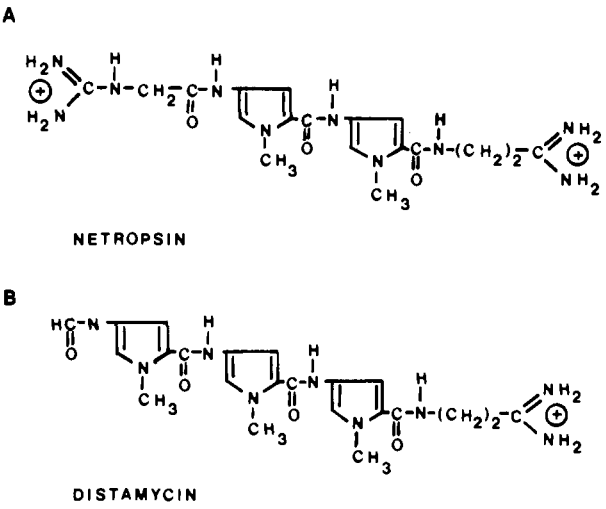

FIGURE 1: Chemical structures of the antitumor compounds used in this study. (A) Netropsin. A pyrrole amidine antibiotic consisting of two pyrrole rings with guanidinium and propylamidinium end groups. (B) Distamycin. A pyrrole amidine antibiotic consisting of three pyrrole groups with formamide and propylamidinium end groups.

constants (McGhee, 1974; Wartell et al., 1974; Zimmer & Wahnert, 1986). In addition, studies with native DNA isolated sources such as calf thymus generated drug affinities averaged over multiple sites (Zimmer & Wahnert, 1986; Wartell et al., 1974). More recently, footprinting analysis was developed to study DNA sequence binding specificity of antitumor drugs with DNA cleaving agents such as DNase I[1] (Lane et al., 1983; Fox & Waring, 1984) or MPE–Fe(II) (Van Dyke et al., 1982). However, only the relative affinities of a drug at different sites on the same DNA fragment were determined in these early studies.

Recently, Brenowitz et al. (1986a,b) described an application of DNase I footprinting to quantitate the equilibrium binding constant for the interaction of the *Escherichia coli* λ repressor protein to an operator-containing DNA fragment having three separate binding sites. However, the data analysis required the assumption that each site could bind only one protein independent of the other sites. Such a data analysis cannot be generally used for studies of drug–DNA interactions, since a particular DNase I protected region may contain multiple drug binding sites each with a different affinity.

In this paper, we describe an additional method, utilizing quantitative DNase I footprinting, which can be used to determine both the equilibrium binding constant and the thermodynamic parameters associated with drug binding to a specific DNA site. This technique is applied to a study of the binding of distamycin A and netropsin to the deoxynucleotide sequence $d(GGTATACC)_2$. Netropsin and distamycin have both been shown to preferentially bind to A–T-rich sequences of B-form DNA (Lane et al., 1983; Luck et al., 1974, 1977; Wartell et al., 1974). They bind to the minor groove of DNA by hydrogen bonding and do not interact by intercalation (Patel, 1979; Patel et al., 1981; Berman et al., 1979; Kopka et al., 1985a,b). Both compounds are pyrrole amidine antibiotics which differ in the number of pyrrole groups in their structure, two for netropsin and three for distamycin, and in their terminal functional groups (as shown in Figure 1). Each has a propylamidinium group at one end, and netropsin has a guanidinium group while distamycin a formamide group at the other end. This results in a net charge of +2 for netropsin and +1 for distamycin at physiological pH. Thus, we determine the equilibrium affinity constants and thermodynamic parameters for the binding of distamycin and netropsin to a specific DNA site within the sequence $^{5'}GGTATACC^{3'}$.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; DNase I, deoxyribonuclease I.

Materials and Methods

Materials. Netropsin and distamycin were gifts from Bristol Laboratories and Dr. Frederico Arcamone, Farmitalia (Milano, Italy), respectively. Solution concentrations were determined spectroscopically with extinction coefficients of $\epsilon_{296} = 20\,200\ M^{-1}\ cm^{-1}$ for netropsin and $\epsilon_{303} = 37\,000\ M^{-1}\ cm^{-1}$ for distamycin.

The self-complimentary octamer $^{5'}d(GpGpTpApTpApCpC)^{3'}$ was obtained as a gift from N. Zanatta and P. Borer, Syracuse University, and was synthesized manually by a procedure using dimethyltrityl nucleoside phosphoramides on a silica solid support (Tanaka & Letsinger, 1982).

The buffer used throughout these experiments (DNase I buffer) contains 10 mM Tris, 8 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.5. Bovine pancreatic deoxyribonuclease I was stored in stock solutions of 50% glycerol and 50% 2× DNase I buffer at −20 °C.

Oligomer Purification, Annealing of Complementary Strands, and 5′ End Labeling. The deoxyoligomers were purified from any smaller oligomer produced by incomplete synthesis by preparative acrylamide gel electrophoresis. A portion of each isolated oligomer was precipitated and loaded onto a 20 cm × 40 cm × 1.5 mm 25% denaturing polyacrylamide gel after addition of 25 μL of a 7 M urea solution with bromophenol blue and xylene as marker dyes. Each full-length oligomer was identified by UV backshadowing with ultraviolet light onto PEI plates containing a fluorescent indicator. Oligomers were further purified by removing the acrylamide by elution through glass wool, dialysis against distilled water in cellulose tubing with a M_r 2000 cutoff pore size, and evaporation. Annealing of self-complementary strands to form duplexes was accomplished by first heating the solution to 80 °C and then letting the solution cool overnight to room temperature in an insulated water bath. The concentration of reconstituted solution of the oligomer was determined by 260-nm absorbance with an extinction coefficient $\epsilon_{260} = 19\,976\ (M\ base\ pairs)^{-1}$ as calculated according to the procedure of Borer (1975). Verification of the double-stranded nature of the oligomer was accomplished by utilizing an S1 digestion. A portion of the oligomer solution was 5′ end labeled with [γ-^{32}P]dATP by a phosphate exchange reaction according to a standard method using T4 polynucleotide kinase (Maxam & Gilbert, 1980).

DNase I Digestion Conditions. Analyses of the binding of drugs to the oligomer DNA were performed at equilibrium conditions at a specified temperature. For each experiment, the amount of drug in the digestion solution was decreased in successive reactions as indicated in Figure 2. This corresponds to a successive decrease in the drug:DNA duplex ratio since the DNA concentration is kept constant throughout. The DNase I enzyme concentration was also kept constant at a level previously determined to maintain "one-hit" kinetics conditions (Lane et al., 1983). For 5 °C experiments, the DNase I concentration was generally 100-fold higher than that for 25 °C experiments. The oligomer concentration had little effect on the enzyme concentration required for digestion (data not shown).

The total reaction volume of 6 μL consisted of 2 μL of an oligomer stock solution, 2 μL of 5′ end labeled oligomer solution, and 2 μL of the appropriate drug concentration. Digestion solutions containing DNase I buffer in place of a drug

solution are indicated as "control lanes" in Figure 2. The amount of labeled DNA constituted approximately 1% of the total DNA concentration. The reaction mixture was allowed to equilibrate at a specified temperature for at least 15 min prior to DNase I digestion. A digestion time of 1 min upon the addition of 2 μL of DNase I solution was used for all experiments. Reactions were stopped by the addition of 10 μL of a solution consisting of 50 mM EDTA, 20 mM Tris, and 7 M urea with marker dyes for electrophoresis. Each tube was heated for 5 min at 80 °C to denature the DNA strands and was quenched in an ice/water bath. Separation of reaction products to single nucleotide resolution was accomplished by electrophoresis on a 25% denaturing polyacarylamide DNA sequencing gel which typically run at 1000 V for 12 h. The gel was exposed to Kodak X-Omat film for autoradiography.

Densitometric Analysis of the Autoradiogram. The band densities on the autoradiograms were quantitated by conversion to optical absorbance data and integrated to obtain peak areas with a Jarrell Ash (Model 23-100) densitometer combined with a NEC CP/M-86 microprocessor. A linear relationship of the band intensities and the integrated peak areas was obtained, with the technique described by Dabrowiak et al. (1986). Integrated peak areas were calculated by averaging the results of three one-dimensional scans, for all experiments. These peak areas were corrected by subtracting any peak area present in lanes where the site is fully protected, which is determined at the limit of no further protection in the presence of increasing amounts of drug. The corrected peak areas are then normalized for the maximum amount of enzymatic cleavage produced by DNase I in the absence of drug, as indicated in the control lanes.

Determination of Stoichiometry and Equilibrium Binding Constants. The drug–DNA equilibrium, as probed by DNase I, can be described as

$$\text{ds oligomer} + \text{drug} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ds oligomer–drug} \qquad (1)$$

The corresponding equilibrium dissociation constant for this process can be expressed as

$$K_d = \frac{k_{-1}}{k_1} = \frac{[\text{free drug}][\text{free sites}]}{[\text{bound drug–site complex}]} = \frac{(L_0 - x)(nD_0 - x)}{x} \qquad (2)$$

where L_0 is the total drug concentration, D_0 is the total DNA duplex, n is defined as the number of sites capable of binding one drug molecule on each duplex, and x is the concentration of bound drug.

Analysis of the binding behavior, and determination of the equilibrium binding constant, depends on an understanding of the value of n, the stoichiometry of the complex per duplex molecule (Lane, 1985b). The fraction of a specific DNA binding site complexed with drug is determined experimentally by DNase I footprinting. The stoichiometry (n) was determined from experimental data by plotting the fraction of sites that contain bound drug (ν) against the molar ratio r of total drug to double-stranded oligomer. It can be shown that at the low drug limit of such a curve (where $nD_0 \gg x$), the fraction of sites bound is equal to

$$\nu = \frac{x}{nD_0} = \frac{L_0/D_0}{n + K_d/D_0} \qquad (3)$$

which gives an initial slope equal to

$$1/(n + K_d/D_0) \qquad (4)$$

This binding curve is dependent on the DNA concentration used in the experiment and K_d, which typically remains constant at all points in the drug titration. The intercept of the tangent to the initial curve and $\nu = 1.0$ is sometimes greater, but never less, than the value of n. Under conditions where higher oligomer concentrations or lower temperatures are used, the curve reaches a limit where the tangent to the initial portion of the curve intercepts $\nu = 1.0$ at the value of n.

From the stoichiometry determined for the equilibrium, the concentration of DNA binding sites in solution is calculated as the product of the stoichiometry, n, and the concentration of the duplex oligomer. The free drug concentration, as written in eq 5 is calculated from the total concentrations of drug and

$$[\text{free drug}] = [\text{total drug}] - \nu[\text{total DNA binding sites}] \qquad (5)$$

DNA in the experiment and from the fraction of sites that are complexed with a drug molecule. This value is determined experimentally, as indicated under Materials and Methods [also as in Brewnowitz et al. (1986a)]. The dissociation binding constant at equilibrium conditions for each drug is determined by means of a Bjerrum plot (as first described for metal amine formation by J. Bjerrum in 1941), which can be described as

$$\text{p[free drug]} = \text{p}K + \log [(1 - \nu)/\nu] \qquad (6)$$

Therefore, pK can be determined from the midpoint of the curve, where $\nu = 0.5$, and pK = p[free drug] = − log [free drug]. In analysis of the densitometric data, the binding constant was calculated for each experimental data point. Values reported indicate the mean of the calculated binding constants, with the standard deviation shown as the error.

Determination of Thermodynamic Parameters Associated with Drug Binding to Oligomers. Equilibrium binding constants are determined at two temperatures, 5 and 25 °C, for each drug to enable calculation of the enthalpy of the binding process. The standard enthalpy change for binding is described by van't Hoff analysis for two temperatures

$$\frac{\ln K_{eq(2)}}{\ln K_{eq(1)}} = \frac{-\Delta H_b^\circ}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \qquad (7)$$

and solved for ΔH_b°, the enthalpy change for drug binding. Previous studies of the binding of netropsin at different temperatures using batch calorimetry have shown that the change in heat capacity is insignificant (Marky et al., 1985).

The standard Gibbs free energy change (ΔG°) associated with binding was calculated for the solution studies at 25 °C, according to the relation $\Delta G^\circ = -RT(\ln K)$. Contributions to the free energy of binding attributed to a change in entropy at 25 °C were then be obtained by solving for ΔS.

Results

A typical autoradiogram from a DNase I footprinting experiment for complex formation of distamycin and the oligomer d(GGTATACC)$_2$ is shown in Figure 2. Similar results are observed for DNase I footprinting of netropsin with this oligomer. The lanes labeled "−Enzyme" include the DNA oligomer but no DNase I and are generally free of cleavage products, in all cases. No bands having a mobility less than that of the denatured full-length oligomer are evident, indicating that no partially base paired oligomers are present in the gel.

The product of DNase I cleavage of the oligomer d(GGTATACC)$_2$ is a single band, which is used for subsequent quantitation of the reaction by densitometry. This band

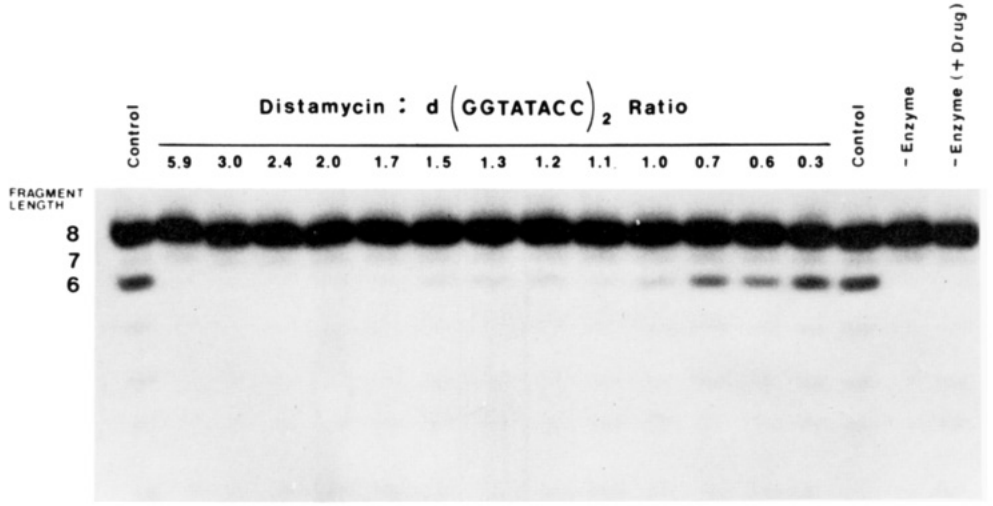

FIGURE 2: Autoradiogram of a DNase I footprinting experiment of distamycin titrated with the synthetic oligomer d(GGTATACC)$_2$ at 25 °C. The total concentration of double-stranded oligomer for each reaction is 8.85 μM. The amount of drug used for each reaction is such that the resulting molar ratio of drug to duplex deoxyoligomer is indicated above the corresponding lane. Control lanes indicate DNase I digests of oligomer solutions without added distamycin. Lanes corresponding to DNA not digested with DNase I are indicated as "−Enzyme", with and without the presence of distamycin. Numbers to the left of the figure indicate the length of the DNA fragment band, as measured from the 5' end of the molecule.

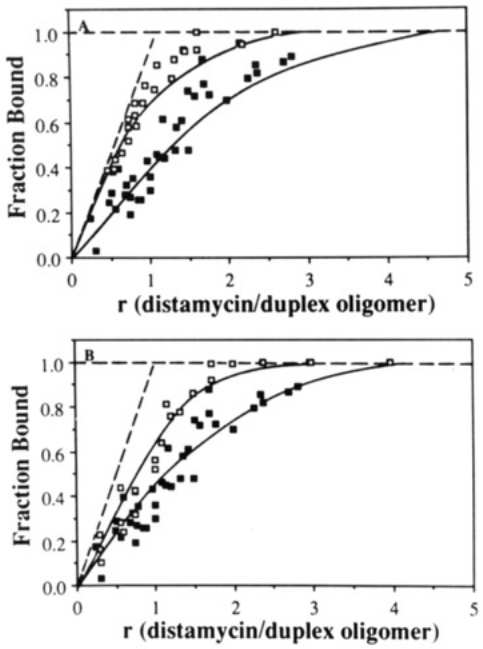

FIGURE 3: Mole ratio plots of distamycin titrations with d(GGTATACC)$_2$. The fraction of total drug binding sites on the DNA oligomer molecules containing bound drug, as quantitated by densitometric analysis of footprinting gels, is plotted against the molar ratio of distamycin to double-stranded oligomer. Dotted lines are drawn corresponding to the limit of the initial slope of the curve where n = 1 and the value of K_d/D_0 is neglected (see text). (A) Effect of DNA concentration on the mole ratio plot. Solutions were digested at 25 °C with concentrations of the double-stranded oligomer of 8.85 (■) and 31.3 μM (□). (B) Effect of temperature on the mole ratio plot. Solutions consisting of 8.85 μM double-stranded oligomer were digested at 5 (□) and 25 °C (■).

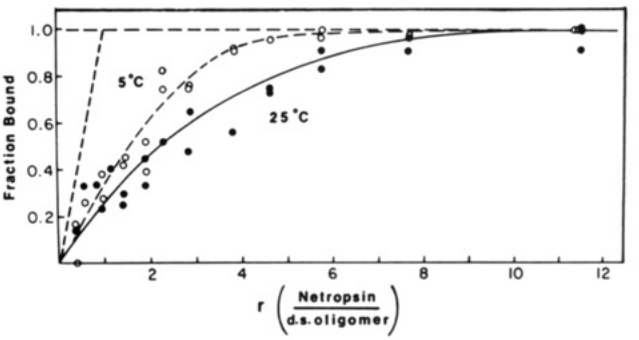

FIGURE 4: Mole ratio plot of the netropsin titration with d(GGTATACC)$_2$. The fraction of total drug binding sites on the DNA oligomers containing bound drug, as quantiated by densitometric analysis of footprinting gels, is plotted against the molar ratio of netropsin to double-stranded oligomer. Points on each curve correspond to three separate experiments for measurement of the equilibrium binding constants, at solution temperatures of 5 (○) and 25 °C (●).

corresponds to DNase I cleavage at a position three nucleotides from the 3′ end of the oligomer. In each set of experiments a range of drug concentrations is employed from a concentration greater than that necessary for maximum DNA protection (minimum cleavage) to a concentration of drug below an amount which corresponds to the minimum detectable degree of protection.

The stoichiometry of each drug complex with d(GGTATACC)$_2$ is interpreted from the mole ratio plots in Figures 3 and 4 for distamycin and netropsin, respectively, on the basis of eq 3 and 4. The initial slope of the curve in these figures is described theoretically by eq 4 as the value of the ν = 1.0 intercept. The tangent to the initial slope for the ratio of drug to duplex oligomer equal to 1 is indicated by the dotted line in each figure. This corresponds to the limit slope of the set of curves where the K_d/D_0 (see eq 4) term goes to zero.

The shape of the curves in the mole ratio plots is a function of DNA concentration and temperature of the equilibrium solution. The curve corresponding to the highest oligomer concentration has an initial slope closest to $1/n$, as shown in Figure 3A, in agreement with the theoretical prediction of eq 4. Curves for footprinting studies at the two different temperatures for both distamycin and netropsin, Figures 3B and 4, respectively, also change the shape of the mole ratio plots due to the temperature dependence of the equilibrium binding constant (K_d). At 5 °C the binding constant is smaller than at 25 °C; therefore, the K_d/D_0 term is also smaller, and the slope of the mole ratio plot at low drug concentrations is closer to the tangent line in the figure.

At a drug and duplex oligomer stoichiometry of 1 there is an equivalence in eq 2 as follows: [free sites] equals [free oligomer duplex]. This, therefore, enables the calculation of the [free drug] in solution. It is, in addition, assumed that since the binding sites are on separate molecules, the binding

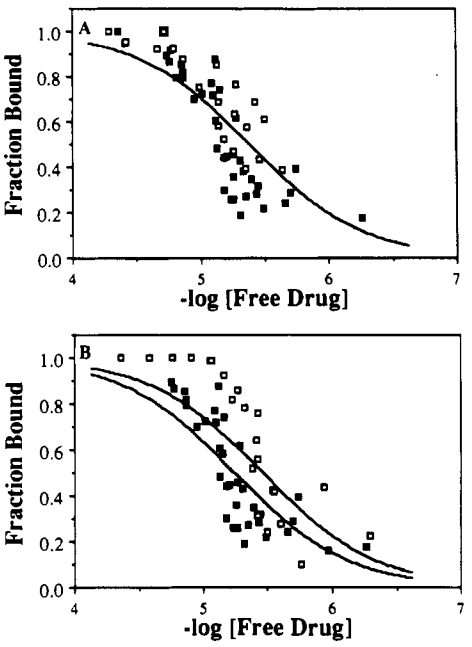

FIGURE 5: Bjerrum plot of distamycin titrations to d(GGTATACC)$_2$. (A) Effect of DNA oligomer concentration on the shape of the curve. Curves are determined from the theoretical relationship of fraction bound and − log [free drug] with the mean value of K_d. Data are plotted for solutions consisting of 8.85 (■) and 31.1 μM (□) deoxyoligodecamers. (B) Effect of temperature on the Bjerrum plot for distamycin binding to a 8.85 μM solution of d(GGTATACC)$_2$. Curves are shown for titrations at equilibrium temperatures of 5 (□) and 25 °C (■).

Table I: Equilibrium Binding Constants of Netropsin and Distamycin Binding to d(GGTATACC)$_2$

	K_a(25 °C) (M^{-1})[a]	K_a(5 °C) (M^{-1})[a]
netropsin	0.99×10^5 (±0.42)	2.32×10^5 (±0.70)
distamycin	1.99×10^5 (±0.73)	2.88×10^5 (±0.64)

[a] K_a, association equilibrium constant.

of the drug is independent and noncooperative.

Bjerrum plots for distamycin binding to d(GGTATACC)$_2$ are shown in Figure 5. Equilibrium binding constants were calcualted for each data point. The mean value of the binding constant was substituted into eq 6 to generate the curves shown. Results of DNase I footprinting experiments with solutions containing oligomer duplex concentrations of 8.85 and 31.3 μM are presented in Figure 5A. Within the range of DNA concentrations used in this experiment, no variation in the value of the equilibrium binding constant was detected. The temperature dependence of the equilibrium constant is indicated by Bjerrum plots for distamycin binding to the oligomer duplex at 5 and 25 °C, shown in Figure 5B. The shift in the position of the curve reflects the difference in the binding affinity of distamycin at the two temperatures.

The equilibrium binding constants measured for both drugs at the two temperatures are listed in Table I. Distamycin is shown to have an affinity for the oligomer duplex slightly greater than twice the affinity for netropsin at both 5 and 25 °C. Equilibrium binding constants were determined at two solution temperatures in order to be able to calculate the enthalpy associated with the binding process.

Thermodynamic parameters calculated for each drug are listed in Table II. These values indicate that the binding is an enthalpy-driven process. The enthalpy stabilization of the drug–oligomer complex associated with distamycin binding is approximately twice that observed for netropsin binding. Changes in entropy calculated for the binding process of both drugs are small.

Discussion

In order to develop a technique that would allow the determination of binding constants at different specific sites in native DNA, it became necessary to design an appropriate synthetic double-stranded oligonucleotide that would be used as carrier which would interact with the free drug concentration in a predetermined manner. Such a molecule should bind the drug with a stoichiometry of 1 and should have a binding constant of approximately 10^5. When such a compound is used as carrier in a DNase I footprinting experiment to study the binding of the drug to native DNA, it allows the determination of free drug concentration and thereby the absolute binding constant at all binding sites in the native molecule. In this study, the DNA oligonucleotide d(GGTATACC)$_2$ was chosen as a model carrier molecule, and therefore, its equilibrium and thermodynamic binding parameters for complex formation with distamycin and netropsin were determined.

A wide range of association constants for netropsin and distamycin have been reported for binding to natural DNAs and synthetic polynucleotides (Zimmer & Wahnert, 1986). With the methods previously available, it had not been possible to measure binding constants at individual sites within natural DNAs, but only average affinities over all DNA binding sites. For example, netropsin binding to the homopolymer poly(dA-dT)·poly(dA-dT) was shown to have a K_a of 4.0×10^5 M^{-1} derived from theoretical calculations and melting data (Wartell et al., 1974) and 5.0×10^8 M^{-1} on the basis of equilibrium dialysis data in a solution with a 100-fold higher ionic strength (McGhee, 1974). Such a discrepancy may be due to the different ionic strengths of the solution which would affect the electrostatic contribution of the drug affinity to DNA. Therefore, none of the values previously reported can be directly compared to the above results obtained by quantitative DNase I footprinting because the buffer solution required for DNase I activity is different than those used in studies by other workers.

The DNA binding of netropsin and distamycin is shown in this study to be an exothermic process, in agreement with previous calorimetric results (Marky et al., 1983, 1985; Marky & Breslauer, 1985). Differences among the published enthalpies of binding between this and previous work are likely due to differences in the ionic conditions of the various experiments.

The negative enthalpy associated with the binding process has been primarily attributed to hydrogen bonding between the pyrrole groups of the antibiotics and the DNA bases adenine and thymine (Marky et al., 1987). Distamcyin contains one more pyrrole group than netropsin. The resulting increase in hydrogen bonding is evident in the higher K_a and larger negative enthalpy for distamycin binding (Breslauer, 1987). The electrostatic interaction of the positively charged propylamidinium group on each drug and the negatively charged DNA phosphate backbone has been shown to be only a minor contributor to the stability of binding (Luck et al., 1974; Wartell et al., 1974) or to the enthalpy change (Marky et al., 1985) but has been shown to have an effect on the entropy change associated with the drug–DNA interaction (Breslauer, 1987).

Distamycin has been predicted to have a binding site size of five base pairs rather than four, which would include a GC as the fifth base pair of the distamycin binding site in the oligomer used in these experiments (Kopka et al., 1985b).

Table II: Thermodynamic Parameters Associated with the Binding of Netropsin and Distamycin to d(GGTATACC)$_2$[a]

	$\Delta G°$(assoc) (kcal mol^{-1})	$\Delta H°$(assoc) (kcal mol^{-1})	$\Delta S°$(assoc) (kcal mol^{-1})
netropsin	−6.29 (±0.19)	−3.75 (±0.95)	0.85×10^{-2} (±0.39)
distamycin	−7.22 (±0.18)	−8.48 (±1.01)	-0.42×10^{-2} (±0.40)

[a] $\Delta G°$(assoc), standard Gibb's free energy change for association, 25 °C. $\Delta H°$(assoc), standard enthalpy change for association, 25 °C. $\Delta S°$(assoc), standard entropy change for association, 25 °C.

Circular dichroism measurements have indicated that there is some affinity of distamycin for dG-dC base pairs (Zimmer et al., 1983), and X-ray analysis has shown that there is no significant sequence specificity for the fifth base of a distamycin binding site. Therefore, a direct comparison of the values of K_a for netropsin and distamycin is meaningful despite the presence of a GC base pair included in the distamycin binding site (Berman et al., 1979; Kopka et al., 1985b).

There are two major considerations in assessing the use of DNase I footprinting as a technique for measuring equilibrium binding constants of small ligands, including (1) the relative affinities of the drug and the probe, DNase I, for the oligomer and (2) the concentration of the oligomer in solution. An artifically low value of ν, the fraction of DNA sites containing bound drug, would be measured if the enzyme had a comparable affinity for the DNA helix as the drug and was partially displacing drug molecules when recognizing a cleavage site on DNA. Measurements of a long residence time of these drug molecules to DNA when compared to DNase I (S. R. La Plante, G. C. Levy, N. Zanatta, and P. N. Borer, unpublished experiments; Fox & Waring, 1984; Pardi et al., 1983; Klevitt et al., 1985) is evidence suggesting that such a displacement does not occur.

The concentration of the oligomer in solution is also an important consideration in the drug binding experiment. The amount of oligomer in solution should be large enough so that the oligomer is in the double-stranded form. For the results described above, oligomer concentrations of 8.85 and 31.3 μM were used. As shown in Figure 5A, the value of the equilibrium binding constant was constant within experimental error for this range of oligomer concentration. Above a concentration of 45 μM, however, the measured value of the binding constant was higher (data not shown). This effect may be due to an unknown aggregation of multiple drug and double-stranded molecules, thereby making the theoretical analysis of the binding behavior invalid.

It has been demonstrated above that studying in detail the interaction of drugs to small DNA oligomers enables the measurement of a equilibrium binding constant for a drug to a unique DNA sequence and the calculation of the associated thermodynamic parameters. This binding process has been shown to be a local drug–DNA interaction by comparison of data obtained by calorimetric and spectroscopic techniques for homopolymers and oligomers (Breslauer, 1987). Experiments to be published elsewhere describe the use of such oligomers as carrier DNA to regulate the free drug concentration in order to quantitate the binding affinity of a drug to each binding site within a large multisite DNA molecule. These studies serve as a basis for understanding the interactions of drugs with biologically relevant DNA molecules.

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Registry No. DNase, 9003-98-9; octamer, 80407-93-8; netropsin, 1438-30-8; distamycin, 39389-47-4.

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Construction and Analysis of Monomobile DNA Junctions†

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ABSTRACT: Immobile DNA junctions are complexes of oligomeric DNA strands that interact to yield branched structures in which the branch point cannot migrate. This is achieved by minimizing the sequence symmetry in the flanking arms, so that base pairs lock at the branch site. Here, we report the design, synthesis, and analysis of two semimobile junctions, structures in which a controlled extent of branch point migratory freedom is deliberately introduced. We have constructed two minimally symmetric four-arm semimobile junctions from synthetic deoxy 17-mers. These junctions, termed "monomobile", contain a single pair of base pairs (A-T or C-G) which can migrate at the site of branching, while the rest of the junction is immobile. We have demonstrated by gel electrophoresis techniques that these junctions form and that they have the predicted 1:1:1:1 stoichiometry. We have compared these junctions with the immobile junction on which they are based, by means of hydroxyl radical protection experiments. From these data, both migratory conformers can be seen to coexist in solution. The semimobile junction with the C-G base pair has the same crossover and stacking pattern observed for the immobile junction, while the junction with the A-T base pair has the opposite pattern. We conclude that crossover and stacking patterns are a direct consequence of the base pairs which flank the junction. In addition, the data indicate that the crossover pattern biases for these junctions are much greater than are the migratory biases.

Genetic recombination involving two DNA duplexes is one of the fundamental processes that generates genetic diversity. Central to duplex exchanges is the Holliday (1964) branched intermediate, formed from four strands of DNA. In naturally occurring recombination, the two double helices which combine to form the Holliday intermediate are identical, or nearly so, thus yielding a structure with 2-fold sequence symmetry. Such an intermediate is unstable and can isomerize to change the particular bases that are paired, as shown in Figure 1. This isomerization process relocates the site of branching and is therefore known as "branch point migration". Branch point migration has been demonstrated experimentally (Lee et al., 1970; Broker & Lehman, 1971; Kim et al., 1972), the rate has been measured (Thompson et al., 1976; Warner et al., 1978, Courey & Wang, 1983), and aspects of the process have been modeled (Meselson, 1972; Robinson & Seeman, 1987).

An understanding of the details of branch point migration is critical if we are to comprehend the detailed chemistry of recombination. This is a difficult process to treat experimentally, since the symmetry of the system renders the entire complex unstable. It has been possible to study the structural features of branched DNA by constructing sets of oligodeoxynucleotides with minimal sequence symmetry (Seeman, 1981, 1982; Seeman & Kallenbach, 1983). Junctions with no sequence symmetry at the site of branching are called immobile junctions. In previous studies, we have demonstrated the formation of immobile junctions with three or four arms (Kallenbach et al., 1983a,b; Ma et al., 1986). A particular immobile four-arm junction, J1, has been explored in depth: Its DNA structure remains primarily B-form (Seeman et al., 1985; Marky et al., 1987), and all bases, including those that flank the junction, are paired (Wemmer et al., 1985). Recently, by hydroxyl radical protection experiments (Churchill et al., 1988), J1 has been shown to be 2-fold symmetric; it appears to consist of two stacked helical domains, such as the parallel (Sigal & Alberts, 1972) or antiparallel helices shown in Figure 2. This is in agreement with the work of Cooper and Hagerman (1987), who have shown that the six pairs of arms of a junction similar to J1 are not equivalent in their electrophoretic mobilities.

In order to study the details of the branch point migration process, it is necessary to relax the constraints on symmetry minimization about the branch site. A branched junction that

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