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ABSTRACT: Sequence-dependent thermodynamic quantities for the antiviral agent netropsin and a related bis(*N*-methylimidazole) dipeptide, lexitropsin, have been determined by DNase I footprinting techniques. The primary data are autoradiographic spot intensities derived from 10 footprinting experiments carried out in the temperature range 0–45 °C. After exclusion effects due to overlapped drug sites on DNA and redistribution phenomena associated with the enzyme were accounted for, sequence-dependent binding constants for the two ligands were calculated. Our approach does not require an independent determination of the free drug concentration, which is calculated, with individual site binding constants, by using only footprinting data. The temperature dependence of the binding constants for netropsin implied that the binding enthalpies for all the sites but one on a 139 base pair restriction fragment of pBR 322 DNA are exothermic. Their values roughly correlate with the free energies of binding, which are smaller for sites including a 5′-TA-3′ sequence. The binding enthalpies for the lexitropsin to all its sites were exothermic and more negative than those of netropsin. This may be due to the greater ability of the lexitropsin, when compared to netropsin, to form hydrogen bonds with sites on DNA. The binding constants of the lexitropsin toward its GC interaction sequences were much lower than those of netropsin, as can be explained by the reduced charge of the former ligand. Although it is difficult to determine the specific origin of the thermodynamic effects measured, comparison between netropsin and the lexitropsin suggests that the degree of solvation in the minor groove of DNA may be a factor influencing the entropy of the binding process.

In the typical footprinting experiment, a cleavage agent is used to partially digest a DNA fragment in the presence and absence of DNA-binding ligand (Galas & Schmitz, 1978; Schmitz & Galas, 1979; Van Dyke et al., 1982; Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984). If the ligand is able to inhibit cleavage at the site of binding, the locations of these sites appear as omissions or "footprints" on a DNA sequencing autoradiogram. Aside from simply revealing the sites of binding, the footprinting experiment has the potential of generating individual site binding isotherms. This information, which cannot be obtained by any other experimental technique, allows the determination of ligand binding constants as a function of sequence on natural DNA molecules. Ackers and co-workers (Brenowitz et al., 1986a,b; Senear, et al., 1986), as well as others (Carey, 1988; Letovsky & Dynen, 1989), showed that footprinting data could be used to measure the binding constants of proteins bound to DNA. Although the low sequence specificity of drugs and other small ligands leads to complications not found in footprinting experiments involving proteins, it has been demonstrated that quantitative footprinting analysis can be used to determine the binding constants of the antiviral agent netropsin (**1**; Figure 1) bound to DNA (Ward et al., 1987, 1988a; Fish et al., 1988). Additional studies revealed the relationships between drug–DNA and probe–DNA equilibria and the manner in which the probe is able to report the occupancy of a binding site by a drug molecule in the quantitative footprinting experiment (Ward et al., 1988b; Dabrowiak et al., 1989a,b; Dabrowiak & Goodisman, 1989, 1990; Rehfuss et al., 1990). In this report we show that it is possible to obtain a full thermodynamic profile ($\Delta G°$, $\Delta H°$, and $\Delta S°$) of drug binding as a function of sequence on polymeric DNA by using only footprinting data.

The antiviral agent netropsin (**1**) binds within the minor groove of DNA to sites of type $(A·T)_4$. The specificity of the agent appears to be related to the fact that the floor of the minor groove in AT-rich regions of DNA is relatively flat and featureless (Kopka et al., 1985; Coll et al., 1989). This allows deep penetration of the drug into the minor groove, producing close van der Waals contacts and hydrogen bonds between the agent and DNA. An additional factor influencing specificity of netropsin is the fact that the minor groove in AT-rich regions is charged negatively (Lavery & Pullman, 1985). Since the drug is a dication, electrostatic interactions may also play a role in the AT specificity of the antiviral agent.

The sequence-reading peptide or lexitropsin (**2**) was designed to target GC-rich regions of DNA. Previous footprinting studies with the compound (Kissinger et al., 1987; Dabrowiak et al., 1989a) revealed that it exhibits a high affinity for sites possessing guanine and cytosine in the binding sequence. By analogy with the single-crystal X-ray analyses of netropsin and distamycin bound to small segments of DNA (Kopka et al., 1985; Coll et al., 1989) and from NMR and nuclear Overhauser effect (NOE)[1] evidence (Lee et al., 1988), the preference of **2** for GC-rich sites was rationalized in terms of its ability to accept hydrogen bonds, through the imidazole nitrogens, from the 2-amino group of guanine located in the minor groove of DNA. This ability, and the ligand's reduced cationic charge, are very likely the reasons for enhanced binding of **2** at GC-rich sites.

Materials and Methods

Quantitative footprinting studies involving **1** and **2**, the latter a generous gift from J. W. Lown, University of Alberta, were carried out with DNase I (Ward et al., 1988a). The restriction fragment, a *Hin*dIII/*Nci*I 139 base pair (bp) cleavage product

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[1] Abbreviations: NOE, nuclear Overhauser effect; bp, base pairs.

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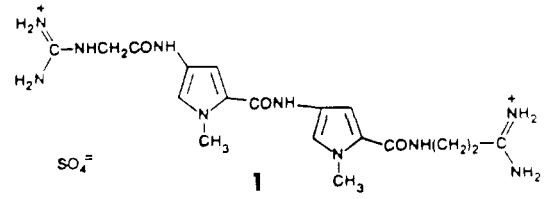

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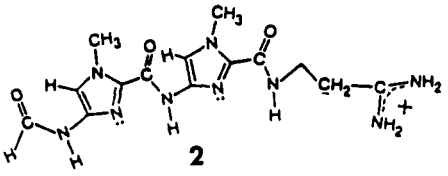

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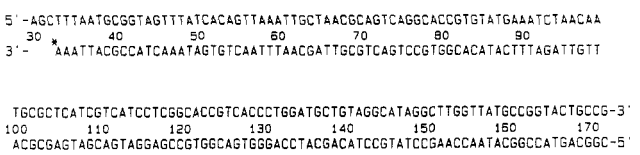

FIGURE 1: Structures of netropsin (**1**) and the sequence reading peptide lexitropsin (**2**) and the *Hin*dIII/*Nci*I restriction fragment from pBR-322 DNA.

from pBR 322 DNA, was singly end-labeled on its noncoding strand at position 33 by using [α-^{32}P]ATP in the presence of reverse transcriptase (Lown et al., 1986). The footprinting experiments were conducted in a total volume of 8 μL in the buffer 37.5 mM Tris-HCl, 8 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.5, containing 193 μM bp of sonicated calf thymus DNA, $\sim$1 μM labeled fragment, $\sim$0.1–$\sim$0.01 μM DNase I, and various concentrations of drug. The amount of DNase I necessary to cleave 20–30% of the labeled fragment (70–80% uncut) was determined by carrying out a series of calibration experiments in the absence of drug at various temperatures. After electrophoresis in a 12% denaturing polyacrylamide gel and brief autoradiography to locate the full-length band, the section of the gel containing the band was excised and subjected to scintillation counting. The amount of cleavage as determined by counting and also by scanning of the autoradiogram with a microdensitometer was less than $\sim$30% (single hit regime). The footprinting reactions for each ligand were carried out at 0, 15, 25, 37, and 45 °C in a buffer medium containing 37 μM Tris-HCl at pH 7.5 for 10 min. The reactions for lexitropsin were carried out at the 16 different drug concentrations shown at the top of Figure 2. While the netropsin experiments at 0, 15, 25, and 45 °C were carried out in this study, the data obtained at 37 °C for the antiviral agent are those collected and analyzed in an earlier report (Ward et al., 1988a).

Microdensitometric scanning of the data (Dabrowiak et al., 1986) showed that the total amount of cleavage on the 139-mer remained constant as drug was added to the system. After minor corrections of the density data for gel loading errors, which were <10%, plots showing the behavior of the intensity of each individual band on the autoradiogram as a function of total drug concentration, termed footprinting plots, were constructed for each temperature studied. These plots formed the basis of the analysis (Dabrowiak & Goodisman, 1989, 1990).

For the calculations on netropsin, we used spot intensities measured for sites 48, 49, 50–55, 58, 59, 62–67, 83 ,87, 89, 90, 96, 102, and 106 (23 sites). Eight of these were (see below) enhancement sites; the sites showing drug binding were 48, 50, 51, 53–55, 58, 59, 62, 63, 87, 89, and 90. Intensities for nine drug concentrations in the range 1–10 μM as well as an experiment at zero drug concentration were used for the digests at 0, 15, 25, and 45 °C. This gave rise to 230 intensities, which were used for each temperature. The footprinting analysis at 37 °C consisted of a slightly larger data set involving 256 intensities (Ward et al., 1988a). In the case of the lexitropsin, 22 sites were monitored: 59, 63, 65–67, 73, 76, 77, 79, 80, 83, 87, 94, 96, 106, 108, 112, 120, 124, 126, 133 and 138, including 8 for the enhancements. Sites showing drug binding were as follows: 65–67, 76, 77, 79, 80, 83, 94, 96, 106, 108, 112, 120, 124 and 126. Intensities for nine drug concentrations in the range 0–23 μM were used. This gave rise to 198 intensities used in the minimization.

In order to examine only the earliest loading events on the restriction fragment and thus minimize any possible drug–drug cooperativity effects that might be present, only the low drug concentrations were used in the analysis. The concentrations employed were determined by noting the level of added drug for which the strong sites begin to accept ligand, but the weak sites do not experience binding. In the case of netropsin, this concentration corresponded to $\sim$10 μM drug. For the lexitropsin used in the study, only four distinct regions of inhibition were observed. Since a clear distinction between their "strong" and "weak" binding character could not be made, i.e., their respective values of K_i were more similar in magnitude than were the "strong" and "weak" binding domains of netropsin, a decision was made to include concentrations in the range 0–23 μM. Although this allowed access to only the beginning of the titration curves for the weaker sites, it provided sufficient data to determine their binding constants. This is because the footprinting plot, after correction for the mass action effect associated with DNase I, can be described by a single parameter, the binding constant. Thus, any part of the curve suffices to determine the binding constant.

Results

The footprinting plots showing the cleavage intensity as a function of drug concentration for either drug were of three types. Within a binding site DNase I cleavage *decreased* as drug was added to the system. This classic footprinting phenomenon can be seen for lexitropsin (**2**) at positions 63–72, 76–84, 106–114, and 119–127 of Figure 2. At numerous sites between drug binding sites the cleavage rate was found to *increase* as drug was added to the system. For lexitropsin (**2**), these increases can be seen at positions 85–110 and at other sites on the 139-mer (Figure 2). For certain positions, footprinting plots were observed to increase at low drug concentration and later, at high drug concentration, decrease. These positions, associated with secondary drug binding sites, were not addressed in this study.

Discussion

Model Used To Analyze the Footprinting Data. The general approach for calculating binding constants from footprinting data has been earlier described (Ward et al., 1988a; Dabrowiak & Goodisman, 1989, 1990). Briefly, the autoradiographic spot intensity for a particular cleavage product is proportional to the cleavage rate at the relevant site, $(\text{rate})_i$, which is given by expression 1. In expression 1, k'_i is the

$$(\text{rate})_i = k'_i[\text{DNase I}]_i(1 - \nu_i) \qquad (1)$$

cleavage rate constant at the site, $[\text{DNase I}]_i$ is the effective concentration of enzyme at the site, and ν_i is the fraction of the DNA fragments that have the site blocked by drug. With increasing drug concentration, ν_i increases for all sites that can be blocked by bound drug. Drug binding to certain sites on

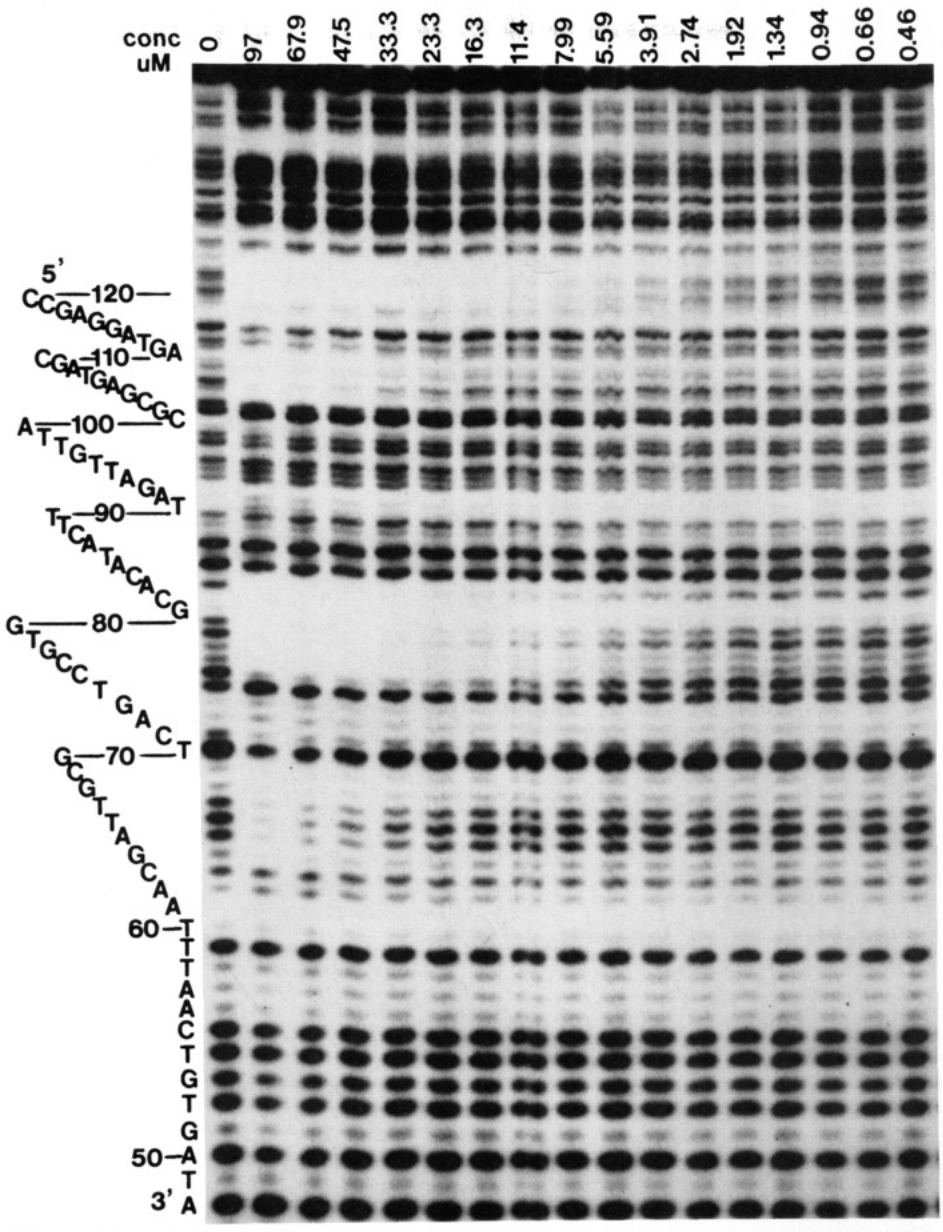

FIGURE 2: Results of DNase I footprinting experiments with lexitropsin (**2**) interacting with the 139-mer at 25 °C. A partial sequence of the noncoding strand of the restriction fragment and position numbers are shown on the left margin of the figure. See Figure 1 for the complete sequence.

a fragment causes an increasing fraction of DNA to be unavailable to the enzyme, making the effective concentration of DNase I increase at all other DNA sites. Earlier studies involving netropsin and the intercalating anticancer drug actinomycin D (Ward et al., 1988b) showed that increases in cleavage are proportional to the amount of DNA blocked by drug. This mass action effect means a footprinting plot is not the true isotherm for the site. Thus, the value of $[\text{DNase I}]_i$ in expression 1 is taken proportional to $(1 - \alpha c_b)^{-1}$, where c_b is the concentration of bound drug and α is a constant describing the enhancement, related to the fraction of sites that can be blocked by drug from cleavage by DNase I. The value of α is found along with the drug binding constants in the minimization (see below).

The relationship between the spot intensity associated with any oligonucleotide fragment produced in the digest, I_i, taking into account the mass action effect associated with DNase I, and the fraction of the site occupied by drug is now given by (2). In expression 2, k_i is a new constant, including k'_i. In the minimization leading to site-specific binding constants, the quantity ν_i is calculated in terms of the relevant drug binding constant K_i and the free drug concentration c_f by use of conventional equilibrium constant expressions. For an isolated drug binding site

$$I_i = k_i(1 - \alpha c_b)^{-1}(1 - \nu_i) \quad (2)$$

$$\nu_i = K_i c_f/(1 + K_i c_f) \quad (3)$$

The *free* drug contribution c_f is determined from the *total* drug concentration by the equilibria between the free drug and the drug bound to labeled fragment and unlabeled carrier DNA. Since the carrier concentration (193 μM) is much larger than that of fragment (1 μM), the equilibrium between the drug and carrier determines the amounts of free and bound drug present in the system, and the concentration of fragment need not be known accurately.

The concentration of strong sites on carrier DNA for lexitropsin (**2**), c_c, can be estimated by noting that there are 4 binding sites for **2** in the 120 base pairs out of a total of 139 observed on the radiolabeled fragment. If the same ratio of

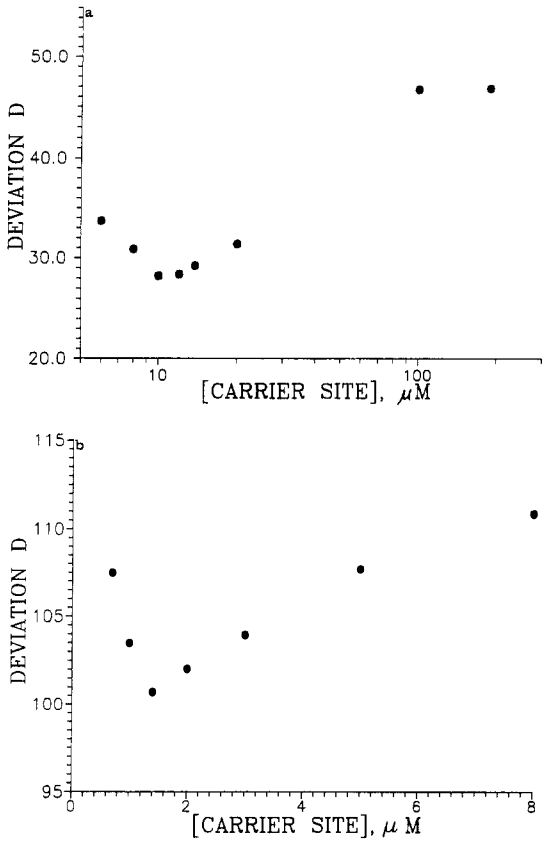

FIGURE 3: Deviation D (eq 5) with best value of $\{K_i\}$, K_e, and K_c for different assumed values of c_c. (a) Netropsin; (b) lexitropsin.

binding sites to base pairs obtains for the carrier, the concentration of sites on the carrier would be (4/120)(193 μM) $\sim$ 6 μM. Similarly, and considering only the strong sites for netropsin, a value of about (8/120)(193 μM) $\sim$ 13 μM is expected for the concentration of strong netropsin sites on the carrier. In our previous work (Ward et al., 1988a) we incorrectly assumed a strong-site concentration of c_c = 194 μM for netropsin on the carrier DNA. Furthermore, we argued that the product $K_c c_c$, where K_c is the binding constant toward the carrier DNA, and not the value of c_c itself, is important in determining the amount of drug bound to the carrier. While this is sometimes true, we now find that K_c and c_c behave as *independent parameters* when c_c becomes comparable to or less than the ligand concentrations used. Thus, the concentration of strong sites on the carrier, c_c, and their average binding constant, K_c, can be determined from the footprinting data derived from the radiolabeled fragment.

The value of K_c will be an average of the strong-site binding constants on the carrier and will also depend on the value assumed for c_c (see below). One cannot directly compare K_c derived from quantitative footprinting analysis with the binding constant obtained by applying a Scatchard analysis to the results of optical, filter binding, or phase-partition experiments. First, as noted by McGhee and Von Hippel (1974), Scatchard analysis can give binding constants "substantially in error" when applied to a substrate possessing overlapping binding sites. Second, although the binding constant obtained in this way is, like our K_c, a weighted average of binding constants for different sites, the weighted average in the case of the Scatchard analysis differs from the weights entering K_c derived from footprinting data, thus complicating the comparison between the two quantities.

To see what these weights are in the case of the footprinting data, suppose the actual carrier sites C1, C2, etc. have binding

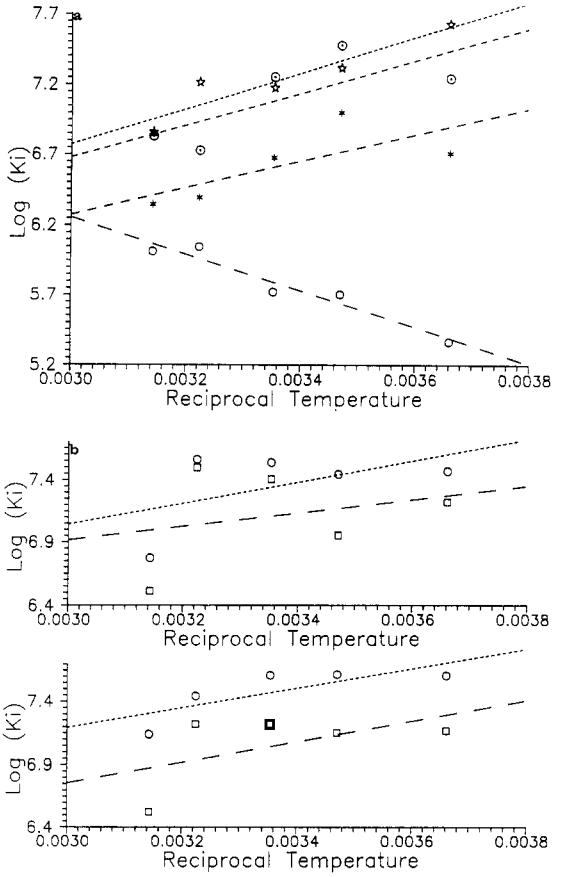

FIGURE 4: Van't Hoff plots of the site-specific binding constants for netropsin (**1**). (a) Sites 56 (○); 57 (☆ and top line); 47 (⊙ and second line); 46 (*). (b) Sites 58 (○), 59 (□). (c) Site 87 (○); carrier (□).

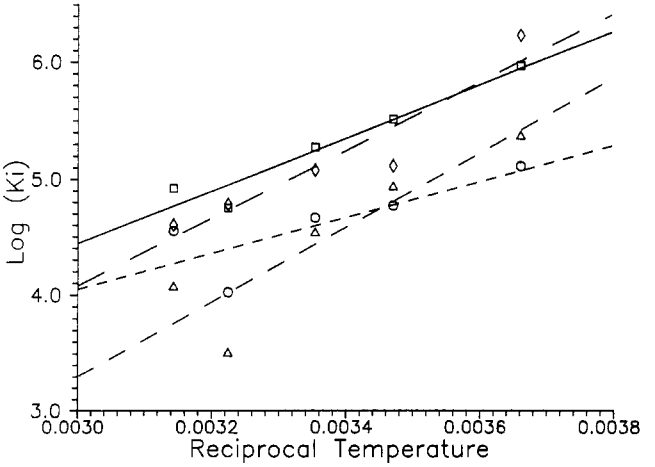

FIGURE 5: Van't Hoff plots of the site-specific binding constants for lexitropsin (**2**) for sites 76 (□, solid line), 119 (◇, long-dash line), 106 (△, medium-dash line), 63 (○, short-dash line).

constants K_{c1}, K_{c2}, etc. and concentrations c_1, c_2, etc., so the bound-drug concentration, c_b, is actually

$$c_b = \sum_i c_i K_{ci} c_f/(1 + K_{ci} c_f) \quad (4a)$$

(This assumes carrier sites i do not overlap. If they do, the equation is somewhat more complicated.) We write instead

$$c_b = K_c c_f/(1 + K_c c_f) \quad (4b)$$

For small c_f, (4a) becomes

$$c_b = (\sum_i c_i K_{ci}) c_f$$

and (4b) becomes

$$c_b = c_c K_c c_f$$

Thus, K_c represents $(\sum_i c_i K_{ci})/c_c$, for small drug concentrations. The equivalence of (4a) and (4b) at low values of the total drug concentration is another reason for restricting the analysis to the earliest parts (low drug) of the footprinting plots.

A third point to make in connection with the values of K_c derived from footprinting data with those obtained through Scatchard analyses is that in the former case only the earliest loading events on the carrier are being examined. Typically, the footprinting-derived values of K_c are obtained from data where the ratio of bound drug to available DNA base pairs, r, is small $<\sim 0.05$. Unfortunately it is this region of the Scatchard plot, r of $<\sim 0.05$, where uncertainties are large and an accurate determination of K from the plot is difficult to make. Thus, comparisons between K_c from footprinting data and other values obtained through conventional methods may not be straightforward.

Determination of Binding Constants. After addressing exclusion effects due to overlapped sites on DNA, the quantities I_i may be calculated for each total ligand concentration, given values for α, K_c, and the K_i. These values are then determined as those which minimize

$$D = \sum_i \sum_j (I_{ij} - \tilde{I}_{ij})^2 \qquad (5)$$

where I_{ij} is the experimental spot intensity for site i, proportional to oligonucleotide concentration, at a total drug concentration of c_j, and $\tilde{I}_{ij}$ is the corresponding calculated spot intensity. The search for values that minimize D is carried out according to the Simplex search algorithm (Ward et al., 1988a; Dabrowiak et al., 1989a,b; Dabrowiak & Goodisman, 1989). Direct knowledge of the free drug concentration is not required: its value for any total drug concentration is calculated in terms of c_j and K_c. The analysis assumes noncooperative independent binding events on the 139-mer. For the generally low binding density over the concentration range studied this assumption is reasonable.

In our first quantitative analysis of a footprinting experiment (Ward et al., 1988a), we fixed the concentration of strong netropsin sites on the calf thymus DNA used as a carrier before minimizing D, the deviation between experimental and calculated data (eq 5). It is now clear that the value of D depends on the value assumed for c_c (Figure 3). Although D varies little with c_c when the latter quantity is large, there is a clear minimum for c_c of ~ 10 μM for netropsin (Figure 3a). If K_c and c_c, along with the other constants, are determined by minimization of D, the values of the site-specific binding constants for netropsin toward the fragment are somewhat lower than those earlier published (Ward et al., 1988a). As is shown in Figure 3b, the value of c_c for the lexitropsin, as determined by the minimum in D, is ~ 1.4 μM. Once the values of c_c for both ligands were determined at one temperature, 37 °C for **1** and 15 °C for **2**, this value was fixed for the remaining minimizations at the various temperatures. In view of the modest sensitivity of the individual K_i's to the value of c_c (Figure 3), this appears to be a valid approximation.

From analysis of the footprinting experiments at each temperature, we get the binding constants K_i toward the individual sites on the labeled fragment. These are shown in Figures 4 and 5 as a function of temperature. Linear fits of log K_i versus $1/T$ are also shown. From $\Delta G° = -RT \ln K$ at 25 °C and $d(\log D)/d(1/T) = -\Delta H°/2.303R$, we get the thermodynamic parameters given in Table I. The stated errors in $\Delta H°$ and $\Delta S°$ come from the correlation coefficients of the least-square fits. We also obtain a value for K_c, the average binding constant to the strong sites on the carrier DNA (Figure 4). In the case of netropsin, this value is $\sim 1 \times 10^7$ M^{-1}, which is ~ 2 orders of magnitude higher than those derived by Scatchard methods (Zimmer & Wahnert, 1986). However, as was earlier stated, direct comparison between the value of K_c derived from footprinting data and those earlier reported may not be possible. The value of K_c for the lexitropsin was found to be 6.7×10^6 M^{-1} at 25 °C.

Table I: Thermodynamic Parameters for Netropsin (**1**) and Lexitropsin (**2**) at 25 °C

position	sequence[a]	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol K)
		Netropsin (**1**)		
46	TTTA AAAT	−37.7 ± 0.4	−18 ± 8	65 ± 26
47	TTAT AATA	−41.3 ± 0.7	−25 ± 4	54 ± 14
56	TTAA AATT	−33.1 ± 0.6	25 ± 3	195 ± 10
57	TAAA ATTT	−40.7 ± 0.7	−22 ± 9	62 ± 30
58	AAAT TTTA	−41.9 ± 0.8	−16 ± 12	87 ± 40
59	AATT TTAA	−40.6 ± 0.6	−10 ± 10	102 ± 32
89	AAAT TTTA	−42.6 ± 0.3	−12 ± 6	103 ± 20
		Lexitropsin (**2**)[b]		
63	GCTAACGCAG CGATTGCGTC	−26.2 ± 0.5	−29.7 ± 9.9	−12 ± 33
76	GGCACCGTG CCGTGGCAC	−30.0 ± 0.3	−43.6 ± 5.3	−46 ± 18
106	CATCGTCAT GTAGCAGTA	−25.3 ± 0.7	−61.4 ± 13.0	−121 ± 44
119	GGCACCGTC CCGTGGCAG	−29.2 ± 0.5	−55.9 ± 8.7	−89 ± 29

[a] Sequence is 5′→3′ on the coding (top) strand. [b] Specific sequences of ligand contact for **2** are not given. However, the most likely contact sequence within the entire inhibition region is indicated. The region was modeled as a single ligand binding event.

Thermodynamic Data. Explaining the thermodynamic data is made difficult by the fact that contributions to $\Delta G°$, $\Delta H°$, and $\Delta S°$ can come from at least three sources (Chaires, 1985). One includes hydrogen bonding, van der Waals effects, and hydrophobic/electrostatic interactions between the drug and its DNA binding site. A second source is conformational changes that take place within the DNA site or the drug as binding occurs. Finally, since both the drug and DNA are highly solvated and have associated counterions, water release and ion redistribution very likely accompany binding. It is not possible to unequivocally determine which factor influences which thermodynamic parameter. However, the reduced charge of **2** compared to netropsin is probably a major contributing factor to the lower binding constants and less negative $\Delta G°$ for the former compound (Table I). A parallel argument can be advanced to explain the lower binding constant of distamycin, which is a monocation, compared to netropsin, which is a dication (Zimmer & Wahnert, 1986).

The presence of a 5′-TA-3′ sequence in the netropsin binding site gives rise to a distortion in the minor groove of DNA (Calladine, 1982). This may not only discourage the binding of the drug but may also affect the degree of solvation within the groove (Drew & Dickerson, 1985; Kennard et al., 1986). Without additional study it is not possible to ascertain the effects of flanking sequence on the thermodynamic parameters. However, the sequence 5′-AAAT-3′, which occurs imbedded in an AT tract at position 58, has essentially the same thermodynamic parameters as does the isolated site of the same sequence at 89.

For netropsin, $\Delta S°$ is positive, as expected for association of two solvated species, the drug and DNA, with ordered solvent being released from DNA. Negative values of $\Delta S°$ are found for **2**, which may reflect the disorderly binding of water at GC sites (Kennard et al., 1986). This water is liberated from DNA to the more ordered structure of the bulk solvent.

The values for $\Delta H°$ for netropsin are all negative and about the same size (from −10 to −25kJ/mol) except for site 56, having the sequence 5′-TTAA-3′, which is positive. The earlier, direct, calorimetric measurements of $\Delta H°$ by Breslauer et al. (1988) primarily used poly(dAdT)·poly(dAdT), poly(dA)·poly(dT) and certain oligonucleotide duplexes as host DNA for netropsin. The small oligonucleotide duplex, d-$(GCGAATTCGC)_2$, studied calorimetrically, possesses the netropsin binding sequence 5′-AATT-3′, which is found at position 59 of the 139-mer (Figure 1). However, despite the fact that the free energy of netropsin binding to this sequence, obtained by us (Table I), is similar to that obtained by other means (Marky & Breslauer, 1987), our enthalpy of -10 ± 10kJ/mol is quite different from that obtained via calorimetric measurements, −38.9 kJ/mol. It is possible that the restriction fragment undergoes a structural change over the temperature range of the study and that this is a factor affecting the thermodynamic parameters, but this does not appear likely. DNase I, which is sensitive to DNA structural changes (Drew & Travers, 1984), showed no change in its cleavage pattern of the 139-mer as a function of temperature. It is also possible that the length of DNA is important in the interaction. In our experiments, the sequence 5′-AATT-3′ is imbedded in a long polymer, while in the earlier calorimetric studies (Breslauer et al., 1988) it was part of a decamer. Quantitative footprinting experiments with actinomycin D using a small DNA oligomer as well as the 139-mer have suggested that DNA length may affect the binding constant of the anticancer drug to DNA (Rehfuss et al., 1990; Dabrowiak & Goodisman, 1990). Clearly, additional work involving quantitative footprinting methods and other techniques will be necessary to fully understand how polymer length, flanking sequences, degree of solvation, and other factors influence the thermodynamic properties of drug/DNA interactions at the sequence level.

As is evident from Table I, the errors associated with the sites for netropsin are quite large. This appears to be due to the number of overlapped netropsin sites on the 139-mer and the small (relative to the number of binding constants) number of concentration points used in the footprinting titrations, giving rise to fewer data points to fit more parameters. Since there are no overlaps and fewer parameters to fit, the errors associated with the lexitropsin are generally lower than those found for netropsin (Table I).

Conclusions

In this report we show that, by carrying out multiple footprinting experiments at a number of different temperatures, it is possible to obtain the full thermodynamic profile of a drug, $\Delta G°$, $\Delta H°$, and $\Delta S°$, as a function of sequence on polymeric DNA. The technique requires the measurement of autoradiographic spot intensities on a DNA sequencing autoradiogram. With a parametrized model for the various equilibria present in the system, the difference between calculated and measured intensities is used in a minimization approach to derive site-specific drug binding constants and other parameters characterizing the system. The temperature dependence of the binding constants, given as van't Hoff plots, allows the determination of site-specific enthalpies and entropies for the drug. In the case of netropsin, the enthalpies for various sequences on the 139-mer are mostly negative and agree in size, but are smaller (less negative) than the few sequence-specific enthalpies determined by other means. The large negative $\Delta H°$ values observed for the bis(*N*-methylimidazole) lexitropsin (**2**) as compared with netropsin may be due to the greater ability of the former compound to hydrogen bond to sites located in the minor groove of DNA.

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Preferential Binding of Daunomycin to 5′$\frac{A}{T}$CG and 5′$\frac{A}{T}$GC Sequences Revealed by Footprinting Titration Experiments†

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ABSTRACT: Results from a high-resolution deoxyribonuclease I (DNase I) footprinting titration procedure are described that identify preferred daunomycin binding sites within the 160 bp *tyr T* DNA fragment. We have obtained single-bond resolution at 65 of the 160 potential binding sites within the *tyr T* fragment and have examined the effect of 0–3.0 μM total daunomycin concentration on the susceptibility of these sites toward digestion by DNase I. Four types of behavior are observed: (i) protection from DNase I cleavage; (ii) protection, but only after reaching a critical total daunomycin concentration; (iii) enhanced cleavage; (iv) no effect of added drug. Ten sites were identified as the most strongly protected on the basis of the magnitude of the reduction of their digestion product band areas in the presence of daunomycin. These were identified as the preferred daunomycin binding sites. Seven of these 10 sites are found at the end of the triplet sequences 5′$\frac{A}{T}$GC and 5′$\frac{A}{T}$CG, where the notation $\frac{A}{T}$ indicates that either A or T may occupy the position. The remaining three strongly protected sites are found at the ends of the triplet sequence 5′$\frac{A}{T}$C$\frac{A}{T}$. Of the preferred daunomycin binding sites we identify in this study, the sequence 5′$\frac{A}{T}$CG is consistent with the specificity predicted by the theoretical studies of Chen et al. [Chen, K.-X., Gresh, N., & Pullman, B. (1985) *J. Biomol. Struct. Dyn. 3*, 445–466] and is the very sequence to which daunomycin is observed to be bound in two recent X-ray crystallographic studies. Solution studies, theoretical studies, and crystallographic studies have thus converged to provide a consistent and coherent picture of the sequence preference of this important anticancer antibiotic.

The cellular target of many clinically useful anticancer compounds is thought to be DNA (Gale et al., 1981; Chagas & Pullman, 1987). A necessary step toward the rational design of new anticancer compounds with enhanced potency and selectivity is to characterize the DNA binding specificity of these existing compounds of proven clinical utility and to elucidate the molecular mechanisms that govern their sequence-selective binding to DNA. It is hoped that this activity will produce, ultimately, a set of rules to guide the rational design of a new generation of anticancer compounds targeted to specific DNA sites.

With this larger goal in mind, our laboratory has studied the interaction of daunomycin with DNA by a variety of physical and biochemical methods (Britt et al., 1986; Chaires, 1983a,b, 1985a,b; Chaires et al., 1982a,b, 1983, 1985, 1987; Herrera & Chaires, 1989; Fritzsche et al., 1982, 1987). Daunomycin is the prototype anthracycline antibiotic, a class of compounds that has well-documented utility in cancer chemotherapy (Arcamone, 1981; Crooke & Reich, 1980; Remers, 1984). Daunomycin binds to DNA by the process of intercalation, and the fundamental aspects of the physical chemistry of its binding to DNA have been well characterized [for reviews, see Fritzsche and Berg (1987) and Chaires

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