Volume 11 Number 9 1983 Nucleic Acids Research

Calorimetric and spectroscopic investigation of drug – DNA interactions. I. The binding of netropsin to poly d(AT)

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Received 25 February 1983; Revised and Accepted 4 April 1983

ABSTRACT

We report the first calorimetric investigation of netropsin binding to poly d(AT). Temperature-dependent uv absorption, circular dichroism (CD), batch calorimetry, and differential scanning calorimetry (DSC) were used to detect, monitor, and thermodynamically characterize the binding process. The following results have been obtained: 1) Netropsin groove binding is accompanied by a large exothermic enthalpy of 9.2 kcal/mol of drug bound at 25°C. This indicates that a large negative binding enthalpy may be a necessary but not a sufficient criterion for drug intercalation. We suggest that the exothermic binding might be correlated with specific H-bonding interactions. 2) From the difference in DSC transition enthalpies in the presence and absence of netropsin, we calculate a binding enthalpy of -10.7 kcal/mol of netropsin at 88°C. 3) We calculate a positive ΔS for netropsin binding to poly d(AT) at 25°C. This positive entropy change may reflect netropsin-induced release of condensed cations and/or bound water. 4) The netropsin-saturated duplex monophasically melts 46°C higher than the free duplex. The unsaturated duplex melts through two thermally-resolved transitions that correspond to netropsin-free and netropsin-bound regions. These two regions interact dynamically with no substantial influence on the thermal stabilities of the separate domains. 5) Netropsin binding decreases the cooperativity of the duplex to single strand transition.

INTRODUCTION

Netropsin is a basic oligopeptide that exhibits a wide range of antibiotic activities against bacteria, fungi, and viruses.[1] The structure of netropsin is shown in Figure 1. To understand better the molecular basis for its biological activity, the interaction of netropsin with DNA molecules has been studied by several laboratories using various experimental techniques.[2-15] The results of these investigations have provided a rather detailed structural picture of the netropsin-DNA complex as outlined below:

1) Netropsin binds only to double helical DNA. It does not bind to most DNA-RNA hybrids or most single strands. This binding inhibits *in vitro* RNA synthesis.

2) Netropsin binds preferentially to A-T base pairs. Clusters of AT

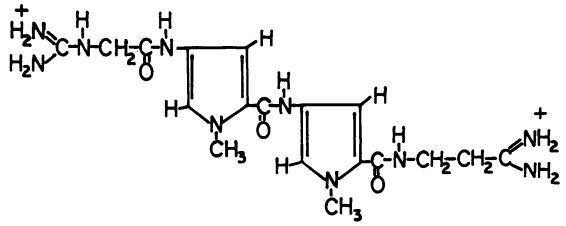

Figure 1. The structure of netropsin.

base pairs bind netropsin more strongly than isolated AT base pairs. A bound netropsin molecule spans between three and five base pairs.

3) Netropsin binds to duplex DNA by a nonintercalative mode in which it lies in the minor groove. This binding does not disrupt the Watson-Crick base pairs but does perturb resonances of the protons located on the base pair edges that face the minor groove.

4) The netropsin-DNA complex appears to be stabilized by three hydrogen bonds between the peptide protons of the netropsin and the adenosine nitrogen (N-3) and/or the thymidine oxygen (O-2) of the DNA.

The description of netropsin binding outlined above represents an essential step on the road to understanding the structural basis for the antibiotic activity of this DNA-binding drug. However, to define fully the nature of the netropsin-DNA interactions, one must thermodynamically characterize the molecular forces that stabilize the complex. To date, the only thermodynamic data on the binding of netropsin to DNA have been several indirectly determined binding constants. In this paper we report the first calorimetric study of the interactions between netropsin and a DNA duplex structure. The resulting thermodynamic data are discussed and interpreted in terms of the specific interactions associated with models that have been proposed for the binding of netropsin to DNA.

EXPERIMENTAL SECTION

Materials

Poly d(AT) was purchased from P.L. Biochemicals, Inc. Netropsin was obtained from Farmitalia (Milano, Italy). The extinctions used for poly d(AT) and netropsin were ε_{260} = 6650 $M^{-1}cm^{-1}$ in phosphate and ε_{296} = 21500 $M^{-1}cm^{-1}$, respectively.[4,16] The buffer used contained 10mM sodium phosphate and 1mM EDTA adjusted to pH 7.0.

Methods

Circular Dichroism Spectroscopy. All CD spectra were recorded using a Cary 60 instrument equipped with a programmable, thermoelectrically controlled cell holder (Aviv Associates). CD titrations were performed by incrementally adding aliquots of netropsin to a cell containing a known amount of poly d(AT). The final spectra were normalized so that the curves could be compared directly.

Ultraviolet Spectrophotometry. Absorbance versus temperature profiles of poly d(AT) and poly d(AT)-netropsin complexes were performed on a thermoelectrically controlled Perkin Elmer 575 programmable spectrophotometer interfaced with a Tektronix 4051 computer. Samples were heated at a rate of 0.5°C/minute while the absorbance at 260 nm and the temperature were recorded every 30 seconds.

Batch Calorimetry. The batch calorimeter used in this study is based upon the design of Prosen and Berger and has been previously described in detail.[17] The apparatus basically consists of a bi-compartment cell surrounded by thermoelectric elements embedded within a massive heat sink. The entire instrument is kept in a temperature-controlled environment. A typical experiment involves filling each compartment of the calorimeter cell with aliquots of the reagents of interest. Reaction is initiated by rotation of the cell chamber which results in mixing the reagents. Any heat either liberated or absorbed is quantitatively conducted through the thermopiles to the massive heat sink. The output of the thermopiles, which measures the rate of heat transfer, is amplified and recorded.

The calorimeter was chemically calibrated at 25°C using the heat of neutralization resulting from the reaction between NaOH and HCl. These experiments provided an average calibration constant of 3.62×10^{-3} mcal/cm^2.

Differential Scanning Calorimetry (DSC). DSC runs on poly d(AT) and poly d(AT)-netropsin complexes were performed on a Microcal-1 instrument as previously described.[18,19]

RESULTS

Optical Titrations

Circular Dichroism (CD). Free netropsin in solution does not exhibit a CD effect. However, the binding of netropsin to poly d(AT) induces a CD signal which is centered at 315 nm.[3,8,14] We have used this induced Cotton effect to monitor the binding of netropsin to poly d(AT). Figure 2a shows the family of CD curves that results when netropsin is titrated into a

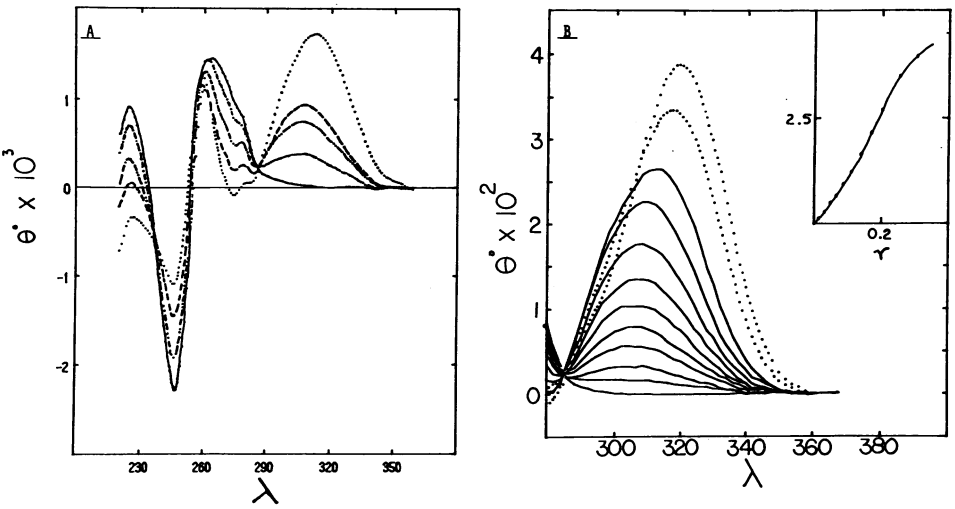

Figure 2. a) Circular dichroism spectra of poly d(AT) (1.2 x 10^{-4}M) with different amounts of added netropsin: r=0 (---); r=0.04 (-•-•-); r=0.08 (-•••-); r=0.11 (---); and r = 0.2 (••••). The buffer is 10mM sodium phosphate, 1mM EDTA, adjusted to pH 7. The temperature is 25°C.
b) Circular dichroism titration of poly d(AT) (1.77 x 10^{-4}M) with netropsin. Inset: Ellipticity at 315 nm versus r.

solution of poly d(AT). The discrete isoelliptic point at 287nm suggests that only one type of binding occurs under the indicated conditions. The significant feature to note is that the Cotton effect at 315 nm increases as netropsin is added. This signal provides a convenient window for monitoring the extent of netropsin binding as illustrated in Figure 2b. We find the induced CD signal has only a small dependence on salt concentration. This observation is consistent with previous reports[4] and indicates the importance of nonionic interactions in the binding process.

The temperature dependence of the induced CD signal at 315 nm is shown in Figure 3. The sharp reduction in the signal occurs at a temperature where the duplex begins to melt. This observation is consistent with previous studies[4] which conclude that netropsin binds only to duplex and not to single strand structures.

Temperature-Dependent UV Absorbance. A second method for monitoring netropsin binding to poly d(AT) does not window netropsin itself but rather takes advantage of the effect of netropsin binding on the thermal stability of the duplex. The thermal stability of the duplex is characterized by the

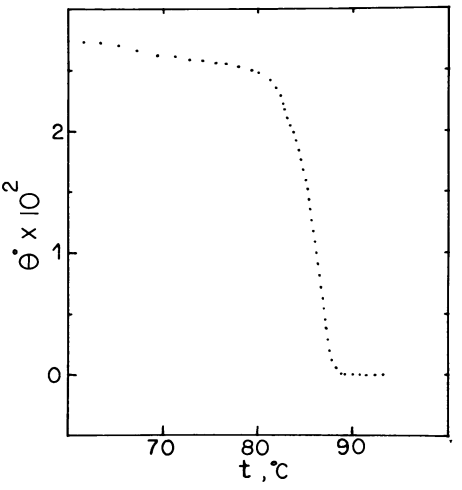

Figure 3. Ellipticity at 315 nm versus temperature for poly d(AT)-netropsin (r=0.2) in 10mM sodium phosphate buffer, 1mM EDTA at pH = 7.0.

temperature midpoint observed in a uv melting curve at 260 nm. Figures 4a and 4b show the families of integral and differentiated melting curves we obtain for poly d(AT) in the presence of increasing concentrations of netropsin. The significant features to note are:

1) Below saturation, netropsin binding causes the duplex structure to melt in a biphasic manner.

2) Increasing the netropsin concentration causes the size of the low temperature peak to decrease and the high temperature peak to increase.

3) The extent of netropsin binding has only a small effect on the t_m's of the low and high temperature transitions. Only the transition areas change.

4) At saturation, the duplex melts monophasically with a t_m some 46°C above that of the free duplex.

Possible structural interpretations of these features will be presented in the Discussion Section.

Calorimetry

Batch Calorimetry. Figure 5 shows a recorder tracing of the exothermic heat burst we obtain at 25°C when poly d(AT) is mixed with netropsin at a drug to base pair ratio (r) of 0.2. Following small corrections for dilution effects, this heat corresponds to the association reaction

$$\underset{\text{(duplex)}}{\text{poly d(AT)}} + \underset{\text{(free)}}{\text{netropsin}} \rightarrow \underset{\text{(complex)}}{\text{poly d(AT)-netropsin}}$$

Integration of the area under the calorimetric curve allows calculation of

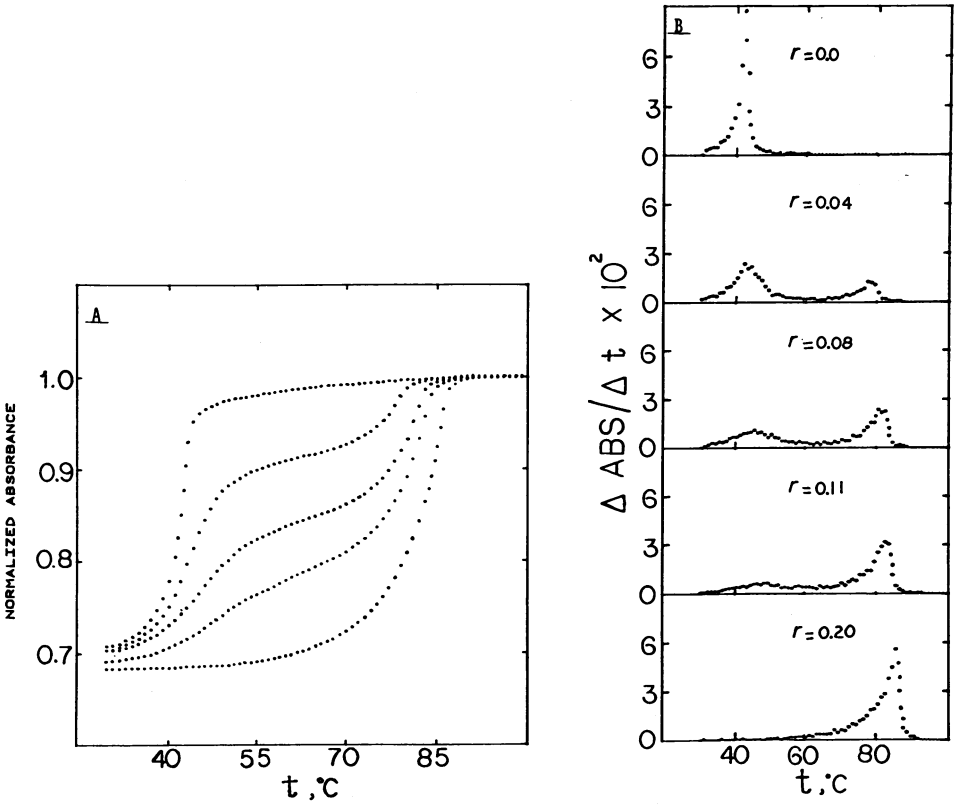

Figure 4. UV melting curves at 260nm of poly d(AT)-netropsin complexes in 10mM sodium phosphate buffer, 1mM disodium EDTA adjusted to pH=7.0, (poly d(AT)=1.21 x 10^{-5}M).
a) The curves are normalized to an absorbance of 1 at high temperature. The r values range from 0.0 to 0.2.
b) Differentiated melting curves. These curves were obtained by taking differences of the absorbance values at intervals of one degree.

the netropsin binding enthalpy, ΔH_b. In Table 1 we list the results of four independent calorimetric determinations of the binding enthalpy at r = 0.2. The significant feature is that the molar binding enthalpy is very large and exothermic. The implications of this result will be described in the Discussion Section.

Differential Scanning Calorimetry. Figures 6a and 6b show the calorimetric transition curves for the duplex to single strand transition of free poly d(AT) and netropsin-saturated poly d(AT), respectively. Straightforward analysis of these calorimetric curves yield the transition enthalpies

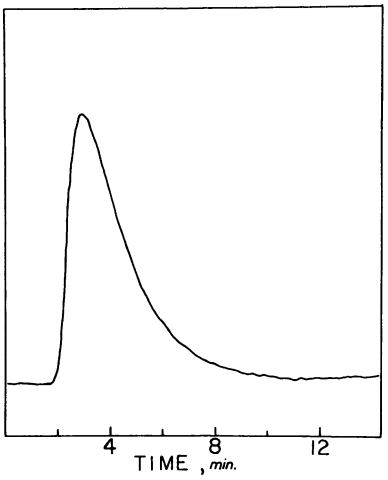

Figure 5. Tracing of the recorder output from a typical exothermic batch calorimetry experiment. A total of 1.44mcal was measured upon mixing of 200µl of poly d(AT) (7.63×10^{-3}M) with 200µl of netropsin solution to give a final r value of 0.2.

ΔH_t, and melting temperatures, t_m, listed in Table 2. The transition enthalpy listed for the free poly d(AT) duplex compares favorably with a previous determination by Scheffler and Sturtevant.[34] The two significant observations are:

1) Netropsin binding at an r value of 0.2 (saturation) increases the thermal stability of the duplex by 46.5°C. This observation is consistent with the optically monitored transitions.

2) The netropsin-induced increase in thermal stability is paralleled by an increase in the overall transition enthalpy.

The transition enthalpies listed in Table 2 can be used to calculate the binding enthalpy of netropsin in the manner described below.

The DSC curve shown in Figure 6b corresponds to the thermally-induced transition

TABLE 1
BATCH CALORIMETRY ON THE BINDING OF NETROPSIN
TO POLY d(AT) AT 25°C AND r = 0.2

Experiment #		ΔH_b
1		-9.3
2		-9.4
3		-8.9
4		-9.1
	Average	-9.2

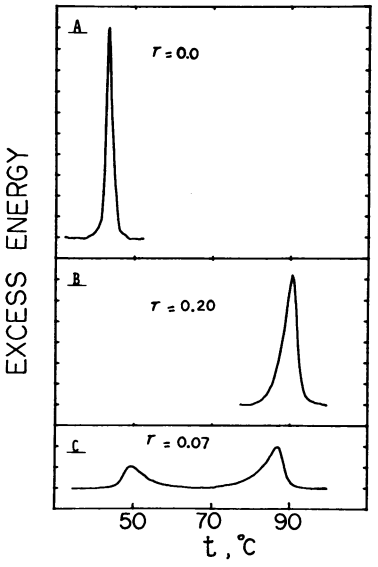

Figure 6. Tracing of the differential scanning calorimeter transition curves for poly d(AT)-netropsin complexes at different r values. For direct comparison, all the curves were normalized.

poly d(AT)-netropsin → poly d(AT) + netropsin
complex (25°C) single strands (100°C)

It is well established that netropsin does not bind to the final single-stranded state. Thus, as shown below, the netropsin dissociation enthalpy (ΔH_3) can be calculated by subtracting the transition enthalpy of the free duplex (ΔH_1) from the transition enthalpy of the netropsin-bound duplex (ΔH_2)

poly d(AT)-netropsin	→ poly d(AT) single strands +	netropsin	ΔH_2
poly d(AT) single strands	→	poly d(AT) duplex	ΔH_1
poly d(AT)-netropsin	→ poly d(AT) +	netropsin	$\Delta H_3 = \Delta H_2 - \Delta H_1$

TABLE 2
DIFFERENTIAL SCANNING CALORIMETRY ON THE NETROPSIN-FREE (r=0) AND NETROPSIN-SATURATED (r=0.2) POLY d(AT) DUPLEX

r (netropsin/base pair)	t_m (°C)	ΔH_t (kcal/mole base pair)	ΔH_3 (kcal/mole of netropsin)
0.0	43.5	7.6	+10.7
0.2	90.0	9.8	

The dissociation enthalpy, ΔH_3, calculated in this manner is listed in the last column of Table 2. If heat capacity effects are small, this dissociation enthalpy at 90°C should be similar in magnitude but opposite in sign to the netropsin binding enthalpy obtained directly by isothermal batch calorimetry at 25°C. Comparison of the dissociation enthalpy (ΔH_3) derived above with the average binding enthalpy listed in Table 1 shows that this is in fact the case. The small difference corresponds to a heat capacity of -24 cal/deg/mole for the binding process.

Figure 6c shows the calorimetric transition curve for the duplex to single strand transition of the unsaturated complex (r = 0.07). The significant observations are:

1) In contrast to the monophasic transitions of the free and the netropsin-saturated duplex, the overall transition of the unsaturated duplex proceeds by two distinct sub-transitions.

2) The low-temperature transition has a t_m only slightly higher than that observed for the free duplex (Figure 6a) while the high-temperature transition has a t_m nearly identical with that observed for the saturated duplex (Figure 6b).

3) Both the low and high-temperature transitions are broader than the transitions of the free duplex.

These features are consistent with the trends noted earlier from the optical melting curves (Figure 3).

Van't Hoff Transition Enthalpies. A van't Hoff transition enthalpy can be calculated from the shape of a calorimetric transition curve by application of the equation:

$$\Delta H_{v.H.} = \frac{-4.37}{\frac{1}{T_{1/2}} - \frac{1}{T_{3/4}}}$$

where $T_{1/2}$ and $T_{3/4}$ are the half and three quarters temperatures at the half height, respectively. Table 3 lists the van't Hoff transition enthalpies obtained by application of this equation to the transitions of the free duplex (Figure 6a) and the netropsin-saturated duplex (Figure 6b). The significant observation is that the netropsin-saturated and the netropsin-free duplexes have simliar van't Hoff transition enthalpies despite the difference in their calorimetrically measured values. The implications of this result will be described in the Discussion Section.

TABLE 3
COMPARISON OF THE CALORIMETRIC AND THE VAN'T HOFF TRANSITION ENTHALPIES FOR THE NETROPSIN-FREE (r=0) AND THE NETROPSIN-SATURATED (r=0.2) POLY d(AT) DUPLEX

r	$\Delta H_{v.H.}$ (kcal)	ΔH_{cal} (kcal/mole base pair)	$\Delta H_{v.H.}/\Delta H_{cal}$ (size of cooperative unit)
0.0	400	7.6	53
0.2	387	9.8	39

DISCUSSION

Our spectroscopic and calorimetric results demonstrate that netropsin binding to poly d(AT) is a highly exothermic process that dramatically increases the thermal stability of the duplex. In the sections that follow, we discuss these results in the context of structural models that have been proposed for the poly d(AT)-netropsin complex.

The Binding Enthalpy and the Mode of Binding

Quadrifoglio *et al.*[21] have used batch calorimetry to determine the binding enthalpy of two intercalaters, ethidium bromide and proflavine, to DNA. For both drugs they measure a binding enthalpy of about -7 kcal/drug bound. Based upon these results they suggest that such a large exothermic binding enthalpy might serve as a useful diagnostic sign-post for drug intercalation. Our calorimetric data on netropsin indicates that such a generalization could be misleading. As reported here, we measure a binding enthalpy of -9.2 kcal/drug bound for netropsin, a known groove binding drug. It is unlikely that binding enthalpies alone will prove diagnostically useful for differentiating between general modes of drug binding.

The Binding Enthalpy and Molecular Interactions

The calorimetrically-determined exothermic enthalpy change accompanying the binding of netropsin to poly d(AT) conceptually can be divided into two parts: The intrinsic binding enthalpy associated with local netropsin-duplex interactions and the enthalpy associated with any netropsin-induced conformational changes. Patel has carried out extensive NMR studies of netropsin binding to poly d(AT) and to two oligomers containing AT cores.[6,7,13] Consequently, we can discuss our calorimetric data in terms of the specific interactions and induced conformational changes as characterized by their NMR studies.

The NMR results of Patel and Canuel[6] reveal that the base pairing and

base stacking interactions remain nearly unperturbed by netropsin binding to poly d(AT). The only significant conformational change appears in the glycosidic torsion angles. Such an alteration has been shown to be accompanied by little if any enthalpy change.[22] Consequently, we believe that induced conformational changes do not significantly contribute to the observed binding enthalpy of netropsin. As a result, we can examine local, specific netropsin-poly d(AT) interactions in an effort to identify the molecular basis for the observed large exothermic binding enthalpy.

The NMR results of Patel[6,7,13] along with model building using the netropsin structure of Berman et al.[23] reveal the following local, specific interactions between netropsin and AT base-paired regions when the drug binds in the minor groove:

1) Three hydrogen bonds between the netropsin amide groups and the base pair edges facing the minor groups. (The adenosine N-3 and the thymidine O-2 probably act as hydrogen bond acceptors.)

2) Two interactions between the charged amino ends of netropsin and the base pair edges or the phosphate groups.

3) Three intermolecular contacts between the bases and each pyrrole H-3 proton as well as the protons of the guanidino CH_2 group.

These interactions all are candidates for the molecular basis of the observed exothermic binding enthalpy. However, the relatively small salt-dependence of the induced CD signal suggests that the interactions of the charged amino ends do not significantly contribute to the binding process. Consequently, it seems reasonable to focus on the three hydrogen bonding interactions as the molecular basis for the observed exothermic binding enthalpy. In this connection it is generally argued that inter-solute hydrogen bond formation in aqueous medium is not associated with a significant enthalpy effect since the solutes could form energetically equal hydrogen bonds with water. Perhaps in this case the minor groove has a lower local dielectric constant than the bulk solvent thereby making the "buried" netropsin-poly d(AT) hydrogen bonds energetically more favorable. At this point such a suggestion only can be classified as speculation. However, if we assume that most of the observed -9 kcal binding enthalpy derives from the three hydrogen bonds, then we calculate a value of -3 kcal per hydrogen bond in the minor groove. This value is in good agreement with the results of Bierzynski et al.[24] who report a value of -2 kcal per peptide hydrogen bond in the alpha-helical environment of a protein and the results of Susi et al.[25,26] who report a value of -3 kcal per hydrogen bond in model compounds.

The Binding Entropy

Since netropsin binds so strongly to double-stranded DNA's, the equilibrium concentration of free netropsin in solution is extremely small. For this reason, it has proven difficult to measure directly the equilibrium constant for netropsin binding to duplex DNA. The several values that have been determined indirectly for netropsin binding to poly d(AT)[4,27] cluster around an average value of approximately $10^{+9}M^{-1}$. This average equilibrium constant corresponds to a binding free energy of -12.3 kcal/mole of bound netropsin at 25°C. Using our calorimetrically-determined binding enthalpy of -9.2 kcal and the standard thermodynamic relationship $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, we calculate a positive binding entropy of +10.3 cal per degree per mole of bound netropsin at 25°C. As described below, such a positive binding entropy for our association reaction may reflect a solvent effect.

From their x-ray studies, Dickerson et al.[28] propose that B-DNA duplexes have a "spine" of structured water in the minor groove around AT-rich regions. Since netropsin binds to AT base pairs in the minor groove, its binding to poly d(AT) could disrupt this structured water and release it to bulk solvent. We suggest that this netropsin-induced disruption of the "water spine" in the minor groove contributes to the observed positive entropy of binding. This molecular interpretation of the positive binding entropy is not the only explanation; however, we consider it attractive as a focus for further discussions. Undoubtedly, netropsin-induced release of condensed sodium ions contributes to the observed positive entropy of binding.[29,30]

The Biphasic Transition and Allosteric Effects

In Figures 3 and 6c we noted that under nonsaturating conditions the netropsin-bound duplex exhibits biphasic behavior in which the t_m of the lower temperature transition is similar to that of the free duplex. This observation suggests that netropsin binding occurs in clusters that create distinct netropsin-free (the low-temperature transition) and netropsin-bound regions (the high-temperature transition) with different thermal stabilities. This interpretation is consistent with the NMR results of Patel and Canuel. As shown in Figure 3, increasing the amount of bound netropsin affects the magnitude and width but not the position of the two transitions. This observation suggests that the netropsin-bound regions do not significantly "communicate" with the netropsin-free regions in so far as stability (t_m) is concerned but do "communicate" in a manner that alters the nature of the transition as monitored by the transition widths. In this connection it should be noted that Patel and Canuel[6] conclude from their NMR data that the

segmental mobility of the netropsin-free regions is decreased by the influence of adjacent netropsin-bound regions. Taken together, these observations suggest that drug-bound and drug-free regions that exhibit independent stabilities can dynamically communicate. This interesting behavior contrasts with that which we have observed for the binding of the steroid diamine, dipyrandium, to poly d(AT).[31]

Nature of the Transition

Inspection of Figures 6a-c reveals that the transitions of the netropsin-bound duplexes are considerably broader (have greater transition widths) than the transition of the netropsin-free duplex. Specifically, the netropsin-free duplex has a transition width of 2°C at the half-height while the corresponding transition width of the netropsin-saturated duplex is 4°C. Thus, in addition to affecting the transition enthalpy and the melting temperature, netropsin binding also alters the nature of the transition as reflected by the increase in transition width.

This netropsin-induced effect on the transition width of the netropsin-bound versus netropsin-free duplex can be quantified by taking the ratio of the corresponding van't Hoff and calorimetric transition enthalpies listed in Table 3. For a given transition, this ratio provides a measure of the size of the cooperative unit.[32,33] The calculated ratios are listed in the final column of Table 3.

The significant observation is that the size of the cooperative unit is larger for the free duplex than for the netropsin-saturated duplex. Based upon this observation we conclude that in addition to increasing the transition enthalpy and the t_m, netropsin binding also reduces the cooperativity of the poly d(AT) transition.

Our calorimetric and spectroscopic studies of netropsin binding are being extended to other synthetic as well as natural DNA polymers. The results of these investigations should allow us to assess the influence of base composition and sequence on the overall thermodynamic profile of the binding process.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health, GM23509, the Charles and Johanna Busch Memorial Fund, and the Research Corporation.

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