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Binding of netropsin to several DNA constructs: Evidence for at least two different 1:1 complexes formed from an –AATT-containing ds-DNA construct and a single minor groove binding ligand

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Abstract

Isothermal titration calorimetry, ITC, has been used to determine the thermodynamics (ΔG, ΔH, and $-T\Delta S$) for binding netropsin to a number of DNA constructs. The DNA constructs included: six different 20–22mer hairpin forming sequences and an 8-mer DNA forming a duplex dimer. All DNA constructs had a single –AT-rich netropsin binding with one of the following sequences, $(A_2T_2)_2$, $(ATAT)_2$, or (AAAA/TTTT). Binding energetics are less dependent on site sequence than on changes in the neighboring single stranded DNA (hairpin loop size and tail length). All of the 1:1 complexes exhibit an enthalpy change that is dependent on the fractional saturation of the binding site. Later binding ligands interact with a significantly more favorable enthalpy change ($\partial\Delta H_{1-2}$ from 2 to 6 kcal/mol) and a significantly less favorable entropy change ($\partial(-T\Delta S_{1-2})$) from −4 to −9 kcal/mol). The ITC data could only be fit within expected experimental error by use of a thermodynamic model that includes two independent binding processes with a combined stoichiometry of 1 mol of ligand per 1 mol of oligonucleotide. Based on the biophysical evidence reported here, including theoretical calculations for the energetics of "trapping" or structuring of a single water molecule and molecular docking computations, it is proposed that there are two modes by which flexible ligands can bind in the minor groove of duplex DNA. The higher affinity binding mode is for netropsin to lay along the floor of the minor groove in a bent conformation and exclude all water from the groove. The slightly weaker binding mode is for the netropsin molecule to have a slightly more linear conformation and for the required curvature to be the result of a water molecule that bridges between the floor of the minor groove and two of the amidino nitrogens located at one end of the bound netropsin molecule.

Keywords: Isothermal tiltration calorimetry; Minor groove binding; Netropsin; DNA hydration; Enthalpy entropy compensation

1. Introduction

It is generally believed that water plays an important role in a number of biological reactions including the binding of small molecules to proteins [1–5], the interactions of small molecules with nucleic acids [6–14], the folding or unfolding of proteins [15,16] and the interaction of proteins with DNA [17–22]. In most cases the water under discussion is water of solvation or hydration of the macromolecule and to a smaller degree the ligand. In the case of DNA, the water of interest is the ordered water along the spine of the nucleic acid.

While it has been observed for a number of ligand DNA interactions that binding is accompanied by large negative changes in heat capacity, it is unclear whether the heat capacity changes reflect an increase or a decrease in the amount of structured water [15–17]. In both groove binding and intercalation the ΔCp values are typically large and negative [23,24]. In general groove binding reactions are accompanied by large favorable entropy changes while intercalation is more

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typically accompanied by large favorable enthalpy changes [24]. Dissection of the free energy of binding for both groove binders and intercalators does not give a simple clear cut representation of whether entropically driven reactions are groove binding in nature or intercalation like, it appears to be compound and sequence specific.

Sturtevant was the first to begin the discussion of the influence of water and water structure on the thermodynamics of macromolecular association [25]. In particular, it was Sturtevant who described the large negative heat capacity changes that accompanied changes in solvation that were associated with protein folding [25] and/or the desolvation that accompanies DNA minor groove binding interactions [26,27]. In this study we focus on not only the solvation changes but also on the incorporation of a specifically bound water molecule in the formation of a netropsin/DNA complex.

The minor groove specificity of the antitumor antibiotic netropsin has been recognized for decades [28–30]. Other compounds with similar geometry have also been identified to have similar minor groove affinity. Some examples of these compounds include the furamidine, DB75 [31], berenil [10,32], Hoechst 33258 [27], distamycin [30,33] and DAPI [34,35]. Even though it is too toxic for clinical use, netropsin has been of interest for biophysical studies attempting to delineate the origins of minor groove drug binding specificity. The binding selectivity of this compound has been under intense examination using multiple techniques. Some examples of structural techniques employed are NMR, circular dichroism, and X-ray crystallography. There has been some inconsistency with respect to the exact conformation of this dicationic compound when binding to the minor groove [33,36]. The vast majority of evidence from both empirical and computational means points to the formation of hydrogen bonds and van der Waal's interactions between the amidino and amide groups present on the ligand with adjacent AT pairs in the minor groove of DNA [26,37–39]. It has also been reported that there is a large difference in enthalpy for netropsin binding to (AAAA/TTTT) sequences vs. $(ATAT)_2$ sequences [40].

In the course of an earlier project comparing the binding affinities determined from Isothermal Titration Calorimetry (ITC), Surface Plasmon Resonance (SPR), and Differential Scanning Calorimetry (DSC) it was observed that the binding of netropsin to a model DNA hairpin structure demonstrated a complex 2 sites profile for a simple 1:1 binding system [41]. The model hairpin DNA used in the previous study was a 20-mer having an $(A_2T_2)_2$ netropsin binding site and a complete sequence of 5′-d(CGAATTCGTCTCCGAATTCG)-3′. Calorimetric titration experiments performed previously using netropsin and other minor groove binding ligands have shown similar complexity in the enthalpy data indicative of more than one binding process in play [24,42–44]. To date, no one has offered an explanation for the presence of multiple binding processes in the 1:1 interaction of minor groove binding ligands to –AT-rich sequences in DNA.

In this paper we report on a systematic thermodynamic (ITC) study of the binding of netropsin to a series of $(A_2T_2)_2$, $(ATAT)_2$, and (AAAA/TTTT) containing hairpin and duplex DNA constructs. The specific oligonucleotides were chosen to probe the impact of stem length, the number of bases in the loop, the sequence of the binding region ($(A_2T_2)_2$ vs. $(ATAT)_2$ and (AAAA/TTTT)), and 3′-tail length on the overall thermodynamics of netropsin binding. The current study was done using the original hairpin, (O·HP), five additional hairpin forming oligonucleotides, and an 8-mer duplex dimer (complete sequences for all of the subject oligonucleotides are listed in Table 1).

We conclude by presenting a complex binding model which is consistent with all of the thermodynamic data, including ΔCp data from a temperature dependent study, and a theoretical estimate of the energetics for trapping a water molecule in one of the two product complex structures [4,15,18]. This model is also supported by docking computations that show similar binding energies for the two complexes, netropsin bound with or without a trapped water molecule.

2. Materials and experimental procedures

2.1. Isothermal titration calorimetry

Oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR). The oligonucleotide "code" or reference names, e.g. original hairpin, (O·HP), *n*-mer length, e.g. 20-mer, complete sequences, e.g. 5′-d(CGAATTCGTCTCCGAATTCG)-3′, and netropsin binding site sequence, e.g. AATT/AATT, are given in Table 1. Netropsin was obtained from Sigma Aldrich (St. Louis, MO). Oligonucleotides were prepared in MES buffer (0.01 M MES, 0.001 M EDTA, 0.2 M NaCl, pH 6.2) and dialyzed against two changes of buffer (24 h each) at 4 °C. Oligonucleotide concentrations were nominally 10 μM. The concentrations of all of the DNA solutions were verified using UV/VIS spectrophotometry with molecular extinction coefficients determined using the nearest neighbor calculation for single strand DNA [45] and the absorbance of thermally denatured constructs extrapolated back to 25 °C, and/or a total phosphate

Table 1
The original hairpin, (O·HP), along with alternate hairpin sequences including base additions in the stem, loop, and tail regions as well as the duplex structure

Code name	Complete hairpin sequence	*n*-mer	Added bases
O·HP	5′-d(CG**AATT**CGTCTCCG**AATT**CG)-3′	20-mer	
AT·HP	5′-d(CG**ATAT**CGTCTCCG**ATAT**CG)-3′	20-mer	
A4·HP	5′-d(CG**AAAA**CGTCTCCG**TTTT**CG)-3′	20-mer	
LS·HP	5′-d(CG**AATT**CGCTCTCGCG**AATT**CG)-3′	22-mer	CG
LL·HP	5′-d(CG**AATT**CGTCTCTCCG**AATT**CG)-3′	22-mer	TC
LT·HP	5′-d(CG**AATT**CGTCTCCG**AATT**CGTTTT)-3′	24-mer	TTTT
Duplex	$(5'$-d(CG**AATT**CG)-$3')_2$	8-mer	

The code names stand for original hairpin, (O·HP), long stem hairpin, (LS·HP), large loop hairpin, (LL·HP) long tail hairpin, (LT·HP) and hairpins having a (A_4/T_4), (A4·HP) or (ATAT), (AT·HP) netropsin binding sites in comparison to the (A_2T_2) binding site of the original hairpin. The code names are given to simply the results and discussion references to individual DNA constructs. The bases located in the netropsin binding region are shown in bold. The bases added to the O·HP in order to lengthen the stem above the binding site, increase the size of the loop, or increase the length of the tail region are indicated by underlining and listed in the right-hand column.

analysis technique [46]. The extinction coefficient determined for the original 20-mer hairpin by these techniques was $\varepsilon_{260}=1.76\times10^{5}\ M^{-1}\ cm^{-1}$.

All ligand solutions were prepared using the final dialysate from the oligonucleotide solutions. Concentrations of netropsin were nominally 1.5×10^{-4} M. Concentrations for netropsin were verified with UV/VIS spectrophotometry using a published molar extinction coefficient of $\varepsilon_{296}=2.15\times10^{4}\ M^{-1}\ cm^{-1}$ [47].

ITC experiments were performed using a Microcal VP-ITC. Titrations were typically done at 25 °C (298 K) and involved overfilling the ITC cell with approximately 1.5 ml of dilute oligonucleotide solution and adding as many as 50 to 60 injections (5 μl) the dilute solution ligand. Typically three replicate measurements were made. The thermograms (integrated heat/injection data) obtained in ITC experiments were fit with our own two-site model fitting algorithm developed for use with Mathematica 5.0 software. The ITC binding data obtained for netropsin could only be fit within expected experimental error using our "two-fractional-sites" binding model and our Mathematica based nonlinear regression algorithm. (The model equations and representative fits are described in Results and shown in Figs. 3 and 4.) Values for ΔG_1 (K_1), ΔG_2 (K_2), ΔH_1, ΔH_2, $-T\Delta S_1$, $-T\Delta S_2$, n_1, and n_2 were extracted directly from the fits obtained for our "two-fractional-sites" model. The $\partial\Delta$ parameters were obtained by simple subtraction of the second site parameter from the first site parameter (ΔG, ΔH, or $-T\Delta S$). ΔCp_1 and ΔCp_2 values were obtained by plotting ΔH_i vs. temperature (°C) and fitting the temperature data with a simple linear regression model over the appropriate narrow temperature ranges within the entire temperature dependent data set (2–45 °C).

2.2. Computational chemistry

Netropsin was docked into the minor groove of the original DNA hairpin molecule using ArgusLab (Planaria Software, L.C.C., Seattle, WA) [48]. The molecule was built using the molecule builder of PC Spartan (Wavefunction, Inc., Irvine, CA), and geometry was minimized using the PM3 semiempirical method, and it was then saved in PDB format for docking [49].

The DNA hairpin was also constructed in PC Spartan. A duplex DNA was made using the PC Spartan molecular builder to pair 5′-d(CGAATTCGTCTC)-3′ with its complimentary strand 5′-d(**GAGA**CGAATTCG)-3′. Next, the 5′-GAGA-3′ was deleted from the 5′ end of complimentary strand and the C-3′ base of the long strand was then connected to the 5′-C base of the shortened complimentary strand. The unpaired bases (TCTC) form the hairpin loop. The constructed hairpin is O·HP sequence as listed in Table 1. The DNA hairpin construct was energy minimized using the MMFF molecular mechanics force field for 1000 cycles to optimize the geometry. Distance constraints were placed between the atoms of the DNA base pairs taking part in the hydrogen bonding interactions to prevent the helix from unwinding. A water molecule was also constructed and placed in the DNA hairpin between the amino group nitrogen $-NH_2$ of the G14 base and the deoxyribose O3 of the A15 base. This initial position for the "trapped" water molecule was based on a published X-ray crystallographic structure for water bound in the minor groove and interacting with another DNA minor groove binding ligand [33]. The DNA hairpin was then saved in PDB format, both with and without the water for docking.

The molecules were transferred to the ArgusLab software, hydrated with ArgusLab, and docked using the ArgusDock docking engine and the flexible ligand option. Scoring was done using the AScore function, as previously described [49]. UFF charges were calculated in ArgusLab for both the ligand (netropsin) and binding site (DNA hairpin). A grid resolution of 0.4 Å was used, and a box size of 20.9 Å × 21.4 Å × 29.5 Å was used to create the grid around the DNA hairpin structure. Subsequently, the two netropsin/DNA structures with the most favorable AScore energy, both with and without the specifically bound water molecule, were placed in a water filled box measuring 30 × 30 × 60 ?, the base pairing constraints removed,

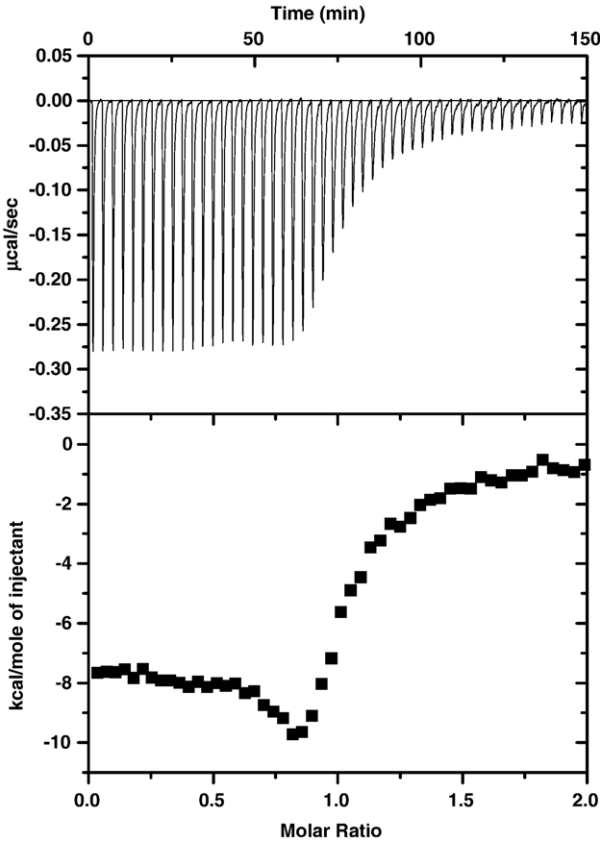

Fig. 1. Representative raw ITC and integrated heat data for netropsin binding to the O·HP (AATT containing DNA construct). The titration data shown were collected at 25 °C in MES buffer (pH 6.25). A typical titration consisted of anywhere from 25 to 60, 5 μl injections of 150 μM netropsin into 1.5 ml of 25 μM DNA. The top panel corresponds to raw data in which the calorimeter compensating power is reduced in response to the heat produced for each injection of reactant during the course of the titration. The bottom panel shows the integrated heat for each injection with respect to the mol ratio of total ligand to total DNA. These are shown with respect to the molar enthalpy (kcal/mol) and mol ratio of added ligand. The integrated heat data shown have been corrected for the heat of dilution of netropsin and the DNA.

and the structures reminimized with 2000 cycles using the MMFF force field.

3. Results

3.1. Isothermal titration calorimetry

The ITC data for binding netropsin to all of the subject DNA constructs indicated the formation of a 1:1 complex with high affinity (e.g. in the range of 10^8 M^{-1}). The interaction of netropsin with each of the subject DNA hairpin constructs and the DNA duplex construct is more complicated than is typically observed for simple 1:1 binding interactions. Fig. 1 shows both raw ITC data and integrated heat data for the titration of the original hairpin, O·HP, with netropsin. It is obvious from these data that the enthalpy change for formation of the 1:1 netropsin complex is not described by a single value of ΔH and that ΔH_2 is more exothermic than ΔH_1. The thermograms for all of the systems studied could only be fit within expected experimental error by employing a "two-fractional-sites" model, where the total number of sites is one netropsin binding site per DNA construct. In this model, the two fractional sites sum to one but have relative stoichiometries that are determined as a result of the nonlinear regression fit. The representative ITC data shown in Fig. 1 result in one of the fits shown in Fig. 2, with the two fractional sites having approximate stoichiometries of 0.7 and 0.3 mol of drug/per mol DNA for sites 1 (n_1) and 2 (n_2) respectively. Similar binding models could result, for example from at least three situations: (1) two different DNA conformers binding a single drug conformer with different affinity to yield a single 1:1 DNA/drug product complex, (2) two different drug conformers binding to a single DNA conformer, again with different affinity and again forming a single DNA/drug product complex, and (3) the binding of a single drug species to a single DNA species but yielding two different product complexes, e.g. one product in which the DNA minor groove is completely

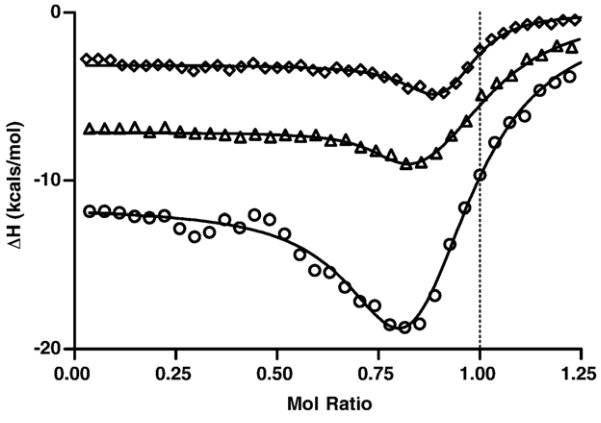

Fig. 2. Representative nonlinear regression fits of the ITC integrated heat data for netropsin binding to the AATT containing O·HP DNA construct at 2 °C (-◇-), 25 °C (-△-), and 45 °C (-○-). The fits shown as solid lines are for the "two-fractional-sites" model described by Eqs. (1) and (2). This model is represented by two fractional sites that are constrained to have a combined stoichiometry of 1.0 mol of ligand/mol of DNA. The fit lines shown above were obtained with a user defined fitting routine coded in Mathematica 5.0 by our laboratory.

Table 2
ITC derived thermodynamic parameters, K_1, ΔG_1, K_2, and ΔG_2 for the target DNA constructs binding netropsin at 25 °C (298 K)

Oligo	K_1 (M^{-1})	ΔG_1 (kcal/mol)	K_2 (M^{-1})	ΔG_2 (kcal/mol)
O·HP	$1.7\ (\pm 0.08)\times 10^8$	−11.2	$1.1\ (\pm 0.08)\times 10^6$	−8.3
AT·HP	$1.9\ (\pm 0.05)\times 10^8$	−11.4	$4.8\ (\pm 0.20)\times 10^5$	−7.8
A4·HP	$1.6\ (\pm 0.06)\times 10^8$	−11.6	$1.1(\pm 0.05)\times 10^6$	−8.4
LS·HP	$1.9\ (\pm 0.05)\times 10^8$	−11.3	$5.0\ (\pm 0.02)\times 10^6$	−9.2
LL·HP	$5.7\ (\pm 0.60)\times 10^7$	−10.5	$3.2\ (\pm 0.05)\times 10^5$	−7.6
LT·HP	$2.5\ (\pm 0.10)\times 10^7$	−10.1	$5.4\ (\pm 0.05)\times 10^5$	−7.9
Duplex	$4.2\ (\pm 0.10)\times 10^7$	−10.4	$1.1\ (\pm 0.01)\times 10^6$	−8.3

The parameters are for the "two-fractional-sites" binding model. The uncertainties listed for K_i are ±2 standard deviations from the mean from the Monte Carlo analysis. The oligonucleotide code names, complete sequences, oligonucleotide length, base additions to the O·HP, and netropsin binding sequence are as listed in Table 1.

desolvated in the complex, (Complex I) and one product which includes bridging water molecules (Complex II). In each case the two DNA structures, two drug structures or two product complex structures must be separated by a large energy barrier so that the paired structures are not at equilibrium, at least at the temperatures and on the time scale of the ITC experiments. It is important to point out that the simple fitting of the ITC data cannot choose between these three possible binding reaction schemes.

Eqs. (1) and (2) are written to represent two different pathways for binding the drug to the minor groove resulting in two different final products (see Fig. 4).

$$\mathrm{Hp} + \mathrm{D} \leftrightarrow \mathrm{Hp\cdot D}, K_1 = [\mathrm{Hp\cdot D}]/[\mathrm{Hp}][\mathrm{D}] \quad (1)$$

$$\mathrm{Hp} + \mathrm{D} + \mathrm{H_2O} \leftrightarrow \mathrm{Hp\cdot D\cdot H_2O}, K_2 = [\mathrm{Hp\cdot D\cdot H_2O}]/[\mathrm{Hp}][\mathrm{D}] \quad (2)$$

K_1 and K_2 are the equilibrium constants for binding to the higher and lower affinity sites respectively. The nonlinear regression is constrained to yield values for n_1 and n_2 that sum to 1 as the stoichiometry is fixed at 1:1 for the complexation

Table 3
ITC derived thermodynamic parameters, ΔH_i, $-T\Delta S_i$ and n_i for the target DNA constructs binding netropsin at 25 °C (298 K)

Oligo	ΔH_1 (kcal/mol)	$-T\Delta S_1$ (kcal/mol)	n_1	ΔH_2 (kcal/mol)	$-T\Delta S_2$ (kcal/mol)	n_2
O·HP	−7.1±0.2	−4.1	0.72	−12.0±0.2	3.7	0.28
AT·HP	−10.0±0.1	−1.4	0.75	−12.3±0.1	4.5	0.25
A4·HP	−7.8±0.1	−3.8	0.67	−12.2±0.1	3.8	0.33
LS·HP	−6.0±0.1	−5.3	0.75	−9.9±0.1	−1.0	0.25
LL·HP	−4.3±0.1	−6.2	0.66	−9.1±0.1	0.4	0.34
LT·HP	−5.7±0.1	−4.4	0.61	−9.9±0.1	2.0	0.39
Duplex	−11.5±0.5	1.1	0.71	−29.0±0.5	21.7	0.29

The parameters are for the "two-fractional-sites" model. The uncertainties listed for ΔH_i are ±2 standard deviations from the mean from the Monte Carlo analysis. The oligonucleotide code names, complete sequences, oligonucleotide length, base additions to the O·HP, and netropsin binding sequence are as listed in Table 1.

Table 4
Calculated thermodynamic difference data from the ITC results given in Tables 2 and 3

Oligo	$\delta\Delta G_{1-2}$ (kcal/mol)	$\delta\Delta H_{1-2}$ (kcal/mol)	$\delta(-T\Delta S_{1-2})$ (kcal/mol)
O·HP	−1.7	3.4	−7.8
AT·HP	−3.6	2.3	−5.9
A4·HP	−3.2	4.4	−7.6
LS·HP	−2.1	3.9	−4.3
LL·HP	−2.9	4.8	−6.6
LT·HP	−2.2	4.2	−6.4
Duplex	−2.1	17.5	−20.6

The $\partial\Delta X_i$ values were obtained by subtracting the site 2 values from the site 1 values for ΔG, ΔH, and $-T\Delta S$ respectively at 298 K. The parameters are for the "two-fractional-sites" model. The oligonucleotide code names, complete sequences, oligonucleotide length, base additions to the O·HP, and netropsin binding sequence are as listed in Table 1.

reaction. The initial K value for netropsin binding is approximately 1.7×10^8 M^{-1} and it decreases approximately 20-fold to 9.6×10^6 M^{-1} near site saturation. The corresponding enthalpy values for site 1 and site 2 interactions are −6.6 and −10.0 kcal/mol respectively.

The ITC derived values for the "two-fractional-sites" model parameters; K_1, K_2, ΔG_1, and ΔG_2, are given for all seven of the hairpin and duplex DNA constructs interacting with netropsin in Table 2. Table 3 lists the values obtained for ΔH_1, $-T\Delta S_1$, ΔH_2, $-T\Delta S_2$, for the interaction of netropsin with all of the target DNAs. Table 4 lists differences in the site 1 and site 2 parameters, $\partial\Delta X_{1-2}$, for each of the seven DNA constructs.

The uncertainties listed for the K values in Table 2 and the ΔH values in Table 3 were determined by use of a Monte Carlo procedure in which 1000 virtual ITC experiments were simulated and analyzed with our "two-fractional-sites" model. The Monte Carlo analysis was begun by taking the best fit parameters from a fit of the actual experimental data, then simulating 1000 experiments in which random experimental error was added to the incremental heat data, refitting the virtual experimental data and finally looking at the distribution of best fit parameters. The random error incorporated into the simulated thermograms was determined from the ITC specifications. The error was assumed to be Gaussian in distribution and to randomly vary over two standard deviations in the measured integrated heat (±0.2 μcal/heat increment). Fig. 3 shows plots of the distributions for Monte Carlo estimated values of K_1, K_2, ΔH_1, and ΔH_2 for the binding of netropsin to the O·HP at 25 °C. The beginning data were the same as shown in Fig. 1 and the 25 °C fit as shown in Fig. 2. None of the distributions shown in Fig. 3 are strictly symmetrical. The inference is that for these systems, site 1 parameters are somewhat correlated with site 2 parameters and more significantly K and ΔH trend in opposite

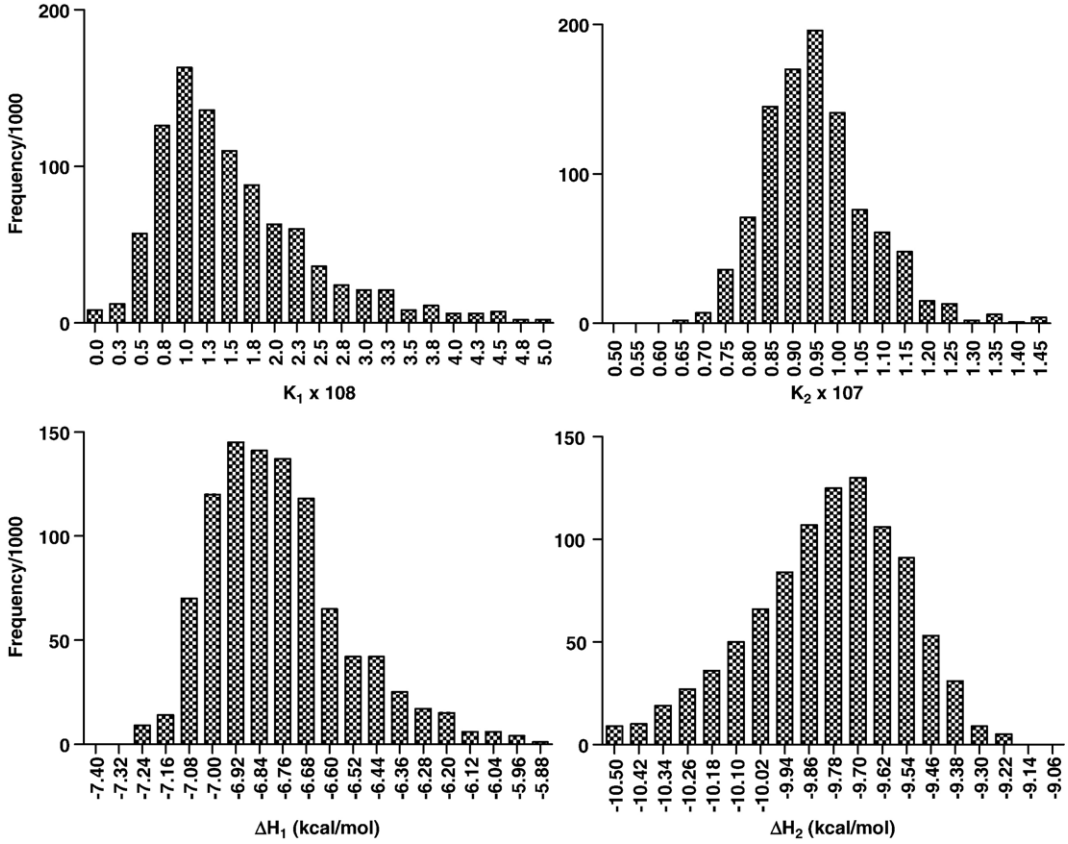

Fig. 3. Representative histograms for the Monte Carlo analysis performed on the ITC titration data obtained for the AATT hairpin and netropsin collected at 25 °C. The Monte Carlo analysis consisted of nominally 1000 simulated experiments based on simulated random error (twice the standard deviation from the ITC fits) added to the same experimental data set included in Fig. 1 and fit in Fig. 2. The histograms demonstrate the distribution of values for simulated binding affinities and enthalpy changes for both binding modes.

directions. In other words, large values for K_1 or K_2 are somewhat compensated by typically being paired with smaller values for ΔH_1 and ΔH_2. Nevertheless, all of the parameters are well determined with the uncertainties in ΔG_i, ΔH_i, and $-T\Delta S_i$ smaller than ±0.2 kcal/mol in all cases.

A schematic of the two-fractional-sites reaction model and the species distribution calculated for the titration of the O·HP DNA with netropsin is shown in Fig. 4. While the reaction scheme is hypothetical (see Discussion) the species distribution accurately reflects the relative concentrations of free hairpin [HP], the two forms of the complex [O·HP] and [HP·D·H_2O], and free netropsin [D] throughout the course of the titration. The energetic implications of the trapped water molecule in the second complex, [HP·D·H_2O], are discussed in detail below.

The thermodynamics of the netropsin interaction with the O·HP DNA were also determined at six temperatures from 2 to 45 °C. The "two-fractional-sites" model fits all of these ITC titration data presented here. Representative data along with the "two-fractional-sites" fits are shown in Fig. 2 for three different temperatures (2, 25, and 45 °C). The general trends for both site 1 and site 2 are that the free energy change is largely unaffected by temperature, the enthalpy change is increasingly more exothermic as the temperature is increased, and the value of (n_1/n_2) decreases with increasing temperature up to 45 °C. The thermodynamic parameters for the interaction of netropsin with the O·HP DNA construct six temperatures between 2 and 45 °C are given in Table 5. The differences in the site 1 and site 2

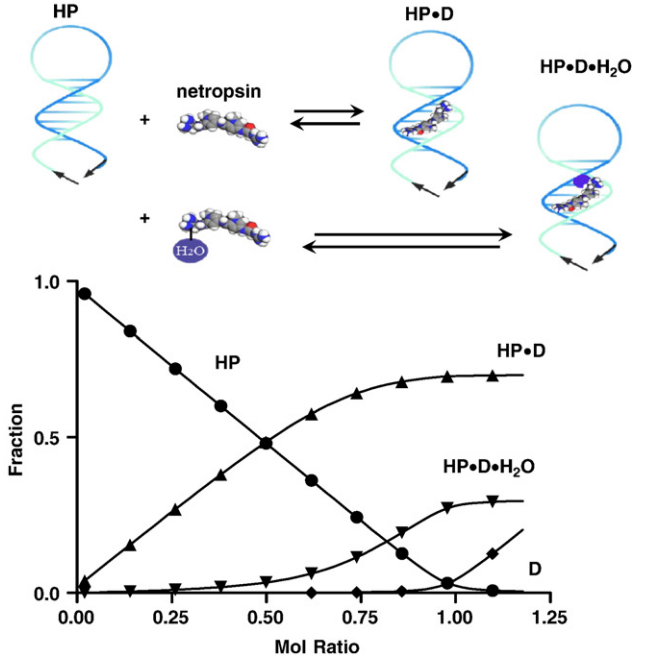

Fig. 4. (Top) Proposed molecular mechanism to describe the observed complex binding of netropsin to the hairpin constructs. The hairpin is represented as a double helix containing a loop, netropsin is represented in a CPK model, and the additional water molecule is represented by a sphere. (Bottom) Species distribution calculated for the titration of the O·HP with netropsin. The relative concentration of each species is calculated from the best fit parameters for the "two-fractional-sites" model. K_1, K_2, n_1 and n_2 are listed in Tables 2 and 3. The lines illustrate the changing concentration for each hairpin or drug containing species, [HP], [HP·D], [HP·D·H_2O], and [D], throughout the titration.

Table 5
ITC derived thermodynamic data for binding netropsin to the O·HP at temperatures of 2, 5, 15, 25, 37, 45, 50, and 55 °C

Temperature (°C)	ΔG_1 (kcal/mol)	ΔH_1 (kcal/mol)	$-T\Delta S_1$ (kcal/mol)	ΔG_2 (kcal/mol)	ΔH_2 (kcal/mol)	$-T\Delta S_2$ (kcal/mol)
2	−9.7	−3.1	−6.6	−7.7	−10.3	2.6
5	−9.2	−3.3	−5.9	−7.4	−9.2	1.8
15	−9.7	−6.3	−3.4	−8.0	−12.2	4.2
25	−11.2	−7.1	−4.1	−8.3	−12.0	3.7
37	−10.9	−12.4	1.5	−8.6	−21.7	13.1
45	−11.2	−11.9	0.7	−8.8	−26.6	17.8

The listed parameters are from the "two-fractional-sites" fit of the integrated heat data. The data and nonlinear regression fit lines for the 2, 25, and 45 °C experiments are shown in Fig. 3. The O·HP complete sequence, length, and netropsin binding sequence are as listed in Table 1.

parameters, $\partial\Delta X_{1-2}$, are listed in Table 6 for these same six temperatures.

The temperature dependence of the enthalpy change has been used to estimate several values for ΔCp. The site 1 temperature dependence of enthalpy change yields a single estimate for ΔCp (−0.23 kcal/mol K). The site 2 enthalpy change vs. temperature data could be interpreted in terms of two values for ΔCp, one at temperatures below about 30 °C, and a second at temperatures above 30 °C. The O·HP DNA construct does not have sufficiently high thermal stability ($T_M \leq 70$ °C) [41] to examine enough higher temperature data to determine whether a single value of the heat capacity change for site 2 or low temperature and high temperature heat capacity change values are necessary. The average ΔCp value calculated for site two for the data shown in Fig. 5 yields a value of (−0.38 kcal/mol K). These ΔCp values were estimated from the slopes of the linear regression fits of the ΔH vs. temperature data, over the appropriate limited temperature range, as shown in Fig. 5.

3.2. Computational

28 candidate poses were obtained for the docking of netropsin to the DNA hairpin/water structure, with the most favorable energy calculated by AScore to be −3.90 kcal mol^{-1}

Table 6
Calculated thermodynamic difference data from the ITC results given in Table 5

Temperature (°C)	$\delta\Delta G_{1-2}$ (kcal/mol)	$\delta\Delta H_{1-2}$ (kcal/mol)	$\delta(-T\Delta S_{1-2})$ (kcal/mol)
2	−2.0	7.2	−9.2
5	−1.8	5.9	−7.7
15	−1.7	5.9	−7.6
25	−2.9	4.9	−7.8
37	−2.3	9.3	−11.6
45	−2.4	14.7	−17.1
50	−3.0	21.8	−24.8
55	na	na	na

The $\partial\Delta X_i$ values were obtained by subtracting the site 2 values from the site 1 values for ΔG, ΔH, and $-T\Delta S$ respectively at each temperature from 2 °C to 55 °C. The parameters are for the "two-fractional-sites" model. The O·HP complete sequence, length, and netropsin binding sequence are as listed in Table 1.

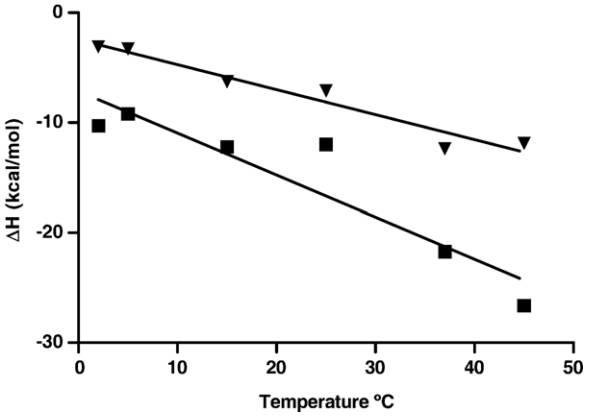

Fig. 5. Temperature dependence of the enthalpy change for netropsin binding to the AATT containing O·HP. The first binding mode demonstrates a linear dependence of the enthalpy change on the temperature over the measurement range of 2 to 55 °C. The slope of the simple linear regression lines shown here correspond to values for the site 1 and site 2 heat capacity changes of $\Delta Cp_1 = -0.23$ kcal mol^{-1} K^{-1} and $\Delta Cp_2 = -0.38$ kcal mol^{-1} K^{-1}.

(Fig. 6A). 51 candidate poses were obtained for the docking of netropsin to the DNA hairpin structure (without water), with the most favorable energy calculated by AScore to be −4.02 kcal mol^{-1} (Fig. 6B).

The docking of netropsin into both the DNA hairpin and DNA hairpin/water structures resulted in two unique conformations for the flexible netropsin molecule (c.f. Fig. 7 with Fig. 6). The difference in the binding score (AScore) is 0.12 kcal mol^{-1}, which is insignificant. Netropsin bound with no water in the minor groove results in a snug fit of the molecule along the wall of the minor groove, allowing for more favorable van der Waal's interactions. The water molecule trapped underneath netropsin in the minor groove appears to cause a significant shift in the conformation of netropsin to a more linear shape. It also results in the placement of the central part of the molecule in the middle of the minor groove with less favorable van der Waal's interactions, as well as more room for movement and potentially less favorable entropic conditions.

There are significant electrostatic interactions between netropsin and the hairpin bases in both complexes. We will discuss the hydrogen bonding in the netropsin/DNA hairpin complex without trapped water first (Fig. 6A). Starting at the top of the figure, one of the di-amidino nitrogens is forming hydrogen bonds with an aromatic nitrogen from base A15 (2.9 Å), and an amino nitrogen from base G14 (3.1 Å). Continuing down the docked netropsin, the first amide nitrogen is interacting with an aromatic nitrogen of base A16 (3.6 Å). The second amide nitrogen is forming a bifurcated electrostatic interaction with both the carbonyl oxygen of base T17 (3.4 Å) and the carbonyl oxygen of base T5 (3.1 Å). The third amide is forming a hydrogen bond to the carbonyl oxygen of base T18 (2.7 Å). Finally, one of the amidino nitrogens of the di-amidino group at the bottom end of the docked netropsin molecule is interacting with both the carbonyl oxygen of base C19 (2.7 Å) and the deoxyribose ether oxygen of base G20 (2.9 Å). The other amidino nitrogen is not interacting with the DNA and is exposed to water.

In the netropsin/DNA complex with a trapped water molecule (Fig. 6B), we need to discuss the interactions between the bound water molecule and DNA base functional groups as well as the interactions between the docked netropsin and the bound water molecule and the docked netropsin and the DNA base functional groups. The water molecule sits in the DNA groove between bases G14 and A15. The water molecule is close enough to several hydrogen bond donor and acceptor groups from these bases, for example the distances between the specifically bound water molecule and either an aromatic nitrogen of base G14 (2.9 Å), or an amino nitrogen of G14 (2.8 Å), and/or the deoxyribose ether oxygen of base A13 (2.7 Å). The water can also interact with both of the di-amidino nitrogens at the top of the docked netropsin molecule (3.3 Å and 3.6 Å, respectively), as well as with the nitrogen of the first amide group (2.8 Å). The second amide is interacting with the carbonyl oxygen of base T6 (3.1 Å), and the third amide is forming a bifurcated interaction with both the carbonyl oxygen of base T17 (2.8 Å) and the carbonyl oxygen of base T5 (3.3 Å). At the bottom end of the docked netropsin, one of the amidino nitrogen atoms is interacting with the carbonyl oxygen of base T18 (2.8 Å), while the other amidino nitrogen is not interacting with the DNA and is exposed to water.

4. Discussion

In contrast to many other minor groove binding ligands [10,24,27,30–35], netropsin binding is accompanied on average by a large favorable enthalpy change. The favorable enthalpy change is seen for both site 1 and site 2 interactions. This is the result of the netropsin molecule being extensively H-bonded to the DNA [26,37,38,50] and/or to the DNA and to the bridging water molecule. Netropsin site 1 interactions have an almost equally favorable entropy contribution to the binding free energy change for all of the netropsin/hairpin oligonucleotide systems studied here (see Table 3). In the site 2 binding interaction, the entropy change becomes small or unfavorable, and the binding process that includes "trapped" water is almost entirely enthalpy driven. The interactions of netropsin with a short duplex DNA are very different from the standpoint of the complete thermodynamic profile in comparison to the series of hairpin DNAs examined here. There are really three subjects that need to be discussed. The first is the influence of oligonucleotide structure and binding site sequence on the thermodynamics of the netropsin interaction with –AT-rich regions of short oligonucleotides. The second involves looking at the temperature dependent data and a discussion of the large negative heat capacity changes that accompany binding to the two fractional sites. The last related topic is an overall discussion of the influence of water both from the standpoint of generic hydration or solvation effects and a closer examination of the proposed trapped or bridging water in Complex II.

The data in Tables 2 and 3 can be examined in light of the sequence at the binding site and the presence or absence of

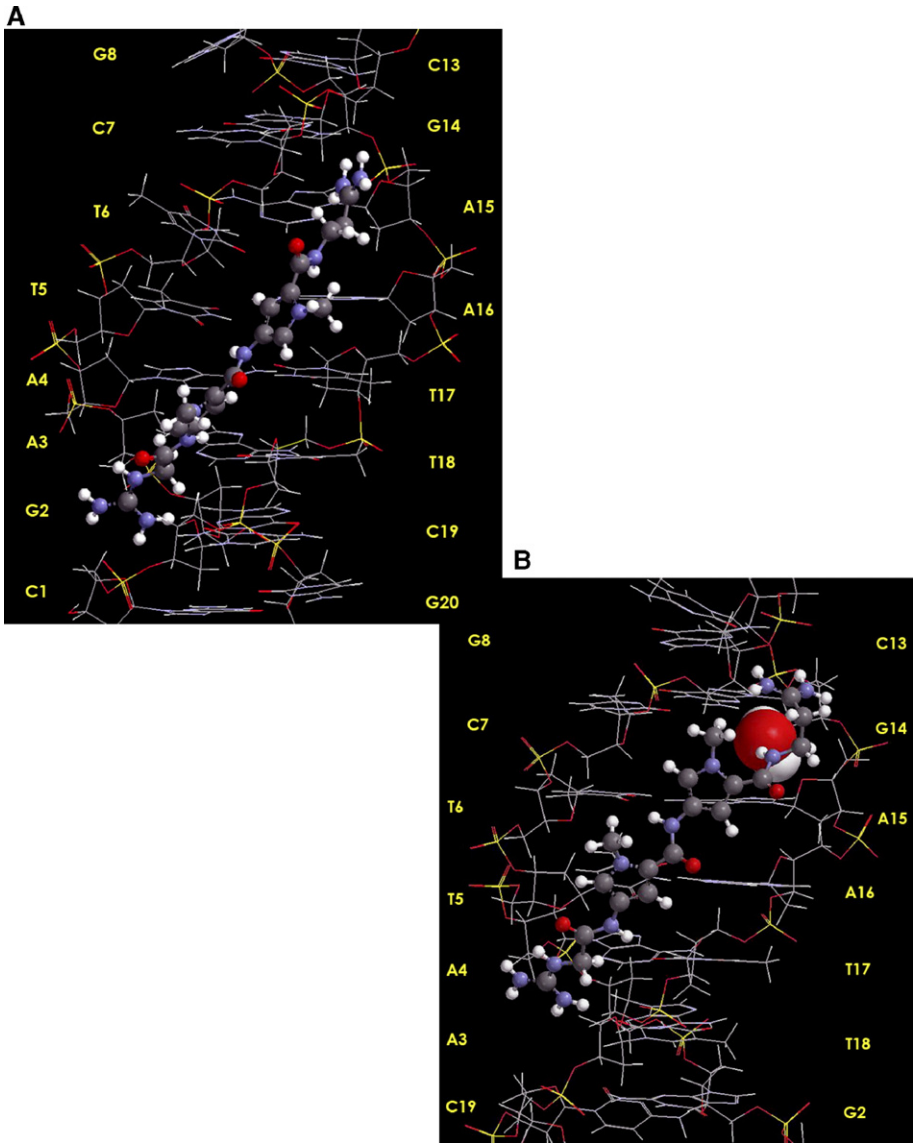

Fig. 6. Molecular models of Netropsin (ball-and-stick view) docked into the minor groove of the original DNA hairpin (wireframe), both without water bound in the minor groove (A) and with one water molecule bound between bases G14 and A15 (B).

nearby single stranded regions e.g. loops and tails. In comparison the O·HP, the AT·HP, A4·HP, and LS·HP constructs exhibit almost the same affinity for netropsin binding to site 1 (ΔG_1 values of −11.2, −11.4, −11.6 and −11.3 kcal/mol respectively). There is certainly more variability in the site 2 affinity with the ATAT sequence exhibiting the weakest interaction and the AATT affinity being the strongest. The ΔG_2 values for the same series of oligonucleotides range from −7.8 kcal/mol for the ATAT hairpin, to −8.4 for the AAAA/TTTT containing hairpin, to −8.3 or −9.2 kcal/mol for the two AATT hairpins. Our hypothetical model (see Fig. 4) is based on site 2 trapping a water molecule. The subtle sequence differences in the site 2 affinities probably result from differences in the solvation of the DNA. The implication is that the AATT sequence contains water that is more tightly bound in the groove than the other sequences, either ATAT or AAAA/TTTT.

There are much larger differences in both the ΔH_1 and ΔH_2 values among the same series of target DNA constructs. The fact that the site 1 free energy change values are almost invariant while the site 1 ΔH values vary from nominally −4 kcal/mol to −11.5 kcal/mol is indicative of enthalpy entropy compensation often seen in water especially at temperatures near 300 K. Again this is the probable result of sequence dependent solvation effects in the target DNA. The ΔH_2 values are less variable, ranging from −9.9 kcal/mol for the O·HP to −12.3 kcal/mol for the AT·HP hairpin construct. Clearly in terms of ΔH_2, any sequence solvation differences are mitigated by the fact that a tightly bound water molecule is left behind in the Complex II structure.

The two oligonucleotides that have additional bases in the single stranded regions of the hairpin, the LL·HP and LT·HP constructs exhibit site 1 and site 2 affinities that more closely resemble the duplex values for K (or ΔG) than those for the

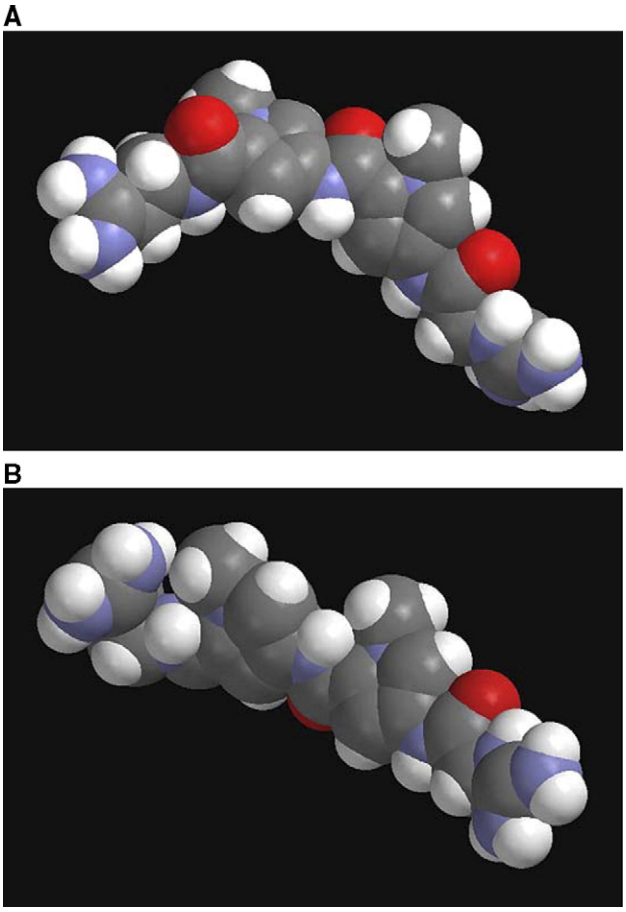

Fig. 7. Space-filling representations of the docked conformation of Netropsin without water bound in the minor groove (A) and with water bound in the minor groove (B). The water molecule in Fig. 6(B) would be found on the left side of the molecule, forming hydrogen bonds with the amines.

O·HP. Both the site 1 and site 2 netropsin affinities for these constructs are lower by approximately 1 kcal/mol in comparison to the O·HP. Again we would argue that the single stranded regions in either the large loop or long tail constructs have a destructuring effect on the groove water and thus less binding energy is available from desolvation of the DNA for either site 1 or site 2 binding. The duplex, having a shorter sequence and free ends, would have similar amounts of structured water in comparison to the LL·HP and LT·HP constructs.

Again there are much larger differences in both the ΔH_1 and ΔH_2 values among the LL·HP, LT·HP, and duplex DNA constructs. The site 1 free energy change values are almost invariant while the site 1 ΔH values vary from −11.5 kcal/mol to −4.3 kcal/mol. This is a classic case of enthalpy entropy compensation. Again this is the probable result of structure and structure length dependent solvation effects in the target DNA. The ΔH_2 values are less variable for the two hairpins ranging from −9.1 kcal/mol for the LL·HP to −9.9 kcal/mol for the LT·HP hairpin construct. The ΔH_2 values are almost constant throughout the series of AATT containing constructs including the O·HP, the LS·HP, LL·HP, and LT·HP. Apparently the binding site sequence largely determines the enthalpy change for site 2 binding. The ΔH_2 value for the netropsin/duplex interaction is significantly more exothermic than the ΔH_2 values for any of the hairpin DNA constructs. This may be the result of some folding enthalpy being included in binding netropsin to some small fraction of unfolded duplex even at 298 K.

The temperature dependent data for binding netropsin to the O·HP are listed in Table 5. The ΔH_2 values reported at 37 and 45 °C appear larger than might be expected. Again, a possible explanation is that at these higher temperatures the hairpin is partially unfolded. Thus the heat for the late binding ligands is composed of both ligand binding and the refolding of the hairpin. The temperature dependent data for binding have been analyzed to yield values of heat capacity change at constant pressure, ΔCp, for both the site 1 and site 2 interactions. The site 1 interaction exhibits a ΔCp1 of −0.23 kcal mol^{-1} K^{-1} over the temperature range of from 2 to 45 °C (275–318 K). This a typical average value that has been observed previously for a number of minor groove binders [5,17,20]. There is not much new in these data for site 1 binding. However, the site 2 enthalpy change may not be a simple linear function of temperature. The site 2 binding interaction may exhibit a larger value for ΔCp_2 above 30 °C. The average value for ΔCp2 is: −0.38 kcal mol^{-1} K^{-1} over the temperature measurement range. There is some evidence that the ΔCp_2 value at temperatures above 30 °C is significantly larger than the value reported here. It almost seems from the data reported in Fig. 5, that there could be a break in ΔCp_2 at approximately 30 °C (≈303 K). The temperature of 300 K is noted to be the isoequilibrium temperature where enthalpy and entropy compensation is known to be at its best [18]. This is almost certainly not a coincidence and the likely consequence of the temperature dependence of water structure. We intend to investigate the possible discontinuity in the ΔCp_2 value at 300 K in future studies of netropsin binding to a hairpin with higher thermal stability(T_M>80 °C).

The differences in the site 1 and site 2 thermodynamic parameters, $\partial\Delta X_{1-2}$, are listed in Tables 4 and 6. The difference in the free energy change for the site 1 vs. site 2 binding interaction is almost invariant for the entire series of oligonucleotides including both hairpins and the target duplex. In comparing the data for all of the DNA targets as shown in Table 4, the mean value for $\partial\Delta G_{1-2}$ is −2.5 kcal/mol, and the mean values for $\partial\Delta H_{1-2}$ and $\partial(-T\Delta S_{1-2})$ are +3.8 and −6.3 kcal/mol respectively. The difference in the enthalpy change for site 1 binding vs. site 2 binding is +3.8 kcal/mol at 298 K. The fact that ΔH_2 is more favorable by −3.8 kcal/mol is in excellent agreement with the enthalpy difference reported by Cooper ($\Delta\Delta H$) for trapping a single water molecule, −6 to −12 kJ/mol (≈−1.5 to −3 kcal/mol) [15]. The difference in the entropy change for site 1 binding vs. site 2 binding is −6.3 kcal/mol at 298 K. The fact that $-T\Delta S_2$ is less favorable by +6.3 kcal/mol is also in reasonable agreement with Cooper's estimate for the entropy change of trapping a single water molecule, $\Delta\Delta S$ of −11 J mol^{-1} K^{-1}, which corresponds to a value for $-T\Delta\Delta S$ of +3.3 kcal/mol at 298 K [15]. Similar discussions of the cost of trapping a water molecule have been applied to protein ligand binding, protein unfolding, protein DNA interactions and a number of biopolymer interactions that

are the result of a large number of weak interactions [5,15,17,18,20]. This seems to support our hypothetical binding model (see Fig. 4) in which the flexible netropsin can bind either with (site 2) or without a trapped water molecule (site 1). In further support of our proposed "two-fractional-sites" binding model for the interaction of netropsin with a series of target oligonucleotides, we computed the binding energies for formation of the two complexes, I and II, as described in Materials and experimental procedures and Results. The AScore binding energies were insignificantly different for the two complexes with netropsin docked in the minor groove and excluding all water molecules from the groove or docked with one water molecule bridging between amidino nitrogens of the drug and H-bonding atoms accessible in the groove. The docked structures are shown in Fig. 6A and B. The netropsin can interact with the minor groove in two different conformations in a bent conformation or with a trapped water molecule completing the curvature of the more linear conformer. This curvature is needed to fill the minor groove. This may be a great example of Ladbury's drug design approach in which he suggests "just add water" [1].

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